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ORIGINAL ARTICLE

L-Citrulline-malate influence over branched chain amino acid utilization during exercise

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Abstract Exhaustive exercise induces disturbances in metabolic homeostasis which can result in amino acid catabolism and limited L-arginine availability. Oral L-citrulline supplementation raises plasma L-arginine concentration and augments NO-dependent signalling. Our aim was to evaluate the effects of diet supplementation with L-citrulline-malate prior to intense exercise on the metabolic handle of plasma amino acids and on the products of metabolism of arginine as creatinine, urea and nitrite and the possible effects on the hormonal levels. Seventeen voluntary male pre-professional cyclists were randomly assigned to one of two groups: control or supplemented (6 g L-citrulline-malate 2 h prior exercise) and participated in a 137-km cycling stage. Blood samples were taken in basal conditions, 15 min after the race and 3 h post race (recovery). Most essential amino acids significantly decreased their plasma concentration as a result of exercise; however, most non-essential amino acids tended to significantly increase their concentration. Citrulline-malate ingestion significantly increased the plasma concentration of citrulline, arginine, ornithine, urea, creatinine and nitrite ($p < 0.05$) and significantly decreased the isoleucine concentration from basal measures to after exercise ($p < 0.05$). Insulin levels significantly increased after exercise in both groups ($p < 0.05$) returning to basal values at recovery. Growth hormone increased after exercise in both groups, although the increase was higher in the citrulline-malate supplemented group ($p < 0.05$). L-citrulline-malate supplementation can enhance the use of amino acids, especially the branched chain amino acids during exercise and also enhance the production of arginine-derived metabolites such as nitrite, creatinine, ornithine and urea.

Keywords Oxidative stress · Insulin · Growth hormone · Nitric oxide · Arginine

Introduction

Arginine displays remarkable metabolic and regulatory versatility as an essential precursor for the synthesis of proteins and other molecules with enormous biological importance (including nitric oxide, urea, ornithine, polyamines, glutamate and creatine) (Wu et al. 2009). Citrulline is a very effective precursor of arginine and consequently it may hold great promise as a nutritional pharmacotherapeutic treatment for a wide array of human diseases (Flynn et al. 2002). It has been clearly demonstrated that L-arginine administration improves endothelial function in various disease states (Creager et al. 1992). In addition, L-arginine infusion at rest induces hormonal changes with increases in plasma insulin, growth hormone, glucagon, catecholamines and prolactin (McConell 2007). However, there has been little examination of the effect of enhanced

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L-arginine availability during exercise or the effects of exercise on the availability of arginine and citrulline (Aguiló et al. 2000). It is important to study whether L-arginine infusion directly or via its precursor citrulline, probably increasing nitric oxide (NO), alters skeletal-muscle metabolism during exercise. Arginine coming from food is mostly withdrawn from the portal blood by the liver; however, the liver is unable to uptake citrulline from portal circulation, which makes it available for the whole body. There is a need for further research, especially to understand the mechanisms of how L-citrulline affects exercise metabolism and also to determine whether the hormonal responses that occur in response to L-arginine at rest are also present to some extent in response to the L-arginine precursor citrulline during exercise.

Nitric oxide (NO) is synthesized from L-arginine by NO synthases in virtually all cell types (Jobgen et al. 2006). NO is a gaseous molecule, highly reactive free radical with multiple and complex roles within many biological systems. Emerging evidence reported that NO at physiological levels regulates the metabolism of glucose, fatty acids and amino acids in mammals (Liaudet et al. 2000). However, at high levels NO acts as an oxidant, inhibiting most enzyme-catalysed reactions through protein oxidation. An inhibition of NO synthesis is evidenced to cause hyperlipidemia and fat accretion in rats (Jobgen et al. 2006).

Plasma arginine levels are a limiting factor for NO synthesis in endothelial cells (Nussler et al. 1994). Upon stimulation, over 80% of L-citrulline is recycled to arginine in endothelial cells to produce NO (Solomonson et al. 2003). L-citrulline supplementation increases plasma L-arginine concentration to a higher level than that achieved by oral L-arginine supplementation (Hickner et al. 2006). Hence, diet supplementation with L-citrulline could avoid the limitations of L-arginine availability for lymphocytes during exhaustive exercise. In fact, the dietary supplementation with watermelon juice (rich in citrulline) enhances arginine availability and ameliorates the metabolic syndrome in Zucker diabetic fatty rats (Wu et al. 2007).

The aim of this study was to describe the influence of acute administration of L-citrulline on its metabolism during exercise, following the changes in the plasma levels of arginine and in some final metabolites produced, i.e. nitrite, ornithine, creatinine and urea. The purpose of the present study also was to describe the effects citrulline supplementation on the changes induced by intense exercise on plasma amino acids and the possible effects on the hormonal levels. This study may have important therapeutic implications for citrulline as there are indications that L-arginine augments the effects of exercise training on insulin sensitivity and capillary growth in muscles (McConell 2007).

