L-Citrulline supplementation improves O2 uptake kinetics and high-intensity exercise performance in humans

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The purpose of this study was to compare the effects of L-citrulline (Cit) and L-arginine (Arg) supplementation on nitric oxide (NO) biomarkers, pulmonary O2 uptake (V˙O2) kinetics, and exercise performance. In a randomized, placebo (Pla)-controlled, crossover study, 10 healthy adult men completed moderate- and severe-intensity cycling exercise on days 6 and 7 of a 7-day supplementation period with Pla, Arg (6 g/day), and Cit (6 g/day). Compared with Pla, plasma Arg concentration was increased by a similar magnitude with Arg and Cit supplementation, but plasma Cit concentration was only increased (P < 0.001) with Cit supplementation. Plasma nitrite (NO2−) concentration was increased with Arg supplementation (P < 0.05) and tended to increase with Cit supplementation (P = 0.08) compared with Pla (83 ± 25, 106 ± 41, and 100 ± 38 nM with Pla, Arg, and Cit, respectively); however, mean arterial blood pressure was only lower (P < 0.05) after Cit supplementation. The steady-state V˙O2 amplitude during moderate-intensity exercise, improved tolerance to severe-intensity exercise (589 ± 101 and 661 ± 107 s with Pla and Cit, respectively), and increased the total amount of work completed in the exercise performance test (123 ± 18 and 125 ± 19 kJ with Pla and Cit, respectively, P < 0.05). These variables were not altered by Arg supplementation (P > 0.05). In conclusion, these results suggest that short-term Cit, but not Arg, supplementation can improve blood pressure, V˙O2 kinetics, and exercise performance in healthy adults.

nitric oxide; blood pressure; near-infrared spectroscopy; metabolism; fatigue

THE MULTIFACETED PHYSIOLOGICAL signaling molecule nitric oxide (NO) can be synthesized endogenously through the nitrate-nitrite (NO3−/NO2−) pathway (37) and through the five-electron oxidation of L-arginine (Arg) in a reaction catalyzed by the NO synthase (NOS) enzymes (55). While studies have shown that dietary nitrate supplementation can increase NO biomarkers, reduce blood pressure, and improve exercise economy/efficiency and exercise tolerance in healthy adults (see Ref. 4 for review), the extent to which these variables are impacted by dietary Arg supplementation is less clear (see Ref. 1 for review). However, when Arg treatment increases NO biomarkers, exercise economy and exercise performance are improved (5, 52), whereas exercise economy and exercise performance are not improved when Arg treatment does not influence NO synthesis (9, 31, 35, 60). Therefore, while there is some evidence to suggest that Arg treatment might improve physiological responses in conjunction with elevated NO synthesis, an optimal Arg administration procedure to enhance NO synthesis and associated physiological responses has yet to be established.

A significant obstacle to increasing Arg delivery to NOS via Arg supplementation is that orally ingested Arg is catabolized by intestinal bacteria and arginases on the first pass (13, 67), with a further 10–15% of systemic Arg extracted and metabolized by the liver (13, 44, 48, 59, 71). While acute (57) and short-term (35) Arg ingestion have been shown to increase plasma Arg concentration ([Arg]), the intracellular utilization of this additional substrate by NOS might be restricted by the competition between Arg, asymmetric dimethylarginine (ADMA), and other Arg analogs for the transporter (1) carrier hCAT-2B (14). This regulation of Arg transport and metabolism could account for the finding that only ~1% of an oral Arg dose is utilized as substrate by NOS (11). On the basis of these restrictions, it appears that oral Arg supplementation might not be the optimal method to stimulate NO production through the NOS pathway.

L-Citrulline (Cit) is coproduced with NO as an end product of NOS activity. It is well documented that NOS-derived Cit is efficiently recycled into Arg for subsequent NO production through the Cit-NO cycle (24). Therefore, exogenous Cit administration might represent an attractive alternative to increase the amount of Arg provided to NOS. An advantage of oral Cit treatment is that, unlike Arg, catabolism of Cit in the intestines is limited, since Cit is not metabolized by arginases and bacteria, and the activity of argininosuccinate synthase, the enzyme that initiates Cit metabolism, is low in enterocytes (68). Moreover, and also in contrast to Arg, Cit is not extracted from the systemic circulation for clearance by the liver (59, 65). Consequently, the majority of an oral Cit bolus passes into the systemic circulation (43). Cit is then extracted by the kidneys to be converted, in sequence, to argininosuccinate and Arg by the enzymes argininosuccinate synthase and argininosuccinate lyase, respectively (15, 24, 59, 65, 70). Synthesis of Arg from Cit is prevalent in other tissues (15, 23, 70), and this process is facilitated since Cit does not compete with ADMA for cell transport (50, 69). Importantly, it has been shown that oral Cit supplementation more effectively increases the circulating (32, 47, 53, 62) and tissue (63) [Arg] than an equivalent dose of Arg and that Cit supplementation can increase NO activation (63) and NO biomarkers (46, 53).
Therefore, these findings suggest that Cit might serve as an important precursor for NO production.

