Effects of L-citrulline oral supplementation on polymorphonuclear neutrophils oxidative burst and nitric oxide production after exercise

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Abstract

Seventeen volunteer male professional cyclists were randomly assigned to control or supplemented (6 g L-citrulline-malate) groups and participated in a cycling stage. Blood samples were taken in basal conditions, after the race and 3 h post-race. Citrulline supplementation significantly increased plasma concentration of both arginine and citrulline after the stage only in the supplemented group. Polymorphonuclear neutrophils (PMNs) from controls responded to exercise with a progressive decrease in ROS production. Supplemented PMNs significantly increased ROS production after exercise compared to basal values and diminished to values lower than basal at recovery. PMN nitrite concentration was significantly higher after exercise and recovery only in the supplemented group. Markers of oxidative damage—CK, LDH, malondialdehyde—and DNA damage remained unchanged in both groups. In conclusion, oral L-citrulline administration previous to a cycling stage increases plasma arginine availability for NO synthesis and PMNs priming for oxidative burst without oxidative damage.

Keywords: *Exercise, L-citrulline, polymorphonuclear neutrophils, nitric oxide, malondialdehyde*

Introduction

Nitric oxide synthases (NOS) convert L-arginine and oxygen into nitric oxide (NO) and L-citrulline [1]. NO promotes vasodilation, regulates platelet activation, inhibits smooth muscle cell proliferation and modulates the expression of redox-regulated genes [2]. In high concentrations NO is cytotoxic because it combines with superoxide, producing the powerful oxidant peroxynitrite (ONOO-) [3,4].

The semi-essential amino acid L-arginine is part of the human diet, but only 5–15% of plasma arginine derives from *de novo* synthesis [5]. L-arginine is continually synthesized in the liver within the urea cycle, but very little of this L-arginine is available for extrahepatic tissues [6]. Oral L-arginine supplementation has been used in several clinical studies to improve endothelium-dependent, nitric oxide (NO)-mediated vasodilation in the presence of certain atherosclerosis risk factors such as hypercholesterolemia, smoking or hypertension [7]. However, after oral administration, L-arginine is subject to extensive pre-systemic and systemic elimination, by bacteria and by arginases [8]. This may limit its bioavailability as a substrate for NOS and subsequent effect on vascular function. The non-essential amino acid L-citrulline is not subject to pre-systemic elimination and it is converted to L-argininosuccinate by argininosuccinate synthase and subsequently to L-arginine by argininosuccinate lyase [9]. It may therefore serve as an L-arginine pre-cursor [10]. Almost all the arginine coming from food is withdrawn from the portal blood by the liver; however, the liver is unable

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to uptake citrulline from portal circulation, which makes it available for the whole body [9,11]. Upon stimulation, over 80% of L-citrulline is recycled to arginine in endothelial cells to produce NO [12]. L-citrulline supplementation increases plasma L-arginine concentration to a higher level than that achieved by oral L-arginine supplementation [13].

Polymorphonuclear neutrophils (PMNs) play a key role in host defences against invading microorganisms [14]. In response to a variety of stimuli, PMNs release large quantities of oxidants such as superoxide anion and hypochlorous acid, in a phenomenon known as the respiratory burst [14]. NO regulates several important functions of PMNs, including chemotaxis, adhesion, aggregation and PMN-mediated bacterial killing or tissue damage [15]. Human PMNs contain iNOS colocalized with myeloperoxidase within neutrophil primary granules, indicating a pivotal cytotoxic role of NO in combating pathogens [16,17]. NO produced by PMNs is responsible for the formation of nitrotyrosine around phagocytosed bacteria, through the production of peroxynitrite [16].

Exhaustive exercise induces oxidative stress [18–20] and oxidative damage to muscles and to immune cells and may impair immune function [20,21]. An increased risk of catching upper respiratory tract infection has been reported after continuous exhaustive exercise [22,23]. Exercise has been shown to induce inflammatory-like changes in immune cells resembling the acute phase immune response (APIR) to infection [24]. The release of ROS by activated PMNs after exercise may contribute to the appearance of oxidative stress. Increasing evidence suggests that free intracellular amino acid turnover is especially important for the metabolic and physiological state of PMN as well as to the special functions in the inflammatory response performed by these cells [25–27].