Materials and methods

Subjects and study design

This work was in accordance with the Declaration of Helsinki, and the protocol for this study was approved by the Bioethical Committee of the University of the Balearic Islands (Palma de Mallorca, Balearic Islands, Spain). Seventeen voluntary male pre-professional cyclists participated in this study which took place in the Mallorca Cinturó Ciclista 2005 (Balearic Islands). The study participants were Semi-professional cyclists (Elite category) according to the International Cycling Union (ICU) and with an average of a 5-year experience in cycling competitions. Subjects were informed of the purpose of this study and the possible risks involved before receiving their written consent to participate. Before starting the study, cyclists were submitted to clinical exams and blood tests according to the ICU protocols, and they showed to be in good health. The sportsmen's mean ± SEM age was 22.3 ± 0.9 years, weight 70.6 ± 1.3 kg determined with a precision scale, 10 g, Model Cobos 50 K and VO_2max 81.9 ± 2.6 ml/kg min. The participants were randomly assigned in a double-blind fashion to one of two treatment groups: supplemented group ($n = 8$) and non-supplemented (control) group ($n = 9$). 2 h before the beginning of the stage subjects ingested 6 g of citrulline-malate dissolved in lemon juice in order to mask the supplement taste. The control group consumed the lemon juice vehicle alone. There is no recommendation for the citrulline intake but, it has been estimated that a 70-kg human subject should be able to tolerate long-term parenteral and enteral supplemental doses of 6 and 15 g/day arginine, respectively, in addition to a daily dose of arginine from a regular diet (4–6 g/day) (Wu et al. 2007). Then, the dose of 6 g of citrulline could be equivalent to the daily intake of arginine in only once. The cycling stage was 137.1 km long with only one significant mountain difficulty (considered second category). Identical amounts of selected foods and beverages were provided to the subjects along the race to control their intake before, during and after the race. All 17 participants finished the stage at exactly the same time of 2:59:31 [h:min:s]. During the intervention time cyclists followed a controlled diet. Subjects were instructed to ingest a standardized food and fluid plan based on individual body mass during the training and preceding each race or laboratory test. The diet was constantly supervised by the medical group of the team.

Three weeks before the beginning of the investigation, each cyclist reported to the laboratory and participated in an incremental maximal cycling test. A mechanically braked cycle ergometer (Monark 818 E, Varberg, Sweden) adapted with a racing saddle, drop handlebars and clip-in

pedals was used. The test started with an initial resistance of 110 W, with further increments of 35 W every 3 min. Subjects kept a constant 75 rpm pedal cadence with the help of a metronome. Testing concluded when the required pedal cadence was no longer maintained by the cyclist. Heart rate was recorded at 5-s intervals during the entire test (Polar S720i, Polar Electro Oy, Finland). Gas-exchange data were continuously monitored with a breath-by-breath metabolic cart (CPX-Plus, Medical Graphics Corporation, St. Paul, Minnesota, USA).

Experimental procedure

EDTA-treated venous blood samples were collected from each subject by antecubital venipuncture with Vacutainer system 3 h before the cycling stage after overnight fasting (basal), 15-min after finishing the race and after 3 h of recovery. Blood samples were used to obtain plasma. Blood cells and haematological parameters—haemoglobin concentration, haematocrit, corpuscular mean volume (VCM), mean corpuscular haemoglobin concentration (CHCM) and the size distribution of erythrocytes (RDW)—were quantified in an automatic flow cytometer analyser Technicon H*2 (Bayer) VCS system. Amino acid and nitrite concentrations were determined in plasma. We also determined the total protein, bilirubin, glucose, creatinine, urea, uric acid and total protein levels by standard procedures used in clinical biochemistry laboratories using an autoanalyser Technicon DAX® System. We also determined insulin and growth hormone (GH) following an adaptation of previously described method based on indirect quimioluminescence immunoassays (Immulite 2000, Siemens M.S. Diagnostics) (Wood 1984). All chemicals and reagents were obtained from Sigma® (Madrid, Spain) unless otherwise specified.