Chronic supplementation with L-citrulline malate has been shown to enhance skeletal muscle power output in concert with a greater oxidative energy turnover and a lower pH-to-power ratio (7) and a lower ATP cost of muscle force production (19). These data suggest that short-term L-citrulline malate supplementation might improve skeletal muscle metabolism and/or contractile efficiency, which would be expected to predispose to greater fatigue resistance. However, since Cit was administered as L-citrulline malate in these experiments and since malate is an important tricarboxylic acid cycle intermediate that might itself influence muscle function (61), it is unclear whether these beneficial effects can be attributed to Cit, per se. Hickner et al. (26) reported compromised endurance performance in concert with a lower plasma concentration of nitrate + NO\textsubscript{3} (\([\text{NO}_3]\)) in humans following the acute ingestion of pure Cit. In contrast, 7 days of supplementation with pure Cit has been shown to improve endurance exercise performance in mice (56). While these data suggest that chronic Cit supplementation has greater potential to improve endurance exercise performance than acute Cit ingestion, this has yet to be investigated in humans. Moreover, since NO biomarkers have not been assessed in studies reporting positive effects of Cit on muscle function and metabolism (7, 19, 56), it is unclear whether these improvements are linked to an increase in NOS-derived NO. It is also unclear whether the improvements in muscle metabolism and performance with chronic Cit (7, 19, 56) are linked to improved \(O_2\) uptake (\(\dot{V}O_2\)) kinetics, as is the case following dietary nitrate supplementation (6). Therefore, further research is required to assess whether short-term oral Cit can influence NO synthesis and exercise performance and determine the underlying mechanisms for any performance gains with Cit.

The purpose of this study was to investigate the effects of short-term Arg and Cit supplementation on plasma [Arg], L-citrulline concentration ([Cit]), and NO\textsubscript{2} concentration ([NO\textsubscript{2}]\textsuperscript{−}), a sensitive marker of NOS activity (34), as well as blood pressure, \(\dot{V}O_2\) kinetics, and exercise performance, compared with a taste- and energy-matched placebo (Pla). We hypothesized that, compared with Pla, 1) plasma [Arg] would be increased to a greater extent with Cit than Arg and 2) Cit, but not Arg, would elevate plasma [NO\textsubscript{2}]\textsuperscript{−}, reduce blood pressure, and improve \(\dot{V}O_2\) kinetics, cycling efficiency, and exercise performance.

METHODS

Subjects. Ten healthy, recreationally active men (mean ± SD: age 19 ± 1 yr, height 1.80 ± 0.08 m, body mass 79 ± 11 kg) volunteered to participate in the study. None of the subjects smoked tobacco or used dietary supplements. The procedures employed in the study were approved by the Institutional Research Ethics Committee. All subjects gave their written informed consent prior to the commencement of the study, after the experimental procedures, associated risks, and potential benefits of participation had been explained. Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, ≥ 3 h postprandial, and to avoid strenuous exercise in the 24 h preceding each testing session. Each subject was also asked to abstain from caffeine and alcohol for 6 and 24 h, respectively, before each test. All tests were performed at the same time of day (± 2 h).

Experimental design. Subjects were required to report to the laboratory on eight occasions over 6–7 wk to complete the experimental testing. On the first visit to the laboratory, subjects completed a ramp incremental exercise test for determination of the gas exchange threshold (GET) and peak \(\dot{V}O_2\) (\(\dot{V}O_2\)\textsubscript{peak}). Subjects were familiarized with the two exercise performance tests employed in the study during the second laboratory testing session. After these preliminary exercise tests, subjects returned to the laboratory on days 6 and 7 of 7-day supplementation periods with Pla, Arg, and Cit to complete the experimental testing. During these tests, resting blood pressure, pulmonary \(\dot{V}O_2\) kinetics, muscle oxygenation, and exercise performance were assessed, and a resting venous blood sample was obtained. The supplements were administered orally in a randomized order as part of a double-blind, crossover experimental design. Each supplementation period was separated by 7–10 days of washout. A food diary was provided for the first supplementation intervention, and the subjects were instructed to replicate their diet over subsequent supplementation periods.

Incremental test. During the first laboratory visit, subjects completed a ramp incremental cycle test on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). Initially, subjects performed 3 min of baseline cycling at 0 W; then the work rate was increased by 30 W/min until the limit of tolerance. The subjects cycled at a self-selected pedal rate (70–90 rpm), which, along with saddle and handle bar heights and configuration, was recorded and reproduced in subsequent tests. Breath-by-breath pulmonary gas-exchange data were collected continuously during the incremental tests and averaged over consecutive 10-s periods. The maximum \(\dot{V}O_2\) (\(\dot{V}O_2\)\textsubscript{max}) was taken as the highest 30-s mean value attained prior to the subject’s volitional exhaustion in the test. The GET was determined from a cluster of measurements, including 1) the first disproportionate increase in \(CO_2\) production (\(\dot{V}CO_2\)\textsubscript{1}) from visual inspection of individual plots of \(\dot{V}CO_2\) vs. \(\dot{V}O_2\), 2) an increase in expired ventilation (\(\dot{VE}\)/\(\dot{V}O_2\) with no increase in \(\dot{VE}/\dot{V}CO_2\), and 3) an increase in end-tidal \(PCO_2\) with no fall in end-tidal \(PO_2\). The work rates that would require 90% of the GET (moderate-intensity exercise) and 70%Δ (GET + 70% of the difference between the work rate at the GET and \(\dot{V}O_2\)\textsubscript{max} severe-intensity exercise) were subsequently calculated with account taken of the mean response time (MRT) for \(\dot{V}O_2\) during ramp exercise (i.e., two-thirds of the ramp was deducted from the work rate at GET and peak \(\dot{V}O_2\)\textsubscript{max} “individualization test”). To avoid any order effect on the performance results as a consequence of a potential “learning effect,” subjects were familiarized with all performance tests prior to the experimental testing. Subjects completed a severe-intensity step exercise test terminating with an all-out sprint (exercise performance test) followed, after a 45-min passive recovery period, by a severe-intensity constant-rate step exercise test that was continued until the limit of tolerance (exercise tolerance test).