Synthesized NO, in the lumen, is oxidized by oxygen resulting in the formation of nitrite or is taken up by erythrocytes. Nitrite is relatively stable under intracellular reducing conditions and has recently been been identified as a storage pool for NO synthesis in erythrocytes [28]. Strenuous exercise decreases the functional capacity of PMNs [29] and also produces tissue hypoxia. The high oxygen demands of muscles during exercise could produce low oxygen availability for other tissues. This observation suggests that exercise increases the use of nitrite as NO precursor to produce vasodilation [30]. Considering that the ingestion of L-citrulline, as by-product of NO formation from L-arginine, is more effective than L-arginine ingestion in increasing plasma L-arginine concentration, the aim of this study was to document the effects of L-citrulline oral supplementation prior to intense exercise on PMN NO production. PMNs oxidative burst was investigated to assess the effects of L-citrulline on PMNs function. We also evaluated the effects of L-citrulline on the induction of exercise-derived oxidative damage.

Materials and methods

Subjects and study design

Seventeen volunteer male pre-professional cyclists participated in this study which took place in the Mallorca Cinturó Ciclista 2005 (Balearic Islands). Subjects were informed of the purpose of this study and the possible risks involved before giving their written consent to participate. The sportsmen's mean $\pm$ SEM age was 22.3 ± 0.9 years, weight 70.6 ± 1.3 Kg and VO_{2max} 81.9 ± 2.6 ml/kg min without significant differences between groups. The participants were randomly distributed in a double-blind fashion to one of two treatment groups: supplemented group ($n = 8$) and control group ($n = 9$). Two hours 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. 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 during the race to control their intake before, during and after the race. All participating cyclists completed the stage with the same time of 179 min. This study 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).

Experimental procedure

EDTA-treated venous blood samples were collected from each subject the morning previous to the cycling stage after overnight fasting (basal), immediately after the stage (post-exercise) and 3 h after the end of the stage (recovery). Blood samples were used to purify PMNs and to obtain plasma [31]. PMNs were quantified in an automatic flow cytometer analyser Technicon H*2 (Bayer) VCS system. Nitrite concentration, luminol-chemiluminescence, malondialdehyde (MDA) levels and DNA damage (DNA ladder kit) were determined in PMNs. Arginine and citrulline concentration was determined in plasma. Creatine kinase (CK) and lactate dehydrogenase (LDH) were measured in serum.

Arginine and citrulline determination

Arginine and citrulline concentrations were determined in plasma by HPLC method with fluorescence detection. A solution of 20% 5-sulphosalicylic acid in

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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 sulphosalicylic acid and then centrifuged for 20 min at 10 000 × g at 4°C. The protein-free supernatant fraction was used for 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 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 were 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 arginine and citrulline vs the internal standard.

Luminol-chemiluminescence assay

(Luminol)-dependent chemiluminescence response of PMNs indicates MPO-mediated formation of highly reactive oxidants. Opsonized zymosan (OZ) was used as a PMN stimulant. Zymosan A (Sigma) was suspended in Hank's balanced salt solution (HBSS) at a concentration of 1 mg/ml and incubated with 10% human serum at 37°C for 30 min, followed by centrifugation at 750 × g, 10 min, 4°C. The precipitate was washed twice in HBSS and finally resuspended in HBSS at 1 mg/ml. OZ suspension (100 μl) was added to a 96-well microplate containing 50 μl PMNs suspension and 50 μl luminol solution (2 mM in PBS, pH 7.4). Chemiluminescence was measured at 37°C for 90 min in a FLx800 Microplate Fluorescence Reader (Bio-tek Instruments, Inc.). Each sample was determined in duplicate.

Nitrite concentration

Nitrite levels were determined in PMNs by the acidic Griess reaction using a spectrophotometric method. Lysed cells were deproteinised with acetone and kept overnight at −20°C. Samples were centrifuged for 10 min at 15000 × g at 4°C and supernatants were recovered. A 96-well plate was loaded with the samples or standard nitrite solutions (100 μl) in duplicate; 50 μl sulphanilamide (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.

MDA determination

PMNs MDA levels as a marker of lipid peroxidation were analysed by a specific colorimetric assay kit (Calbiochem, San Diego, CA).

DNA ladder assay

DNA damage was assessed by the DNA ladder kit (Roche Diagnostics) following the manufacturer's instructions. Isolated DNA (3 μg) was applied to 1% agarose gel for 1.5 h at 75 V. DNA was stained with ethidium bromide and then visualized by placing the gel onto an UV light source.

CK and LDH determinations

These determinations were made using commercial clinical kits in an autoanalyser system (Technicon DAX System).

In the determination of CK activity the enzyme reacts with creatine phosphate and ADP to form ATP, which is coupled to the hexokinase/GDP reaction generating NADPH [32]. LDH activity determination is based on measuring the conversion of pyruvate to L-lactate by monitoring the oxidation of NADH [33]. All these activities were monitored by measuring the change in absorbance at 340 nm.