Amino acid determination

Amino acid concentrations were determined in plasma by HPLC method with fluorescence detection. A solution of 20% 5-sulfosalicylic acid in ethanol was introduced into eppendorf tubes and was evaporated at 37°C. In order to deproteinise plasma samples, 500 µl of plasma was added to the tubes containing the sulfosalicylic acid and centrifuged for 20 min at 10,000×*g* at 4°C. The protein-free supernatant fraction was used for individual amino acid measurements. Samples were reacted with fluoraldehyde (*o*-phthaldehyde, OPA) reagent and injected into a HPLC system attached to a fluorescence reader and an integrator. The HPLC was a Waters Inc with a fluorescent detector operating at an excitation wavelength of 330 nm and an emission wavelength of 380 nm. The column was a Waters Sunfire, C18 3.5 µm, 4.6 × 150 mm. The separation was carried out by using an isocratic gradient with a mobile phase A consisting of phosphate buffer 9 mM, acetonitrile, methanol and tetrahydrofurane (96:2:2:0.2) and a mobile phase B with the same constituents (54:30:16:0.4). L-Norvaline was used as the internal standard and to calibrate the system a standard of neutral, acid and basic amino acids was used diluted in phosphate buffer to a final concentration of 157 µM. Sample amino acid levels were calculated from the peak area, taking into account the internal standard L-norvaline and the individual response of each standard amino acid versus the internal standard. The assessed amino acids were Trp, Phe, Tyr, Hys, Lys, Glu, Gln, Gly, Met, Val, Leu, Ile, Asp, Asn, aminobutirate, Ser, Thr, Ala, Tau, Arg, Orn and citrulline.

Plasma amino acid loss was calculated from the difference between plasma amino acid concentration before and after exercise. This difference was expressed as a percentage when divided by the concentration before exercise.

Nitrite determination

Nitrite levels, as metabolic product of NO, were determined in plasma and lymphocytes by the acidic Griess reaction using a spectrophotometric method. Lysed cells and plasma were deproteinised with acetone and kept overnight at −20°C. Samples were centrifuged for 10 min at 15,000×*g* at 4°C and supernatants were recovered. A 96-well plate was loaded with the samples or nitrite standard solutions (100 µl) in duplicate. 50 µl sulfanilamide (2% w/v) in 5% HCl was added to each well, and 50 µl *N*-(1-napthyl)-ethylenediamine (0.1% w/v) in water was then added. The absorbance at 540 nm was measured following an incubation of 30 min. Standard curve was performed to determine nitrite concentration, which was normalized to cell number in lymphocyte determinations.

Statistical analysis

Statistical analyses were performed on SPSS version 15.0. Data are expressed as the means ± standard error. The degree of significance of differences between means was calculated using two-way ANOVA: The statistical factors analysed were citrulline-malate supplementation (*S*), time (*T*) and interaction of supplementation and time (*S***T*). The sets of data in which there was significant *S***T* interaction were tested by ANOVA one-way test. When significant effects of *S* or *T* factor were found, a Student's *t*-test for unpaired data was used to determine the differences between the groups involved; it was also used to find significant differences between supplemented and control groups. One-way ANOVA was also used to determine differences between the groups involved in the analysis of anthropometric characteristics and to determine differences

in percentage of amino acid loss. A probability level of significance of $p < 0.05$ was accepted.

Results

The assignment to the experimental or placebo group was randomly made at the beginning of the cycling stage. No evidence of any adverse effects as a result of citrulline-malate supplementation was observed.

Participant characteristics

The initial characteristics of the subjects participating in the present study presented no significant differences between control and supplemented groups. No differences in age (22.6 ± 1.0 years in control vs. 22.5 ± 1.8 years in supplemented), body weight (69.5 ± 1.0 kg in control vs. 71.3 ± 3.1 kg in supplemented), or VO_2max (82.6 ± 4.4 ml/kg min in control vs. 79.0 ± 3.5 ml/kg min in supplemented) were observed between sportsmen belonging to the control or supplemented groups. All participants had the same performance time.

Haematological and biochemical data

The cycling stage maintained the basal values (Table 1) of erythrocytes, blood haemoglobin concentration, haematocrit, corpuscular mean volume (VCM) and the size distribution of erythrocytes (RDW). The only significant change in haematological parameters was in the mean corpuscular haemoglobin concentration (CHCM) which increased slightly, 2%, in the control group during the recovery from the cycling stage. No effects of citrulline-malate diet supplementation were observed in these haematological parameters. Serum bilirubin concentration and total proteins also maintained the basal values. No blood concentration or dilution was produced as results of the cycling stage in both the placebo and citrulline group. However, both the cycling stage and the citrulline-malate diet supplementation significantly altered the urate values. The cycling stage increased urate concentration in the control group which was maintained high at recovery, while in the citrulline-malate group there were no significant differences. Serum glucose was significantly increased immediately after cycling and returned to basal values during recovery in the control group. This picture was more attenuated in the supplemented group, but without significant differences.

Plasma creatinine significantly rose in both groups after the stage, and theses values were maintained high at recovery (Table 1). However, the increase evidenced in supplemented group was significantly higher when compared with the control group. Insulin levels significantly increased after exercise in both supplemented and control groups, and returned to basal values during recovery. No significant differences in insulin concentrations were reported between groups. GH significantly rose after exercise and returned to initial values during recovery in both groups. The increase after exercise was significantly higher in supplemented group than in the control.

