Effects of Citrulline Supplementation on Fatigue and Exercise Performance in Mice

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\textbf{Summary} During high-intensity exercise, the concentration of ammonia is augmented in skeletal muscle. Ammonia activates phosphofructokinase and prevents oxidation of pyruvate to acetyl CoA, thus leading to exhaustion. Citrulline is an amino acid component of the urea cycle in the liver, along with ornithine and arginine. The aim of this study was to examine the effect of citrulline supplementation on fatigue and performance during high-intensity exercise. We constructed a swimming exercise protocol, in which mice were subjected to exhaustive swimming with a load of 5% body weight, and measured the time until exhaustion, the blood levels of lactate and ammonia, and the glycogen content of the gastrocnemius and biceps femoris muscles. Citrulline supplementation significantly increased the swimming time until exhaustion. Exercise-induced blood ammonia elevation was repressed by citrulline supplementation, and exercise-induced blood lactate increment in the citrulline-supplemented group was significantly lower than that in the non-supplemented group. Citrulline supplementation may facilitate the detoxification of ammonia via the urea cycle and inhibit additional glycolysis. Our findings suggest that citrulline supplementation may be useful for improving the exercise performance of athletes.

\textbf{Key Words} exercise, citrulline, ammonia, fatigue, supplementation

It is well known that exercise-induced fatigue affects performance, but fatigue is attributable to many factors, including accumulation of metabolites, and depletion of muscle glycogen (1–3). The blood level of ammonia, a product that accumulates in skeletal muscle when AMP is deaminated to IMP during the resynthesis of ATP, is elevated by exercise and is thought to be one of the causes of exercise-induced fatigue (4, 5). Wilkinson et al. reported that the blood level of ammonia was increased about 1.8-fold after exhaustive treadmill running (6). Ammonia activates phosphofructokinase (PFK) and inhibits the oxidation of pyruvate to acetyl CoA (7, 8). Activation of PFK facilitates production of lactate, causing a decline of intracellular pH, a decrease of Ca\textsuperscript{2+} release from the sarcoplasmic reticulum, and consequently a decrease of contractility (9). Inhibition of pyruvate oxidation hinders the supply of ATP to skeletal muscle, thus causing exhaustion. Therefore, accumulation of ammonia has an unfavorable effect on exercise tolerance, the urea cycle being responsible for the detoxification of ammonia to urea in the liver (10).

Citrulline, one of the non-essential amino acids, has recently received attention as an ammonia detoxifier, working as a component of the urea cycle. In a study using mice, Meneguello et al. found that supplementation with a citrulline/arginine/ornithine mixture suppressed the accumulation of blood ammonia after exercise and prolonged the time until exhaustion in a swimming task (11). Although arginine or ornithine supplementation is reported to suppress the accumulation of ammonia, few studies have examined whether this improves exercise performance in mice or humans (12–14). Therefore, we hypothesized that citrulline supplementation would facilitate ammonia detoxification during exercise and improve exercise performance. In the present study using mice, we measured the blood ammonia level after exhaustive swimming and the time until exhaustion in a swimming task after administration of citrulline.

\textbf{MATERIALS AND METHODS}

\textbf{Experimental approval.} Animal experiments were carried out in a humane manner after receiving approval from the Institutional Animal Experiment Committee of the University of Tsukuba, and in accordance with the Regulations for Animal Experimentation at the University and Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

\textbf{Animals.} Eight-week-old male ICR mice were used. They were kept at 22±1°C and a relative humidity of 60±10% under a light/dark cycle of 12/12 h. with lights on at 07:00. All mice were provided with a nor-
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mal diet (MF, Oriental Yeast Co., Ltd., Japan) and water ad libitum.

Exercise protocol. Fifty-six mice were divided into four groups (n=14 per group), and trained to perform a swimming exercise for 10 min without any weight burden every other day. The swimming exercise was carried out in a tank (42×64×38 cm) filled with water to 30-cm depth and kept at 30±1˚C. On the seventh day, an exhaustion swimming test was carried out (Fig. 1). The mice swam to exhaustion with a load of 5% body weight attached to their tails. Each mouse was considered to have reached exhaustion when it failed to rise his face to the surface of the water for inhale within 5 s.

