

EFFECT OF EXERCISE ON GLUTAMINE SYNTHESIS AND TRANSPORT IN SKELETAL MUSCLE FROM RATS

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SUMMARY

1. Reductions in plasma glutamine are observed after prolonged exercise. Three hypotheses can explain such a decrease: (i) high demand by the liver and kidney; (ii) impaired release from muscles; and (iii) decreased synthesis in skeletal muscle. The present study investigated the effects of exercise on glutamine synthesis and transport in rat skeletal muscle.

2. Rats were divided into three groups: (i) sedentary (SED; $n = 12$); (ii) rats killed 1 h after the last exercise bout (EX-1; $n = 15$); and (iii) rats killed 24 h after the last exercise bout (EX-24; $n = 15$). Rats in the trained groups swam 1 h/day, 5 days/week for 6 weeks with a load equivalent to 5.5% of their bodyweight.

3. Plasma glutamine and insulin were lower and corticosterone was higher in EX-1 compared with SED rats ($P < 0.05$ and $P < 0.01$, respectively). Twenty-four hours after exercise (EX-24), plasma glutamine was restored to levels seen in SED rats, whereas insulin levels were higher ($P < 0.001$) and corticosterone levels were lower ($P < 0.01$) than in EX-1. In the soleus, ammonia levels were lower in EX-1 than in SED rats ($P < 0.001$). After 24 h, glutamine, glutamate and ammonia levels were lower in EX-24 than in SED and EX-1 rats ($P < 0.001$). Soleus glutamine synthetase (GS) activity was increased in EX-1 and was decreased in EX-24 compared with SED rats (both $P < 0.001$).

4. The decrease in plasma glutamine concentration in EX-1 is not mediated by GS or glutamine transport in skeletal muscle. However, 24 h after exercise, lower GS may contribute to the decrease in glutamine concentration in muscle.

Key words: aerobic exercise, glutamine synthetase, glutamine transport, moderate exercise, soleus.

INTRODUCTION

Glutamine is a conditionally essential amino acid that comprises 20% of total plasma amino acids and is actively produced in organs such as the liver, kidneys, lungs and skeletal muscle.^{1–5} Skeletal muscle is the major tissue involved in glutamine synthesis and storage; it

is known to release glutamine into the blood and to influence plasma glutamine concentrations,¹ as well as the metabolism of glutamine in other tissues.²

In vivo studies on humans have shown that physical exercise is initially accompanied by increased muscle glutamine release and hence an increase in plasma glutamine concentration.⁶ However, a subsequent reduction in plasma glutamine concentration has been observed, such as during prolonged exhaustive exercise in humans and rodents.^{2,4,5,7–10} The mechanism by which this decrease in plasma glutamine concentration occurs during physical exercise is not well understood. Three hypotheses have been proposed: (i) the decrease in plasma glutamine concentration occurs because of high demand by both the liver and kidney for glucose synthesis accompanied by an increased rate of glutamine utilization by several other cell types, such as those of the immune system;^{5,11} (ii) under exhaustive conditions, the rate of glutamine release from muscles is impaired, resulting in a decrease in plasma glutamine concentrations;¹¹ and (iii) exhaustive exercise decreases glutamine synthesis in skeletal muscle.

Glutamine synthetase (GS) is an enzyme that catalyses the ATP-dependent condensation of glutamate and ammonia to form glutamine.¹² Endurance training can result in attenuation of the usual increase in GS expression under catabolic conditions.^{13,14} In contrast with what is known about changes in enzyme expression after training, little is known about the effect of acute exercise on GS activity.¹²

Glutamine release by skeletal muscle is regulated in a precise and specific manner, possibly in response to the demand for glutamine. In skeletal muscle, glutamine release is mediated by a transport system (system N^m), which is affected by concentrations of corticosterone (release) and insulin (uptake). This transport mechanism is bidirectional, controlling both the release and uptake of glutamine in skeletal muscle.¹⁵ It may also modulate glutamine metabolism throughout the entire body, as seen during periods of rest, under pathological conditions and after both moderate and exhaustive physical exercise.^{11,16}

Therefore, the aim of the present study was to investigate the effects of exercise on glutamine synthesis and release in rat skeletal muscle. We hypothesized that the decrease in glutamine plasma concentration after exercise was caused, at least in part, by impairment of glutamine synthesis in and release from skeletal muscle.