Supplementation procedures. Experimental testing was conducted during a 7-day supplementation period with Pla, Arg, and Cit. The Pla supplement consisted of 10.7 g of maltodextrin, the Arg supplement consisted of 6 g of Arg + 4.3 g of maltodextrin, and the Cit supplement consisted of 6 g of Cit + 4.3 g of maltodextrin. All supplements were energy-matched, containing 40 kcal per serving. Pure maltodextrin, Arg, and Cit powders (NOW Sports Nutrition, NOW Foods, Bloomingdale, IL) were mixed with 500 ml of water and 75 ml of blackcurrant cordial in the proportions described above to produce the Pla, Arg, and Cit supplements. On days 1–5 of supplementation, subjects were instructed to drink the beverage slowly over the course of the day. On days 6 and 7 of supplementation, subjects were instructed to consume the beverage over a 10-min window, such that the entire beverage had been consumed 60 min before the subject was required to report to the laboratory.

Experimental tests. After reporting to the laboratory on days 6 and 7 of the supplementation interventions, subjects were required to rest in a seated position for 10 min in an isolated room. Thereafter, while the subject was seated, blood pressure of the brachial artery was measured using an automated sphygmomanometer (Dinamap Pro, GE
Medical Systems, Tampa, FL). Four measurements were taken, and the mean of the measurements was calculated. A venous blood sample was then drawn into a lithium-heparin tube and centrifuged at 4,000 rpm and 4°C for 10 min, within 3 min of collection. Plasma was subsequently extracted and immediately frozen at −80°C for later analysis of [NO2] in duplicate via chemiluminescence (6), and [Arg], [Cit], and l-ornithine (Orn) concentration ([Orn]) were determined using high-performance liquid chromatography (HPLC; see below).

At 30 min after arrival at the laboratory (90 min after ingestion of the supplement), subjects completed a series of cycle exercise tests. We elected to commence exercise testing 90 min after supplement consumption, since published pharmacokinetic data have shown that this time frame should coincide with peak plasma [Arg] after oral ingestion of 6 g of Cit (53) or 6 g of Arg (10). The exercise protocol consisted of three “step” exercise tests: two moderate-intensity step tests followed by one severe-intensity exercise bout. Moderate-intensity step tests were completed to assess VO2 kinetics and cycling economy in the absence of a VO2 slow component, while severe-intensity step tests were completed to assess VO2 kinetics in the presence of a VO2 slow component, where VO2max is attained and the tolerable duration of exercise is <20 min (49, 66). We conducted repeated step tests on the same laboratory visit, since a prior moderate-intensity step exercise bout does not affect VO2 kinetics during subsequent moderate- or severe-intensity cycle exercise (12, 17). Therefore, all subjects performed a total of four bouts of moderate-intensity exercise and two bouts of severe-intensity exercise for each experimental condition.

Each transition began with 3 min of baseline cycling at 20 W before an abrupt transition to the target work rate. A passive recovery of 5 min separated the transitions. The duration of each moderate-intensity step was 6 min. On day 6 of each supplementation condition, subjects cycled for 6 min at a severe-intensity constant work rate (70%Δ) followed immediately by a 60-s all-out sprint. The resistance on the pedals during the 60-s all-out effort was set using the linear mode of the Lode ergometer, so that the subject would attain the power output calculated to be 50%Δ if he attained his preferred cadence (linear factor = power/preferred cadence2). Subjects were provided with a 5-s countdown prior to the sprint and were instructed to attain the peak power as quickly as possible and to continue exercising maximally for the duration of the sprint. No time feedback was given to the subjects at any point during the sprint. On day 7 of the supplementation period, the severe-intensity constant-work-rate bout was continued to the limit of tolerance. The time to task failure was used as a measure of exercise tolerance and was recorded when the pedal rate fell by >10 rpm below the required pedal rate.

Measurements. During all tests, pulmonary gas exchange and ventilation were measured breath-by-breath, with subjects wearing a nose clip and breathing through a low-dead-space, low-resistance mouthpiece-and-impeller turbine assembly (Triple V, Jaeger, Hoechberg, Germany). The inspired and expired gas volume was continuously sampled at 100 Hz; gas concentration signals were continuously sampled at 100 Hz using paramagnetic (O2) and infrared (CO2) analyzers (Oxycon Pro, Jaeger) via a capillary line connected to the mouthpiece. The gas analyzers were calibrated before each test with analyzers (Oxycon Pro, Jaeger) via a capillary line connected to the sample at 100 Hz; gas concentration signals were continuously sampled at 100 Hz using paramagnetic (O2) and infrared (CO2) analyzers (Oxycon Pro, Jaeger) via a capillary line connected to the mouthpiece. 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Table 1. Resting plasma [NO$_2$], [l-Arg], [l-Cit], and [l-Orn] following Pla, l-Arg, and l-Cit supplementation

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<th>Pla</th>
<th>l-Arg</th>
<th>l-Cit</th>
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<tbody>
<tr>
<td>Plasma [NO$_2$], nM</td>
<td>83 ± 25</td>
<td>106 ± 41*</td>
<td>100 ± 38</td>
</tr>
<tr>
<td>Plasma [l-Arg], µM</td>
<td>57 ± 14</td>
<td>151 ± 31*</td>
<td>135 ± 22*</td>
</tr>
<tr>
<td>Plasma [l-Cit], µM</td>
<td>23 ± 5</td>
<td>26 ± 6</td>
<td>665 ± 205†</td>
</tr>
<tr>
<td>Plasma [l-Orn], µM</td>
<td>26 ± 8</td>
<td>62 ± 14*</td>
<td>50 ± 6*†</td>
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</table>

Values are means ± SD. Pla, placebo. *Significantly different from Pla (P < 0.05). †Significantly different from l-Arg (P < 0.05).

