How L-Tyrosine Benefits Your Mental and Physical Health

L-tyrosine is an amino acid that's often used in supplements to improve cognitive function affected by stress. Supplements containing L-tyrosine supplements may also help treat phenylketonuria (PKU).

Your body uses L-tyrosine (also known as tyrosine) to produce dopamine and norepinephrine. These neurohormones (catecholamines) are quickly depleted when you're under stress. Though L-tyrosine supplements may restore them to healthy levels, they're not safe for everyone. Consult your healthcare provider before taking them.

Uses

L-tyrosine is made in the body from another amino acid called phenylalanine. Then, your body uses tyrosine to make three catecholamines: dopamine, norepinephrine, and epinephrine.[1]

Catecholamines are neurohormones, which means they have roles in your brain as neurotransmitters and also serve as hormones in your body. Periods of prolonged or severe stress often deplete these essential substances.

You may improve the following conditions by taking L-tyrosine supplements:

Cognitive Function During Stress

Dopamine helps the brain manage stress. Whether you face mental, psychological, emotional, or physical stress, dopamine activates brain areas that support your ability to deal with the pressure.

When you endure intensive, prolonged, or chronic stress, dopamine levels drop.[23] If that happens, studies suggest that L-tyrosine supplements may help.

A systematic review published in *Pharmacology Biochemistry and Behavior* reported that a single dose of L-tyrosine significantly improved a decline in working memory and information processing. Both cognitive problems were caused by situational stress, such as intense mental work or extreme weather.[4]

In another study, L-tyrosine supplementation improved cognitive functioning, such as response inhibition, task switching, and working memory in young adults. However, this was primarily seen in short-term stressful situations or cognitively demanding scenarios.[5]

However, there's no evidence that L-tyrosine will help you feel less stressed, which some may mistakenly interpret from the marketing claims.

Phenylketonuria

Phenylketonuria is an inherited disorder in which the person cannot process phenylalanine appropriately.

Since phenylalanine converts to L-tyrosine, this could lead to low levels. People with PKU may be advised to consume a diet that's low in phenylalanine, but this meal plan can be very restrictive.[6]

Three studies evaluated the effect of taking L-tyrosine instead of or together with a low-phenylalanine diet. In people with PKU, L-tyrosine blood levels significantly increased when taking supplements. However, the results didn't verify whether L-tyrosine supplements improve other issues associated with PKU, such as growth, neuropsychological performance, and quality of life.[6]

Tyrosine supplements should not be used in place of a low phenylalanine diet or medications to help manage PKU. If you have PKU, your healthcare provider can help you determine if you might benefit from a tyrosine supplement.

Other Possible Benefits

L-tyrosine supplements are often marketed to improve attention-deficit/hyperactivity disorder (ADHD), athletic performance, depression, and anxiety, but evidence is lacking.

ADHD: L-tyrosine may improve some symptoms of ADHD through its role in the production of dopamine. However, scientific studies supporting a direct link between L-tyrosine supplements and ADHD have yet to be completed. Additionally, researchers found that 83 children (aged 6-13) with ADHD did not have deficiencies in L-tyrosine.[7]

Athletic performance: Though L-tyrosine boosts leucine production (an amino acid crucial for building muscles), studies don't support its ability to improve athletic performance.[18]

Depression: Depression is linked with imbalanced neurotransmitters like serotonin and dopamine. L-tyrosine supplements may improve your mental

health by boosting neurotransmitter levels. Studies in lab animals suggest L-tyrosine may improve depression, but more research is needed in people.[9]

Anxiety: A 2019 study suggests that L-tyrosine may reduce the brain's fear response. Though fear is closely related to anxiety, L-tyrosine's direct role in anxiety hasn't been studied. More research is needed to determine if supplements containing L-tyrosine can reduce anxiety.

Precautions

There is insufficient data on the safety of supplementing L-tyrosine in pregnant or breastfeeding people. Therefore, sticking to food sources is the safest.

People with hyperthyroidism (overactive thyroid gland) should avoid L-tyrosine supplements as they can increase thyroid hormone production and worsen symptoms of hyperthyroidism (including Graves' disease, the autoimmune form of hyperthyroidism).

L-tyrosine can also trigger migraine headaches. Avoid taking supplements if you struggle with migraines.[1]

Interactions

Some medications may interact with supplemental L-tyrosine, including the following:

- Levodopa is a medication used for Parkinson's disease. Levodopa and L-tyrosine can compete for absorption in the small intestine, which could influence how well they work. This can be avoided by taking supplements two hours apart from the levodopa dose.[12]
- Monoamine oxidase inhibitors (MAOIs) combined with high-tyrosine foods can increase blood pressure to dangerous levels.[1] People taking MAOIs should avoid high-tyrosine foods and supplements.
- Synthetic thyroid hormones are used to treat hypothyroidism by raising hormone levels. L-tyrosine helps produce thyroid hormone.

Taking supplements together with synthetic hormones can raise thyroid hormone levels too high.[1]

How to Take L-Tyrosine

Food Sources

L-tyrosine is available in the foods that we eat. Most people get enough from their diet unless they're following a low-protein meal plan.

For adults, the estimated amino acid requirement for phenylalanine and tyrosine combined is 14 mg/kg body weight daily.[13]

High L-tyrosine foods include:

- Meat, such as chicken or turkey
- Fish
- Dairy products, like cheese, or yogurt
- Eggs
- Soy
- Avocado
- Wheats and oats
- Pumpkin seeds
- Peanuts

In supplements, L-tyrosine can be found in its free form and as N-acetyl L-Tyrosine (NALT). The conversion rate of NALT to tyrosine is lower so you may need higher doses of NALT.

Dosage for Supplements

Dosage varies depending on what you're taking it for and your L-tyrosine levels. Talk to your healthcare provider before taking a supplement to determine if it will help you, and if so, the appropriate dose for your individual needs.

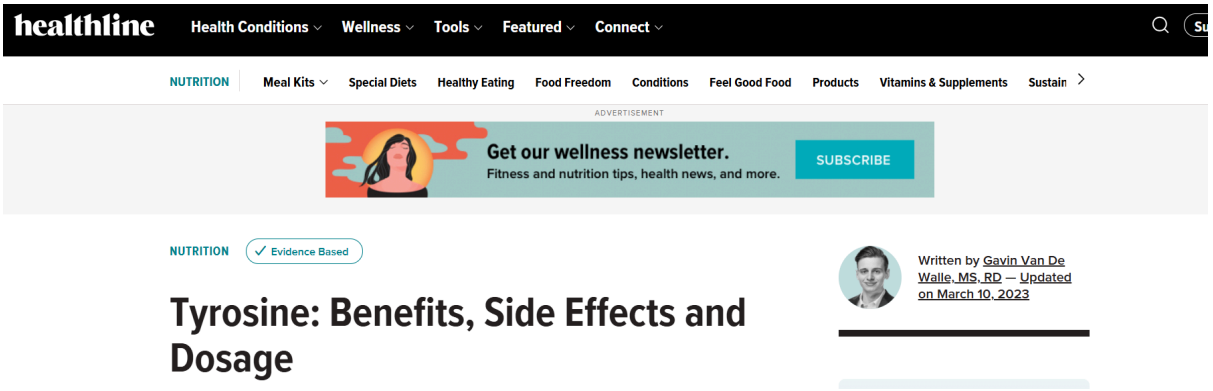
There is not a lot of data on the effects of high doses of L-tyrosine or toxicity. The possible ill effects of such high doses over the long term are not known.

In the United States, the Food and Drug Administration (FDA) does not regulate dietary supplements the way it regulates prescription medications. As a result, some supplement products may not contain the ingredients listed on the label. When choosing a supplement, look for products independently tested or certified by organizations such as the National Sanitation Foundation (NSF), United States Pharmacopeia (USP), or ConsumerLab. For personalized guidance, consult your healthcare provider, registered dietitian nutritionist (RD or RDN), or pharmacist.

Summary

L-tyrosine is a nonessential amino acid that is produced in the body. It is also easily available in many of the foods we eat. For this reason, a food-first approach to getting more L-tyrosine is usually preferred.

Tyrosine is marketed to relieve stress, ease symptoms of hypothyroidism, and improve brain health, but there is very little scientific evidence to support any of these claims. Speak with a healthcare provider if you are interested in using supplements of any sort.



Tyrosine: Benefits, Side Effects and Dosage

Tyrosine is a supplement that may help improve alertness, attention, and focus. Depending on the dose, it may help boost physical and mental performance. But, not all research is conclusive, and there may be side effects.

Tyrosine produces important brain chemicals that help nerve cells communicate and may even regulate mood Despite these benefits, supplementing with tyrosine can have side effects and interact with medications.

This article tells you all you need to know about tyrosine, including its benefits, side effects, and recommended dosages.

What Is Tyrosine and What Does It Do?

Tyrosine is an amino acid that is naturally produced in the body from another amino acid called phenylalanine.

It’s found in many foods, especially in cheese, where it was first discovered. In fact, “tyros” means “cheese” in Greek

It is also found in chicken, turkey, fish, dairy products and most other Tyrosine helps make several important substances, including (4):

- Dopamine: Dopamine regulates your reward and pleasure centers. This important
  brain chemical is also important for memory and motor skills (5
- Trusted Source
-).
- Adrenaline
  and noradrenaline: These hormones are responsible
  for the fight-or-flight response to stressful situations. They prepare the
  body to “fight” or “flee” from a perceived attack or harm (5
- Trusted Source
-).
- Thyroid
  hormones: Thyroid hormones are produced by the thyroid
  gland and primarily responsible for regulating metabolism (6
- Trusted Source
-).
- Melanin: This pigment gives your skin, hair and eyes their color. Dark-skinned people have more melanin in their skin than light-skinned
  people

It’s also available as a dietary supplement. You can purchase it alone or blended with other ingredients, such as in a pre-workout supplement.

Supplementing with tyrosine is thought to increase levels of the neurotransmitters dopamine, adrenaline and norepinephrine.

By increasing these neurotransmitters, it may help improve memory and performance in stressful situations (4).

Summary Tyrosine is an amino acid that the body produces from phenylalanine. Supplementing with it is thought to increase important brain chemicals, which affect your mood and stress response.

labcorp OnDemand

Build an understanding of your nutritional intake

Labcorp OnDemand's Micronutrient Test may help you uncover nutritional deficiencies and make informed decisions about what you eat.

It May Improve Mental Performance in Stressful Situations

Stress is something that everyone experiences.

This stress can negatively affect your reasoning, memory, attention and knowledge by decreasing neurotransmitters For example, rodents who were exposed to cold (an environmental stressor) had impaired memory due to a decline in neurotransmitters However, when these rodents were given a tyrosine supplement, the decline in neurotransmitters was reversed and their memory was restored.

While rodent data does not necessarily translate to humans, human studies have found similar results.

In one study in 22 women, tyrosine significantly improved working memory during a mentally demanding task, compared to a placebo. Working memory plays an important role in concentration and following instructions

In a similar study, 22 participants were given either a tyrosine supplement or placebo before completing a test used to measure cognitive flexibility. Compared to the placebo, tyrosine was found to improve cognitive flexibility

Cognitive flexibility is the ability to switch between tasks or thoughts. The quicker a person can switch tasks, the greater their cognitive flexibility.

Additionally, supplementing with tyrosine has been shown to benefit those who are sleep deprived. A single dose of it helped people who lost a night's sleep stay alert for three hours longer than they otherwise would What's more, two reviews concluded that supplementing with tyrosine can reverse mental decline and improve cognition in short-term, stressful or mentally demanding situations And while tyrosine may provide cognitive benefits, no evidence has suggested that it enhances physical performance in humans

Lastly, no research suggests that supplementing with tyrosine in the absence of a stressor can improve mental performance. In other words, it won't increase your brainpower.

Summary Studies show that
tyrosine can help maintain your mental capacity when taken before a stressful

activity. However, there is no evidence that supplementing with it can improve your memory.

It Might Help Those With Phenylketonuria

Phenylketonuria (PKU) is a rare genetic condition caused by a defect in the gene that helps create the enzyme phenylalanine hydroxylase Your body uses this enzyme to convert phenylalanine into tyrosine, which is used to create neurotransmitters (4).

However, without this enzyme, your body cannot break down phenylalanine, causing it to build up in the body.

The primary way to treat PKU is to follow a special diet that limits foods containing phenylalanine However, because tyrosine is made from phenylalanine, people with PKU can become deficient in tyrosine, which can contribute to behavioral problems (21

Trusted Source

).

Supplementing with tyrosine may be a viable option for alleviating these symptoms, but the evidence is mixed.

In one review, researchers investigated the effects of tyrosine supplementation alongside or in place of a phenylalanine-restricted diet on intelligence, growth, nutritional status, mortality rates and quality of life The researchers analyzed two studies including 47 people but found no difference between supplementing with tyrosine and a placebo.

A review of three studies including 56 people also found no significant differences between supplementing with tyrosine and a placebo on the outcomes measured The researchers concluded that no recommendations could be made about whether tyrosine supplements are effective for the treatment of PKU.

Summary PKU is a serious
condition that may cause tyrosine deficiency. More studies are needed before
recommendations can be made about treating it with tyrosine supplements.

Evidence Regarding Its Effects on Depression Is Mixed

Tyrosine has also been said to help with depression.

Depression is thought to occur when the neurotransmitters in your brain become unbalanced. Antidepressants are commonly prescribed to help realign and balance them Because tyrosine can increase the production of neurotransmitters, it's claimed to act as an antidepressant However, early research doesn't support this claim.

In one study, 65 people with depression received either 100 mg/kg of tyrosine, 2.5 mg/kg of a common antidepressant or a placebo each day for four weeks. Tyrosine was found to have no antidepressant effects Depression is a complex and varied disorder. This is likely why a food supplement like tyrosine is ineffective at combating its symptoms.

Nevertheless, depressed individuals with low levels of dopamine, adrenaline or noradrenaline may benefit from supplementing with tyrosine.

In fact, one study among individuals with dopamine-deficient depression noted that tyrosine provided clinically significant benefits

Dopamine-dependent depression is characterized by low energy and a lack of motivationUntil more research is available, the current evidence does not support supplementing with tyrosine to treat symptoms of depression Summary Tyrosine can be converted into neurotransmitters that affect mood. However, research doesn't support supplementing with it to combat symptoms of depression.

Side Effects of Tyrosine

Tyrosine is “generally recognized as safe” (GRAS) by the Food and Drug Administration (28).

It has been supplemented safely at a dose of 68 mg per pound (150 mg per kg) of body weight per day for up to three months While tyrosine is safe for most people, it can cause side effects and interact with medications.

Monoamine Oxidase Inhibitors (MAOIs)

Tyramine is an amino acid that helps regulate blood pressure and is produced by the breakdown of tyrosine.

Tyramine accumulates in foods when tyrosine and phenylalanine are converted to tyramine by an enzyme in microorganisms (31).

Cheeses like cheddar and blue cheese, cured or smoked meats, soy products and beer contain high levels of tyramine (31).