Plasma amino acids

Neither the cycling stage nor citrulline-malate diet supplementation altered the values of plasma Trp, Phe, Tyr, Hys (Table 2), Lys (Table 3), Gln (Table 4), Gly and Met (Table 5). The cycling stage significantly decreased plasma concentrations of Val, Leu and Ile (only in citrulline group) (Table 3), and Asp, Asn and aminobutirate (Table 4). These amino acids returned to basal values during recovery in both the control and supplemented groups, except Asn which maintained the low levels at that moment. Although no significant differences were observed in plasma Val, Leu and concentrations between placebo and citrulline group, the percentage of loss after stage, considering the basal value as 100%, in the concentration of Val (19.6% control, 30.8% supplemented) and Leu (24.1% control, 38.9% supplemented) and Ile (22.0% control, 30.7% supplemented) was greater in the citrulline-malate supplemented group than in the control (Table 6). The plasma Ile concentration (Table 3) was unaffected by the cycling stage in the placebo group; however, citrulline supplementation significantly decreased the plasma Ile concentration after exercise, returning to basal values at recovery. The cycling stage significantly decreased Ser and Thr plasma concentrations—a mean of 39% and 37%, respectively—during recovery (Table 5). The levels of Ala, Glu (Table 4) and Tau (Table 5) significantly increased after the cycling stage, but the values returned to the basal levels during recovery. The plasma concentration of the amino acids related to the urea cycle (Arg and Orn) and also the plasma citrulline and urea concentration were affected by both citrulline-malate ingestion and by the cycling stage. The ingestion of citrulline-malate prior to the cycling stage significantly increased the plasma concentration of citrulline, Arg and Orn after exercise. These values returned to basal levels during recovery. The control group maintained the basal values of citrulline, Arg and Orn after exercise and recovery. The plasma concentrations of citrulline, Arg and Orn of the supplemented group were significantly higher than the control after cycling (2.6×, 3.1× and 2.4×, respectively). Plasma urea concentration significantly increased in both control and supplemented groups after exercise and these values were

Table 1 Haematological and biochemical parameters

	Basal	After exercise	3-h Recovery	*S*	*T*	*S***T*
Erythrocytes (10^6/μl)						
Control	5.15 ± 0.14	5.08 ± 0.11	4.94 ± 0.12			
Citrulline	5.12 ± 0.20	5.01 ± 0.14	4.80 ± 0.19			
Haemoglobin (gr/dl)						
Control	15.7 ± 0.5	156 ± 0.5	15.3 ± 0.5			
Citrulline	16.0 ± 0.6	16.0 ± 0.5	15.2 ± 0.5			
Haematocrit (%)						
Control	46.8 ± 1.5	46.1 ± 1.2	44.4 ± 1.4			
Citrulline	47.1 ± 1.7	46.7 ± 1.5	44.4 ± 1.8			
VCM (Fl)						
Control	90.9 ± 1.9	90.8 ± 1.5	89.8 ± 1.4			
Citrulline	92.0 ± 2.5	93.2 ± 2.0	92.3 ± 2.2			
CHCM (g/dl)						
Control	33.6 ± 0.1	33.9 ± 0.2	34.3 ± 0.2*	X		
Citrulline	33.9 ± 0.2	34.3 ± 0.3	34.2 ± 0.3			
RDW (%)						
Control	13.4 ± 0.3	13.3 ± 0.3	13.2 ± 0.3			
Citrulline	12.7 ± 0.2	12.6 ± 0.2	12.5 ± 0.2			
Bilirubin (mg/dl)						
Control	1.12 ± 0.26	0.86 ± 0.13	0.70 ± 0.09			
Citrulline	0.90 ± 0.12	0.85 ± 0.16	0.70 ± 0.12			
Total protein (g/dl)						
Control	7.40 ± 0.10	7.86 ± 0.06	7.70 ± 0.13			
Citrulline	7.32 ± 0.24	7.90 ± 0.29	7.70 ± 0.31			
Urate (mg/dl)						
Control	6.26 ± 0.21	8.12 ± 0.52*	7.40 ± 0.46	X		
Citrulline	5.65 ± 0.56	7.05 ± 0.61	6.35 ± 0.55			
Glucose (mg/dl)						
Control	79.6 ± 2.2	115 ± 14*	73.0 ± 1.7	X		
Citrulline	88.7 ± 1.1	101 ± 17	83.2 ± 1.7			
Creatinine (mg/dl)						
Control	0.95 ± 0.03	1.24 ± 0.04*	1.06 ± 0.02*	X	X	
Citrulline	0.97 ± 0.02	1.42 ± 0.06*#	1.12 ± 0.05*			
Insulin (μUI/ml)						
Control	0.61 ± 0.04	1.12 ± 0.15*	0.66 ± 0.11$		X	
Citrulline	0.77 ± 0.09	1.56 ± 0.33*	0.61 ± 0.08$			
GH (ng/ml)						
Control	0.58 ± 0.16	5.25 ± 0.60*	1.10 ± 0.43$	X	X	X
Citrulina	0.72 ± 0.23	8.76 ± 1.41*#	1.41 ± 0.59$			