RESULTS

The Pla, Arg, and Cit supplements were well tolerated by all subjects, and no negative side effects were reported. Subjects consumed all doses of the supplement for each experimental condition, and their diet was consistent across all the dietary interventions. The VO$_2$ peak attained in the ramp incremental test was 3.94 ± 0.51 l/min, which equated to a relative VO$_2$ peak of 50 ± 9 ml·kg$^{-1}$·min$^{-1}$. The work rates that corresponded to 90% GET and 70%Δ were 120 ± 23 and 284 ± 40 W, respectively.

Plasma [NO$_2$], [l-Arg], [l-Cit], and [l-Orn]. The plasma [NO$_2$], [l-Arg], [l-Cit], and [l-Orn] data for the Pla, Arg, and Cit conditions are reported in Table 1. The ANOVA revealed a significant main effect for supplement on plasma [l-Arg], [l-Cit], and [l-Orn] (all P < 0.001). Plasma [l-Arg] was increased above Pla (57 ± 14 µM) with Arg (151 ± 31 µM) and Cit (135 ± 22 µM, both P < 0.001), with no differences between Arg and Cit (P > 0.05; Table 1). Plasma [l-Cit] was not significantly different between Pla (23 ± 5 µM) and Arg (26 ± 6 µM, P > 0.05) but was significantly greater than both these conditions with Cit (665 ± 205 µM, P < 0.001; Table 1). Plasma [l-Orn] was significantly greater with Cit (50 ± 6 µM) than Pla (26 ± 8 µM, P < 0.001) and significantly greater with Arg (62 ± 14 µM) than Pla and Cit (P > 0.05; Table 1). Plasma [NO$_2$] was significantly increased with Arg (106 ± 41 nM, P < 0.05), but not Cit (100 ± 38 nM, P = 0.08), compared with Pla (83 ± 25 nM; Table 1).

Blood pressure. The blood pressure data for the Pla, Arg, and Cit conditions are reported in Table 2. There was a significant main effect for supplement on systolic blood pressure (P < 0.05), with follow-up analyses showing that systolic blood pressure was lower after Cit (118 ± 6 mmHg, P < 0.05), but not Arg (120 ± 7 mmHg, P > 0.05), than after Pla (122 ± 7 mmHg; Table 2). While there was no main effect for supplement on diastolic blood pressure (P > 0.05), there was no negative side effects were reported. Subjects consumed all doses of the supplement for each experimental condition, and their diet was consistent across all the dietary interventions. The VO$_2$ peak attained in the ramp incremental test was 3.94 ± 0.51 l/min, which equated to a relative VO$_2$ peak of 50 ± 9 ml·kg$^{-1}$·min$^{-1}$. The work rates that corresponded to 90% GET and 70%Δ were 120 ± 23 and 284 ± 40 W, respectively.

Table 2. Resting blood pressure measures following Pla, l-Arg, and l-Cit supplementation

<table>
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<th>Pla</th>
<th>l-Arg</th>
<th>l-Cit</th>
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<tr>
<td>Systolic blood pressure, mmHg</td>
<td>122 ± 7</td>
<td>121 ± 5</td>
<td>118 ± 6*</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>65 ± 6</td>
<td>64 ± 4</td>
<td>64 ± 5</td>
</tr>
<tr>
<td>Mean arterial blood pressure, mmHg</td>
<td>87 ± 3</td>
<td>86 ± 2</td>
<td>85 ± 2*</td>
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Values are means ± SD. *Significantly different from Pla (P < 0.05).
significant differences in the fundamental $\dot{V}O_2$ amplitude but not Arg (86 ± 0.87 l/min vs. 0.23 l/min with Pla, Arg, and Cit, respectively, $P < 0.05$). There were no between-condition differences in the fundamental $\dot{V}O_2$ amplitude was not significantly different between the conditions (2.23 ± 0.87 vs. 0.23 ± 0.87 l/min with Pla, Arg, and Cit, respectively, $P > 0.05$; Table 3). The phase II $\tau$ was also not significantly different between conditions (24 ± 7, 22 ± 7, and 21 ± 6 s with Pla, Arg, and Cit, respectively, $P > 0.05$; Table 3).

The baseline $\dot{V}O_2$ and phase II $\dot{V}O_2$ kinetics during severe-intensity exercise were not significantly impacted by the dietary interventions employed in this investigation ($P > 0.05$ for all comparisons). The $\dot{V}O_2$ at exhaustion was not significantly different between experimental conditions and was also not significantly different from the $\dot{V}O_2$ peak attained in the ramp incremental test ($P > 0.05$ for all comparisons). No significant differences in the fundamental $\dot{V}O_2$ amplitude (2.23 ± 0.34, 2.26 ± 0.42, and 2.29 ± 0.45 l/min with Pla, Arg, and Cit, respectively) or $\dot{V}O_2$ slow component (0.66 ± 0.09, 0.60 ± 0.12, and 0.58 ± 0.13 l/min with Pla, Arg, and Cit, respectively, $P > 0.05$; Table 3) were observed across the experimental conditions. However, there was a significant main effect for supplement on the MRT ($P < 0.05$), with faster overall $\dot{V}O_2$ kinetics after Cit than Pla supplementation (60 ± 8 s vs. 54 ± 5 s, $P < 0.05$; Fig. 1). There were no significant differences in $\dot{V}CO_2$ and respiratory exchange ratio between the Pla, Arg, and Cit conditions during moderate- or severe-intensity cycle exercise ($P > 0.05$ for all comparisons; data not shown). There were also no between-condition differences in blood [lactate] at any time comparison in this study ($P > 0.05$, data not shown).