Antidepressant medications known as monoamine oxidase inhibitors (MAOIs) block the enzyme monoamine oxidase, which breaks down excess tyramine in the body

Combining MAOIs with high-tyramine foods can increase blood pressure to a dangerous level.

However, it is unknown if supplementing with tyrosine may lead to a buildup of tyramine in the body, so caution is necessary for those taking MAOIs

Thyroid Hormone

The thyroid hormones triiodothyronine (T3) and thyroxine (T4) help regulate growth and metabolism in the body.

It's important that T3 and T4 levels are neither too high nor too low.

Supplementing with tyrosine may influence these hormones This is because tyrosine is a building block for the thyroid hormones, so supplementing with it might raise their levels too high.

Therefore, people who are taking thyroid medications or have an overactive thyroid should be cautious when supplementing with tyrosine.

Levodopa (L-dopa)

Levodopa (L-dopa) is a medication commonly used to treat Parkinson's disease In the body, L-dopa and tyrosine compete for absorption in the small intestine, which can interfere with the drug's effectiveness (38).

Thus, doses of these two drugs should be separated by several hours to avoid this.

Interestingly, tyrosine is being investigated for alleviating some of the symptoms associated with cognitive decline in older adultsSummary Tyrosine is safe for the majority of people. However, it may interact with certain medications.

How to Supplement With Tyrosine

As a supplement, tyrosine is available as a free-form amino acid or N-acetyl L-tyrosine (NALT).

NALT is more water-soluble than its free-form counterpart, but it has a low conversion rate to tyrosine in the body

This means that you would need a larger dose of NALT than tyrosine to get the same effect, making the free-form the preferred choice.

Tyrosine is commonly taken in doses of 500–2,000 mg 30–60 minutes before exercise, even though its benefits on exercise performance remains inconclusive (42, 43).

It does seem to be effective for preserving mental performance during physically stressful situations or periods of sleep deprivation when taken in doses ranging from 45–68 mg per pound (100–150 mg per kg) of body weight.

This would be 7–10 grams for a 150-pound (68.2-kg) person.

These higher doses may cause gastrointestinal upset and be split into two separate doses, taken 30 and 60 minutes prior to a stressful event.

Summary Tyrosine as a free-form amino acid is the best form of the supplement. Its greatest
anti-stress effects have been observed when it's taken in doses of 45-68 mg per pound (100–150 mg per kg) of body weight about 60 minutes before a stressful event.

The Bottom Line

Tyrosine is a popular dietary supplement used for a variety of reasons.

In the body, it's used to make neurotransmitters, which tend to decrease under periods of stressful or mentally demanding situations.

There is good evidence that supplementing with tyrosine replenishes these important neurotransmitters and improves mental function, compared to a placebo.

Supplementing with it has been shown to be safe, even in high doses, but has the potential to interact with certain medications, warranting caution.

While tyrosine has many benefits, their significance remains unclear until more evidence is available.

Molecular Nutrition & Food Research
www.mnf-journal.com

L-Citrulline Supplementation-Increased Skeletal Muscle PGC-1α Expression Is Associated with Exercise Performance and Increased Skeletal Muscle Weight

*Myra O. Villareal, Toshiya Matsukawa, and Hiroko Isoda**

Scope: L-citrulline has recently been reported as a more effective supplement for promoting intracellular nitric oxide (NO) production compared to L-arginine. Here, the effect of L-citrulline on skeletal muscle and its influence on exercise performance were investigated. The underlying mechanism of its effect, specifically on the expression of skeletal muscle peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α), was also elucidated.
Methods and results: Six-week-old ICR mice were orally supplemented with L-citrulline (250 mg kg^{-1}) daily, and their performance in weight-loaded swimming exercise every other day for 15 days, was evaluated. In addition, mice muscles were weighed and evaluated for the expression of PGC-1α and PGC-1α-regulated genes. Mice orally supplemented with L-citrulline had significantly higher gastrocnemius and biceps femoris muscle mass. Although not statistically significant, L-citrulline prolonged the swimming time to exhaustion. PGC-1α upregulation was associated with vascular endothelial growth factor α (*VEGFα*) and insulin-like growth factor 1 (*IGF-1*) upregulation. *VEGFα* and *IGF-1* are important for angiogenesis and muscle growth, respectively, and are regulated by PGC-1α. Treatment with NG-nitro-L-arginine methyl ester hydrochloride (L-NAME), a nitric oxide synthesis inhibitor, suppressed the L-citrulline-induced *PGC-1α* upregulation in vitro.
Conclusion: Supplementation with L-citrulline upregulates skeletal muscle PGC-1α levels resulting in higher skeletal muscle weight that improves time to exhaustion during exercise.

1. Introduction

Regular exercise and being active physically enhance endurance during exercise and more importantly, improve metabolic dysfunction that may help prevent lifestyle-related diseases.[1,2] Physical inactivity increases the risk of development of obesity, type 2 diabetes, sarcopenia, hypertension, and cardiovascular diseases.[3] Physically active people's life expectancy, without long-standing illness, is in fact 8–10 years longer compared to that of inactive people.[4] Several studies have demonstrated that enhancement of the skeletal muscle functions is observed to be the major positive impact of exercise.[1,2] This means that enhancing the skeletal muscle functions, in terms of improved mitochondrial biogenesis, capillaries, and fatty acid transporters, significantly improve exercise capacity.

Peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α) is the transcriptional coactivator responsible for the regulation of mitochondrial biogenesis, angiogenesis, oxidative metabolism, and muscle growth, and has a crucial role in the adaptation of muscles to exercise.[5,6] Muscle-specific overexpression of PGC-1α increases exercise capacity and improves maximal oxygen uptake (VO_{2max}) by increasing mitochondrial biogenesis and capillary density in skeletal muscle.[7] Skeletal muscle PGC-1α is known to have an important role in exercise adaptation and enhancement of exercise capacity.[8,9]

Nitric oxide (NO) is a reactive nitrogen molecule synthesized enzymatically through the catalytic action of nitric oxide synthase (NOS). NO is expressed in the skeletal muscles of mammals and acts as a second messenger in transduction pathways associated with the expression of genes for oxidative metabolism, vasodilation, and skeletal muscle contraction. NOS inhibition also reduces the maximal oxygen uptake during exercise in humans.[10] Conversely, increased intracellular NO production leads to phosphorylation of CREB that in turn induces PGC-1α expression.[11] Thus, several studies have indicated that the physiological amount of NO positively impacts adaptation to exercise, much so that nitrate supplementation has been

Dr. M. O. Villareal, Dr. H. Isoda
Faculty of Life and Environmental Sciences
University of Tsukuba
Tsukuba City, 305-8572, Japan
E-mail: isoda.hiroko.ga@u.tsukuba.ac.jp
Dr. M. O. Villareal, Dr. H. Isoda
Alliance for Research on Mediterranean and North Africa
University of Tsukuba
Tsukuba City, 305-8572, Japan
Dr. T. Matsukawa
Graduate School of Life and Environmental Sciences
University of Tsukuba
Tsukuba City, 305-8572, Japan

DOI: 10.1002/mnfr.201701043

considered to be an ergogenic supplementation for athletes or in sports.[12,13]

Normally, physiological NO is derived from L-arginine. However, absorption of orally administered L-arginine is hampered by first-pass and systemic metabolism. L-citrulline, on the other hand, is only subject to systemic metabolism. L-citrulline, a nonessential amino acid, has recently been recognized as an effective alternative source of NO, and therefore can be taken as a dietary supplement to increase intracellular NO production.[14] Oral supplementation with L-citrulline increases NO levels by increasing endothelial NO synthase (eNOS) expression that results in improved endothelial function.[15,16] Neuronal NOS (nNOS) and eNOS are also expressed in the skeletal muscle.[11] L-citrulline is a precursor of L-arginine but has several functional advantages over L-arginine.[14,15] L-citrulline is superior to L-arginine in terms of ease of handling and palatability since it is tasteless, odorless, and non-hygroscopic, whereas L-arginine tends to be extremely bitter and highly water absorbent.[17] Therefore, L-citrulline is considered to be more effective in enhancing exercise performance by virtue of its effect on skeletal muscle regulation. Several studies have reported that L-citrulline supplementation enhances exercise performance[18–20]; however, the underlying mechanism of its effect on skeletal muscles and exercise performance, has yet to be elucidated. In this study, the effect of L-citrulline supplementation on exercise and the underlying cause of those effects were investigated in vivo, using exercised mice, and in vitro, using skeletal muscle cells.

2. Experimental Section

2.1. Chemicals

Rhodamine 123, L-NAME, and sodium dodecyl sulfate (SDS) were purchased from Wako (Tokyo, Japan). ISOGEN was purchased from Nippongene (Tokyo, Japan). DMEM, RIPA buffer, protease inhibitor cocktail, and β-Actin antibody were purchased from Sigma (MO, USA). Fetal bovine serum (FBS) and house serum (HS) were purchased from Gibco (NY, USA). MTT or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and L-NAME were purchased from Dojindo (Kumamoto, Japan). L-citrulline was supplied by Kyowa Hakko Bio Co., Ltd., Tokyo, Japan) while PGC-1α (3G6) antibody was purchased from Cell Signaling Technology (Hertfordshire, UK). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (6C5) was purchased from Santa Cruz Biotechnology (CA, USA).

2.2. Animal Experiments

Five-week-old male ICR mice were obtained from Charles River Laboratories (Kanagawa, Japan) and maintained under a 12-h light/dark cycle, with free access to water and a normal diet (MF, Oriental Yeast Co., Ltd., Japan). Body weight was measured daily while food intake was measured once a week. After 1 week of acclimatization, the mice were divided into three groups. Group 1 ("no exercise" group) was orally administered with distilled water (D.W.) without swimming exercise ($n = 6$). Group 2 (control group) was orally administered with water. Group 3 (L-citrulline group) was orally administered with 250 mg kg^{-1} day of L-citrulline. The L-citrulline dose used in this study was based on Takeda et al.'s[21] study. Both Groups 2 and 3 performed swimming exercises ($n = 7$ per group). Oral administration of 100 μL sample solution was done using animal-feeding needles (Group 1 and 2: D.W. or Group 3: 250 mg kg^{-1} L-citrulline dissolved in water) every day for 15 days, 1 h before the swimming exercise, and at the same time on "no exercise" days. The exercise protocol was adapted from Takeda et al.[21] with some modifications. Briefly, mice with a load corresponding to 5% of their body weight attached to their tails, were trained to perform the swimming exercise for 10 min, in a tank (30 × 30 × 40 cm) filled with water to a depth of 25 cm., with water temperature kept at 30 ± 1 °C. The swimming exercise was performed every other day for 14 days. On day 15, the mice were made to swim to exhaustion with a load corresponding to 10% of their body weight. Each mouse was considered to have reached its point of exhaustion when it failed to raise its face from the water surface to breathe within a period of 5 s. Blood lactate levels were measured before and after exercise (0 and 60 min) using Lactate Pro 2 (Arkrey, Japan). Blood glucose levels were measured after exercise (0 min) using Glucose Pilot system (Iwai Chemicals Company, Japan). The mice were sacrificed and blood and tissues from the liver, gastrocnemius, and biceps femoris were collected. The serum was separated from the blood by centrifugation at 3000 × g for 10 min and the serum biochemical parameters (BUN, creatinine, total ketone bodies, AST, ALT, ALP, nonessential fatty acid [NEFA]) were analyzed by Oriental Yeast Co., Ltd., (Japan) using test kits obtained from Wako Pure Chemical Industries (Osaka, Japan). All animal experiments performed are in compliance with the guidelines and regulations for Animal Experiments of the University of Tsukuba (No. 16-044), and were approved by the International Animal Care and Use Committee of the University of Tsukuba.

2.3. Real-Time PCR Analysis

Total RNA was isolated from tissue samples (50 mg) and C2C12 myotubes using ISOGEN. For C2C12 myotubes, cells were treated with or without L-citrulline or NOS inhibitor L-NAME. Total RNA isolation and TaqMan real-time PCR amplification reactions were performed as previously reported.[22] For the quantification of the gene expression in muscle tissues and C2C12 myotubes, the following specific TaqMan probes purchased from Applied Biosystems (CA, USA) were used: *β-actin* (Mm00607939_s1), *PGC-1α* (Mm01208835_m1), *LDHa* (Mm01612132_g1), *LDHb* (Mm01267402_m1), *MCT1* (Mm01306379_m1), *CPT-1β* (Mm00487191_g1), *TFAM* (Mm00447485_m1), vascular endothelial growth factor α (*VEGFα*) (Mm00437306_m1), and insulin-like growth factor 1 (*IGF-1*) (Mm00439560_m1). The mRNA levels of all genes were normalized to *β-actin* mRNA levels (internal control).

2.4. Western Blotting

Total protein was isolated from tissue samples (10 mg) using RIPA buffer containing a protease inhibitor cocktail according to

the manufacturer's instructions. Protein samples (15 μg) were separated using 10% SDS-PAGE and transferred to a PVDF membrane (Merck Millipore, USA). Membranes were incubated with primary antibody at 4 °C overnight, then washed, and incubated with secondary antibodies (IRDye 800CW donkey antirabbit IgG or IRDye 680LT goat antimouse [LI-COR, Inc., NE, USA]) at room temperature for 30 min. The signal was detected using the Odyssey Fc Imaging System (LI-COR, Inc., NE, USA).

2.5. Cell Culture and Differentiation

The mouse C2C12 myoblasts (ATCC, USA) were cultured in DMEM supplemented with 10% FBS and 1% penicillin (5000 μg mL^{-1})–streptomycin (5000 IU mL^{-1}) (Lonza, Tokyo, Japan). To induce C2C12 myoblasts to differentiate into C2C12 myotubes, C2C12 cells were cultured until confluent and then transferred to DMEM containing 2% horse serum, and incubated further for 5 days with the growth medium changed every other day.

2.6. MTT Assay

Following treatment and incubation with L-citrulline at different concentrations, MTT solution (5 mg mL^{-1}) was added to the C2C12 myotubes culture and incubated further for 3 h until formazan crystals were formed. Formazan crystals were then dissolved by adding 10% SDS and the plates incubated further for 16 h. The absorbance at 570 nm was measured using a Powerscan HT plate reader (Dainippon Sumitomo Pharma Co, Ltd., Japan).

2.7. Statistical Analysis

All the results are expressed as the mean ± standard deviation, and statistical evaluation was performed using the Student's *t*-test when two value sets were compared. Analysis that includes multiple comparisons were carried out using one-way analysis of variance or ANOVA, followed by Tukey's multiple comparison test using SPSS (IBM Statistics for Windows, version 22.0. IBM Corp., Armonk, NY). $p \leq 0.05$ was considered to be statistically significant.