METHODS

Animals

Male Wistar rats, weighing 150–200 g, were obtained from the Institute of Biomedical Science animal house (University of São Paulo, São Paulo, Brazil). Rats were maintained under a constant 12 h light–dark cycle (lights on 0700 hours), at $22 \pm 2^\circ\text{C}$ and $60 \pm 5\%$ humidity. Rats were kept in

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collective cages, with five animals per cage, and were allowed water and food *ad libitum*.

All protocols were authorized by the Committee for Ethics in Animal Research of the Institute of Biomedical Science in agreement with the ethical principles for animal research adopted by the Brazilian College of Animal Experimentation.

Groups and training protocol

Forty-seven rats were divided randomly into two groups, sedentary (SED; $n = 12$) and trained ($n = 35$). During training, five animals were excluded from the study because they were not able to train for 60 min daily. After 6 weeks of training, the trained rats were divided into two subgroups, rats sacrificed 1 h after the last exercise bout (EX-1; $n = 15$) to enable comparisons with previous studies that reported decreases in plasma glutamine concentrations 1 h after acute exercise^{6–10} and rats killed 24 h after the last exercise bout (EX-24 h; $n = 15$) because 24 h may be sufficient time for the recovery of several parameters that change during exercise, such as glutamine and hormones.^{10,17,18} Rats in the EX-1 and EX-24 groups trained 1 h/day, 5 days/week for 6 weeks. During the exercise bouts, the rats swam with an extra load equivalent to 5.5% total bodyweight attached to their tail to aid aerobic training.^{10,19} Swimming is known to be an efficient training method that promotes several biochemical, physiological and metabolic adjustments.^{17,18} The swimming apparatus we used was designed to control the duration, load and frequency of exercise during the training regimen. Maximal oxygen consumption ($\text{VO}_{2\text{max}}$) of sedentary rats ranges between 85 and 100 mL/min per kg bodyweight. When a weight equal to 5% of the rat's total bodyweight is attached at the tail, it performs with 70–75% of its $\text{VO}_{2\text{max}}$.¹⁸ Rats were killed by decapitation between 0800 and 1000 hours after a 6 h fast.

Plasma and skeletal muscle sampling

After rats had been killed, blood was placed into test tubes containing sodium heparin and kept on ice. Blood was centrifuged at 690 g for 25 min at 4°C and the plasma was collected and stored at –80°C until biochemical assays were performed. Muscle tissue samples were removed from the soleus muscles of one leg. Tissues were homogenized in phosphate-buffered saline (PBS) at 1 : 9 w/v and the supernatant was extracted after centrifugation at 2500 g for 10 min at 4°C and stored at –80°C until the assays were performed.

Biochemical assays

Glutamine in muscle and plasma was assayed enzymatically in buffer containing 10 mmol/L KH_2PO_4 , 50% glycerol, 4 mmol/L NADH, 10% bovine serum albumin (BSA), 5 U/mL glutamate dehydrogenase, 4 mol/L 2-oxoglutarate and 5.0 U/mL asparaginase, pH 8.0 and 20–25°C.²⁰ In this reaction, the decrease in NADH measured spectrophotometrically at 340 nm is proportional to the amount of glutamine in the medium. Plasma and muscle glutamine concentrations were determined by comparison against a standard curve over the range 100–1000 nmol and 1–50 μmol glutamine, respectively. All samples were run in duplicate with a coefficient of variation of 4%.

Glutamate in muscle and plasma was assayed enzymatically in buffer containing 300 mmol/L glycine, 250 mmol/L hydrazine, 1 mmol/L ADP, 1.6 mmol/L NAD and 4.5 U/mL glutamate dehydrogenase, pH 9.0 and 20–25°C,²¹ according to the method described previously.²¹ In this reaction, the increase in NADH measured spectrophotometrically at 340 nm is proportional to the amount of glutamate in the medium. Plasma and muscle glutamate concentrations were determined by comparison against a standard curve over the range 100–1000 nmol and 1–50 μmol glutamate, respectively. All samples were run in duplicate with a coefficient of variation of 5%.

