

Effect of arginine, ornithine and citrulline supplementation upon performance and metabolism of trained rats

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During intense exercise there is an augmented production of ammonia and IMP in the exercised muscle that could be related to the establishment of peripheral fatigue. In order to prevent this accumulation, the urea cycle in the liver eliminates ammonia in the form of urea and the skeletal muscle buffers the increase of ammonia via transamination reactions. In the present study we evaluated the effect of arginine, citrulline and ornithine supplementation, intermediates of the urea cycle, on the performance of sedentary and swimming-trained rats submitted to a single bout of exhaustive exercise. We also measured the glycogen content of the soleus and gastrocnemius muscles and of the liver, as well as the plasma concentrations of ammonia, urea, glutamine, glucose and lactate. The results indicate that arginine, citrulline and ornithine supplementation increased the flux of substrate through the reaction catalysed by glutamine synthetase, leading to increased glutamine production after an exhaustive bout of exercise, and of the mechanism involved in ammonia buffering. Copyright © 2002 John Wiley & Sons, Ltd.

KEY WORDS — ammonia; glutamine; skeletal muscle; lactate; amino acids; exhaustion

INTRODUCTION

During intense exercise multiple grades of disturbance in metabolic homeostasis are noticed, according to the training status of the subject.¹ In all of them, however, despite individual levels of training, the changes follow the same pattern, and therefore, vary only in intensity.¹ It is well established that during intense exercise, there is an augmented production of ammonia and IMP in the exercised muscle. The deamination of AMP to IMP and ammonia seems to facilitate the reaction catalysed by the enzyme adenylate kinase.² The production of IMP and ammonia can be substantial,³ increasing with muscle incapacity to produce ATP via oxidative phosphorylation, and hence being linked to the establishment of muscle fatigue.^{4,5}

The accumulation of ammonia is very toxic for the organism, since it interferes in the activity of some

flux-generating enzymes, and alters cell permeability to ions, as well as the ratio of NAD⁺/NADH.^{6,7} To avoid this accumulation, the urea cycle in the liver is responsible for ammonia elimination in the form of urea.⁸ Another important site for ammonia detoxification is skeletal muscle, which presents a high capacity to buffer ammonia by generating alanine from pyruvate and glutamine from glutamate and α -oxoglutarate in transamination reactions.⁹

In the present study we investigated the effect of the supplementation by arginine, citrulline and ornithine, intermediates of the urea cycle, on the performance of sedentary and swimming-trained rats submitted to a single bout of exhaustive exercise. We also measured the glycogen content of the soleus and gastrocnemius muscles, of liver and the plasma concentration of ammonia, urea, glutamine, glucose and lactate.

MATERIALS AND METHODS

Animals

Male albino Wistar rats weighing 150 g were obtained from the Institute of Biomedical Sciences, University

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of São Paulo. The rats were kept at $23 \pm 1^\circ\text{C}$ in a light/dark cycle of 12/12 h; lights on at 07.00 hours.

Chemicals and enzymes

All chemicals and enzymes were obtained from Boehringer Mannheim, GmbH, Lewes, East Sussex, UK. Laboratorios Baldacci S/A kindly provided the amino acids mixture.

Training procedure

The rats were submitted to a pre-training period of 3 weeks, in which they were put to swimming for 1 h, 5 days per week, in individual tanks with flowing water at 30°C . The rats exercised with an extra load of 5% body weight attached to their tails. After this period, the rats were submitted to a 1-week period of a variable intensity training schedule, as shown:

Day	1	2	3	4	5	6	7
Intensity (% body weight extra load)	5%	6%	7%	8%	6%	rest	experiment

During this week the rats received, daily, a single dose of the mixture containing: 0.4 g kg^{-1} body weight arginine, 0.2 g kg^{-1} body weight ornithine and 0.026 g kg^{-1} body weight citrulline. The amino acids were given by gavage in a single dose 30 min after the exercise session, and at the same time on the rest day (sixth day). On the day of the experiment the rats received one dose of the amino acid mixture 30 min prior to the exercise session. Sedentary rats received the same amount of the amino acid solution, at the same time of the day, following the same protocol of administration.