NIRS variables. The NIRS-derived muscle [HHb], [HbO2], and TOI data during moderate- and severe-intensity cycle exercise with Pla, Arg, and Cit supplementation are reported in Table 4. There were no significant differences between the experimental conditions for the [HbO2] and TOI responses during moderate-intensity exercise ($P > 0.05$ for all comparisons). However, the [HHb] amplitude during moderate-intensity cycling exercise was significantly lower after Cit supplementation (8 ± 4 and 6 ± 4 arbitrary units with Pla and Cit, respectively, $P < 0.05$; Fig. 2). While there were no significant between-supplement differences in [HHb] dynamics or muscle [HbO2] during severe-intensity cycle exercise in this study, the muscle TOI was significantly elevated over the first 360 s of severe-intensity exercise with Cit supplementation ($P < 0.05$; Table 4, Fig. 3).

Exercise performance. The power profiles for the three experimental conditions during the 60-s all-out sprint that followed the 6-min bout of severe-intensity exercise (the exercise performance test) are shown in Fig. 4, while the times to exhaustion during the severe-intensity constant-work-rate cycle trials (the exercise tolerance test) are shown in Fig. 5. A significant main effect for supplement was observed for the peak power attained and total work completed during the 60-s all-out sprint that concluded the exercise performance test ($P < 0.05$ vs. Pla. Note significant decrease in $\dot{V}O_2$ MRT after Cit, but not Arg, supplementation.)

Table 3. Pulmonary gas exchange measures during moderate- and severe-intensity cycle exercise after Pla, l-Arg, and l-Cit supplementation

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<tr>
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<th>Pla</th>
<th>l-Arg</th>
<th>l-Cit</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Moderate-intensity exercise</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\dot{V}O_2$, l/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.07 ± 0.83</td>
<td>1.09 ± 0.11</td>
<td>1.09 ± 0.13</td>
</tr>
<tr>
<td>End-exercise</td>
<td>1.94 ± 0.29</td>
<td>1.96 ± 0.28</td>
<td>1.93 ± 0.30</td>
</tr>
<tr>
<td>Phase II time constant, s</td>
<td>24 ± 7</td>
<td>22 ± 7</td>
<td>21 ± 6</td>
</tr>
<tr>
<td>Mean response time, s</td>
<td>37 ± 7</td>
<td>38 ± 7</td>
<td>36 ± 6</td>
</tr>
<tr>
<td>Fundamental amplitude, l/min</td>
<td>0.87 ± 0.21</td>
<td>0.87 ± 0.22</td>
<td>0.86 ± 0.23</td>
</tr>
<tr>
<td><strong>Severe-intensity exercise</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\dot{V}O_2$, l/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.12 ± 0.10</td>
<td>1.13 ± 0.13</td>
<td>1.13 ± 0.11</td>
</tr>
<tr>
<td>360 s</td>
<td>3.94 ± 0.49</td>
<td>3.95 ± 0.49</td>
<td>3.94 ± 0.51</td>
</tr>
<tr>
<td>Exhaustion</td>
<td>4.12 ± 0.50</td>
<td>4.09 ± 0.49</td>
<td>4.13 ± 0.56</td>
</tr>
<tr>
<td>Phase II time constant, s</td>
<td>26 ± 7</td>
<td>26 ± 6</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>Fundamental amplitude, l/min</td>
<td>2.23 ± 0.34</td>
<td>2.26 ± 0.42</td>
<td>2.29 ± 0.45</td>
</tr>
<tr>
<td>Slow component amplitude</td>
<td>0.66 ± 0.09</td>
<td>0.60 ± 0.12</td>
<td>0.58 ± 0.13</td>
</tr>
<tr>
<td>%</td>
<td>23 ± 7</td>
<td>21 ± 4</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>Overall mean response time, s</td>
<td>60 ± 8</td>
<td>56 ± 7</td>
<td>54 ± 5*</td>
</tr>
</tbody>
</table>

Values are means ± SD. *Significantly different from Pla ($P < 0.05$).
completed during the 60 s sprint by 7% (21 [HHb], AU) moderate- and severe-intensity cycle exercise after Pla, L-Arg, and L-Cit supplementation. The changes in exercise tolerance after Cit supplementation increased the test peak power by 9% (480 J) 0.05). The total work 107 s, 0.07). Neither peak power output (482 ± 102 W) nor total sprint work completed (21 ± 5 kJ) was significantly impacted by Arg supplementation (P > 0.05). The total work completed over the entire exercise performance test was greater with Cit (125 ± 19 kJ, P < 0.05), but not Arg (124 ± 19 kJ, P > 0.05), than with Pla (123 ± 18 kJ, P < 0.05). There was a strong trend for a main effect of supplement on the time to exhaustion during the exercise tolerance test (P = 0.07). When between-condition analyses were conducted, there was a significant 12% increase in exercise tolerance time after Cit supplementation relative to Pla (589 ± 101 vs. 661 ± 107 s, P < 0.05; Fig. 5). Exercise tolerance was not significantly improved with Arg (612 ± 150 s) compared with Pla (P > 0.05). The changes in exercise tolerance after Cit supplementation were not related to changes in plasma [NOx], VO2 kinetics, or muscle oxygenation (P > 0.05 for all comparisons).