Ammonia in the muscle was assayed in buffer containing 0.5 mol/L Tris-aminomethane-HCl, 0.1 mmol/L oxoglutarate acid, 8 mmol/L NADH and 6 U/mL glutamate dehydrogenase. The solution was buffered at pH 8.0 and maintained between 20 and 25°C.¹⁰ After the first absorbance reading, samples were incubated for 20 min and then absorbance was read again. The

concentration of the ammonia in samples was determined by calculating the difference between the two absorbances.¹⁰ In this method, the reductive amination of 2-oxoglutarate is catalysed by the enzyme glutamate dehydrogenase. NADH and ammonia are consumed and glutamate, NAD and water are produced; the decrease in NADH is proportional to the ammonia concentration. In the present study, plasma ammonia concentrations were determined by comparison against a standard curve over the range 1–20 μmol . Absorbance was determined spectrophotometrically at 340 nm. All samples were run in duplicate with a coefficient of variation of 7%.

Hormones

Plasma insulin concentrations were determined by radioimmunoassay using a commercially available kit (Coat-a-count; DPC, São Paulo, Brazil). Corticosterone plasma concentrations were determined by a colourimetric method, as described by Guillemain *et al.*²² in a medium containing dichloromethane, 0.1 mol/L NaOH and distilled water. After incubation for 20 min in a 7 : 3 v/v solution of 10 mol/L H_2SO_4 and 99% ethyl alcohol, fluorescence was determined at an excitation wavelength of 425 nm and an emission wavelength of 530 nm.

Glutamine synthetase

Maximal GS activity was determined enzymatically using the method described by Fray *et al.*²³ This method estimates GS activity indirectly. The process takes place in two steps with enzyme-bound γ -glutamyl phosphate as an intermediate. Homogenized samples of soleus muscle were incubated in 0.01 mol/L imidazole-HCl (pH 7.2), 0.02% mercaptoethanol, 10 mmol/L glutamate, 10 mmol/L ATP, 20 mmol/L magnesium chloride (MgCl_2) and 126 mmol/L hydroxylamine. After 20 min, the reaction was stopped by the addition of 0.75 mL ice-cold acid/ FeCl_3 , consisting of 0.2 mol/L trichloroacetic acid, 0.67 mol/L HCl and 0.37 mol/L FeCl_3 . Samples were vortexed and precipitated protein was removed by centrifugation at 7000 g for 10 min. The supernatant was removed and its absorbance at 535 nm measured against a blank of imidazole buffer and acid/ FeCl_3 using a standard curve for γ -glutamyl hydroxamate over the range 50–500 μmol . The absorbance values were converted to μmol product formed. Glutamine synthetase activity was calculated as μmol product formed/min per mg protein for each sample.

Glutamine transport

Glutamine transport was determined in whole soleus muscle from one leg incubated according to a method described by Parry-Billings *et al.*²⁴ Briefly, the muscle was dissected longitudinally and the distal and proximal tendons were ligated and attached to stainless steel holders such that the muscle was maintained at *in situ* resting tension. Several lines of evidence have demonstrated that this setup is a viable *in vitro* muscle preparation with which to study the rate of glutamine release: after 60 min of incubation, mitochondrial morphology is normal, no significant hypoxia is observed, the glycogenolytic rate is low and ATP, ADP and AMP are maintained at near *in vivo* levels.²⁵ After 30 min incubation at 37°C in incubation medium containing PBS, 1.5% BSA, 5 mmol/L glutamine and 10 $\mu\text{UI/mL}$ insulin, the rate of glutamine uptake from incubated soleus muscle was determined by enzymatic analysis of the change in glutamine concentration in the incubation medium over the incubation period.