Statistical analysis

Statistical analysis was carried out using a statistical package (SPSS 15.0 for Windows). Results are expressed as mean $\pm$ SEM and $p < 0.05$ was considered statistically significant. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analysed were supplementation with L-citrulline (C) and cycling stage (E). When significant effects of C or E factors were found, a Student's t-test for unpaired data was used to determine the differences between the groups involved.

Results

Citrulline supplementation before the cycling stage significantly increased the plasma concentration of both arginine and citrulline after the stage ($p < 0.05$) (Figure 1). These values returned to basal levels after 3-h recovery. In the placebo group, basal levels of both amino acids were maintained in all situations.

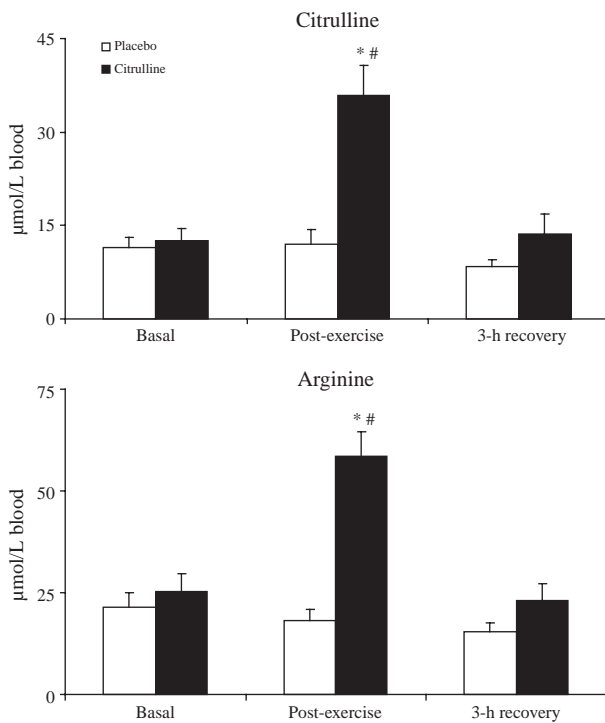The cycling stage induced an increase in the number of total circulating PMNs immediately

Figure 1. Effects of exercise and citruline-malate supplementation on blood citrulline and arginine (μmol/L) determined before the race in basal conditions, immediately after the race and after 3 h of recovery. Two-way ANOVA, $p < 0.05$. (★) Indicates significant differences respect to basal values. (#) Indicates significant differences between control and L-citrulline supplemented groups. Values are mean $\pm$ SEM.

post-exercise, as well as during the recovery period (Table I). We wanted to verify whether this increase in the number of circulating PMNs was accompanied by an oxidative burst. PMNs from the control group responded to the cycling stage with a progressive decrease of ROS production measured by luminol chemiluminescence assay; which was significantly different in the recovery period compared to basal values ($p < 0.05$). Supplemented PMNs significantly increased ROS production immediately post-exercise

($p < 0.05$) and returned to baseline at 3 h post. ROS production by PMNs from the supplemented group was significantly higher than the values measured in the control group at post-exercise and recovery ($p < 0.05$). No significant differences were reported in the time where the maximal chemiluminescence was attained.

Measurement of PMN nitrite was utilized as a surrogate marker of NO production (Figure 2). Nitrite values were unchanged in the control group after exercise and recovery. However, in the supplemented group, nitrite concentration was significantly higher after exercise in comparison to basal concentration ($p < 0.05$); these values returned to initial basal values after 3-h recovery.

In order to verify the presence of oxidative unbalance induced by the cycling stage, we measured serum CK as a marker of muscle damage and serum LDH as a marker of haemolysis (Table II). CK and LDH activities remained unchanged in both placebo and L-citrulline supplemented groups. MDA levels (Table II) and DNA damage (Figure 3) measured in PMNs also remained unchanged.

Discussion

The basal amount of L-arginine from regular diets is $\sim$ 4–6 g per day. Although citrulline intake recommendation has not been estimated, a human subject weighing 70 Kg can tolerate long-term parenteral and enteral supplement of 6 and 15 g per day of arginine, respectively. Taking into account these considerations, the present study used 6 g of citrulline which could be equivalent to the daily intake of this amino acid. In the present study, L-citrulline administration before a cycling stage increased plasma levels of arginine, citrulline and NO concentration in PMNs at post-exercise sample. These results are in accordance with previous studies which reported that

Table I. Polymorphonuclear neutrophil counts and luminol chemiluminescence.