3. Results

3.1. L-Citrulline Supplementation before Exercise Prevented Exercise-Induced Elevation of Blood Lactate and Decrease in Glucose Levels

To examine the effect of L-citrulline supplementation on blood lactate and glucose levels during exercise, mice were orally administered with L-citrulline or water (control), 1 h before exercise, for 15 days. On day 13, mice were made to perform weight-loaded forced swimming test for 10 min, and 1 h later, were given L-citrulline supplementation (**Figure 1**). Changes in the blood lactate levels during exercise are associated with exercise performance. Lactate that accumulates in the muscles produces H^+ ions that cause fatigue, impairing muscle function and performance.[23] As shown in **Figure 2**A, before exercise, the blood lactate levels between the control and L-citrulline groups were not significantly different. However, after exercise, the lactate level of the L-citrulline group was lowered (9.7 ± 2.0 mM vs. 7.3 ± 0.5 mM, $p = 0.055$). The difference in the lactate levels before and after exercise however, was bigger in the control group. Lactate is produced when glucose or glycogen is used as a fuel source, so that an increase in the glucose levels would lead to higher lactate levels. Additionally, the L-citrulline group had significantly higher glucose levels after exercise on day 13 compared to the control (142 ± 26.8 mg dL^{-1} vs. 174 ± 21.5 mg dL^{-1}, $p = 0.04$) (Figure 2B).

3.2. L-Citrulline Supplementation before Exercise Increased Muscle Weight and Increased Time to Exhaustion in Swimming

To evaluate the effect of L-citrulline supplementation on exercise performance, mice were made to perform a weight-loaded forced swimming test until exhaustion, with a load corresponding to 10% of their body weight, 1 h after L-citrulline supplementation (Figure 1). As shown in **Figure 3**A, the L-citrulline group had longer time to exhaustion, though it was not statistically significant, compared to that of the control groups (270 ± 64.4 s vs. 537 ± 285.2 s, $p = 0.07$). Although the mice in the L-citrulline supplementation group swam for a longer period of time than the control group, the lactate levels between these groups were not significantly different (12.6 ± 0.7 mM vs. 14.0 ± 2.6 mM, $p = 0.27$) (Figure 3B). Also, compared to the control, the L-citrulline group had significantly lower blood glucose levels (153 ± 5.4 mg dL^{-1} vs. 119 ± 7.9 mg dL^{-1}, $p < 0.01$) (Figure 3C). Additionally, L-citrulline supplementation showed an increase in the weight of the gastrocnemius (0.23 ± 0.02 g vs. 0.28 ± 0.02 g, $p = 0.02$) and biceps femoris muscles (0.34 ± 0.01 g vs. 0.52 ± 0.07 g, $p < 0.01$) compared to the control, even though the body weights of both groups were not significantly different (**Table 1**). The food intake, including the amino acids from feeds, was also not significantly different between the control and the L-citrulline-administered groups (data not shown). Although other studies have reported that L-citrulline elevates the levels of urea nitrogen, creatinine, and total ketone bodies in the blood after exercise,[24,25] this study shows that L-citrulline supplementation lowered the creatinine level (0.20 ± 0.05 mg dL^{-1} vs. 0.14 ± 0.02 mg dL^{-1}, $p = 0.06$) and total ketone bodies (1163.3 ± 245.7 μM vs. 948.4 ± 206.6 μM, $p = 0.15$) over time (**Table 2**).

3.3. L-Citrulline Supplementation Upregulated the PGC-1α Expression in Gastrocnemius and Biceps Femoris

PGC-1α in the skeletal muscle has significant regulatory role in the muscles' several adaptations to exercise such as lactate metabolism, angiogenesis, and muscle growth.[1,5] Since the gastrocnemius and biceps femoris are extensively used during swimming, we specifically evaluated the effects of L-citrulline supplementation on the gastrocnemius and biceps femoris. Supplementation with L-citrulline greatly increased *PGC-1α*

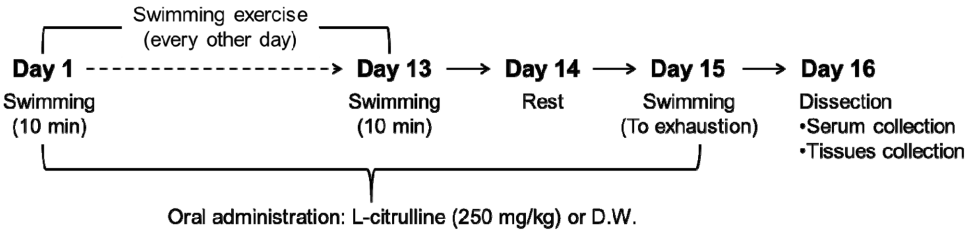


Figure 1. Study design. Mice were made to perform a swimming exercise every other day for 14 days. A swimming-until-exhaustion test was carried out on day 15. During the experimental period, mice were orally administrated with L-citrulline (250 mg kg^{-1}) or distilled water (D.W.) every day.

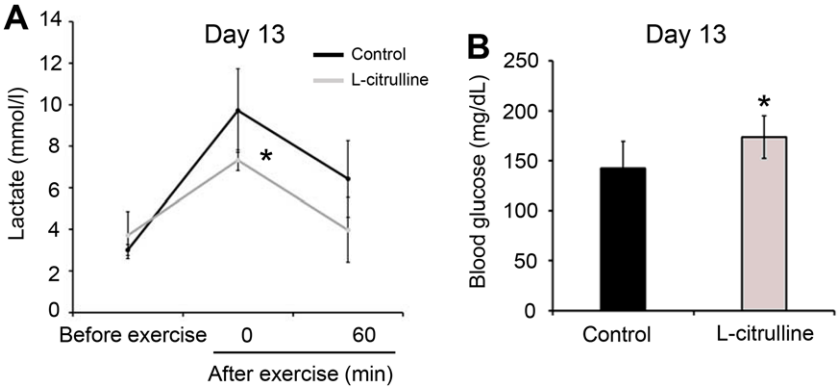


Figure 2. Effect of L-citrulline supplementation on the blood lactate and glucose levels after weight-loaded exercise performance. Mice orally administrated with L-citrulline (250 mg kg^{-1}) or distilled water (D.W.) were made to perform a weight-loaded forced swimming test for 10 min on day 13. A) Blood lactate levels before and after exercise (0 and 60 min) and B) blood glucose levels after exercise (0 min) were evaluated. Values are expressed as the mean ± standard deviation. $*p \leq 0.05$ and $**p \leq 0.01$ indicate a significant difference compared to the control group.

expression in the gastrocnemius (**Figure 4**A; 3.6 ± 0.5-fold, $p < 0.01$) and biceps femoris (Figure 4B; 2.8 ± 0.6-fold, $p < 0.01$). As shown in **Figure 5**, the protein expression level of PGC-1α in the gastrocnemius and biceps femoris (2.6 ± 0.7-fold, $p < 0.01$ and 2.3 ± 0.8-fold, $p = 0.02$, respectively) was also increased with L-citrulline supplementation. VEGFα and *IGF-1*, cytokines released from skeletal muscle are important factors for angiogenesis and muscle growth, respectively, and are under the regulation of PGC-1α.[5] As shown in Figure 4, L-citrulline-supplemented mice had significantly higher levels of *VEGFα* and *IGF-1* in their gastrocnemius (1.8 ± 0.3-fold, $p < 0.01$ and 1.3 ± 0.1-fold, $p < 0.01$, respectively) and biceps femoris (1.4 ± 0.2-fold, $p < 0.01$ and 1.5 ± 0.2-fold, $p < 0.01$, respectively) compared to the control group. The role of PGC-1α in the promotion of lactate metabolism through increased lactate dehydrogenase (LDH) B and monocarboxylate transporter 1 (MCT1) expression in the skeletal muscle has already been established.[26] L-citrulline supplementation also increased the *MCT1* expression in the gastrocnemius (1.5 ± 0.4-fold, $p = 0.05$) and biceps femoris (1.3 ± 0.2-fold, $p < 0.05$). The upregulation of *LDH B*, however, was only observed in the gastrocnemius (1.6 ± 0.3-fold, $p < 0.01$) (Figure 4).

3.4. L-Citrulline-Induced *PGC-1α* Upregulation in C2C12 Myotubes was Suppressed by NO Synthesis Inhibition

To investigate the effect of L-citrulline on the expression of *PGC-1α* in the skeletal muscle cells, the effective and non-cytotoxic concentrations of citrulline were first determined by performing MTT assay using C2C12 myotubes, a cellular model of skeletal muscle. L-citrulline treatment significantly increased the proliferation of C2C12 myotubes but a gradual decrease was observed starting at concentrations of more than 500 μM L-citrulline (**Figure 6**A). Considering the MTT assay results, 100 μM L-citrulline was chosen as the best concentration to use in the succeeding experiments. Treatment with 10, 50, and 100 μM L-citrulline for 1 h increased the *PGC-1α* expression in C2C12 myotubes by 1.2 ± 0.1-fold, 1.4 ± 0.1-fold, and 1.5 ± 0.1-fold, respectively ($p < 0.01$) (Figure 6B). L-citrulline is known to elevate intracellular NO levels by promoting NOS activity.[14–16] Consequently, an elevation of intracellular NO production increases muscle cell's *PGC-1α* expression.[27] In the current study, the upregulation of *PGC-1α* expression in C2C12 myotubes by L-citrulline treatment was suppressed in the presence of NOS inhibitor L-NAME (Figure 6C).

4. Discussion

In the field of sports physiology, nitrate supplementation is believed to be an effective strategy for the enhancement of exercise performance. Increased intracellular NO production increases PGC-1α expression via phosphorylation of CREB.[11] The transcriptional activator PGC-1α has an important role in the regulation of genes associated with adaptation to exercise such as oxidative metabolism, angiogenesis, and muscle growth.[1] It has been reported that non-protein amino acids, (e.g., ornithine, citrulline,

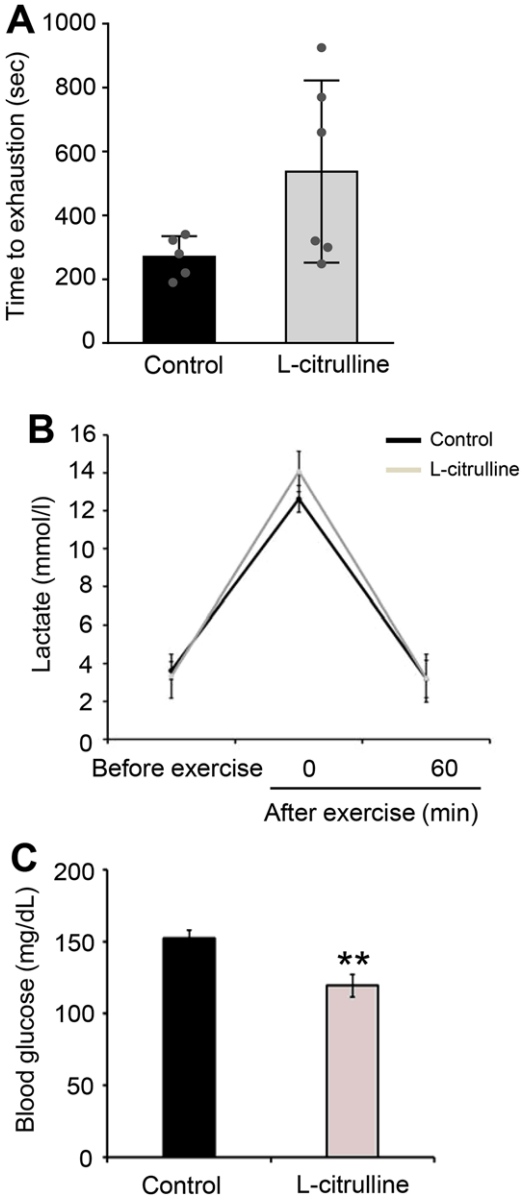


Figure 3. Effect of L-citrulline supplementation on swimming endurance. Mice were trained to perform swimming exercise every other day for 14 days, then a swimming-until-exhaustion test was carried out on day 15. A) Swimming time to exhaustion, B) blood lactate levels before and after exercise (0 and 60 min), and C) blood glucose levels after exercise (0 min) were evaluated. Values are expressed as the mean ± standard deviation. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ indicate a significant difference compared to the control group. The individual raw data was plotted over the bar graph.

Table 1. Mice body weight, food intake, and tissue weight.

		Swimming	
	No exercise	Control	L-citrulline
Initial body weight [g]	28.7 ± 1.3	28.2 ± 1.4	28.5 ± 0.8
Final body weight [g]	30.7 ± 1.2	31.6 ± 2.0	32.5 ± 1.7
Food intake [g day^{-1}]	3.67 ± 0.12	3.64 ± 0.23	3.92 ± 0.32$^{\#}$
Kidney [g]	0.43 ± 0.01	0.50 ± 0.03**	0.45 ± 0.02$^{\#}$
Liver [g]	1.19 ± 0.04	1.18 ± 0.04	1.21 ± 0.06
Gastrocnemius [g]	0.24 ± 0.03	0.23 ± 0.02	0.28 ± 0.02$^{*,\#\#}$
Biceps femoris [g]	0.26 ± 0.03	0.34 ± 0.01**	0.52 ± 0.07$^{**,\#}$

Values are expressed as the mean ± standard deviation. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ indicate a significant difference from the "no exercise" group. $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.01$ indicate a significant difference compared to the control group.

Table 2. Level of serum biochemical parameters at the end of the study.

		Swimming	
	No exercise	Control	L-citrulline
BUN [mg dL^{-1}]	32.3 ± 0.9	23.9 ± 2.1**	23.3 ± 2.7**
Creatinine [mg dL^{-1}]	0.15 ± 0.01	0.20 ± 0.05	0.14 ± 0.02
Total ketone bodies [μM]	593.3 ± 53.3	1163.3 ± 245.7**	948.4 ± 206.6*
AST [IU L^{-1}]	1782.5 ± 280.9	1922.0 ± 461.2	1750.4 ± 238.1
ALT [IU L^{-1}]	226.3 ± 3.5	226.8 ± 40.4	201.7 ± 23.7
ALP [IU L^{-1}]	345.0 ± 17.9	433.7 ± 37.6**	369.4 ± 40.9$^{\#}$
NEFA [μEq L^{-1}]	1125.8 ± 140.9	1298.2 ± 129.8	1180.3 ± 158.1

Values are expressed as the mean ± standard deviation. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ indicate a significant difference compared to the "no exercise" group. $^{\#}p \leq 0.05$ indicates a significant difference compared to the control group. BUN, urea nitrogen; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; NEFA, non-esterified fatty acid.

and homocysteine) which are not used for protein synthesis, play an important role in cell metabolism.[28]

L-citrulline, a non-essential amino acid, has recently been demonstrated to be effective in increasing of intracellular NO production by increasing NOS expression.[14–16] The major findings of this study are that the upregulation of PGC-1α in the skeletal muscle by L-citrulline is associated with increased exercise performance and muscles weight. L-citrulline is a precursor of L-arginine that is considered to be preferable than L-arginine in terms of solubility and taste, as well as several functional advantages.[14,15,17] Moreover, L-arginine can enhance NO production but it is rapidly metabolized in the small intestine and liver when administered orally.[14] Therefore, when compared to L-citrulline, L-citrulline comes out as a more effective sports supplement for enhancing sports performance compared to L-arginine. Some studies, have reported that single supplementation of L-citrulline does not help improve exercise performance[21,29] suggesting the need for long-term supplementation of L-citrulline (more than 1 week) to effectively enhance exercise tolerance.[20,21] The current study also provides data to validate assumptions of past researches that L-citrulline supplementation for 15 days can enhance exercise performance and increase muscle mass. The results of this study therefore suggest that long-term intake of L-citrulline has a positive impact on exercise performance.