Protein

Protein levels in muscle tissue homogenates were determined using the method described by Lowry *et al.*²⁶

Statistical analysis

All results are presented as the mean \pm SEM. Data were evaluated using Prisma V Program (GraphPad Software, San Diego, CA, USA). Statistical

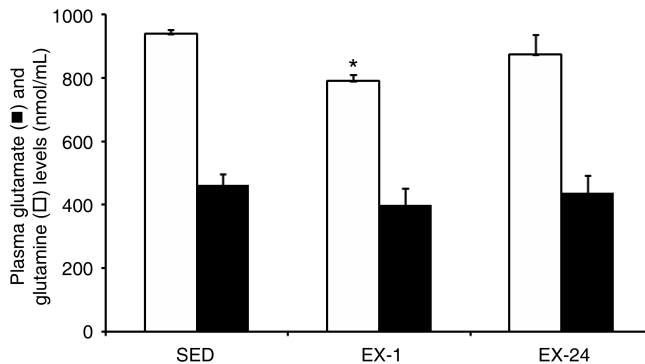


Fig. 1 Plasma glutamine and glutamate concentrations in sedentary rats (SED; $n = 12$) and in trained rats 1 and 24 h after the last exercise bout (EX-1 and EX-24, respectively; $n = 15$ in both groups). Values are the mean \pm SEM. * $P < 0.05$ compared with the SED group.

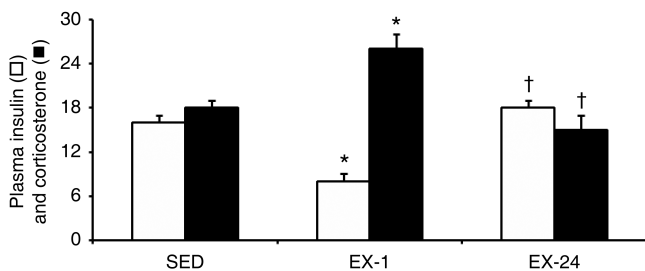


Fig. 2 Plasma insulin and corticosterone concentrations (in $\mu\text{IU}/\text{mL}$ and ng/mL , respectively) in sedentary rats (SED; $n = 12$) and in trained rats 1 and 24 h after the last exercise bout (EX-1 and EX-24, respectively; $n = 15$ in both groups). Values are the mean \pm SEM. * $P < 0.05$ compared with the SED group; † $P < 0.05$ compared with EX-1.

differences were determined by one-way ANOVA and Tukey post hoc test with significance set at $P < 0.05$.

RESULTS

Biochemical parameters in plasma

Figure 1 shows plasma glutamine concentrations. One hour after exercise, the glutamine concentration was lower in EX-1 rats than in sedentary rats (790 ± 21 vs 940 ± 13 nmol/mL, respectively; $P < 0.05$; $F = 3.27$; d.f. = 41), whereas 24 h after exercise, the plasma glutamine concentration did not differ significantly between trained and sedentary rats. As shown in Fig. 1, plasma glutamate concentrations did not differ between sedentary and trained animals at any time-point ($P > 0.5$; $F = 3.27$; d.f. = 41).

The corticosterone concentration was higher in EX-1 than in SED rats (26.16 ± 2.15 vs 18.45 ± 1.18 ng/mL, respectively; $P < 0.05$; $F = 10.30$; d.f. = 41); conversely, in EX-24 rats, corticosterone concentrations (15.56 ± 2.44 ng/mL) were similar to those in SED rats and lower than those in EX-1 rats ($P < 0.01$; $F = 10.30$; d.f. = 41; Fig. 2). There was a significant decrease in plasma insulin concentrations 1 h after the last exercise bout (EX-1) compared with sedentary rats (8.84 ± 1.12 vs 16.32 ± 1.33 $\mu\text{IU}/\text{mL}$, respectively; $P < 0.01$; $F = 29.20$; d.f. = 41). However, insulin concentrations in the EX-24 group (18.08 ± 1.81 $\mu\text{IU}/\text{mL}$) were increased compared with concentrations in EX-1 rats ($P < 0.001$; $F = 29.20$; d.f. = 41), but were similar to those in the SED group (Fig. 2).