DISCUSSION

The principal novel findings from this study are that short-term supplementation with pure Cit enhanced endurance exercise performance and resulted in faster overall VO2 kinetics and a 21% increase in the sensitive NO biomarker plasma [NO3]. These findings differ from previous research demonstrating that acute Cit supplementation lowers plasma [NOx] and compromises exercise tolerance (26) but are consistent with studies showing that short-term (15 days) supplementation with l-citrulline malate can positively affect skeletal muscle power output and metabolic responses (7). These findings are important, since they suggest that Cit might be responsible for the positive effects previously reported following l-citrulline malate supplementation and offer new insights into the mechanisms by which Cit supplementation might be ergogenic. Conversely, no significant differences in VO2 kinetics and exercise performance were observed following short-term Arg supplementation, consistent with previous findings (60). These findings suggest that short-term Cit, but not Arg, supplementation might be an exercise enhancement supplement.
 effective dietary intervention to improve oxidative metabolism and exercise performance in healthy adults.

Influence of Arg and Cit supplementation on plasma [Arg], [Cit], [Orn], and [NO2]. In an attempt to overcome the well-developed interorgan system for Arg clearance, recent studies have investigated the efficacy of oral Cit supplementation as an alternative method to enhance NO production via NOS. Oral Cit supplementation is appealing in this regard, since Cit is not significantly metabolized in the gut (68) and liver (59, 65) and <1% of orally ingested Cit is excreted in the urine (50). As such, the majority of an oral Cit load passes into the systemic circulation, as reflected by a significant increase in plasma [Cit] after Cit ingestion in the current study and numerous previous reports (8, 16, 42, 51, 53). Thereafter, the bulk of plasma Cit is converted to Arg, mostly in the kidneys (15, 24, 59, 65, 70), but also in several other tissues (15, 23, 70). This is compatible with the significant increase in plasma [Arg] in this study and several previous studies (32, 47, 53, 62) following Cit supplementation. It is important to note that not only does Cit increase systemic [Arg] by avoiding catabolism along the intestinal-renal axis, but Cit might also be expected to enhance Arg bioavailability, given that Cit can function as an allosteric inhibitor of arginase (54). This is supported by our finding of a lower plasma [Orn], the product of Arg metabolism by arginase (70), after Cit than Arg supplementation in this study. However, despite this potential for greater systemic Arg bioavailability following oral Cit than oral Arg supplementation and in contrast to previous studies reporting a greater increase in plasma [Arg] after Cit than Arg ingestion (32, 47, 53, 62), plasma [Arg] was increased by a similar magnitude when the same dose of Cit and Arg was orally administered in this study. These conflicting findings might be a function of between-study differences in the experimental subjects and supplementation regimens. Therefore, our results do not support the notion of a greater systemic Arg availability after Cit than Arg supplementation, at least in healthy adult men undergoing the Arg and Cit supplementation procedures employed in this study.

In addition to an increase in NOS substrate provision, there is some evidence to suggest that NO production is enhanced after Cit treatment (46, 53, 63) and that Cit can restore NO production in conditions where NO production is compromised (16, 36). However, there is also a suggestion that Cit ingestion tends to lower NO production, as inferred from plasma [NOx] (26). Plasma [NO2] better reflects human NOS activity than plasma [NOx] (34) and is likely to provide a more accurate assessment of NOS-derived NO. In this study Cit supplementation increased plasma [NO2] by 21%, but this increase did not attain statistical significance \( (P = 0.08) \). On the other hand, Arg supplementation resulted in a statistically significant (28%) increase in plasma [NO2]. Taken together, these data suggest that short-term Arg supplementation might be more effective than Cit supplementation at increasing the sensitive biomarker of NOS activity, plasma [NO2] (28, 34). However, the extent to which plasma [NO2] reflects skeletal muscle NOS activity is unclear. In this study plasma [Orn] was lower following Cit than Arg. It is known that Orn competes with Arg for cellular uptake via the \( y^+ \) carrier system (70). This might have facilitated greater skeletal muscle Arg uptake after Cit than Arg supplementation in this study. The finding of a greater increase in tissue [Arg] (63) after Cit than Arg ingestion supports this postulate. Moreover, the lower plasma [Orn] following Cit than Arg, despite a similar increase in plasma [Arg], implies a lower arginase activity following Cit (70).

Fig. 4. Group mean power profiles during a 60-s all-out cycle sprint commenced immediately after 6 min of severe-intensity cycle exercise following Pla, Arg, and Cit supplementation. Note significant increase in peak and mean power output during 60-s all-out sprint after Cit, but not Arg, supplementation compared with Pla.