After swimming until exhaustion with an extra load of 9% body weight attached to the tail, the rats were killed by decapitation and plasma, muscles and liver were appropriately collected.

Time of exhaustion

The rats were considered to have reached exhaustion when they remained submerged for a period longer than 12 s.¹⁰

Metabolite measurements

The glycogen content of the soleus and gastrocnemius muscles and of liver were measured.¹¹ Neutralized

samples of the plasma were analysed to measure ammonia,¹² urea (by the reaction of Berthelot,¹³ using a Mercktest), glutamine,¹⁴ glucose¹⁵ and lactate.¹⁶

Glutamine synthetase assay

Mitochondria were isolated,¹⁷ in a buffer containing 250 mM sucrose, 1 mM EGTA, 1 mM dithiothreitol, 5 mM ATP, 10 mM sodium glutamate, 0.1 mM phenylmethanesulfonyl fluoride (added from a 10 mM stock in 100% ethanol) and 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), pH 7.4. The enzyme was assayed¹⁸ with an assay mixture which contained 100 mM HEPES, pH 7.4, 100 mM NaCl, 5% ethylene glycol, 10 mM NH_4Cl , 10 mM ATP, 25 mM MgCl_2 and 50 mM glutamate in a final volume of 1.0 ml, containing 100 μl of mitochondrial preparation. After 30 min the reaction was terminated by the addition of 50 μl 2 N HCl, followed by neutralization

with 50 μl 2 N Tris base. The ADP content of this mixture was determined by incubating 500 μl with 500 μl of a solution of 150 mM Tris/HCl, pH 7.6, 50 mM KCl, 20 mM MgSO_4 , 2.7 mM phosphoenolpyruvate, 0.6 mM NADH and 10 units ml^{-1} each of pyruvate kinase and lactate dehydrogenase. The samples were read at 340 nm in a Gilford Response spectrophotometer, after 20 min incubation at room temperature.

For evaluating the importance of glutamine synthetase in our model, five rats in each group received, intravenously, a single dose of methionine sulfoximine (MSO) 14 mM,¹⁹ a potent inhibitor of the enzyme.²⁰

Muscle incubation

Immediately after the sacrifice, a small portion of soleus muscle, weighing 35–45 mg, was obtained by separating the cells without damage, with two small needles. The muscle strip was attached through the tendons, to a small clip to keep the fibres extended. The cells were incubated in a solution of phosphate buffered saline, 10% defatted bovine serum albumin, 20 U ml^{-1} insulin, 5 mM glucose (final volume of 5 ml), under an atmosphere of 5% CO_2 in air, at 37°C , in a Dubnoff water bath. After 30 min of pre-incubation, muscles slices were incubated for 1 h with

different concentrations of NH_4Cl (0.1, 0.2, 0.5 and 1.0 mM).

The same protocol was used to determine the effect of arginine, citrulline and ornithine upon the flux of $[\text{U-}^{14}\text{C}]$ -glucose through the Krebs cycle in muscle slices and the rate of $^{14}\text{CO}_2$ production from $[\text{1-}^{14}\text{C}]$ -pyruvate. In this case, the incubation was carried out in a stoppered Erlenmeyer flask and the amount of $^{14}\text{CO}_2$ produced was trapped in a solution of phenyl-ethylamine:methanol (1:1). Radioactivity was measured with a Packard Tricarb counter with Ecolume Liquid Scintillation Cocktail (ICN, Costa Mesa, CA, USA).

Statistical analysis

The results are presented as mean \pm SEM. Differences were detected by using Student's *t*-test or the two-way analysis of variance using Scheffe's method. The significance level was set for $p < 0.05$.

RESULTS

Administration of the amino acid mixture provoked an increase (50%) in the time to reach exhaustion of sedentary rats and of 72.5% for trained animals. The training programme induced, *per se*, an increase of 40% in the time to exhaustion (Table 1).