Lactate is produced during intense exercise when the supply of O_2 is insufficient. The blood lactate level during exercise is dependent on the ratio of lactate production to lactate clearance.[23,30] Hydrogen ions (H^+) dissociate from lactic acid and accumulate in the muscles, causing fatigue and depressing muscle function and contraction.[23] In addition, decreased blood glucose levels

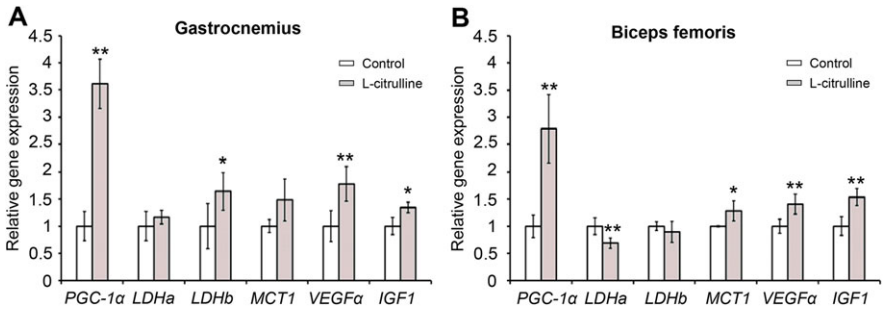


Figure 4. Effect of L-citrulline supplementation on the expression of *PGC-1α* and PGC-1α-related genes in the gastrocnemius and biceps femoris. Expression levels of *PGC-1α* and PGC-1α-targeted genes in the A) gastrocnemius and B) biceps femoris were evaluated. Expression levels of mRNA were normalized to the *β-actin* expression level. Values are expressed as the mean ± standard deviation and relative to the "no exercise" group. $*p \leq 0.05$ and $**p \leq 0.01$ indicate a significant difference compared to the control group.

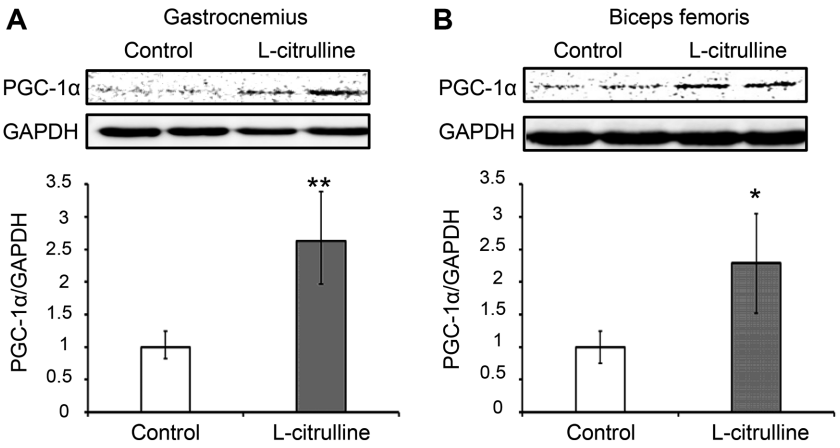


Figure 5. Effect of L-citrulline supplementation on the protein expression of PGC-1α in the gastrocnemius and biceps femoris. Protein expression levels of PGC-1α in the A) gastrocnemius and B) biceps femoris were evaluated. All gels were run under the same experimental conditions and the representative blots were shown. The protein expression levels were normalized to GAPDH expression. Values are expressed as the mean ± standard deviation and relative to the unexercised group. $*p \leq 0.05$ and $**p \leq 0.01$ indicate a significant difference from the control group.

(hypoglycemia) during exercise also causes fatigue and low energy, leading to exercise cessation.[31] The L-citrulline supplemented mice group had lower blood lactate levels and higher glucose levels immediately after exercising with the same load (Figure 2). These results therefore suggest that the observed inhibition of lactate production and/or increased lactate metabolism during exercise can be attributed to L-citrulline supplementation. However, the blood lactate levels after exercise were not different from the after the swimming-to-exhaustion test blood lactate levels (Figure 3). Following a swimming-to-exhaustion test, the blood lactate levels gradually increase depending on the duration of the exercise.[32] In this study, L-citrulline supplemented group had longer average swimming to exhaustion time compared to the control (270 s vs. 537 s, respectively) (Figure 3A). Therefore, it can be assumed that blood lactate levels before and after the swimming-to-exhaustion test remained the same. Skeletal muscle PGC-1α promotes lactate metabolism by increasing the expression of LDH B and MCT1, and conversely, prevents lactate production by suppressing the expression of LDH A that catalyzes the conversion of pyruvate to lactate.[1,26] A decrease in *LDH A* expression and increase in *MCT1* and *LDH B* levels were also observed following L-citrulline supplementation (Figure 3). Several studies reported have established that L-citrulline has antidiabetic and antiobesity effects[33–35] but does not affect the blood glucose level. So, in this study, it can then be assumed that L-citrulline did not affect the blood glucose levels and instead suggests that a decrease in lactate production and an increase in lactate metabolism are therefore involved in the regulation of blood lactate and glucose levels. PGC-1α mRNA level is elevated after performing exercise but the level returns back to its "before exercise level" during the rest period, specifically within 24 h after exercise.[36,37] Even though the muscle samples were collected 24 h after exercise, an increase in PGC-1α mRNA and protein expression due to L-citrulline supplementation was still observed (Figure 2). Therefore, it is clear that the increase in lactate metabolism can be attributed to the longitudinal PGC-1 α upregulation by L-citrulline rather than a transient upregulation by exercise.

Increased blood flow enhances not only exercise performance, by improving nutrient and oxygen delivery in muscle, but also by boosting protein synthesis and muscle fiber repair.[38] Several reports have suggested that L-citrulline supplementation can improve blood pressure, VO_2 kinetics, and exercise performance in healthy adults,[19,20] and believed to be due to PGC-1α upregulation in the muscles that promoted formation of new blood vessels (angiogenesis), and thus, integrating oxygen/nutrient

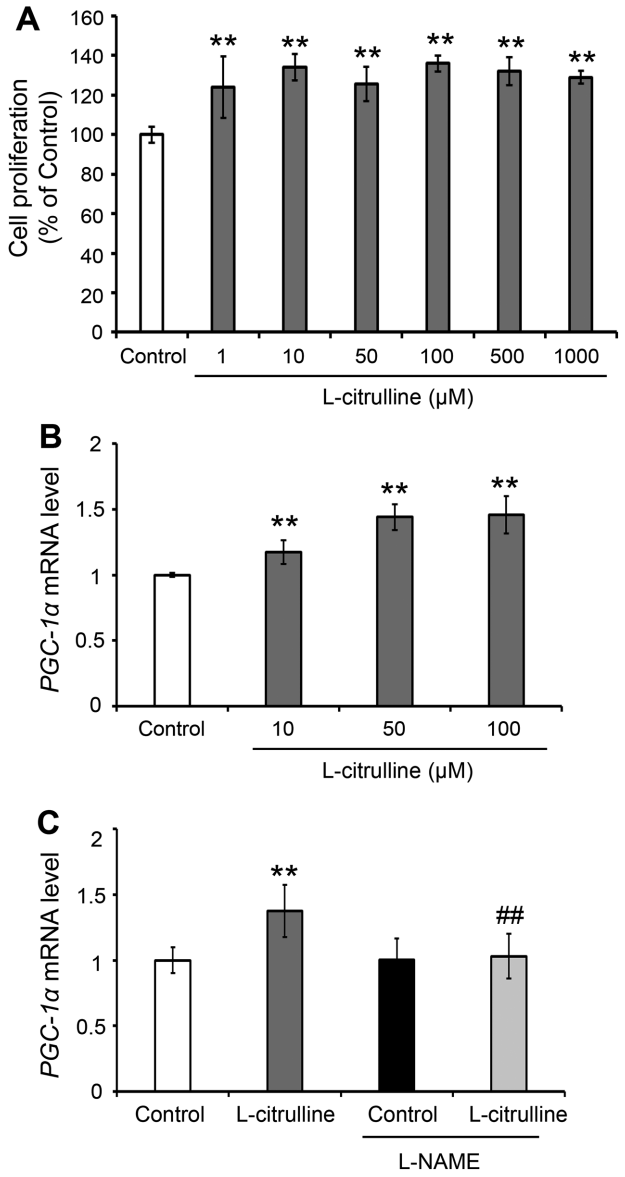


Figure 6. Effect of L-citrulline on the gene expression of *PGC-1α* in C2C12 myotubes. Differentiated C2C12 myotubes were treated with or without L-citrulline for 24 h. A) After that, cell viability was evaluated and value expressed as percentage (%) of control. B) C2C12 myotubes were treated with or without L-citrulline (10, 50, 100 μM) for 1 h. C) L-citrulline (50 μM) treatment was performed with or without 100 μM NG-nitro-L-arginine methyl ester hydrochloride (L-NAME) for 1 h. Following treatment, *PGC-1α* mRNA levels were quantified using real-time PCR and the values normalized to the expression level of *β-actin*. Values are expressed as the mean ± standard deviation of triplicate experiments. $^{**}p \leq 0.01$ indicates a significant difference from the control group. $^{\#\#}p \leq 0.01$ indicate a significant difference from the L-NAME-treated L-citrulline group.

consumption and supply. PGC-1α expression in cultured muscle cells and in skeletal muscle promotes the expression of several angiogenic factors, including VEGF, which plays a crucial role in vascular development. At the same time, muscle vascularization by VEGFα increases blood supply and oxygen availability in the muscle, increasing exercise time and endurance.[39] In the current study, an L-citrulline-induced increase in skeletal muscle *VEGFα* expression was observed (Figure 4), suggesting that L-citrulline supplementation-induced angiogenesis in skeletal muscle can be associated with the observed increase in swimming time. It is a well-known fact that oral supplementation with L-citrulline elevates NO levels by increasing NOS expression, resulting in improved endothelial function.[15] On the other hand, NOS inhibition has been established to reduce maximal oxygen uptake during exercise in humans.[13] However, increased intracellular NO production induces PGC-1α expression.[11] In this study, L-citrulline-induced *PGC-1α* upregulation in C2C12 myotubes was suppressed by L-NAME, a NOS inhibitor (Figure 6), suggesting, therefore, that L-citrulline-increased skeletal muscle PGC-1α level was due to the rise in the intracellular NO production.

Several PGC-1α variants are expressed from alternative gene promoter, namely PGC-1α-b and PGC-1α4, and have been shown to induce VEGF expression in skeletal muscle and angiogenesis.[40] Transgenic expression of PGC-1α4 in skeletal muscle in mice induces angiogenesis in vivo.[40] PGC-1α4 also activates a hypertrophic gene program in skeletal muscle. In addition, PGC-1α4 also regulates skeletal muscle growth by inducing the anabolic hormone IGF-1 and repressing myostatin, a powerful inhibitor of muscle differentiation and growth.[7,41] In this study, skeletal muscle weight was increased corresponding to PGC-1α and IGF-1 upregulation by L-citrulline. It can therefore be inferred from these results that L-citrulline induces PGC-1α4 in association with L-citrulline-induced *VEGFα* and *IGF-1* upregulation. Elevated PGC-1α in muscle dramatically protects against the sarcopenia, obesity, and diabetes that normally accompanies aging.[42] Therefore, the use of L-citrulline to increase PGC-1α expression may be useful in the management of diseases such as obesity, diabetes, and sarcopenia, as well as in the enhancement of exercise performance.

5. Concluding Remarks

L-citrulline supplementation before exercise upregulates PGC-1α expression in the skeletal muscle, resulting in a significant increase in skeletal muscle weight. A longer average time before mice became exhausted during exercise was also observed in L-citrulline-supplemented animals, but the significance of this effect needs further verification in a clinical trial. Furthermore, inhibition of NOS expression suppresses the L-citrulline-induced *PGC-1α* upregulation. Further experiments would aim to compare the effect of other amino acids to the effect of L-citrulline on enhancing exercise performance and increasing muscle weight. This study has demonstrated that L-citrulline supplementation resulted in a significant improvement in exercise performance and increased skeletal muscle weight, and therefore may be used to enhance sports or exercise performance.

Acknowledgments

This work was supported by the Center of Innovation Program (COI) of the University of Tsukuba and financially supported by the Ministry of Education Culture and Sports (MEXT), Japan, and Japan Science and Technology

(JST). M.O.V., H.I., and T.M. designed the study; T.M. conducted the experiments, and analyzed the data with M.O.V. and H.I.; M.O.V. and T.M. wrote the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

exercise performance, L-citrulline, *PGC-1α*, skeletal muscle weight, supplementation