Fig. 5. Time to exhaustion during severe-intensity constant-work-rate cycle exercise after Pla, Arg, and Cit supplementation. Top: time to exhaustion following Pla and Arg supplementation. Bottom: time to exhaustion following Pla and Cit supplementation. Solid gray lines, individual responses to supplementation. Values are means ± SE. * \( P < 0.05 \) vs. Pla. Note significant increase in exercise tolerance after Cit, but not Arg, supplementation.
Providing muscle [Orn] was also lower following Cit than Arg supplementation in this study, muscle arginase activity may have been downregulated. While there appears to be some controversy regarding the levels of arginase in human skeletal muscle (20, 45), the potential for Cit to inhibit arginase might be important, since arginase II content in human skeletal muscle can be similar to that in the kidney, i.e., relatively high (45). Therefore, the functional effects of Cit (see below) may be muscle-specific and not detected as changes in plasma [NO2\textsubscript{\textsuperscript{-}}], which may be more indicative of gross changes in NOS activity throughout the body. Similarly, the Arg-induced increase in plasma [NO2\textsubscript{\textsuperscript{-}}], observed in the present study, may be due to nonendothelial NOS-mediated NO production or NOS-mediated NO production at sites other than skeletal muscle.

**Influence of Arg and Cit supplementation on blood pressure.**

A hallmark of enhanced NO synthesis is a reduction in blood pressure owing to NO-induced smooth muscle relaxation (22). It has also recently been demonstrated that circulating NO2\textsubscript{\textsuperscript{-}} itself can act as a source for NO synthesis via endogenous human nitrite reductase activities, associated with proteins such as xanthine oxidase and deoxyhemoglobin (see Ref. 37 for review). However, despite a significant increase in plasma [NO2\textsubscript{\textsuperscript{-}}] after Arg supplementation, resting blood pressure was not significantly lowered. This suggests that the increase in plasma [NO2\textsubscript{\textsuperscript{-}}] after short-term Arg supplementation might not have been sufficient to lower resting blood pressure in normotensive adults. Conversely, Cit supplementation, which did not significantly increase plasma [NO2\textsubscript{\textsuperscript{-}}], significantly reduced resting blood pressure. Although previous studies have shown a reduction in arterial stiffness (46), enhanced endothelium-dependent vasorelaxation in response to acetylcholine (25), and an association between the change in the Arg-to-ADMA ratio and flow-mediated dilation (53) with Cit, we have shown for the first time that pure Cit supplementation can reduce blood pressure in healthy normotensive adults. An increase in cGMP has been reported after short-term Cit consumption (53), which suggests that the reduction in blood pressure with Cit might result from NO-cGMP-related smooth muscle relaxation. Alternatively, Cit might alter vascular tone through another endothelium-derived relaxing factor, such as prostacyclin or endothelium-derived hyperpolarizing factors, independent of, or alongside, an increase in NO. Further research is required to investigate the mechanisms by which Cit might positively affect vascular and other physiological responses.

**Influence of Arg and Cit supplementation on VO2 kinetics.**

Giannesini et al. (19) reported that short-term l-citrulline malate supplementation lowered the oxidative and phosphocreatine (PCr) cost of skeletal muscle force production in the rat gastrocnemius muscle in situ. We previously showed that short-term dietary nitrate supplementation can also lower skeletal muscle ATP turnover by attenuating ATP flux through oxidative phosphorylation and PCr hydrolysis in association with lower VO2 in humans completing knee-extensor exercise (2). Therefore, we hypothesized that short-term Cit supplementation might lower VO2 in humans completing cycle exercise. However, in contrast to our previous findings with dietary nitrate supplementation (6) and our experimental hypothesis, short-term Cit supplementation did not significantly lower VO2 during moderate-intensity cycle ergometry exercise. Our findings in this study might differ from those reported by Giannesini et al. as a consequence of differences in the experimental model (human skeletal muscle contraction in vivo vs. isolated rat skeletal muscle in situ) or differences in the Cit supplementation procedures (pure Cit supplementation vs. l-citrulline malate supplementation).

In the present study short-term Cit supplementation did not significantly increase plasma [NO2\textsubscript{\textsuperscript{-}}] and moderate-exercise VO2 was not significantly altered. We recently showed that acute Arg ingestion did not increase plasma [NO2\textsubscript{\textsuperscript{-}}] or lower moderate-exercise VO2 (60). However, plasma [NO2\textsubscript{\textsuperscript{-}}] was increased by 28% following short-term Arg in the present study without affecting moderate-exercise VO2. Importantly, neither Arg nor Cit increased plasma [NO2\textsubscript{\textsuperscript{-}}] to the extent observed when moderate-exercise VO2 is lowered by dietary nitrate supplementation (2, 5, 6, 33), and this likely accounts for the similar moderate-exercise VO2 across Pla, Arg, and Cit in the present study. While neither the steady-state VO2 nor the rate at which VO2 increased following the onset of moderate-intensity exercise was significantly impacted by Arg or Cit supplementation, the NIRS-derived muscle [HHb] amplitude was lower with Cit. Since the NIRS-derived muscle [HHb] signal is considered a noninvasive proxy for muscle fractional O2 extraction (21), the lower muscle [HHb] amplitude with Cit in the absence of a change in VO2 suggests that Cit may have improved O2 availability/distribution within the muscle microvasculature.