Sampling. After swimming, the mice were killed by cervical dislocation, and blood samples, together with the gastrocnemius (red and white portions were separated) and biceps femoris muscles, were collected. Previous analyses of the motion of mice during swimming had suggested that these two muscles were extensively used. The blood samples were collected from the heart. Blood lactate was measured using Lactate Pro (Arkrey, Japan). Blood ammonia was assayed using a commercial kit (Ammonia Test WAKO, Wako Pure Chemical Industries, Ltd., Japan). Muscle samples were stored at −80˚C until measurement of muscle glycogen.

Citrulline. The citrulline used in this study was purchased from Kyowa Hakko Kirin Co., Ltd. The mice in the supplemented group were given citrulline at a dose of 250 mg/kg body weight by gavage in a single dose 30 min after the exercise session, and at the same time on the remaining days (days 2, 4 and 6). On the day of the exhaustion test, the mice received the same amount of citrulline 30 min prior to the exercise session. Non-supplemented mice received the same amount of distilled water, under the same administration protocol.

Muscle glycogen assay. Muscle glycogen measurement followed Brodal et al. (1). Muscle samples were homogenized in a buffer at 4˚C containing 1.0 mM HEPES pH 7.6, 150 mM NaCl, 1.5 mM MgCl2, 10 mM EDTA, 10 mM Na-pyrophosphate, 200 mM NaF, 10% glycerol, 1% Triton-X100, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 0.5 µg/mL pepstatin, and then centrifuged at 15,000 rpm for 60 min. The supernatants were added to wo/AG (NaAc buffer= 200 mM NaAcetate pH 5.5, 10% BSA) and w/AG (NaAc buffer with amyloglucosidase), then incubated at 37˚C for 1 h to allow degradation of glycogen to glucose by amyloglucosidase. The incubated samples were then treated with 600 µL glucose reagent (50 mM Tris-HCl pH 8.1, 0.04% BSA, 0.2 mM NADP, 0.3 mM DTT, 0.5 mM ATP, 1.0 mM MgCl2, 0.05 U/mL G6PDH, 1.429 U/mL hexokinase), and absorbance measured with a fluorescence spectrophotometer (F-4500, Hitachi High-Technologies Corporation, Japan). The muscle glycogen content was calculated from the difference in absorbance between the wo/AG and w/AG samples.

Statistical analysis. All results are presented as mean±SE. The data on exercise time to exhaustion were analyzed by the unpaired t-test. The data on blood ammonia, blood lactate, and muscle glycogen were analyzed using two-way ANOVA and the Tukey post-hoc test. The significance level was set at p<0.05. Pearson’s correlation coefficient analysis and simple regression were used to assess the relationship between exercise time and blood lactate. Differences in the correlation coefficients of the regression lines obtained for the non-supplemented and supplemented groups were determined by testing the t-value.

RESULTS

The non-supplemented group became exhausted after about 15 min of swimming exercise with a load equivalent to 5% body weight. The citrulline-supplemented group showed significant prolongation of the swimming time to exhaustion as compared with the non-supplemented group (Fig. 2).
The blood ammonia level was significantly elevated by exhaustive swimming in the non-supplemented group, but no change was observed in the citrulline-supplemented group (Fig. 3). The blood lactate level was significantly increased by swimming exercise in both the citrulline-supplemented and non-supplemented groups, but the increase was significantly lower in the former (Fig. 4).

Comparison of the Con and Ex groups showed that the muscle glycogen contents of the gastrocnemius (R-GAS, W-GAS) and biceps femoris (BF) were decreased by 80%, 75%, and 60%, respectively, by exhaustive swimming. Muscle glycogen was also decreased significantly by swimming exercise in the supplemented group (Fig. 5). There was no difference in the reduction of muscle glycogen between the supplemented and non-supplemented groups.

A strong negative correlation was found between
exercise time and blood lactate level in both the non-supplemented and citrulline-supplemented groups. Both groups show a negative correlation, but the coefficient is higher in the non-supplemented group than in the supplemented group. Ex: swimming exercise group, Ex+cit: swimming exercise with citrulline supplemented group.

**DISCUSSION**

Although it is well known that exercise-induced fatigue influences exercise performance, many factors affect induction of fatigue during exercise. Ammonia is a substance that accumulates during exercise, and is thought to cause fatigue. Ammonia activates PFK, which causes a decline of intracellular pH and suppresses the release of Ca\(^{2+}\) from the sarcoplasmic reticulum (7, 9). Furthermore, ammonia inhibits the oxidation of pyruvate to acetyl CoA, which causes a decline of ATP production from the TCA cycle, and skeletal muscle cannot contract sufficiently (8). Hence, ammonia detoxification is important for prolongation of exercise period.