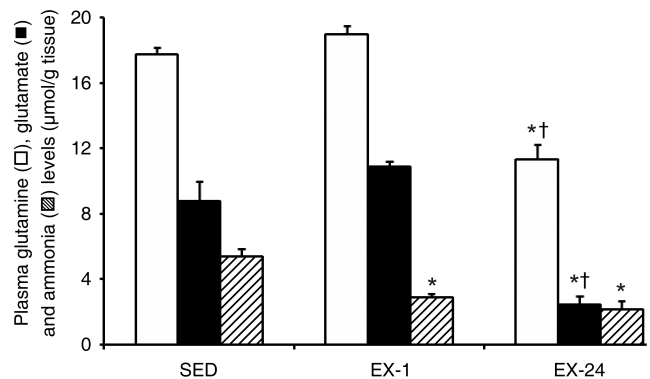


Fig. 3 Glutamine, glutamate and ammonia concentration in soleus muscle from sedentary rats (SED; $n = 12$) and in trained rats 1 and 24 h after the last exercise bout (EX-1 and EX-24, respectively; $n = 15$ in both groups). Values are the mean \pm SEM. * $P < 0.05$ compared with the SED group; † $P < 0.05$ compared with EX-1.

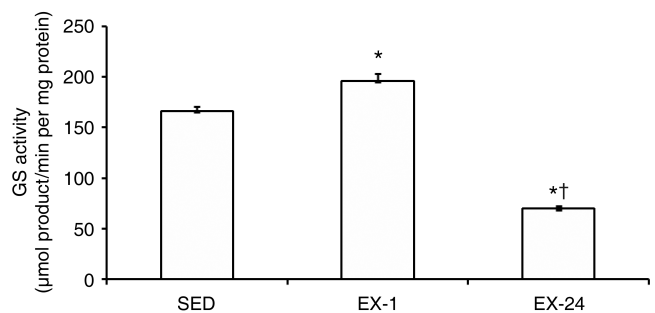


Fig. 4 Glutamine synthetase (GS) activity in soleus muscle from sedentary rats (SED; $n = 12$) and in trained rats 1 and 24 h after the last exercise bout (EX-1 and EX-24, respectively; $n = 15$ in both groups). Values are the mean \pm SEM. * $P < 0.05$ compared with the SED group; † $P < 0.05$ compared with EX-1.

Biochemical parameters in skeletal muscle

Figure 3 shows glutamine, glutamate and ammonia concentrations in soleus muscle. The glutamine concentration was comparable between the SED and EX-1 groups (17.74 ± 0.41 and 18.96 ± 0.51 $\mu\text{mol}/\text{mg}$ tissue, respectively), but was decreased in the EX-24 group (11.0 ± 0.9 $\mu\text{mol}/\text{mg}$ tissue; $P < 0.001$; $F = 39.37$; d.f. = 41 for both comparisons). Similar results were found for glutamate: concentrations in the SED and EX-1 groups were similar, whereas those in the EX-24 group were significantly lower (8.77 ± 1.21 , 10.88 ± 0.34 and 2.45 ± 0.54 $\mu\text{mol}/\text{mg}$ tissue, respectively; $P < 0.001$; $F = 39.94$; d.f. = 41 for both comparisons). The ammonia concentration in both the EX-1 and EX-24 groups was less than that in the SED group (2.87 ± 0.27 , 2.2 ± 0.5 and 5.40 ± 0.45 $\mu\text{mol}/\text{mg}$ tissue, respectively; $P < 0.001$, $F = 14.25$; d.f. = 41 for both comparisons). There were no significant differences in ammonia concentrations between the two trained groups.