	Basal	Post-exercise	Recovery	ANOVA C★E	C	E
Neutrophils (10^3/μl blood)						★
Placebo	3.60 ± 0.22	13.3 ± 2.0★	9.06 ± 1.31★			
Citrulline	2.85 ± 0.54	10.9 ± 0.6★	8.98 ± 0.93★			
Luminol chemiluminescence (RLU/10^6 cells)					★	★
Placebo	3053 ± 166	2601 ± 98	1674 ± 101★ &			
Citrulline	3521 ± 417	4512 ± 442★ #	3283 ± 125& #			
Time max RLU (min)						
Placebo	12.3 ± 0.2	12.9 ± 0.4	13.1 ± 0.3			
Citrulline	12.0 ± 0.2	13.2 ± 0.3	12.9 ± 0.4			

Polymorphonuclear neutrophils, luminol chemiluminescence and the time when the maximum chemiluminescence was attained were measured in basal conditions, after exercise and after 3 h of recovery. Two-way ANOVA, $p < 0.05$. (★) Indicates significant differences respect to basal values. (&) Indicates significant differences respect to post-exercise values. (#) Indicates significant differences between control and L-citrulline supplemented groups. Values are mean $\pm$ SEM.
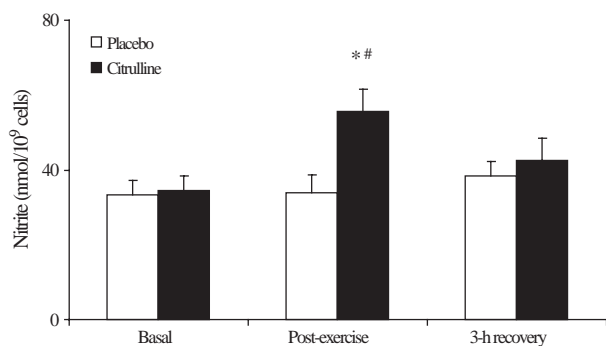
Figure 2.   Nitrite concentration in polymorphonuclear neutrophils ($nmol/10^9$ cells) measured in basal conditions, after exercise and after 3 h of recovery. Two-way ANOVA, $p < 0.05$. (*) Indicates significant differences respect to basal values. (#) Indicates significant differences between control and L-citrulline supplemented groups. Values are mean $\pm$ SEM.
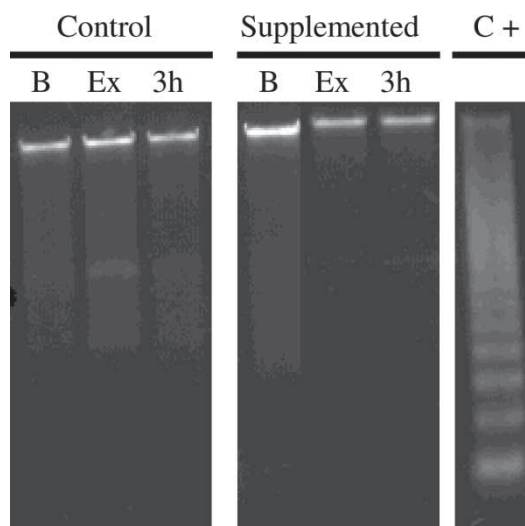

Figure 3.   DNA ladder kit determined in polymorphonuclear neutrophils in basal conditions, after exercise and after 3 h of recovery. No evidence of DNA damage was evidenced.

oral L-citrulline supplementation raises plasma L-arginine concentration and augments NO production by the citrulline-NO cycle [34,35].

Exercise leads to a rise in metabolic oxidative processes, accompanied by ROS generation and tissue damage, commonly resulting in intracellular enzyme leakage [36,37]. Both CK and LDH are found almost exclusively in the muscle tissue, thus their presence in the circulation are commonly used as markers of muscle damage [38]. The high circulating CK and LDH activities could serve as an indicator for the severity of exercise. The increase in circulating PMNs after the cycling stage reflects PMN mobilization due to exercise. It has been pointed out that PMN mobilization is mediated by stress hormones—catecholamine, cortisol and growth hormone—and by some cytokines—IL-6 and IL-8 [39]. Positive correlations were reported between the rise in PMNs after exercise and stress hormones and circulating cytokines [40]. PMN mobilization and priming for oxidative burst could participate in the exercise-induced muscle damage and also in tissue repair [41]. However, in the present study both markers maintained basal values, indicating that the cycling stage was not strenuous enough to induce muscle damage. In a previous study, we reported that

Table II.   Markers of oxidative damage.