Received: December 18, 2017
Revised: May 8, 2018
Published online: May 24, 2018

[1] L. Chen, J. Pei, J. Kuang, H. Chen, Z. Chen, Z. Li, H. Yang, *Metab*. **2015**, *64*, 338.
[2] A. Safdar, J. P. Little, A. J. Stokl, B. P. Hettinga, M. Akhtar, M. A. Tarnopolsky, *J. Biol. Chem*. **2011**, *286*, 10605.
[3] D. E. Warburton, A. Charlesworth, A. Ivey, L. Nettlefold, S. S. Bredin, *Int. J. Behav. Nutr. Phys. Act*. **2010**, *7*, 39.
[4] H. Brønnum-Hansen, K. Juel, M. Davidsen, J. Sørensen, *Scand. J. Public Health*. **2007**, *35*, 510.
[5] A. Safdar, J. P. Little, A. J. Stokl, B. P. Hettinga, M. Akhtar, M. A. Tarnopolsky, *J. Biol. Chem*. **2011**, *286*, 10605.
[6] Z. Yan, M. Okutsu, Y. N. Akhtar, V. A. Lira, *J. Appl. Physiol*. **2011**, *110*, 264.
[7] M. Tadaishi, S. Miura, Y. Kai, Y. Kano, Y. Oishi, O. Ezaki, *PloS One* **2011**, *6*, e28290.
[8] C. Handschin, C. A. Choi, S. Chin, S. Kim, D. Kawamori, A. J. Kupad, N. Neubauer, J. Hu, V. K. Mootha, Y.-B. Kim, R. N. Kulkarni, G. I. Shulman, B. M. Spiegelman, *J. Clin. Invest*. **2007**, *117*, 3463.
[9] M. C. Chan, Z. Arany, *Metabolism* **2014**, *63*, 441.
[10] A. M. Jones, D. P. Wilkerson, I. T. Campbell, *J. Physiol*. **2004**, *560*, 329.
[11] S. Baldelli, D. Lettieri Barbato, G. Tatulli, K. Aquilano, M. R. Ciriolo, *J. Cell Sci*. **2014**, *127*, 4813.
[12] R. Bescos, A. Sureda, J. A. Tur, A. Pons, *Sports Med*. **2012**, *42*, 99.
[13] A. M. Jones, *Sports Med*. **2014**, *44*(Suppl 1), S35.
[14] E. Schwedhelm, R. Maas, R. Freese, D. Jung, Z. Lukacs, A. Jambrecina, W. Spickler, F. Schulze, R. H. Boger, *Br. J. Clin. Pharmacol*. **2008**, *65*, 51.
[15] T. Hayashi, P. A. Juliet, H. Matsui-Hirai, A. Miyazaki, A. Fukatsu, J. Funami, A. Iguchi, L. J. Ignarro, *Proc. Natl. Acad. Sci. U.S.A*. **2005**, *102*, 13681.
[16] G. Wu, J. K. Collins, P. Perkins-Veazie, M. Siddiq, K. D. Dolan, K. A. Kelly, C. L. Heaps, C. J. Meininger, *J. Nutr*. **2007**, *137*, 2680.
[17] K. Akashi, C. Miyake, A. Yokota, *FEBS Lett*. **2001**, *508*, 438.
[18] D. Bendahan, J. P. Mattei, B. Ghattas, S. Confort-Gouny, L. M. E. Guern, P. J. Cozzone, *Br. J. Sports Med*. **2002**, *36*, 282.
[19] S. J. Bailey, J. R. Blackwell, T. Lord, A. Vanhatalo, P. G. Winyard, A. M. Jones, *J. Appl. Physiol. (1985)*. **2015**, *19*, 385.
[20] T. Suzuki, M. Morita, Y. Kobayashi, A. Kamimura, *J. Int. Soc. Sports Nutr*. **2016**, *13*, 1.
[21] K. Takeda, M. Machida, A. Kohara, N. Omi, T. Takemasa, *J. Nutr. Sci. Vitaminol. (Tokyo)*. **2011**, *57*, 246.
[22] T. Matsukawa, T. Inaguma, J. Han, M. O. Villareal, H. Isoda, *J. Nutr. Biochem*. **2015**, *26*, 860.
[23] F. Aldeam, R. Irving, L. Dilworth, *AJSSM* **2013**, *1*, 42.
[24] S. Nozaki, M. Tanaka, K. Mizuno, S. Ataka, H. Mizuma, T. Tahara, T. Sugino, T. Shirai, A. Eguchi, K. Okuyama, K. Yoshida, Y. Kajimoto, H. Kuratsune, O. Kajimoto, Y. Watanabe, *Nutrition* **2009**, *25*, 51.
[25] S. Y. Wang, W. C. Huang, C. C. Liu, M. F. Wang, C. S. Ho, W. P. Huang, C. C. Hou, H. L. Chuang, C.-C. Huang, *Molecules* **2012**, *17*, 11864.
[26] S. Summermatter, G. Santos, J. Pérez-Schindler, C. Handschin, *Proc. Natl. Acad. Sci. U.S.A*. **2013**, *110*, 8738.
[27] V. A. Lira, D. L. Brown, A. K. Lira, A. N. Kavazis, Q. A. Soltow, E. H. Zeanah, D. S. Criswell, *J. Physiol*. **2010**, *588*, 3551.
[28] G. Wu, *Amino Acids* **2009**, *37*, 1.
[29] P. T. Cutrufello, S. J. Gadomski, G. S. Zavorsky, *J. Sports Sci*. **2015**, *33*, 1459.
[30] B. Stallknecht, J. Vissing, H. Galbo, *Scand. J. Med. Sci. Sports*. **1988**, *8*, 127.
[31] J. F. Brun, M. Dumortier, C. Fedou, L. Mercier, *Diabetes Metab*. **2001**, *27*, 92.
[32] S. E. Gaskill, B. C. Ruby, A. J. Walker, O. A. Sanchez, R. C. Serfass, A. S. Leon, *Med. Sci. Sports Exerc*. **2001**, *33*, 1841.
[33] P. Persson, A. Fasching, T. Teerlink, P. Hansell, F. Palm, *Hypertension* **2014**, *64*, 323.
[34] M. Kudo, H. Yashitomi, M. Momoo, S. Suguro, Y. Yamagishi, M. Gao, *Biol. Pharm. Bull*. **2017**, *40*, 524.
[35] S. Ikeda, T. Kizaki, S. Haga, H. Ohno, T. Takemasa, *Biochem. Biophys. Res. Comm*. **2008**, *368*, 323.
[36] H. Pilegaard, B. Saltin, P. D. Neufer, *J. Physiol*. **2003**, *546*, 851.
[37] A. S. Mathai, A. Bonen, C. R. Benton, D. L. Robinson, T. E. Graham, *J. Appl. Physiol (1985)*. **2008**, *105*, 1098.
[38] D. J. Weinert, *J. Can. Chiropr. Assoc*. **2009**, *53*, 186.
[39] H. Delavar, L. Noqueira, P. D. Wagner, M. C. Hoqan, D. Metzqer, E. C. Breen, *Am. J. Physiol. Regul. Integr. Comp. Physiol*. **2014**, *306*, 586.
[40] R. Thom, G. C. Rowe, C. Jang, A. Safdar, Z. Arany, *J. Biol. Chem*. **2014**, *289*, 8810.
[41] J. L. Ruas, J. P. White, R. R. Rao, S. Kleiner, K. T. Brannan, B. C. Harrison, N. P. Greene, J. Wu, J. L. Estall, B. A. Irving, I. R. Lanza, K. A. Rasbach, M. Okutsu, K. S. Nair, Z. Yan, L. A. Leinwand, B. M. Spiegelman, *Cell* **2012**, *151*, 1319.
[42] L. L. Ji, C. Kang, *Gerontology* **2015**, *61*, 139.

Routledge
Taylor & Francis Group

Journal of the International Society of Sports Nutrition

ISSN: 1550-2783 (Online) Journal homepage: www.tandfonline.com/journals/rssn20

Oral L-citrulline supplementation enhances cycling time trial performance in healthy trained men: Double-blind randomized placebo-controlled 2-way crossover study

Takashi Suzuki, Masahiko Morita, Yoshinori Kobayashi & Ayako Kamimura

To cite this article: Takashi Suzuki, Masahiko Morita, Yoshinori Kobayashi & Ayako Kamimura (2016) Oral L-citrulline supplementation enhances cycling time trial performance in healthy trained men: Double-blind randomized placebo-controlled 2-way crossover study, Journal of the International Society of Sports Nutrition, 13:1, 6, DOI: 10.1186/s12970-016-0117-z

To link to this article: https://doi.org/10.1186/s12970-016-0117-z

Published online: 01 Apr 2022.

Suzuki et al. Journal of the International Society of Sports Nutrition (2016) 13:6
DOI 10.1186/s12970-016-0117-z

Journal of the International Society of Sports Nutrition

RESEARCH ARTICLE Open Access

Oral L-citrulline supplementation enhances cycling time trial performance in healthy trained men: Double-blind randomized placebo-controlled 2-way crossover study

Takashi Suzuki[1], Masahiko Morita[1], Yoshinori Kobayashi[2] and Ayako Kamimura[1*]

Abstract

Background: Many human studies report that nitric oxide (NO) improves sport performance. This is because NO is a potential modulator of blood flow, muscle energy metabolism, and mitochondrial respiration during exercise. L-Citrulline is an amino acid present in the body and is a potent endogenous precursor of L-arginine, which is a substrate for NO synthase. Here, we investigated the effect of oral L-citrulline supplementation on cycling time trial performance in humans.

Methods: A double-blind randomized placebo-controlled 2-way crossover study was employed. Twenty-two trained males consumed 2.4 g/day of L-citrulline or placebo orally for 7 days. On Day 8 they took 2.4 g of L-citrulline or placebo 1 h before a 4-km cycling time trial. Time taken to complete the 4 km cycle, along with power output/VO_2 ratio (PO/VO_2), plasma nitrite and nitrate (NOx) and amino acid levels, and visual analog scale (VAS) scores, was evaluated.

Results: L-Citrulline supplementation significantly increased plasma L-arginine levels and reduced completion time by 1.5 % ($p < 0.05$) compared with placebo. Moreover, L-citrulline significantly improved subjective feelings of muscle fatigue and concentration immediately after exercise.

Conclusions: Oral L-citrulline supplementation reduced the time take to complete a cycle ergometer exercise trial.

Trial registration: Current Controlled Trials UMIN000014278.

Keywords: Ergogenic, Human, L-Citrulline, Nitric oxide (NO), Sport performance

Background

NO plays key roles such as maintaining the function and integrity of the endothelium, including vascular tone and structure [1]. In sports physiology, nitrate supplementation is thought to be an ergogenic aid [2–4]. This view is based on evidence that NO is an important modulator of blood flow and mitochondrial respiration under physiological conditions [5]. Some studies have shown that dietary NO related supplements, such as nitrate-rich beetroot juice, enhance human sport performance [6–9]. Dietary supplementation with nitrate thus appears to be beneficial for exercise.

There is growing interest in the use of L-citrulline as an NO-related dietary ingredient. L-Citrulline is present in the body and is a potent endogenous precursor of L-arginine [10], which is a substrate for NO synthase (NOS). NOS catalyzes a complex enzymatic reaction that leads to NO formation from L-arginine and oxygen and generates L-citrulline as a byproduct [11]. L-Citrulline is effectively recycled via the L-citrulline NO cycle to L-arginine and plays an important role in the metabolism and regulation of NO [12]. L-Citrulline supplementation has various beneficial effects, such as ameliorating arterial stiffness [13] and improving erectile function [14], memory [15], O_2 uptake kinetics, and high-

* Correspondence: ayako.kamimura@kyowa-kirin.co.jp
[1]Healthcare Products Development Center, KYOWA HAKKO BIO CO., LTD., 2, Miyukigaoka, Tsukuba, Ibaraki 305-0841, Japan
Full list of author information is available at the end of the article

intensity exercise performance [16] through upregulation of NO synthesis. We and others have demonstrated in animal models that oral supplementation with L-citrulline upregulates endothelial NO synthase (eNOS) expression, improves endothelial function, and plays an atheroprotective role [17–19]. Interestingly, a clinical trial has shown that oral intake of L-citrulline dose-dependently and more effectively increases plasma L-arginine levels than does L-arginine supplementation in healthy human volunteers [20]. Therefore, L-citrulline may be considered an effective L-arginine and NO supplies which might be expected to have potential for enhancing sport performance. Some studies have found that acute L-citrulline supplementation has no effect on exercise [21, 22]. On the other hand, Bailey et al. [16] showed that 6 days of L-citrulline supplementation improved exercise tolerance. This suggests that chronic L-citrulline supplementation (for about 1 week) is needed to enhance exercise tolerance. However, it is not presently known whether chronic small doses of L-citrulline enhance sport performance. Moreover, to our knowledge, no study has comprehensively evaluated the effects of L-citrulline on endurance exercise performance during simulated competition or on subjective feelings of discomfort associated with exercise in humans. We hypothesized that chronic L-citrulline administration would enhance performance during simulated competition.

The aim of this study was to investigate the effect of oral supplementation of L-citrulline on cycling time-trial performance in healthy trained men.

Methods

Subjects

Twenty-five trained healthy Japanese males volunteered to take part in this double-blind, randomized, placebo-controlled, two-way crossover trial. Because of the crossover design, half of the subjects participated under one condition and half under the other at the same time, with a washout period of 3 weeks. Randomization was conducted by using SAS 9.3 (SAS Institute Inc.). The subjects recruited were aged 20 to 39 years and participated in sport twice a week or more. The sports included athletics (long distance running), baseball, cycling, soccer, triathlon, and skiing. Current smokers, subjects taking medication or dietary supplements for chronic conditions, and subjects with injuries that could interfere with their performance were excluded. The participants' health status was assessed by both physical and laboratory examinations, including an electrocardiogram and blood chemistry panel. Three males were excluded from the analysis because they had colds on the test day. We therefore analyzed a final total of 22 males (mean ± SD age, 29 ± 8.4 years; body mass, 74 ± 9.4 kg; height, 175 ± 4.1 cm; body mass index, 24 ± 3.3 kg/m^2). The subjects were instructed not to change their usual training volume or diet during the 7 days of the study. The protocol was conducted according to the Declaration of Helsinki and was approved by the Ethics Committee of Fukuda Clinic (Osaka, Japan). All subjects gave their written informed consent.

Study design

The study was conducted at Fukuda Clinic (Osaka, Japan). After enrollment, the subjects were randomized into two groups to receive the following treatments once a day for 1 week before the experimental day in a double-blind fashion: 9 capsules consisting of 2.4 g of L-citrulline (KYOWA HAKKO BIO CO., Ltd., Tokyo, Japan) or 9 placebo capsules consisting of 2.4 g of cornstarch (Nippon NSC Co., Ltd., Tokyo, Japan) before bedtime. The indistinguishability of the capsules was confirmed by the Ethics Committee of Fukuda Clinic (Osaka, Japan). The purity of L-citrulline was analyzed by using an amino acid analyzer (L-8900 Hitachi High-Technologies Corporation, Tokyo, Japan) [23, 24]. There is no recommended dose for L-citrulline intake to enhance sport performance, but a dose of 6 or 8 g of L-citrulline malate has been used in other studies [25, 26]. On the day before each test day, subjects were all given the same meals, which they were required to finish by 9:00 PM; they then fasted overnight. The following morning, blood pressure and heart rate were measured and blood samples were collected. Thereafter, the subjects had breakfast (a rice ball, about 180 kcal) to provide energy 1 h before intake of the 9 capsules of placebo or L-citrulline. After the intake of placebo or L-citrulline, the subjects rested quietly before takingpart in a 4-km cycling time trial (TT). Before the TT, each subject completed a warm-up. The TT was performed on a cycle ergometer (Aerobike 75XL2; Konami Sports & Life Co., Ltd., Tokyo, Japan) [27] 1 h after of the intake of placebo or L-citrulline. After the TT, blood samples were collected from the brachial vein. The study design is summarized in Fig. 1.

Physical working capacity test

Subjects completed a physical working capacity test to determine work rate during the TT. Physical working capacity is an index employed in performance diagnostics to appraise the tested person's aerobic performance capacity. The protocol began with 3 min of 25-watt (W) cycling, after which 3 min each of 75-W and 125-W cycling was imposed. Physical working capacity at 75 % of the predicted maximum heart rate ($PWC_{75\%HRmax}$) was determined from the relationship between HR in the final 30 s and exercise work load in the above-mentioned incremental exercise test. $PWC_{75\%HRmax}$ was evaluated as work load at 75 % of HR_{max} (=220 − age) [28]. The subjects were then familiarized with the cycle ergometer.