Maximal GS maximal activity and glutamine uptake

Figure 4 shows maximal muscle GS activity, whereas Fig. 5 shows glutamine transport in the soleus muscle. The EX-1 group exhibited increased GS activity compared with the SED group (196 ± 7 and

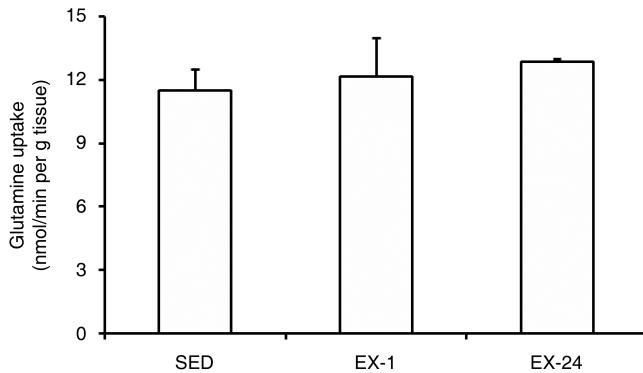


Fig. 5 Glutamine uptake in soleus muscle from from sedentary rats (SED; $n = 12$) and in trained rats 1 and 24 h after the last exercise bout (EX-1 and EX-24, respectively; $n = 15$ in both groups). Values are the mean \pm SEM.

$167 \pm 5 \mu\text{mol product/min per mg protein}$, respectively; $P < 0.001$; $F = 155.9$; d.f. = 41), whereas GS activity in the EX-24 group ($69.74 \pm 2.78 \mu\text{mol product/min per mg protein}$) was less than in both the EX-1 and SED groups ($P < 0.001$; $F = 155.9$; d.f. = 41 for both comparisons). Glutamine uptake was similar across all three groups ($P > 0.05$; $F = 0.53$; d.f. = 41; Fig. 5).

DISCUSSION

The present study examined the effect of exercise on GS activity and skeletal muscle glutamine metabolism in rats. Collectively, the results demonstrate that glutamine metabolism in skeletal muscle is regulated by different mechanisms 1 and 24 h after exercise. One hour after exercise, there was a decrease observed in plasma glutamine concentrations, despite an increase in GS activity and maintenance of glutamine uptake from skeletal muscle. Twenty-four hours after exercise, the plasma glutamine concentration was restored to levels found in sedentary animals. This change was associated with lower muscle glutamine concentrations caused by impaired glutamine synthesis.

Despite the importance of glutamine in different cells and tissues after exercise,^{5,11,27,28} the mechanisms that modulate plasma glutamine concentration after exercise are still poorly understood. In fact, the effects of physical exercise on GS activity and glutamine transport from skeletal muscle after exercise are still not fully known.

In the present study, there was a decrease observed in plasma glutamine concentrations 1 h after exercise (EX-1), confirming the results of previous studies in humans and rodents.^{6–10,29} However, the results indicate an increase in glutamine synthesis because increased GS activity was associated with a decrease in the concentration of ammonia, an important substrate for glutamine synthesis. Despite increased demand for glutamate, its concentration was maintained in the soleus. The increase in GS activity, together with decreased glutamine plasma concentrations, may indicate increased glutamine consumption in other tissues, such as the liver and kidney. This relationship has also been suggested by previous studies.^{5,11,27,28}

In contrast, 24 h after exercise, the restoration of plasma glutamine concentration to the SED baseline was associated with a significant decrease (35%) in muscle glutamine concentrations. In the EX-24 group, GS activity was lower than in either the SED or EX-1 groups, supporting the hypothesis of slow glutamine restoration in muscle.

Glutamine is synthesised from glutamate and ammonia in a process catalysed by the enzyme GS.^{12,15} Skeletal muscle is the main site of glutamine storage and production.¹ Although glutamine accounts for at least 50% of the total amino acid pool in skeletal muscle, the free glutamine in the muscle is not enough to account for the total amount of glutamine released during elevated glutamine demand.³⁰

For adequate glutamine synthesis, it is necessary to maintain physiological concentrations of glutamate, ammonia and ATP.¹² In addition, previous studies have shown that glucocorticoids have an important role in the modulation of glutamine synthesis in several tissues,^{13,30–32} including skeletal muscle.^{30,33–36} It is possible that during and after exercise, the interaction between biochemical and hormonal changes may modulate glutamine synthesis because, during exercise, an increase in hypothalamic–pituitary axis activity promotes a transient increase in plasma cortisol concentrations.^{37–40}