	Basal	Post-exercise	Recovery
CK (U/L)			
Placebo	386 ± 84	408 ± 88	402 ± 98
Citrulline	471 ± 93	433 ± 98	409 ± 86
LDH (U/L)			
Placebo	332 ± 31	351 ± 25	340 ± 18
Citrulline	298 ± 19	360 ± 32	371 ± 23
Neutrophil MDA ($\mu mol/10^6$ cells)			
Placebo	0.14 ± 0.02	0.16 ± 0.03	0.17 ± 0.02
Citrulline	0.13 ± 0.02	0.16 ± 0.02	0.16 ± 0.02

No significant differences were observed in any of the markers.

a mountain cycling stage was capable of inducing oxidative damage by increasing serum activities of CK and LDH [42]. The absence of mountain difficulties and the well trained status of cyclists could have avoided the appearance of oxidative damage [43,44].

PMNs represent 50–60% of total circulating leukocytes in basal conditions and consequently could substantially contribute to the amount of NO in the circulation. It has been reported that iNOS in human PMNs is colocalized with myeloperoxidase in the primary granules [45]. This localization for iNOS appears to be adequate for its microbicidal activity. Reaction of NO with O_2^- results in the formation of ONOO- [3]. Thus, the exact role of PMNs-derived NO, whether it is cytotoxic or cytoprotective, is not clearly understood. However, NO seems to have a biphasic effect on PMN functions: activating at lower concentrations and inhibiting at higher concentrations [15]. Several studies have demonstrated NO-mediated modulation of free radical generation from PMNs in various physiological and pathological conditions [46,47]. L-Arginine supplementation significantly enhances bacterial phagocytosis in human PMNs, via its effects on cytoskeletal phenomena, and this appears to be mediated through NOS activity [46]. In a previous study it was noted that L-citrulline supplementation reduced plasma NO after exercise, probably as a result of end-product inhibition of the L-arginine/NO reaction [13]. In the present study, supplementation with L-citrulline increased PMNs NO synthesis after exercise to levels that avoid exercise-derived immunosupression. We did not measure plasma NO, but the results suggest that PMNs respond to citrulline in a different way than plasma. In fact, PMNs from supplemented cyclists

are primed for the oxidative burst which was activated in the presence of opsonized zymosan.

The typical features of PMN immune response are the release of granule constituents, such as myeloperoxidase (MPO) [48,49], the increased ability to produce ROS through the activation of NADPH oxidase and stimulation of phagocytosis as well as other innate mechanisms [50]. PMN luminol chemiluminescence, as a marker of the oxidative burst, from the placebo group seems to be inhibited during the recovery period group when they were activated. The findings were consistent with previous studies [51–53] which reported that PMN killing capacity was decreased after prolonged strenuous exercise. Chemiluminescence assay measures the reaction between luminol and HOCl. In a previous study we reported that the excess of ROS induced by the exercise-activated PMNs could act by inhibiting the PMN inflammatory response in order to avoid oxidative damage and could inhibit MPO synthesis *de novo* [54]. The drop in PMN chemiluminescence during recovery, probably by the activation of the degranulation process, could explain the decrease in HOCl production. Secreted MPO is also rapidly deactivated in plasma, losing its catalytic activity. MPO release by degranulation may be a strategy for PMNs to avoid self-damaging effects [55]. However, it has been noted that repeated bouts of strenuous exercise without sufficient recovery in between may induce chronic fatigue and further depression of the immune function [56,57], with the athletes being more susceptible to suffering from upper tract illness [22,23]. PMNs appear to be activated by an acute bout of exercise, but show a diminished responsiveness to bacterial stimulation after intense exercise, which can last for many hours [58,59]. The rise in chemiluminescence production after exercise in the L-citrulline supplemented group could reduce the immune suppression or at least delay it, probably via NO signalling, thus reducing the likelihood of suffering from upper tract infections. Further research is required to investigate the factors related to the increased oxidative burst observed in the supplemented group and the real effects on the susceptibility to suffering infections after exhaustive exercise.

It was evidenced that the effects of oral arginine or citrulline supplementation on exercise performance in healthy subjects and well-trained athletes were not clear [13,60]. In fact, Hickner et al. [13] showed a reduction in treadmill time following L-citrulline ingestion over the 24 h prior to performance of a graded treadmill exercise test to exhaustion.

In conclusion, oral L-citrulline administration previous to a cycling stage increases arginine availability in plasma for NO synthesis and PMNs priming for the oxidative burst, without the appearance of oxidative damage in lipids or DNA. NO is an important regulator of PMN functions and its increased production after exercise could be related with the enhanced ROS production in PMNs. However, further research is necessary for a better understanding of the role of NO in PMNs modulation.

Acknowledgements

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