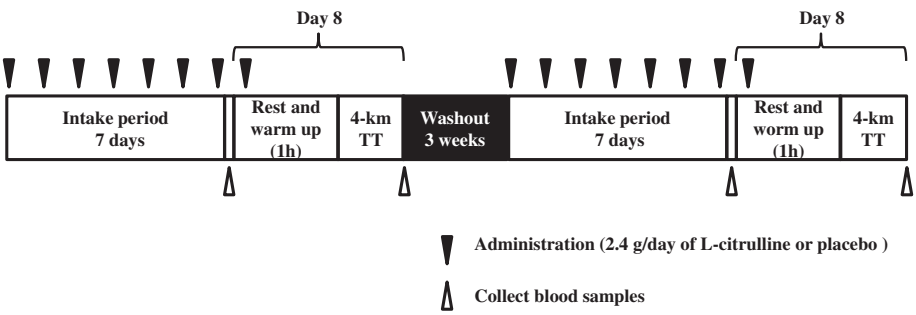


Fig. 1 Study design

Time trial test

On Day 8, one hour after intake of 2.4 g of placebo or L-citrulline, each subject performed the TT. The work rate of each subject was set at 60 rpm and $PWC_{75\%HRmax}$. Time to complete 4 km of cycling, power output (PO), VO_2, plasma nitrite and nitrate (NOx) levels, plasma amino acid concentrations, and visual analog scale (VAS) scores were evaluated. The computrainer ergometry system recorded PO every 10 s, and these values were averaged for every 0.5 km completed in the TT to create a PO profile. During the TT, breath-by-breath pulmonary gas exchange and ventilation were measured continuously (Aero Monitor AE-300S, Minato Medical Science Co., Ltd., Osaka, Japan) [29–31]. These data were also used to produce a PO/VO_2 ratio, namely the PO produced in watts per liter of O_2 consumed per min (W/L/min).

Visual analog scale

Subjects were asked to subjectively rate their degree of discomfort on a VAS from 0 mm (excellent) to 100 mm (poor) after the TT. The VAS was originally developed for measuring pain level [32] and has also been used to assess fatigue level [33].

Blood sample analyses

Blood samples were collected from the brachial vein; 5 mL was collected each time. Plasma samples were prepared by collecting blood in an EDTA-2Na-containing tube and kept on ice until centrifugation at 1700 *g* for 10 min at 4 °C. Plasma NOx was assayed via the Griess reaction by using a colorimetric assay kit (Nitrate/Nitrite Colorimetric Assay Kit, Cayman, Ann Arbor, MI, USA) [34, 35]. To assess amino acids, the plasma sample was deproteinized with 4 % sulfosalicylic acid (plasma to 20 % sulfosalicylic acid ratio = 0.3: 0.075 mL) for 30 min on ice and then centrifuged at 1700 *g* for 10 min at 4 °C. The supernatant was stored at −80 °C until analysis. The concentrations of amino acids (L-valine, L-isoleucine, L-leucine, L-arginine, and L-citrulline) in the plasma were measured with an amino acid analyzer (L-8900 Hitachi High-Technologies Corporation, Tokyo, Japan) [23, 24].

Statistical analyses

Values are shown as means ± S.E.M. Paired *t*-tests were used to evaluate the significance of any differences between the placebo and L-citrulline groups. Analyses were performed with SPSS Statistics 22 (IBM Japan, Ltd., Tokyo, Japan). *P* values of below 0.05 were regarded as statistically significant.

Results

Blood chemistry

Plasma amino acid concentrations are summarized in Table 1. Seven days' intake of L-citrulline significantly increased the plasma L-arginine level. On Day 8, plasma L-citrulline and L-arginine levels after TT were significantly higher in the L-citrulline group than in the placebo group. Levels of plasma branched chain amino acids (BCAAs: L-valine, L-isoleucine, L-leucine) were significantly lower at pre-load and post-load in the L-citrulline group than in the placebo group. There was no significant difference in the level of plasma NOx between the placebo and L-citrulline groups (Fig. 2).

Physical performance test

Mean TT completion times are displayed in Fig. 3, and the accompanying PO profiles are shown in Fig. 4a. L-Citrulline supplementation significantly reduced completion time compared with placebo, with a group mean reduction of 1.5 % (placebo: 578 ± 15 s, L-citrulline: 569 ± 14 s, $p < 0.05$, Fig. 3). Ingestion of L-citrulline increased mean PO by 2 % (placebo = 189 ± 5 W vs. L-citrulline = 193 ± 5 W, $p < 0.05$, Fig. 4b). There was no significant difference in VO_2 response between placebo and L-citrulline (Table 2). PO/VO_2 tended to be higher in the L-citrulline-supplemented group in three of six elapsed distances ($p < 0.1$, Fig. 5).

Table 1 Plasma amino acid concentration on Day 8

	before TT and before intake		after TT and after intake	
	Placebo	L-Citrulline	Placebo	L-Citrulline
L-Citrulline (nmol/ml)	39.3 ± 1.4	54.3 ± 11.0	40.0 ± 1.4	475 ± 37**
L-Arginine (nmol/ml)	110 ± 4	139 ± 7**	110 ± 4	192 ± 9**
BCAA (nmol/ml) (valine, isoleucine, leucine)	565 ± 15	553 ± 21*	518 ± 20	501 ± 15*

Values are means ± S.E.M. $n = 22$, * $p < 0.05$, ** $p < 0.001$, indicating a significant difference from placebo

Visual analog scale

L-Citrulline significantly improved subjective feelings of muscle fatigue, and concentration, immediately after exercise (Fig. 6). A marked but not statistically significant improvement in ease of pedaling was observed with L-citrulline supplementation ($p < 0.1$).

Discussion

We demonstrated that oral supplementation with L-citrulline at 2.4 g/day for 7 days significantly increased plasma L-arginine levels. Moreover, intake of L-citrulline for 7 days and I h before the TT significantly increased plasma L-citrulline and L-arginine levels and enhanced cycling TT performance. In addition, subjective feelings of muscle fatigue, and concentration, right after exercise were significantly improved with L-citrulline.

In this human trial, the subjects engaged in TT cycling to allow us to evaluate their exercise performance. Competitive sports typically require athletes to complete a given distance in the shortest possible time. Time-to-exhaustion tests are primarily measures of "exercise capacity," and because there is no competitive sports event in which competition is based on time and distance before exhaustion, tests of this type have limited physiological validity [36]. It has also been reported that there is no relationship between measured time-to-exhaustion and actual cycling performance [37]. In contrast, the TT protocol used here has a high level of physiological validity [36], provides an accurate simulation of physiological responses during competition [38], and is well correlated with actual race performance [39]. Therefore, L-citrulline intake might be expected to enhance performance in real competitive sport. We used cornstarch as the placebo and the study design was a double-blind crossover; we expected that these factors would reduce the placebo effect.

The improved TT performance after supplementation with L-citrulline was the consequence of significantly greater PO for the same VO_2. Interestingly, there was a

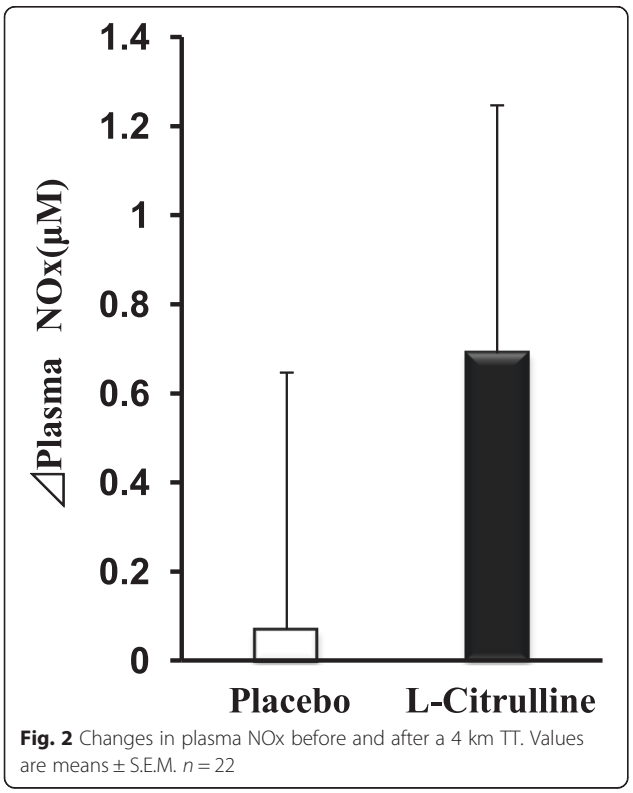


Fig. 2 Changes in plasma NOx before and after a 4 km TT. Values are means ± S.E.M. $n = 22$

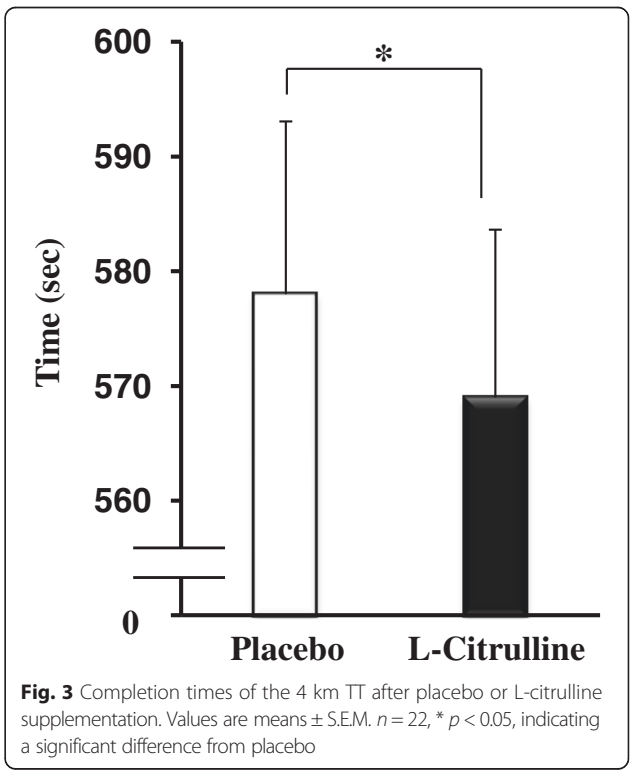


Fig. 3 Completion times of the 4 km TT after placebo or L-citrulline supplementation. Values are means ± S.E.M. $n = 22$, * $p < 0.05$, indicating a significant difference from placebo

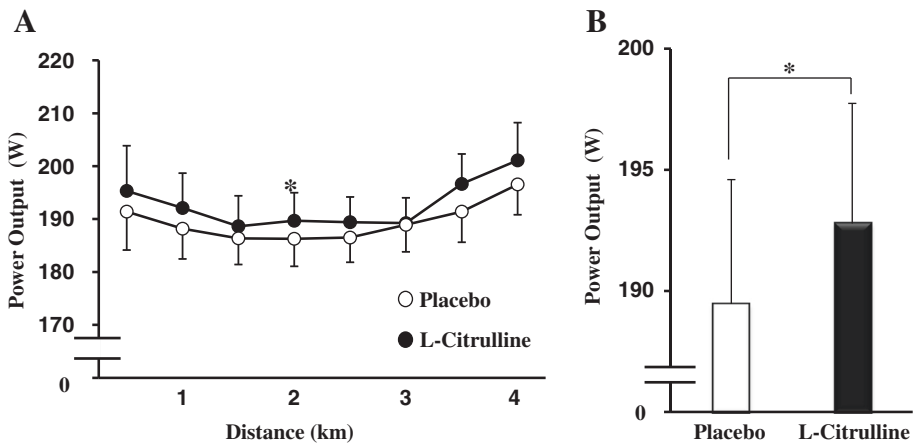


Fig. 4 PO profile during the 4 km TT (**a**) and mean PO (**b**) of the 4 km TT after placebo or L-citrulline supplementation. Values are means ± S.E.M. $n = 22$. * $p < 0.05$, indicating a significant difference from placebo

significant correlation between plasma NOx and PO/VO_2 after supplementation with L-citrulline but placebo had no correlation (data not shown). This finding suggests that the effects of L-citrulline on PO/VO_2 may have been related to improved plasma NO availability, which in turn may have enhanced sport performance. In sports nutrition, NO-related products are attracting a lot of attention for their ergogenic effects. Many applied studies in humans report that NO improves sports performance. This is because NO is a potential modulator of blood flow, muscle energy metabolism, and mitochondrial respiration during exercise [5, 6, 40, 41]. Dietary supplementation with nitrate reduces the O_2 cost of submaximal cycling [6, 42], knee extensor exercise [43], and treadmill walking and running [44]. L-Arginine is the direct precursor of NO via NOS activity. Moreover, oral intake of L-arginine improves sports performance in healthy subjects [45–47]. However, a relatively large dose (6 to 14.2 g/day) of L-arginine would be required for beneficial effects on sports performance, because L-arginine is degraded by arginase in the small intestine and liver [48–50]. For this reason, some studies have shown no effect of L-arginine on O_2 cost and sports performance [51, 52]. In contrast, L-citrulline is not metabolized in the intestine or liver [53]. On entering the kidneys, vascular endothelium, and other tissues, L-citrulline is readily converted to L-arginine, thus raising plasma and tissue levels of L-arginine and enhancing NO production [10]. We found here that oral intake of L-citrulline increased not only L-citrulline levels but also L-arginine levels. It has been reported that oral supplementation with L-citrulline increases plasma L-arginine levels more effectively than does L-arginine supplementation in healthy subjects [20], and increased plasma L-arginine levels before exercise enhance sport performance [16]. However, some studies have found that oral L-citrulline supplementation has no effect on exercise [21, 22]. This is likely because a single dose of L-citrulline is insufficient to enhance sport performance. Bailey et al. [16] demonstrated that 6 days of L-citrulline supplementation improved exercise tolerance. These findings suggest that L-citrulline needs to be taken continuously (for about 1 week) to enhance exercise tolerance. This is why we conducted an 8-day trial, which showed positive effects of L-citrulline. The daily dose of L-citrulline in our study was 2.4 g. This seems smaller than those used in other previous studies [16, 21, 22], but 2 to 3 g of oral L-citrulline has been reported to increase plasma L-arginine levels [20, 54]. Moinard et al. [54] showed that the C_{max} of plasma L-arginine was 146 μM when subjects consumed 2 g of L-citrulline. In the study by Bailey et al. [16], the L-citrulline group had a mean plasma L-arginine level of 135 μM and showed improved exercise performance. Therefore, we had hypothesized that 2.4 g/day of L-citrulline for 8 days might be enough to increase plasma L-arginine levels such that we would obtain ergogenic effects. In fact, Bailey et al.