Effects of exercise and citrulline-malate supplementation on haematological and biochemical parameters determined before the race in basal conditions, immediately after the race and after 3 h of recovery

Values are mean ± s.e.m, $p < 0.05$

X indicates significant effects (*S* or *T*) or significant interaction (*S***T*) of two ANOVA factors

* significant differences with respect to basal values

$ significant differences with respect to after exercise values

significant differences between placebo and citrulline-supplemented groups

Table 2 Plasma aromatic amino acids

	Basal	After exercise	3-h Recovery	*S*	*T*	*S*T*
Tryptophan						
Control	43.3 ± 5.9	40.0 ± 5.7	35.1 ± 2.0			
Citrulline	46.0 ± 0.6	38.9 ± 4.4	36.7 ± 5.1			
Phenylalanine						
Control	62.4 ± 2.4	65.5 ± 2.0	55.3 ± 2.0			
Citrulline	68.8 ± 5.2	63.1 ± 2.9	59.6 ± 3.2			
Tyrosine						
Control	64.6 ± 2.9	70.2 ± 5.3	61.6 ± 3.4			
Citrulline	67.5 ± 1.4	62.5 ± 3.7	56.1 ± 6.4			
Histidine						
Control	84.5 ± 4.7	82.8 ± 3.2	72.6 ± 7.8			
Citrulline	94.2 ± 5.7	79.1 ± 7.6	72.5 ± 9.3			

Effects of exercise and citrulline-malate supplementation on plasma aromatic amino acids concentration (μmol/l) determined before the race in basal conditions, immediately after the race, and after 3 h of recovery. Two-way ANOVA. No significant differences were evidenced. Control $n = 9$, citrulline supplemented $n = 8$. Values are mean ± s.e.m, $p < 0.05$

Table 3 Plasma branched chain amino acids and lysine

	Basal	After exercise	3-h Recovery	*S*	*T*	*S*T*
Valine						
Control	242 ± 18	189 ± 13*	225 ± 17		X	
Citrulline	229 ± 9	160 ± 10*	194 ± 19			
Leucine						
Control	152 ± 7	115 ± 6*	139 ± 10		X	
Citrulline	160 ± 9	98.8 ± 5.7*	138 ± 13			
Isoleucine						
Control	71.0 ± 6.3	55.6 ± 6.0	72.3 ± 2.9		X	
Citrulline	68.5 ± 6.6	47.1 ± 4.2*	76.2 ± 8.2			
Lysine						
Control	173 ± 19	165 ± 9	155 ± 12			
Citrulline	187 ± 20	145 ± 13	142 ± 18			

Effects of exercise and citrulline-malate supplementation on plasma branched chain amino acids and lysine concentration (μmol/l) determined before the race in basal conditions, immediately after the race and after 3 h of recovery

Values are mean ± s.e.m, $p < 0.05$

X indicates significant effects (*S* or *T*) or significant interaction (*S*T*) of two ANOVA factors

* significant differences with respect to basal values

Control $n = 9$, citrulline supplemented $n = 8$

maintained high at recovery. The rose in plasma urea was significantly higher in the supplemented group than in the control.

Nitrite levels

NO production, measured as nitrite plasma concentration, was significantly increased after the cycling stage in the supplemented group and maintained higher values during recovery (Fig. 1). The control group also presented a similar trend; however, the changes were more attenuated, with no significant difference in nitrite values across time.

Discussion

Lack of changes in haematological parameters and in plasma protein concentration after the cycling stage or

Table 4 Urea and amino acids of urea cycle, inter organ nitrogen transport and nitrogen handling