The exercise session induced a decrease in the plasma level of glucose in sedentary rats (9.8%), paralleled by an increase of lactate production (99.1%). The amino acids supplementation provoked only a slight increase in glucose consumption and a small decrease in lactate production, that was not statistically significant. Such changes, were nevertheless much more evident for the trained group, where the rats showed a decrease of 19.0% in the plasma level

Table 1. The effect of arginine, ornithine and citrulline supplementation on the time to reach exhaustion of sedentary and exercise-trained rats submitted to a single exhaustive exercise session

	Time to exhaustion (min)
SC	12.05 \pm 1.08
SS	19.04 \pm 1.70*
TC	17.04 \pm 2.53 [†]
TS	29.41 \pm 3.07* [†]

The results are expressed as mean \pm SEM of 15 animals.

* $p < 0.05$ for the comparison between control and supplemented rats.

[†] $p < 0.05$ for the comparison between exercise-trained and sedentary rats.

SC, sedentary control rats; SS, sedentary rats supplemented with the amino acids mixture; TC, exercise-trained control rats; TS, exercise-trained rats supplemented with the amino acids mixture.

Table 2. The effect of arginine, ornithine and citrulline supplementation on plasma levels of glucose (mg dl⁻¹) and lactate (mM) of sedentary and exercise-trained rats at rest and after a single exercise session until exhaustion

Groups	Glucose (mg dl ⁻¹)	Lactate (mM)
SC	98.7 \pm 1.3	2.43 \pm 0.12
SS	104.1 \pm 3.2	2.01 \pm 0.18
SC after exhaustion	89.1 \pm 3.5 [‡]	4.84 \pm 0.31 [‡]
SS after exhaustion	83.4 \pm 2.1 [‡]	4.81 \pm 0.42 [‡]
TC	102.4 \pm 7.2	2.07 \pm 0.17
TS	107.3 \pm 3.1	1.81 \pm 0.15*
TC after exhaustion	82.9 \pm 2.5 [‡]	4.72 \pm 0.25* [‡]
TS after exhaustion	68.1 \pm 3.1* [†]	3.91 \pm 0.42* [†]

The values are presented as mean \pm SEM of 15 samples.

* $p < 0.05$ for the comparison between control and supplemented rats.

[†] $p < 0.05$ for the comparison between exercise-trained rats and sedentary animals.

[‡] $p < 0.05$ for the comparison between rats submitted to a single session of exhaustive exercise before sacrifice and those sacrificed at rest.

SC, sedentary control rats; SS, sedentary rats supplemented with the amino acids mixture; TC, exercise-trained control rats; TS, exercise-trained rats supplemented with the amino acids mixture.

of glucose accompanied by an increase of 2.2-fold in lactate production (Table 2). The amino acids supplementation in the trained group submitted to the exhaustion regimen induced an even greater consumption of glucose (17.8%), paired with a diminished lactate production (17.1%), when compared with the results observed for the trained rats submitted to the same conditions without the amino acid supplementation (Table 2).

The glycogen content of the soleus and gastrocnemius muscles, as well that of the liver decreased by 29, 15 and 6% respectively, in response to the single exercise bout (Table 3). As expected, the trained rats showed an increased muscle glycogen content at rest, 19% for soleus and 61% for gastrocnemius muscles, respectively, which were not altered by the amino acid supplementation (Table 3). Trained rats also presented increased glycogen consumption after exercise, 40, 33 and 10% for the soleus and gastrocnemius muscle and the liver, respectively (Table 3). In this group, however, the amino acids supplementation caused an even greater glycogen consumption in the gastrocnemius muscle (22%) and liver (13%), compared with that observed for non-supplemented rats after exercise (Table 3).

The amino acid supplementation induced, in sedentary rats, a decrease in the plasma concentration of ammonia (37.7%) and urea (28.5%) and an increase in that of glutamine (40%). Amino acid supplementation induced, in trained animals, a decrease of 39.2%

Table 3. The effect of arginine, ornithine and citrulline supplementation on the glycogen content of the gastrocnemius and soleus muscles and on the liver of sedentary and exercise-trained rats at rest and after a single exhaustive exercise session. The results are expressed as mg (100 g)⁻¹ of fresh tissue and represent the mean \pm SEM of 15 experiments