Table 2 VO_2 profile during the 4 km TT

Distance (km)	Placebo VO_2 (mL/min)	L-Citrulline VO_2 (mL/min)
0.0 ~ 0.5	1662 ± 236	1682 ± 255
0.5 ~ 1.0	2396 ± 311	2400 ± 341
1.0 ~ 1.5	2553 ± 319	2539 ± 386
1.5 ~ 2.0	2692 ± 353	2676 ± 381
2.0 ~ 2.5	2777 ± 346	2801 ± 369
2.5 ~ 3.0	2861 ± 344	2847 ± 363
3.0 ~ 3.5	2933 ± 361	2911 ± 377
3.5 ~ 4.0	3005 ± 381	2971 ± 397

Values are means ± S.E.M. $n = 22$

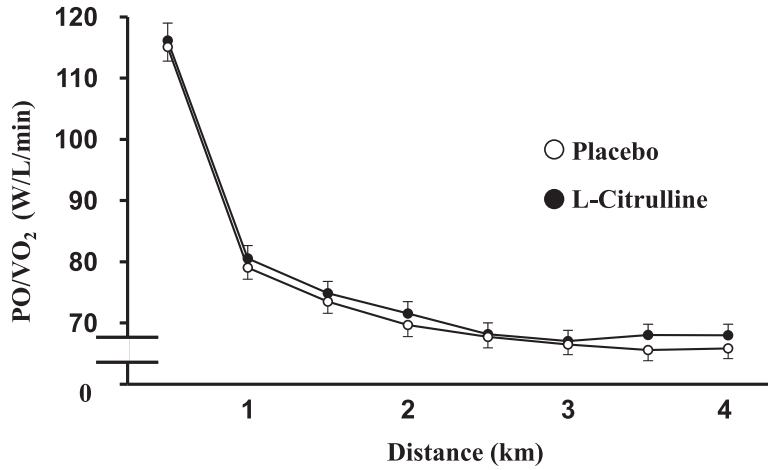


Fig. 5 PO/VO_2 during the 4 km TT after placebo or L-citrulline supplementation. Values are means ± S.E.M. $n = 22$

reported that the plasma L-arginine level in their L-citrulline group was about 2.3 times that in their placebo group [16]. On the other hand, our data demonstrated that the plasma L-arginine level in our L-citrulline group was about 1.7 times that in our placebo group. However, our absolute value of plasma L-arginine after intake of L-citrulline was higher than that in the study by Bailey et al. Therefore, 2.4 g/day of L-citrulline for 8 days is likely enough to obtain ergogenic aid.

We found here that, L-citrulline supplementation significantly increased plasma levels of plasma L-citrulline and L-arginine, which are essential for NO synthesis. In our study the plasma arginine level after supplementation of L-citrulline was as high as that in the study by Bailey et al. [16]. Our results thus suggest that L-citrulline would enhance sport performance through NO synthesis; however, we were not able to observe an increase in plasma NOx level. We measured NOx at only two time points: before and after exercise, not during exercise. These evaluation points might not have been suitable for detecting significant between-point differences in NO generation. Chemiluminescence assay is more sensitive than colorimetric assay for detecting NOx. In this study, we measured NOx by colorimetric assay, and it may not have been sensitive enough to detect changes in plasma NOx.

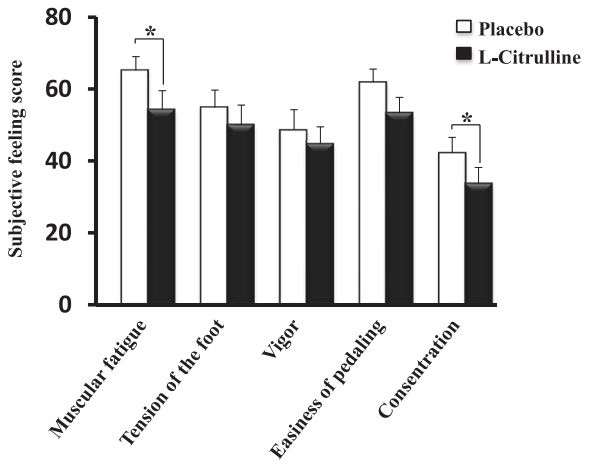


Fig. 6 Subjective feelings immediately after exercise. Values are means ± S.E.M. $n = 22$. * $p < 0.05$, indicating a significant difference from placebo

Sureda et al. [26] showed that oral intake of 6 g of L-citrulline malate 2 h before exercise enhances the use of BCAAs, which are metabolized in the muscles to produce energy. In our study, L-citrulline supplementation decreased plasma BCAA levels. These data indicate that L-citrulline promotes the metabolic use of these amino acids as fuel to support muscular exercise. Moreover, L-citrulline significantly improved subjective feelings of muscular fatigue. BCAA reduces muscle soreness and fatigue [55, 56]. Furthermore, L-citrulline malate reduces fatigue and post-workout muscle soreness [25]; watermelon juice, which is rich in L-citrulline, also reduces muscle soreness [57]. Our data suggest that L-citrulline has the potential to relieve muscle fatigue. Therefore, the effects of L-citrulline on BCAA utilization and muscular fatigue might also contribute to enhanced sport performance. In addition, the subjective feeling of concentration was significantly improved by oral intake of L-citrulline. Hayashi et al. have reported that L-citrulline improves blood flow [17]. The concentration-enhancing effects of L-citrulline are likely due to enhanced blood flow.

A growing number of sport supplements include L-arginine, which is claimed to enhance NO production, despite L-arginine being rapidly metabolized in the small intestine and liver when administered orally. As mentioned above, L-citrulline is a potent precursor of L-

arginine, and several functional advantages of L-citrulline over L-arginine have been elucidated [17, 20]; moreover, Akashi et al. [58] have revealed that L-citrulline is an efficient hydroxyl radical scavenger. L-arginine tends to be extremely bitter and highly water absorbent, whereas L-citrulline is tasteless, odorless, and non-hygroscopic. It would thus appear that L-citrulline is superior to L-arginine in terms of ease of handling and taste as an ingredient of supplements. L-Citrulline is present in large quantities in watermelon but is not abundant in other fruits, vegetables, meat, or fish because it is a free amino acid. It is difficult to obtain L-citrulline from a conventional diet in sufficient amounts to enhance sports performance. Therefore, it may be beneficial to take a few tablets of L-citrulline before exercise as an ergogenic aid.

Our study had several limitations. We instructed the subjects not to change their training volumes and to eat their usual diets during the 7 days of the study. However, we did not make the training volumes and diets identical among the subjects, with the exception of dinner on the evening before the test day and breakfast on the test day. Some of the subjects' performances may have been affected by intense training sessions in the 2 days before the trials. Here, we conducted a double-blind randomized placebo-controlled two-way crossover study in 22 subjects; in future, an additional, larger-scale study will be needed to verify our findings.

Conclusions

We conclude that oral L-citrulline supplementation enhances cycling time trial performance. Moreover, L-citrulline improves subjective feelings (e.g. of muscle soreness) after performance. These data, taken together, suggest that L-citrulline is a promising amino acid for enhancing sport performance.

Abbreviations

BCAAs: branched chain amino acids; eNOS: endothelial NO synthase; NO: nitric oxide; NOS: NO synthase; NOx: nitrite and nitrate; PO/VO_2: power output / VO_2 ratio; TT: time trial; VAS: Visual Analog Scale.

Competing interests

This study was conducted in research funding by KYOWA HAKKO BIO CO., LTD. Takashi Suzuki, Masahiko Morita and Ayako Kamimura are employee of KYOWA HAKKO BIO CO., LTD. The other co-author declare no conflict of interests.

Authors' contributions

TS carried out study design and drafted the manuscript. MM made contributions to design, helped to draft the manuscript. YK made contributions to conception and design. AK supervised manuscript preparation. All authors read and approved the final manuscript.

Author details

[1]Healthcare Products Development Center, KYOWA HAKKO BIO CO., LTD., 2, Miyukigaoka, Tsukuba, Ibaraki 305-0841, Japan. [2]Laboratory of Pharmacognosy, School of Pharmaceutical Sciences, Kitasato University, 5-9-1, Shirokane, Minato-ku, Tokyo 108-0861, Japan.

Received: 11 September 2015 Accepted: 12 February 2016
Published online: 19 February 2016

References

1. Albrecht EW, Stegeman CA, Heeringa P, Henning RH, Van Goor H. Protective role of endothelial nitric oxide synthase. J Pathol. 2003;199:8–17.
2. Petroczi A, Naughton DP. Potentially fatal new trend in performance enhancement: a cautionary note on nitrite. J Int Soc Sports Nutr. 2010;7:25.
3. Bescos R, Sureda A, Tur JA, Pons A. The effect of nitric-oxide-related supplements on human performance. Sports Med. 2012;42:99–117.
4. Jones AM. Dietary nitrate supplementation and exercise performance. Sports Med. 2014;44 Suppl 1:S35–45.
5. Shen W, Xu X, Ochoa M, Zhao G, Wolin MS, Hintze TH. Role of nitric oxide in the regulation of oxygen consumption in conscious dogs. Circ Res. 1994;75:1086–95.
6. Bailey SJ, Winyard P, Vanhatalo A, Blackwell JR, Dimenna FJ, Wilkerson DP, et al. Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. J Appl Physiol (1985). 2009;107:1144–55.
7. Cermak NM, Gibala MJ, van Loon LJ. Nitrate supplementation's improvement of 10-km time-trial performance in trained cyclists. Int J Sport Nutr Exerc Metab. 2012;22:64–71.
8. Lansley KE, Winyard PG, Bailey SJ, Vanhatalo A, Wilkerson DP, Blackwell JR, et al. Acute dietary nitrate supplementation improves cycling time trial performance. Med Sci Sports Exerc. 2011;43:1125–31.
9. Wilkerson DP, Hayward GM, Bailey SJ, Vanhatalo A, Blackwell JR, Jones AM. Influence of acute dietary nitrate supplementation on 50 mile time trial performance in well-trained cyclists. Eur J Appl Physiol. 2012;112:4127–34.
10. Curis E, Nicolis I, Moinard C, Osowska S, Zerrouk N, Benazeth S, et al. Almost all about citrulline in mammals. Amino Acids. 2005;29:177–205.
11. Schmidt HH, Nau H, Wittfoht W, Gerlach J, Prescher KE, Klein MM, et al. Arginine is a physiological precursor of endothelium-derived nitric oxide. Eur J Pharmacol. 1988;154:213–6.
12. Hecker M, Sessa WC, Harris HJ, Anggard EE, Vane JR. The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. Proc Natl Acad Sci U S A. 1990;87:8612–6.
13. Ochiai M, Hayashi T, Morita M, Ina K, Maeda M, Watanabe F, et al. Short-term effects of L-citrulline supplementation on arterial stiffness in middle-aged men. Int J Cardiol. 2012;155:257–61.
14. Hotta Y, Shiota A, Kataoka T, Motonari M, Maeda Y, Morita M, et al. Oral L-citrulline supplementation improves erectile function and penile structure in castrated rats. Int J Urol. 2014;21:608–12.
15. Yabuki Y, Shioda N, Yamamoto Y, Shigano M, Kumagai K, Morita M, et al. Oral L-citrulline administration improves memory deficits following transient brain ischemia through cerebrovascular protection. Brain Res. 2013;1520:157–67.
16. Bailey SJ, Blackwell JR, Lord T, Vanhatalo A, Winyard PG, Jones AM. L-citrulline supplementation improves O2 uptake kinetics and high-intensity exercise performance in humans. J Appl Physiol (1985). 2015;19(4):385–95. doi:10.1152/japplphysiol.00192.2014.
17. Hayashi T, Juliet PA, Matsui-Hirai H, Miyazaki A, Fukatsu A, Funami J, et al. l-Citrulline and l-arginine supplementation retards the progression of high-cholesterol-diet-induced atherosclerosis in rabbits. Proc Natl Acad Sci U S A. 2005;102:13681–6.
18. Romero MJ, Platt DH, Tawfik HE, Labazi M, El-Remessy AB, Bartoli M, et al. Diabetes-induced coronary vascular dysfunction involves increased arginase activity. Circ Res. 2008;102:95–102.
19. Wu G, Collins JK, Perkins-Veazie P, Siddiq M, Dolan KD, Kelly KA, et al. Dietary supplementation with watermelon pomace juice enhances arginine availability and ameliorates the metabolic syndrome in Zucker diabetic fatty rats. J Nutr. 2007;137:2680–5.
20. Schwedhelm E, Maas R, Freese R, Jung D, Lukacs Z, Jambrecina A, et al. Pharmacokinetic and pharmacodynamic properties of oral L-citrulline and L-arginine: impact on nitric oxide metabolism. Br J Clin Pharmacol. 2008;65:51–9.
21. Cutrufello PT, Gadomski SJ, Zavorsky GS. The effect of l-citrulline and watermelon juice supplementation on anaerobic and aerobic exercise performance. J Sports Sci. 2015;33:1459–66.
22. Hickner RC, Tanner CJ, Evans CA, Clark PD, Haddock A, Fortune C, et al. L-citrulline reduces time to exhaustion and insulin response to a graded exercise test. Med Sci Sports Exerc. 2006;38:660–6.
23. Heo KN, Hong EC, Kim CD, Kim HK, Lee MJ, Choo HJ, et al. Growth performance, carcass yield, and quality and chemical traits of meat from