	Basal	After exercise	3-h Recovery	*S*	*T*	*S***T*
Urea (mg/dl)						
Control	35.8 ± 2.0	50.2 ± 3.7*	46.8 ± 3.6*	X	X	
Citrulline	41.0 ± 2.6	65.5 ± 3.9*#	59.5 ± 3.6*#			
Glutamic acid						
Control	17.5 ± 1.4	29.2 ± 3.9*	17.3 ± 4.3		X	
Citrulline	20.5 ± 1.0	28.4 ± 1.7*	16.5 ± 5.7			
Glutamine						
Control	751 ± 17	768 ± 15	635 ± 56			
Citrulline	729 ± 99	764 ± 66	624 ± 86			
Aspartic acid						
Control	4.76 ± 0.12	3.32 ± 0.51*	5.33 ± 0.34		X	
Citrulline	5.45 ± 0.34	3.91 ± 0.74*	5.02 ± 0.26			
Asparagine						
Control	62.1 ± 3.8	44.9 ± 0.6*	40.6 ± 8.0*		X	
Citrulline	67.0 ± 1.7	43.1 ± 2.6*	44.0 ± 9.3*			
Alanine						
Control	332 ± 21	530 ± 49*	307 ± 31		X	
Citrulline	368 ± 24	468 ± 46*	312 ± 38			
Citrulline						
Control	22.4 ± 3.1	25.2 ± 5.7	14.2 ± 2.0	X	X	
Citrulline	24.2 ± 3.7	66.2 ± 22.1*#	23.7 ± 10.4			
Arginine						
Control	43.2 ± 6.0	34.7 ± 5.5	27.0 ± 4.3	X	X	
Citrulline	48.7 ± 8.5	109 ± 38*#	40.7 ± 13.0			
Ornithine						
Control	53.6 ± 5.9	57.5 ± 1.3	86.0 ± 34.7	X	X	
Citrulline	55.5 ± 8.2	140 ± 41*#	88.5 ± 13.5			
Aminobutirate						
Control	24.5 ± 0.3	14.6 ± 0.9*	22.3 ± 0.9		X	
Citrulline	22.6 ± 0.3	12.1 ± 2.5*	18.4 ± 1.0			

Effects of exercise and citrulline-malate supplementation on plasma urea (mg/dl) and amino acids of the urea cycle, inter organ nitrogen transport and nitrogen handling (μmol/l) determined before the race in basal conditions, immediately after the race and after 3 h of recovery

Values are mean ± s.e.m, $p < 0.05$

X indicates significant effects (*S* or *T*) or significant interaction (*S***T*) of two ANOVA factors

* significant differences with respect to basal values

significant differences between control and citrulline-supplemented groups

Control $n = 9$, citrulline supplemented $n = 8$

during recovery was evidenced. It is recommended, when sampling for biochemical and hormonal parameters in blood following an acute bout of exercise that corrections for Plasma Volume Changes should be conducted (Kargotich et al. 1997). However, we can assume that variations in the concentration of plasma parameters in the present cycling stage cannot be attributed to modifications in the plasma volume because of the constancy of the aforementioned blood parameters. This cycling stage does not produce haemolysis nor hem concentration as evidenced by the maintained basal values of erythrocytes and billirubin.

Prolonged heavy exercise is accompanied by increased protein catabolism and changes in plasma amino acid concentrations similar to those observed during prolonged starvation, but differing from those seen at heavy exercise of less than 2 h duration or prolonged exercise of moderate intensity (Aguiló et al. 2000; Refsum et al. 1979). The cycling stage for about 3 h produced a decrease in some

Table 5 Plasma hydroxy and sulphur amino acids and glycine

	Basal	After exercise	3-h Recovery	*S*	*T*	*S***T*
Serine						
Control	105 ± 7	97.5 ± 9.5	77.3 ± 10.2*		X	
Citrulline	113 ± 10	86.8 ± 7.1	65.3 ± 11.3*			
Threonine						
Control	123 ± 7	101 ± 12	80.0 ± 3.2*		X	
Citrulline	139 ± 11	99.1 ± 9.7	84.5 ± 7.0*			
Glycine						
Control	179 ± 16	155 ± 16	140 ± 11			
Citrulline	165 ± 23	139 ± 21	142 ± 14			
Methionine						
Control	25.2 ± 0.8	24.9 ± 2.6	19.6 ± 1.3			
Citrulline	27.1 ± 2.2	23.9 ± 1.1	20.9 ± 3.1			
Taurine						
Control	37.2 ± 2.0	58.1 ± 6.9*	39.2 ± 3.4		X	
Citrulline	40.5 ± 4.9	63.2 ± 6.6*	44.9 ± 1.5			

Effects of exercise and citrulline-malate supplementation on plasma hydroxy and sulphur amino acids and glycine concentration (μmol/l) determined before the race in basal conditions, immediately after the race and after 3 h of recovery. (x) indicates significant effects (*S* or *T*) or significant interaction (*S***T*) of two ANOVA factors. * significant differences with respect to basal values. Control $n = 9$, citrulline supplemented $n = 8$. Values are mean ± s.e.m, $p < 0.05$