Groups	Soleus	Gastrocnemius	Liver
SC	0.338 \pm 0.01	0.8300 \pm 0.02	1.781 \pm 0.12
SS	0.357 \pm 0.02	0.8413 \pm 0.04	1.820 \pm 0.14
SC after exhaustion	0.240 \pm 0.01 [‡]	0.704 \pm 0.03 [‡]	1.683 \pm 0.10 [‡]
SS after exhaustion	0.231 \pm 0.02 [‡]	0.718 \pm 0.02 [‡]	1.701 \pm 0.09 [‡]
TC	0.402 \pm 0.03 [†]	1.337 \pm 0.03 [†]	1.774 \pm 0.12
TS	0.458 \pm 0.04 [†]	1.384 \pm 0.04 [†]	1.804 \pm 0.11
TC after exhaustion	0.242 \pm 0.02 [‡]	0.887 \pm 0.04 [‡]	1.590 \pm 0.15 [‡]
TS after exhaustion	0.231 \pm 0.02 [‡]	0.540 \pm 0.03 ^{*‡}	1.384 \pm 0.10 ^{*‡}

* $p < 0.05$ for the comparison between control and supplemented rats.

[†] $p < 0.05$ for the comparison between exercise-trained rats and sedentary animals.

[‡] $p < 0.05$ for the comparison between rats submitted to a single session of exhaustive exercise before sacrifice and those sacrificed at rest. SC, sedentary control rats; SS, sedentary rats supplemented with the amino acids mixture; TC, exercise-trained control rats; TS, exercise-trained rats supplemented with the amino acids mixture.

for ammonia and 27.2% for urea levels and an increase of 24.8% in plasma glutamine concentration (Table 4). This effect also appeared in the trained rats submitted to the exhaustive exercise. These rats showed a decrease of 54 and 32% in ammonia and urea levels respectively, and an increase of 26.8% in plasma glutamine concentration (Table 4).

Soleus muscle slices incubated in the presence of different concentrations of NH₄Cl consumed more glucose (47%, 2.1-, 2.6- and 2.7-fold, for 0.1, 0.2, 0.5 and 1.0 mM of NH₄Cl, respectively; Table 5). The production of lactate by muscle slices incubated in the presence of NH₄Cl (0.2 and 0.5 mM) was the same as that presented by cells incubated in medium without ammonia. The presence of ammonium ions increased, however, the production of ¹⁴CO₂ from radiolabelled glucose by incubated soleus muscle by 2.5-, 3.2-, 4.5- and 4.6-fold for 0.1, 0.2, 0.5 and 1.0 mM of NH₄Cl added to the incubation medium,

respectively (Table 5). The addition of NH₄Cl to muscle slice incubations obtained from control rats did not change glucose consumption and lactate production (data not shown).

Muscle slices obtained from sedentary and trained rats supplemented with the amino acids, presented an increased production of [¹⁴CO₂] from labelled glucose and pyruvate (2.8- and 3.1-fold, respectively, for glucose and pyruvate), indicating an augmented flux of substrates through the Krebs cycle and an increase in pyruvate dehydrogenase activity (Table 6).

The inhibition of glutamine synthetase (44.4%) by methionine sulfoximine, shown in Table 7, led to a reduction (39%) in the total time to reach exhaustion for the trained supplemented rats. In fact, the arginine, ornithine and citrulline supplementation increased glutamine synthetase activity from 97.4 \pm 4.6 nmol min⁻¹ mg⁻¹ of protein to 198.7 \pm 11.3 nmol min⁻¹ mg⁻¹ of protein in the soleus muscle of trained rats

Table 4. The effect of arginine, ornithine and citrulline supplementation on the plasma concentration of ammonia, urea and glutamine of sedentary and exercise-trained rats at rest and after a single exhaustive exercise session. The results are expressed as mean \pm SEM of 15 experiments