Citrulline, one of the non-essential amino acids, is a component of the urea cycle, which is involved in the detoxification of ammonia in the liver, along with arginine and ornithine. Supplementation with a mixture of citrulline, arginine and ornithine is known to suppress the increased accumulation of ammonia during exercise, and to improve exercise performance during swimming (11). However, neither arginine nor ornithine supplementation improves exercise performance, even though ammonia accumulation is inhibited (12–14). In the present study, therefore, we investigated the effect of citrulline supplementation upon the time taken to reach exhaustion for a single bout of swimming exercise. Citrulline supplementation prolonged the period to exhaustion and suppressed the increase in the blood ammonia level during swimming (Figs. 2, 3). These results suggested that citrulline supplementation was effective for ammonia detoxification during exercise.

Sugino et al. administered ornithine for eight days to human subjects, who then performed bicycle exercise for four hours at 80% of the anaerobic threshold. At the end of the exercise, the ornithine supplemented group showed a lower level of blood ammonia and higher level of blood urea than the placebo group (12). Arginine supplementation has also been reported to increase the level of blood urea significantly (16). These results suggest that ornithine or arginine supplementation promotes the urea cycle, consequently increasing the production of urea. However, the above studies demonstrated little effect in terms of exercise performance. In the present study, the level of urea was thought to have been increased by citrulline supplementation, suggesting that further research is warranted to confirm the effect on the level of blood urea.

The blood lactate level was significantly increased 2-fold and 3-fold in the citrulline-supplemented and non-supplemented groups, respectively, by exhaustive swimming exercise (Fig. 4), in comparison with the control group. This result suggests that PFK activity was suppressed as a result of ammonia detoxification, and that utilization of glycogen was decreased. Immediately after swimming, the level of muscle glycogen was similar in the two groups. However, mice in the citrulline-supplemented group swam longer than those in the non-supplemented group, suggesting that the former utilized glycogen more efficiently.

Gobatto et al. reported that in mice performing swimming exercise, the blood lactate level was about 5.5 mM, reflecting a balance between production of lactate and its removal (=MLSS: maximal lactate steady state) (17). In the present study, the lactate level in the non-supplemented group was also about 5.5 mM. Thus, the intensity of exercise while swimming to exhaustion with a burden of 5% body weight is considered to be comparable to MLSS, which is equivalent to an intermediate level. At intermediate exercise intensity, exhaustion is due mostly to depletion of muscle glycogen (1, 18). In the present study, the muscle glycogen content was significantly decreased in both groups by exhaustive swimming with a 5% body weight load (Fig. 5), and thus muscle glycogen depletion appears to be the main factor responsible for exhaustion in this exercise program. Citrulline supplementation was not involved in depletion of muscle glycogen, but suppressed the increase of blood lactate through prevention of additional glycolysis.
detoxification of ammonia and may suppress the increase in the level of blood lactate. Citrulline supplementation is expected to be especially effective during high-intensity exercise, which relies on glycolysis for energy provision. Future studies will need to focus on the effect of exercise intensity and assess the influence of citrulline supplementation.

Several previous studies have examined the anti-fatigue effects of amino acids that are components of the urea cycle. Meneguello et al. reported that supplementation with a mixture of citrulline, arginine, and ornithine suppressed the accumulation of blood ammonia after exercise and prolonged the time to exhaustion in a swimming task by about 60% (11). Although a single supplementation of arginine or ornithine suppressed the increase in the blood level of ammonia, any effect on exercise performance was obscure (12–14). However, our present study shows not only repression of ammonia during exercise but also improvement of exercise performance. Therefore, in this respect, citrulline may be the most effective amino acid component of the urea cycle for supplementation.

Citrulline is a precursor of arginine, which forms Nitric Oxide (NO). Jones et al. reported NO synthase inhibition reduces maximal oxygen uptake during exercise in humans (20). Arginine supplementation has been reported to increase blood flow in hypercholesterolemic individuals (21). So, there is a possibility that citrulline supplementation would be effective on not only blood ammonia but also vasodilatation during exercise.

Taken together, our results indicate that citrulline supplementation prolongs the period until exhaustion in swimming exercise, and that this effect involves inhibition of blood ammonia accumulation when the intensity of exercise is comparable to MLSS. Muscle glycogen reduction was delayed during exercise by facilitating the detoxification of ammonia, and the blood lactate level was reduced in comparison with the non-supplemented group. Our present findings suggest that citrulline supplementation would be very helpful for individuals performing high intensity exercise.

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REFERENCES