plasma amino acid concentrations which cannot be attributed to changes in the plasma volume. The decrease observed in the levels of the branched chain amino acids Val, Leu and Ile is compatible with the metabolic use of these amino acids as fuel to maintain muscular exercise, and the increased synthesis of Ala as a means of transporting nitrogen from the muscle to the liver as part of the glucose–alanine cycle. Muscle-produced Ala is driven to the liver for glucose synthesis de novo, and the nitrogen is transferred to urea synthesis (Goodman and Ruderman 1982). The amino acids metabolized in muscle provide the amino groups and probably the ammonia required for synthesis of glutamine and alanine (Wagenmakers 1998). The decrease in plasma Asp and Asn concentration could also be related to an increase in urea synthesis because the nitrogen of Asp is used in the biosynthesis of Arg and urea (Husson et al. 2003), in accordance with the increase in plasma urea after cycling. Acute citrulline-malate intake prior to the cycling stage did not influence this picture described for the control group; however, the amino acids involved in the metabolism of Arg—citrulline and Orn—and the urea, creatinine and nitrite products of arginine metabolism were significantly influenced, increasing their plasma concentration after the cycling stage. The overload of L-citrulline, as an intermediate metabolite of the urea cycle, could overload the flux of urea synthesis in a similar way as the anaplerotic substrates with the tricarboxylic cycle. Hence, the acute intake of citrulline increases both urea and Arg synthesis and their plasma concentration. For the synthesis of urea it is necessary to obtain nitrogen from the degradation of amino acids; however, no significant effects of the citrulline overload were observed in the plasma concentration of amino acids, but the decrease in the branched chain amino acids Val, Leu and Ile concentration induced by the cycling stage was higher in the supplemented than control group. These facts are in accordance with an increased use of amino acids, mainly branched chain amino acids, as fuel in the citrulline-malate supplemented group than in the control in order to maintain energy expenditure for cycling.

A mountain cycling stage increases ROS production resulting in oxidative stress (Cases et al. 2005; Cases et al. 2006). Exercise affects NO handling by blood and blood cells (Cases et al. 2006). NO synthesis is attributed to NOS action using Arg as a substrate, although erythrocytes can also synthesize NO from nitrite, which is associated with peripheral vasodilatation in hypoxic tissues (Dejam et al. 2004). The synthesis of NO by NOS is related to ROS production when the substrate Arg is limiting (Colantoni et al. 1998). The cycling stage has no significant effect on nitrite in the control group, whereas its levels significantly increase in the citrulline-supplemented group. This situation is compatible with a limitation of Arg as a substrate of NOS in the control group. Supplementation with citrulline avoids the limited availability of Arg by NOS because citrulline is recycled in a great proportion to Arg, as it was

Table 6 Percentage of amino acids loss after exercise

Amino acid	% Loss		*p* value
	Control	Citrulline	
Tryptophan	7.69 ± 4.86	15.3 ± 5.3	0.372
Phenylalanine	−4.61 ± 3.80	8.27 ± 4.48	0.080
Tyrosine	−8.81 ± 5.61	7.17 ± 5.33	0.085
Histidine	2.36 ± 2.08	16.5 ± 5.2	0.063
Valine	19.6 ± 2.0	30.8 ± 3.8	0.039*
Leucine	24.1 ± 1.2	38.9 ± 0.5	<0.000*
Isoleucine	22.0 ± 1.1	30.7 ± 2.9	0.032*
Lysine	4.43 ± 0.86	23.0 ± 4.4	0.006*
Glutamic Acid	−66.8 ± 12.8	−38.5 ± 9.7	0.218
Glutamine	−2.25 ± 1.20	−4.45 ± 2.30	0.431
Aspartic acid	30.6 ± 6.9	29.6 ± 5.7	0.908
Asparagine	26.0 ± 2.7	36.8 ± 5.0	0.109
Alanine	−59.5 ± 8.5	−26.9 ± 3.5	0.012*
Citrulline	−12.4 ± 2.1	−173 ± 26	0.001*
Arginine	18.8 ± 6.4	−123 ± 18	<0.000*
Ornithine	7.66 ± 1.79	−151 ± 20	<0.000*
Aminobutirate	40.8 ± 2.0	47.6 ± 3.1	<0.117
Serine	7.40 ± 1.75	23.96 ± 8.3	0.098
Threonine	17.8 ± 5.0	28.7 ± 7.2	0.260
Glycine	13.4 ± 2.1	15.4 ± 3.1	<0.631
Methionine	2.34 ± 1.8	11.6 ± 3.4	<0.055
Taurine	−55.9 ± 6.4	−56.2 ± 5.5	<0.966

Percentage of amino acid loss determined immediately after the race respect to initial values in control and in citrulline-malate supplemented groups. One way ANOVA, control $n = 9$, citrulline supplemented $n = 8$. Values are mean ± s.e.m, $p < 0.05$