Groups	Ammonia (μ g ml ⁻¹)	Urea (mg dl ⁻¹)	Glutamine (μ M)
SC	6.1 \pm 0.31	39.2 \pm 0.94	918.4 \pm 34.5
SS	3.8 \pm 0.17*	28.0 \pm 1.20*	1287.6 \pm 42.8*
SC after exhaustion	12.2 \pm 0.81 [‡]	48.5 \pm 2.03 [‡]	1402.3 \pm 54.2 [‡]
SS after exhaustion	8.5 \pm 0.42 ^{*‡}	45.4 \pm 1.84 [‡]	1787.0 \pm 81.9 ^{*‡}
TC	5.6 \pm 0.28	35.2 \pm 0.81	1204.2 \pm 68.2 [†]
TS	3.4 \pm 0.17*	25.6 \pm 0.94*	1503.8 \pm 74.3 ^{*‡}
TC after exhaustion	13.7 \pm 0.28 [‡]	47.5 \pm 1.38 [‡]	1422.5 \pm 44.8 [‡]
TS after exhaustion	6.3 \pm 0.14 ^{*‡}	32.21 \pm 1.40 ^{*‡}	1804.2 \pm 92.1 ^{*‡}

* $p < 0.05$ for the comparison between control and supplemented rats.

[†] $p < 0.05$ for the comparison between exercise-trained rats and sedentary animals.

[‡] $p < 0.05$ for the comparison between rats submitted to a single session of exhaustive exercise before sacrifice and those sacrificed at rest. SC, sedentary control rats; SS, sedentary rats supplemented with the amino acids mixture; TC, exercise-trained control rats; TS, exercise-trained rats supplemented with the amino acids mixture.

Table 5. The effect of different ammonia concentrations (0.1, 0.2, 0.5 and 1.0 mM) on glucose consumption and [U-¹⁴C]-glucose decarboxylation by incubated soleus muscle. The results are expressed as mean ± SEM of 10 incubations

	Glucose (μM)	Lactate (μM)	[U- ¹⁴ C]-glucose (μM)
No addition	137.2 ± 10.1	60.31 ± 5.8	61.4 ± 5.2
NH ₄ Cl (0.1 mM)	203 ± 25.0*	34.51 ± 2.6*	153.2 ± 11.8*
NH ₄ Cl (0.2 mM)	287.5 ± 22.8*	60.37 ± 5.7	185.5 ± 16.8*
NH ₄ Cl (0.5 mM)	357.6 ± 26.8*	67.9 ± 6.1	240.7 ± 22.1*
NH ₄ Cl (1.0 mM)	376.1 ± 31.2*	79.4 ± 5.8	251.5 ± 24.8*

**p* < 0.05 for comparison with the muscle slices incubated in the absence of NH₄Cl.

Table 6. The effect of arginine, ornithine and citrulline supplementation on [U-¹⁴C]-glucose and [1-¹⁴C]-pyruvate decarboxylation by incubated soleus muscle. The results are expressed as mean ± SEM of 10 incubations

	[U- ¹⁴ C]-glucose [μM]	[1- ¹⁴ C]-pyruvate (μM)
SC	56.73 ± 4.46	45.97 ± 5.02
SS	158.65 ± 15.87*	143.22 ± 16.87*

**p* < 0.05 for the comparison between control and supplemented rats.

SC, sedentary control rats; SS, sedentary rats supplemented with the amino acids mixture.

and to 127.6 ± 7.5 nmol min⁻¹ mg⁻¹ of protein in the muscle of sedentary rats.

DISCUSSION

We have investigated the effect of arginine, ornithine and citrulline supplementation upon the time to reach exhaustion of sedentary and swimming-trained rats submitted to a single bout of high intensity exercise. The amino acid supplementation provoked an increase in the time to exhaustion of both groups of rats, a greater effect being observed in the trained animals. The effect of the training protocol is confirmed by the increased time to reach exhaustion observed for the trained rats that were not supplemented. The training protocol also increased citrate synthase activity²¹ in the red portion of the gastrocnemius muscle from 44.7 ± 3.4 μmol min⁻¹ g⁻¹¹⁵ muscle for sedentary rats to 98.7 ± 2.7 μmol min⁻¹ g⁻¹¹⁵ muscle for trained rats.