The results of the present study suggest that elevated plasma concentrations of corticosterone may be associated with an increase in GS activity, as demonstrated in the EX-1 group. However, as soon as the corticosterone concentration decreases, GS activity also declines, as observed in the EX-24 group. In fact, the results from the EX-1 rats showed increased GS activity associated with increased corticosterone (44%), maintenance of glutamate concentration and decreased ammonia concentration in muscle. These results suggest that, under conditions with an adequate physiological concentration of glutamate, the increase in corticosterone could induce increased GS activity and ammonia consumption for glutamine synthesis. However, after 24 h rest, the mechanism of modulation of GS activity is different because the higher corticosterone concentration in EX-1 animals was fully reversed in their EX-24 counterparts. It is possible that the increased GS activity observed after 1 h of recovery induced the depletion of both glutamate and ammonia, as well as the partial impairment of GS activity, observed 24 h after exercise. Beyond 24 h after exercise, the alanine concentration in muscle is increased (data not shown); this amino acid has been described as an important inhibitor of muscle GS in rat skeletal muscle.³⁵

Under stress conditions, glutamine homeostasis in skeletal muscle is dependent on three factors: (i) glutamine uptake from other tissues; (ii) glutamine production; and (iii) glutamine release from skeletal muscle protein breakdown.^{1,24,41} The process of glutamine release from skeletal muscle is the flux-generating step for glutamine metabolism in a number of tissues.^{42,43} Therefore, in the present study, we assayed glutamine uptake in incubated soleus muscles. This technique has a number of advantages; for example, the metabolism of skeletal muscle can be studied in isolation from other tissues, which allows the responses of different muscle types to be studied separately. This method of muscle preparation also allows for the determination of the absolute rate of glutamine uptake, in contrast with the net rate of transport determined from arteriovenous difference studies.²⁴ In addition, this technique provides a viable *in vitro* muscle preparation with which it is possible to investigate rates of glutamine transport at rest and during exercise.^{25,42}

In the skeletal muscle, glutamine transport occurs via bidirectional transport system (system N^m), which controls both the release and uptake of glutamine in this tissue and is regulated by Na⁺ and insulin.⁴⁴ There is evidence that glucocorticoids increase the rate of glutamine release from skeletal muscle in several species, including human.³⁵ However, the effect of exercise on the rate of glutamine release from muscle has not been defined.

Previous studies have shown that adjustments promoted by aerobic training induce changes in amino acid release from skeletal muscle during muscular contraction caused by electrical stimulation.⁴³ Despite the changes in corticosterone and insulin concentrations in exercised animals 1 h after exercise (EX-1), there was no difference in glutamine uptake from skeletal muscle compared with sedentary animals. This result is in contrast with that obtained in a previous study⁴³ and no explanation for this difference is apparent.

Insulin is a potent stimulant of glutamine uptake.⁴⁴ However, 1 h after exercise the insulin concentration is significantly decreased compared with levels in SED animals. Conversely, glucocorticoids stimulate glutamine release¹⁵ and may influence red and white muscle fibres differently, at least with respect to glutamine metabolism. This may be due to a greater number of glucocorticoid receptors in red muscle fibres, because the soleus is a muscle with predominantly red fibres. However, it is possible that the capacity of the glucocorticoid receptor for activation may be significantly decreased in skeletal muscle from the EX-1 group. A similar result has been shown in other cell types, including monocytes⁴⁵ and skeletal muscle from men during high-intensity resistance exercise.⁴⁶ In fact, corticosteroid sensitivity is a dynamic, rather than static, phenomenon; therefore, the increases in plasma corticosterone present 1 h after exercise may downregulate corticosterone receptors in skeletal muscle and so partially impair corticosterone actions on glutamine transport.

Twenty-four hours after exercise, glutamine uptake was similar to that seen in SED rats. Changes in glutamine release are modulated by several factors and glutamine release may be increased only when more glutamine is necessary based on a decrease in the glutamine concentration in the bloodstream.⁴²

In conclusion, the results of the present study demonstrate that the decrease in glutamine plasma concentration 1 h after exercise is not mediated by GS activity or by glutamine transport in skeletal muscle. Conversely, 24 h after exercise, the lower GS activity may contribute to the decrease in glutamine concentration observed in the muscle.

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