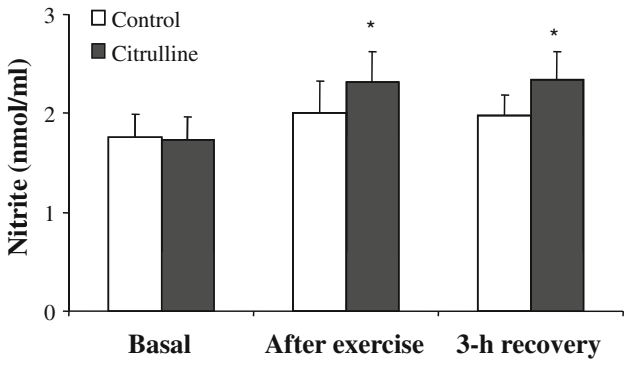

Fig. 1 Nitrite concentration in plasma (nmol/ml plasma) measured in basal conditions, after exercise and after 3 h of recovery. Two-way ANOVA, $p < 0.05$. No significant differences were evidenced between basal values. * Indicates significant differences with respect to basal values. Control $n = 9$, citrulline supplemented $n = 8$. Values represent mean ± s.e.m

evidenced by the increased Arg levels reported after exercise (Goodwin and Solomonson 2004; Mori 2007). Presumably, the synthesis of NO is more effective in the citrulline-supplemented group than the control one because the supplementation avoids the limited availability of arginine. It was evidenced that arginine supplementation increased plasma concentrations of arginine and endothelial NO synthesis. Dietary L-arginine supplementation stimulates endothelial NO synthesis by increasing endothelial tetrahydrobiopterin (BH_4) provision, which is beneficial for vascular function and glucose homeostasis in diabetic subjects (Kohli et al. 2004). L-arginine infusion also increases plasma and urinary nitrate/nitrite content and cGMP concentrations in humans (Bode-Boger et al. 1999), suggesting increased NO production (McConell et al. 2006). Then, directly or indirectly, the diet supplementation with citrulline-malate enhances both the arginine and the endothelial NO synthesis after prolonged intense exercise.

The apparent increase in plasma urea levels more than in the nitrite levels after the cycling stage and recovery in the citrulline-malate supplemented group is in favour of an important production of Orn which increases about 2.5 times after cycling in the supplemented group. The changes in the concentration of citrulline, arginine and ornithine in the control group after exercise emphasize the importance of the synthesis of ornithine in exercise. The cycling stage decreased the plasma concentration of citrulline and Arg to about 47 and 63% of the basal values and increased Orn to about 160% of the basal value in the control group. These plasma changes are consistent with an important use of Arg during recovery, mainly to synthesize urea and Orn; these patterns were enhanced by citrulline-malate supplementation. Presumably, the synthesis of Pro and polyamines could be enhanced as a result of the higher availability of Orn. This activated production of polyamines could be beneficial in the recovery of damaged muscle, due to their cellular proliferative effect (Stechmiller et al. 2005) as pointed out in the recovery from surgical intervention (Gilad et al. 2001; Konturek et al. 1998).

In humans, L-arginine infusion at rest results in an approximately twofold increase in plasma insulin concentration (Bode-Boger et al. 1999). L-arginine infusion during exercise augmented the normal exercise-induced increases in glucose disposal in humans, probably as result as increased NO production, which increased the level of glucose utilization by the contracting muscles (McConell et al. 2006). The similar insulin increase observed in both supplemented and control groups suggests that L-citrulline augments the normal exercise-induced increases in glucose disposal for the muscle via NO, but not via insulin release. It was established that moderate-intensity exercise may enhance cholinergic tone (Kelijman and Frohman 1991), which may potentiate the GH response by suppressing hypothalamic secretion of somatostatin and enhancing the response to GH-releasing hormone (GHRH) (Thompson

et al. 1993). Acute administration of oral arginine has been reported to increase (Isidori et al. 1981) or to have no impact on GH secretion (Marcell et al. 1999). Arginine may stimulate the GH secretion by inhibition of endogenous somatostatin release (Fogelholm et al. 1993). In the present results, we evidenced a positive effect of citrulline on GH levels, probably via arginine, enhancing the release of this hormone after the cycling stage.

A fraction of the body's creatine and creatine phosphate spontaneously degrades to creatinine, which is excreted by the kidneys. In humans, this amounts to about 1–2 g per day and demands a comparable rate of de novo creatine synthesis (Brosnan and Brosnan 2007). L-arginine is used for creatine synthesis, and consequently the increased creatinine evidenced after the cycling stage suggested that increased L-arginine may enhance creatine synthesis (Evans et al. 2004). The higher availability of amino acids could result in an increase in net protein catabolism as a fuel for exercise demands and an increase in creatinine excretion (Calles-Escandon et al. 1984).

In conclusion, citrulline supplementation raises plasma L-arginine concentration and augments arginine-derived metabolites such as nitrite, creatinine, ornithine and urea and hormones such as GH. Citrulline supplementation could also increase whole body nitrogen availability to allow higher protein synthesis and to increase the protein content in muscle during exercise, enhancing the use of amino acids, especially the branched chain amino acids.

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