Table 7. The effect of 14 mM methionine sulfoximine (MSO) on glutamine synthetase activity in the soleus muscle of trained supplemented rats and on performance in an exhaustive bout of exercise. The results are expressed as mean ± SEM of 10 animals

	GS (nmol min ⁻¹ mg ⁻¹ protein)	Time to exhaustion (min)
Control	198.7 ± 11.3	31.4 ± 3.2
MSO	110.4 ± 8.7*	18.9 ± 1.6*

**p* < 0.05 for comparison with control rats.

As is well established, fatigue can be evaluated by the increase in plasma lactate and ammonia levels.^{22–25} In high intensity exercise, the increase in ADP concentration activates the enzyme AMP deaminase, a key enzyme in the purine nucleotide cycle, leading to an increased production of NH₄⁺.²⁴ The most important mechanism for ammonia detoxification is present in the liver, in the form of the urea cycle. Skeletal muscle, however, is also able to detoxify ammonia through the metabolism of pyruvate to alanine and by glutamate and α-oxoglutarate to glutamine (review in reference 7). In the present study, the rats were fed an amino acid supplement consisting of arginine, citrulline and ornithine, i.e. components of the urea cycle. Besides the performance improvement observed, there was, surprisingly, a reduction in the plasma concentration of urea. The increased performance of the supplemented group was accompanied also, by a reduction in plasma lactate and ammonia levels after the exercise bout.

One point of interest is that the training protocol, *per se*, provoked only a small decrease in the plasma ammonia concentration, not statistically significant, but in accordance with that observed by Constable and colleagues¹ and by Dudley and Terjung.²⁶ The lack of significance could, however, be linked to the short period of training and to the different protocols developed by the other groups.

During exercise there is a great release of ammonia, glutamine and alanine by the active muscle,^{27,28} which was observed for ammonia and glutamine in our model. The increased release of glutamine is probably part of the mechanism of ammonia detoxification in skeletal muscle, as previously reported.²⁹

Despite the fact that the amino acids mixture given to the rats was rich in those amino acids that take part in the urea cycle in the liver, we did not observe any increase in plasma urea concentration. Surprisingly, the data showed an increased performance associated with high levels of glutamine and a reduction of ammonia and urea levels. These data suggest a slightly different mechanism for ammonia detoxification,

compared with that proposed by other researchers. As the increased plasma level of glutamine is associated with a decreased content of muscle glycogen and a reduced production of lactate, it seems that the supplementation provoked a diversion of pyruvate to the Krebs cycle, as previously observed for skeletal muscles incubated in the presence of different levels of ammonia. In fact, incubated muscle slices obtained from supplemented rats showed an increased production of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ -pyruvate, indicating an increased activity of pyruvate dehydrogenase, without changes, however, in glucose consumption. The increased activity of the Krebs cycle could lead to an augmented production of α -oxoglutarate which could be diverted to glutamine, receiving two molecules of ammonia. If so, this mechanism will occur in parallel with that proposed by Perriello and colleagues,³⁰ who showed a glucose–glutamine cycle in the skeletal muscle of humans in the post-absorption period.

The glycogen concentration in soleus muscle from supplemented animals could be a strong indication that the amino acid supplementation increased the Krebs cycle activity and provoked a high production of citrate which could inhibit glycolysis. Consequently, we observed a sparing effect on glycogen content in the muscle, probably related to the increased time to exhaustion observed in this group. We also observed that the inhibition of glutamine synthetase by MSO abolished the ergogenic effect of the supplementation, as well as the decreased plasma glutamine concentration after exercise, but increased that of ammonia. These findings reinforce the idea that, under a regimen of supplementation, glutamine synthesis seems to be a key step in ammonia detoxification. In fact, muscle slices from supplemented rats incubated in the presence of different ammonia concentrations produced less lactate and consumed more glucose than muscle slices incubated without the ion in the medium.

Taken together, our results indicate the existence of another mechanism to explain the increased performance observed in rats supplemented with arginine, citrulline and ornithine involving an increased flux of glucose through the Krebs cycle as well as a diversion of α -oxoglutarate for glutamine synthesis. This pathway should be important as it would cause a decrease in lactate release by muscle and improve the ability of the skeletal muscle to detoxify ammonia. The possible effects of the augmented content of glutamine in the plasma of the supplemented rats are under investigation in our laboratory.

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