During severe-intensity exercise, the overall VO2 kinetics were faster following Cit than Pla supplementation. These data support the findings of Bendahan et al. (7), who reported that l-citrulline malate supplementation elevated muscle oxidative ATP production determined in vivo using 31P-MRS. The faster overall VO2 kinetics after Cit supplementation was accompanied by an increased NIRS-derived TOI throughout the exercise bout. This suggests that Cit supplementation improved the O2 delivery to the muscle microvasculature, which, in turn, permitted a greater VO2 over the initial stages of severe-intensity cycle exercise. Previous studies have also reported that overall VO2 kinetics during severe-intensity exercise are accelerated by interventions that enhance muscle O2 availability (3, 64). However, it is known that muscle NIRS measures manifest significant special heterogeneities across the contracting quadriceps (29), so it is unclear whether a lower [HHb] amplitude during moderate-intensity exercise and the increased TOI during severe-intensity exercise at a discrete site of the vastus lateralis is reflective of an improved matching between muscle O2 delivery and muscle VO2 across the contracting quadriceps. Indeed, some studies report good agreement between NIRS markers of muscle oxygenation and mixed venous PO2 or oxygenation (39), while other studies do not (38). It has also been suggested that the interpretation of NIRS data may be complicated by increased skin blood flow (30, 40), as develops during exercise. However, the NIRS-derived [HHb] (30) and TOI (40) that were used to draw inferences on muscle oxygenation in this study are not altered by increased skin blood flow. Alternatively, since short-term l-citrulline malate supplementation has been shown to speed the rate of muscle PCr resynthesis following exercise (7, 18), a process coupled to the maximal rate of ATP derived from mitochondrial oxidative phosphorylation (41), it is also possible that Cit accelerated overall VO2 kinetics through enhancing muscle oxidative metabolism independent of enhanced muscle O2 delivery. There
was no improvement in NIRS-derived TOI or \( \dot{V}O_2 \) dynamics during severe-intensity exercise in this study after Arg supplementation.

**Influence of Arg and Cit supplementation on exercise performance.** Severe-intensity exercise tolerance was increased by 12% and subjects completed 7% more sprint work during an exercise performance test with Cit supplementation. While \( \text{l-citrulline} \) malate supplementation has been shown to increase muscle force production \((7, 19)\), improve muscle contractile efficiency \((19)\), and prevent the decline in muscle force production with endotoxemia \((18)\), studies investigating the effects of Cit on exercise performance or muscle fatigue resistance are limited and equivocal. Of the two studies investigating the influence of Cit supplementation on exercise performance to date, one reported improved exercise tolerance in mice performing swimming exercise \((56)\), while the other showed that Cit compromised incremental exercise performance in humans \((26)\). It is unclear why our findings contrast with those of Hickner et al. \((26)\). While Hickner et al. designed an experiment to assess the influence of acute ingestion of 3 or 9 g of Cit on incremental treadmill exercise performance, we investigated the effects of 7 days of Cit supplementation at 6 g/day on cycling exercise tolerance and performance. These conflicting findings might therefore be a consequence of different exercise performance tests and dosing procedures, with 6–7 days of supplementation being more effective than acute Cit ingestion. Moreover, an important difference between these studies is the influence of Cit supplementation on NO biomarkers. Specifically, we have shown that short-term Cit tended \((P = 0.08)\) to increase plasma \( [\text{NO}_2^-] \) and improve exercise performance, whereas Hickner et al. reported a surprising tendency for acute Cit to lower plasma \( [\text{NOx}] \) \((\text{plasma} \ [\text{NO}_2^-] + [\text{nitrate}]) \) and compromise exercise performance. Therefore, the extent to which Cit influences exercise performance appears to be linked to the duration of the supplementation period and its impact on NO bioavailability.

The improved exercise performance after Cit supplementation was accompanied by faster overall \( \dot{V}O_2 \) kinetics, although there was no significant correlation between the two. An increase in muscle oxidative ATP turnover in concert with a lower pH-to-power ratio after \( \text{l-citrulline} \) malate supplementation has also been reported \((7)\). While we observed no change in blood [lactate] during and after severe-intensity exercise with Cit supplementation in this study, previous studies have reported lower end-exercise blood [lactate] and ammonia concentration \((56)\), as well as a lower rate of muscle PCR degradation \((19)\) with Cit. Taken together, these findings suggest that Cit supplementation might increase the proportional energy contribution from oxidative metabolism, thereby limiting the utilization of the finite anaerobic energy reserves and reducing the accumulation of metabolites linked to the process of muscle fatigue.

The findings presented in this study suggest that short-term supplementation with Cit powder might lower blood pressure, speed \( \dot{V}O_2 \) kinetics, and improve exercise tolerance/performance. An alternative, natural, method to increase dietary Cit intake is consumption of watermelon \((\text{Citrullus lanatus})\), which contains \( \sim 2.33 \) g Cit per liter of unpasturized watermelon juice \((58)\). Accordingly, subjects would be required to consume a daily watermelon dose of \( \sim 2.5 \) liters to ingest the same dose of Cit administered in this study. Further research is required to determine whether the effects reported following short-term Cit supplementation in this study can be reproduced using watermelon juice supplementation.

In conclusion, this study has shown that short-term Cit supplementation can reduce blood pressure, speed \( \dot{V}O_2 \) kinetics, and enhance endurance exercise performance. Supplementation with Arg, on the other hand, did not significantly affect these parameters. Therefore, the results of this study suggest that chronic supplementation with Cit might represent a practical, dietary intervention to reduce blood pressure and enhance oxidative metabolism and exercise performance in young healthy adults.

**ACKNOWLEDGMENTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

S.J.B., A.V., P.G.W., and A.M.J. developed the concept and designed the research; S.J.B., J.R.B., and T.L. performed the experiments; S.J.B., J.R.B., T.L., A.V., P.G.W., and A.M.J. interpreted the results of the experiments; S.J.B. and A.M.J. drafted the manuscript; S.J.B., J.R.B., A.V., P.G.W., and A.M.J. edited and revised the manuscript; S.J.B., J.R.B., T.L., A.V., P.G.W., and A.M.J. approved the final version of the manuscript.

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