commercial korean native ducks with 2-way crossbreeding. Asian-Australas J Anim Sci. 2015;28:382–90.
24. Watts ST. Advancements in Amino Acid Analysis: Introduction of the New Hitachi L-8900 Amino Acid Analyzer. LCGC N Am. 2006;24(Suppl):17–20.
25. Perez-Guisado J, Jakeman PM. Citrulline malate enhances athletic anaerobic performance and relieves muscle soreness. J Strength Cond Res. 2010;24:1215–22.
26. Sureda A, Cordova A, Ferrer MD, Perez G, Tur JA, Pons A. L-citrulline-malate influence over branched chain amino acid utilization during exercise. Eur J Appl Physiol. 2010;110:341–51.
27. Sugino T, Aoyagi S, Shirai T, Kajimoto Y, Kajimoto O. Effects of citric acid and l-carnitine on physical fatigue. J Clin Biochem Nutr. 2007;41:224–30.
28. Nagashima J, Musha H, Takada H, Takagi K, Mita T, Mochida T, et al. Three-month exercise and weight loss program improves heart rate recovery in obese persons along with cardiopulmonary function. J Cardiol. 2010;56:79–84.
29. Abe T, Fujita S, Nakajima T, Sakamaki M, Ozaki H, Ogasawara R, et al. Effects of Low-intensity cycle training with restricted Leg blood flow on thigh muscle volume and VO2MAX in young Men. J Sports Sci Med. 2010;9:452–8.
30. Nagai N, Sakane N, Tsuzaki K, Moritani T. UCP1 genetic polymorphism (−3826 A/G) diminishes resting energy expenditure and thermoregulatory sympathetic nervous system activity in young females. Int J Obes (Lond). 2011;35:1050–5.
31. Yamada K, Suda T, Komoro YS, Kanefuji T, Kubota T, Murayama T, et al. Low fat intake is associated with pathological manifestations and poor recovery in patients with hepatocellular carcinoma. Nutr J. 2013;12:79.
32. Maxwell C. Sensitivity and accuracy of the visual analogue scale: a psycho-physical classroom experiment. Br J Clin Pharmacol. 1978;6:15–24.
33. Leung AW, Chan CC, Lee AH, Lam KW. Visual analogue scale correlates of musculoskeletal fatigue. Percept Mot Skills. 2004;99:235–46.
34. Mayo JN, Beard Jr RS, Price TO, Chen CH, Erickson MA, Ercal N, et al. Nitrative stress in cerebral endothelium is mediated by mGluR5 in hyperhomocysteinemia. J Cereb Blood Flow Metab. 2012;32:825–34.
35. Tuzcu H, Aslan I, Aslan M. The effect of high-dose insulin analog initiation therapy on lipid peroxidation products and oxidative stress markers in type 2 diabetic patients. Oxid Med Cell Longev. 2013;2013:513742.
36. Currell K, Jeukendrup AE. Validity, reliability and sensitivity of measures of sporting performance. Sports Med. 2008;38:297–316.
37. Laursen PB, Rhodes EC, Langill RH, McKenzie DC, Taunton JE. Relationship of exercise test variables to cycling performance in an Ironman triathlon. Eur J Appl Physiol. 2002;87:433–40.
38. Foster C, Green MA, Snyder AC, Thompson NN. Physiological responses during simulated competition. Med Sci Sports Exerc. 1993;25:877–82.
39. Palmer GS, Dennis SC, Noakes TD, Hawley JA. Assessment of the reproducibility of performance testing on an air-braked cycle ergometer. Int J Sports Med. 1996;17:293–8.
40. Tschakovsky ME, Joyner MJ. Nitric oxide and muscle blood flow in exercise. Appl Physiol Nutr Metab. 2008;33:151–61.
41. Larsen FJ, Schiffer TA, Borniquel S, Sahlin K, Ekblom B, Lundberg JO, et al. Dietary inorganic nitrate improves mitochondrial efficiency in humans. Cell Metab. 2011;13:149–59.
42. Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B. Effects of dietary nitrate on oxygen cost during exercise. Acta Physiol (Oxf). 2007;191:59–66.
43. Bailey SJ, Fulford J, Vanhatalo A, Winyard PG, Blackwell JR, DiMenna FJ, et al. Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. J Appl Physiol (1985). 2010;109:135–48.
44. Lansley KE, Winyard PG, Fulford J, Vanhatalo A, Bailey SJ, Blackwell JR, et al. Dietary nitrate supplementation reduces the O2 cost of walking and running: a placebo-controlled study. J Appl Physiol (1985). 2011;110:591–600.
45. Campbell B, Roberts M, Kerksick C, Wilborn C, Marcello B, Taylor L, et al. Pharmacokinetics, safety, and effects on exercise performance of L-arginine alpha-ketoglutarate in trained adult men. Nutrition. 2006;22:872–81.
46. Fricke O, Baecker N, Heer M, Tutlewski B, Schoenau E. The effect of L-arginine administration on muscle force and power in postmenopausal women. Clin Physiol Funct Imaging. 2008;28:307–11.
47. Koppo K, Taes YE, Pottier A, Boone J, Bouckaert J, Derave W. Dietary arginine supplementation speeds pulmonary VO2 kinetics during cycle exercise. Med Sci Sports Exerc. 2009;41:1626–32.
48. Hayashi T, Esaki T, Sumi D, Mukherjee T, Iguchi A, Chaudhuri G. Modulating role of estradiol on arginase II expression in hyperlipidemic rabbits as an atheroprotective mechanism. Proc Natl Acad Sci U S A. 2006;103:10485–90.
49. Munder M. Arginase: an emerging key player in the mammalian immune system. Br J Pharmacol. 2009;158:638–51.
50. Castillo L, Chapman TE, Sanchez M, Yu YM, Burke JF, Ajami AM, et al. Plasma arginine and citrulline kinetics in adults given adequate and arginine-free diets. Proc Natl Acad Sci U S A. 1993;90:7749–53.
51. Alvares TS, Conte CA, Paschoalin VM, Silva JT, Meirelles Cde M, Bhambhani YN, et al. Acute l-arginine supplementation increases muscle blood volume but not strength performance. Appl Physiol Nutr Metab. 2012;37:115–26.
52. Vanhatalo A, Bailey SJ, DiMenna FJ, Blackwell JR, Wallis GA, Jones AM. No effect of acute L-arginine supplementation on O(2) cost or exercise tolerance. Eur J Appl Physiol. 2013;113:1805–19.
53. Romero MJ, Platt DH, Caldwell RB, Caldwell RW. Therapeutic use of citrulline in cardiovascular disease. Cardiovasc Drug Rev. 2006;24:275–90.
54. Moinard C, Nicolis I, Neveux N, Darquy S, Benazeth S, Cynober L. Dose-ranging effects of citrulline administration on plasma amino acids and hormonal patterns in healthy subjects: the Citrudose pharmacokinetic study. Br J Nutr. 2008;99:855–62.
55. Kim DH, Kim SH, Jeong WS, Lee HY. Effect of BCAA intake during endurance exercises on fatigue substances, muscle damage substances, and energy metabolism substances. J Exerc Nutrition Biochem. 2013;17:169–80.
56. Matsumoto K, Koba T, Hamada K, Sakurai M, Higuchi T, Miyata H. Branched-chain amino acid supplementation attenuates muscle soreness, muscle damage and inflammation during an intensive training program. J Sports Med Phys Fitness. 2009;49:424–31.
57. Tarazona-Diaz MP, Alacid F, Carrasco M, Martinez I, Aguayo E. Watermelon juice: potential functional drink for sore muscle relief in athletes. J Agric Food Chem. 2013;61:7522–8.
58. Akashi K, Miyake C, Yokota A. Citrulline, a novel compatible solute in drought-tolerant wild watermelon leaves, is an efficient hydroxyl radical scavenger. FEBS Lett. 2001;508:438–42.

L-Arginine

L-arginine is an amino acid that helps your body produce proteins. Proteins are essential because every cell in your body contains proteins. You can add l-arginine into your diet by eating foods high in protein like meat and nuts. You can also take l-arginine as a supplement by mouth or through an IV under your provider's supervision.

Overview

What is l-arginine?

L-arginine is an amino acid that you can find naturally in foods like fish, meat and nuts. L-arginine is semi-essential (conditional), which means that your body can produce the amino acid, but you also need to include some sources of the amino acid in your diet.

What does the "l" in l-arginine stand for?

The letter L in front of arginine stands for "levorotatory," which is a chemistry term that means the amino acid didn't bond with a protein molecule (free form). This helps providers categorize amino acids based on how similar they are to the amino acids humans produce in their own bodies. The L designates that it easily absorbs into your body because it's most similar to amino acids already in your body.

What are amino acids?

Amino acids are molecules within your body that produce proteins when they combine with other molecules. An amino acid chain forms a protein. Proteins are an essential part of what makes humans function because every cell in the human body contains protein.

Your body also uses amino acids to produce energy.

Do I need protein in my diet?

Protein in your diet helps your body function. Each cell in your body contains protein. Proteins help your body:

- **Build and repair new cells essential for growth.**
- **Repair damaged tissues.**
- **Help cells complete their assigned function.**
- **Break down and digest food.**

What is l-arginine approved for?

L-arginine can treat several conditions including:

- **Chest pain or pressure (angina).**
- **Erectile dysfunction.**
- **Heart failure.**
- **High blood pressure (hypertension).**
- **Migraines.**

What foods contain l-arginine?

Foods that are high in protein naturally contain l-arginine including:

- **Meat (red meat, chicken, turkey).**
- **Fish (salmon, haddock).**
- **Nuts and seeds (almonds, cashews, pumpkin seeds).**
- **Legumes (soybeans, chickpeas).**
- **Whole grains (brown rice, oats).**
- **Dairy products (milk, yogurt, cheese).**

Additional Common Questions

What are the forms and brand names of l-arginine?

L-arginine is available in two forms: fluid injected inside of your vein through an IV (intravenously) or taken by mouth (capsules or tablets).

The brand name for IV-form l-arginine is R-Gene 10 ®.

There are several types of l-arginine supplements available over the counter. Talk with your healthcare provider before starting l-arginine supplements.

What dosage strengths does l-arginine come in?

Dosage varies for each brand of l-arginine and the specifications are marked on the label. The average dosage of l-arginine is between 6 grams to a maximum of 30 grams per day. The daily dose is normally divided into three smaller doses per day that won't exceed the maximum dosage. Do not take more than the maximum dosage of l-arginine.

Check with your healthcare provider before taking over-the-counter l-arginine supplements to see if they are right for you.

If your provider prescribes treatment with l-arginine via an IV, they will administer the correct dosage according to your age, the reason for treatment and other factors.

How should I take l-arginine?

You can take l-arginine by incorporating foods that are high in l-arginine into your diet. Foods that are high in protein are great ways to increase your l-arginine levels.

If you aren't getting enough l-arginine through your diet, your provider might suggest taking over-the-counter l-arginine supplements. You can take l-arginine supplements by mouth, following the dosage on the label of the supplement and the recommendation from your provider.

If you need to take l-arginine via IV, your provider will monitor and administer the treatment in a healthcare facility.

How long does it take for l-arginine to work?

Depending on your reason for taking l-arginine and which type of amino acid you need, it takes a minimum of 24-hours for the l-arginine to absorb into your body. In some cases, to see the full effects of regular l-arginine treatment, it could take up to three months.

What are the side effects of l-arginine?

Side effects are possible with l-arginine treatment and could include:

- **Bloating.**
- **Diarrhea.**
- **Dizziness.**
- **Nausea or vomiting.**

Life-threatening side effects include:

- **Allergic reaction (hives, itching or rash).**

- **Difficulty breathing or a tight feeling in your chest.**
- **Heart failure.**

If you experience any side effects, reach out to your healthcare provider or visit your nearest emergency room immediately.

Are there any serious interactions with l-arginine?

L-arginine interacts with other medicines. Don't take l-arginine without first talking to your provider about medicines you currently take. L-arginine interacts with the following:

Medicine	Type
ACE inhibitors.	Benazepril, enalapril, lisinopril.
Alpha-blockers.	Doxazosin, prazosin.
Angiotensin receptor blockers.	Candesartan, irbesartan, losartan, valsartan.
Beta-blockers.	Atenolol, carvedilol, labetalol, metoprolol.
Calcium channel blockers.	Amlodipine, diltiazem, nifedipine, verapamil.
Nitrates.	Isosorbide, nitroglycerin.
Propranolol.	Hemangeol ®.
Vitamins or natural remedies.	Fish oil.

L-arginine can cause interactions if you have certain health conditions. You shouldn't take l-arginine if you:

- **Recently had a heart attack.**
- **Have a guanidinoacetate methyltransferase deficiency.**
- **Are a child under 16 years of age, are pregnant or breastfeeding or are an adult older than 65 years of age without approval from your provider.**

Always check with your provider before starting l-arginine supplements or treatment.

Can I take l-arginine if I'm pregnant or breastfeeding?

Don’t take l-arginine supplements if you’re pregnant or breastfeeding without first talking with your provider to see if the amino acid is right for you. L-arginine could cause unexpected complications during pregnancy, and there is not enough research to specify whether or not l-arginine supplements pass through breastmilk.

What should I tell my healthcare provider before starting l-arginine?

Talk to your healthcare provider about the medicines, vitamins and supplements that you’re currently taking before starting l-arginine. You’ll also want to discuss your health history with your provider, especially if you recently had a heart attack, are pregnant or breastfeeding or if you have an underlying medical condition. Your provider will assess your symptoms and let you know if it is safe to start taking l-arginine.

A note from Cleveland Clinic

Always talk with your healthcare provider before starting any new supplements like l-arginine. L-arginine is safe for most adults, especially if you incorporate more protein-forward products into your diet like meat and nuts. If you have any serious reactions to the amino acid, contact your provider or visit the emergency room immediately.

Molecular Nutrition & Food Research
www.mnf-journal.com

L-Citrulline Supplementation-Increased Skeletal Muscle PGC-1α Expression Is Associated with Exercise Performance and Increased Skeletal Muscle Weight

*Myra O. Villareal, Toshiya Matsukawa, and Hiroko Isoda**

Scope: L-citrulline has recently been reported as a more effective supplement for promoting intracellular nitric oxide (NO) production compared to L-arginine. Here, the effect of L-citrulline on skeletal muscle and its influence on exercise performance were investigated. The underlying mechanism of its effect, specifically on the expression of skeletal muscle peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α), was also elucidated.
Methods and results: Six-week-old ICR mice were orally supplemented with L-citrulline (250 mg kg^{-1}) daily, and their performance in weight-loaded swimming exercise every other day for 15 days, was evaluated. In addition, mice muscles were weighed and evaluated for the expression of PGC-1α and PGC-1α-regulated genes. Mice orally supplemented with L-citrulline had significantly higher gastrocnemius and biceps femoris muscle mass. Although not statistically significant, L-citrulline prolonged the swimming time to exhaustion. PGC-1α upregulation was associated with vascular endothelial growth factor α (*VEGFα*) and insulin-like growth factor 1 (*IGF-1*) upregulation. *VEGFα* and *IGF-1* are important for angiogenesis and muscle growth, respectively, and are regulated by PGC-1α. Treatment with NG-nitro-L-arginine methyl ester hydrochloride (L-NAME), a nitric oxide synthesis inhibitor, suppressed the L-citrulline-induced *PGC-1α* upregulation in vitro.
Conclusion: Supplementation with L-citrulline upregulates skeletal muscle PGC-1α levels resulting in higher skeletal muscle weight that improves time to exhaustion during exercise.

1. Introduction

Regular exercise and being active physically enhance endurance during exercise and more importantly, improve metabolic dysfunction that may help prevent lifestyle-related diseases.[1,2] Physical inactivity increases the risk of development of obesity, type 2 diabetes, sarcopenia, hypertension, and cardiovascular diseases.[3] Physically active people's life expectancy, without long-standing illness, is in fact 8–10 years longer compared to that of inactive people.[4] Several studies have demonstrated that enhancement of the skeletal muscle functions is observed to be the major positive impact of exercise.[1,2] This means that enhancing the skeletal muscle functions, in terms of improved mitochondrial biogenesis, capillaries, and fatty acid transporters, significantly improve exercise capacity.

Peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α) is the transcriptional coactivator responsible for the regulation of mitochondrial biogenesis, angiogenesis, oxidative metabolism, and muscle growth, and has a crucial role in the adaptation of muscles to exercise.[5,6] Muscle-specific overexpression of PGC-1α increases exercise capacity and improves maximal oxygen uptake (VO_{2max}) by increasing mitochondrial biogenesis and capillary density in skeletal muscle.[7] Skeletal muscle PGC-1α is known to have an important role in exercise adaptation and enhancement of exercise capacity.[8,9]

Nitric oxide (NO) is a reactive nitrogen molecule synthesized enzymatically through the catalytic action of nitric oxide synthase (NOS). NO is expressed in the skeletal muscles of mammals and acts as a second messenger in transduction pathways associated with the expression of genes for oxidative metabolism, vasodilation, and skeletal muscle contraction. NOS inhibition also reduces the maximal oxygen uptake during exercise in humans.[10] Conversely, increased intracellular NO production leads to phosphorylation of CREB that in turn induces PGC-1α expression.[11] Thus, several studies have indicated that the physiological amount of NO positively impacts adaptation to exercise, much so that nitrate supplementation has been

Dr. M. O. Villareal, Dr. H. Isoda
Faculty of Life and Environmental Sciences
University of Tsukuba
Tsukuba City, 305-8572, Japan
E-mail: isoda.hiroko.ga@u.tsukuba.ac.jp
Dr. M. O. Villareal, Dr. H. Isoda
Alliance for Research on Mediterranean and North Africa
University of Tsukuba
Tsukuba City, 305-8572, Japan
Dr. T. Matsukawa
Graduate School of Life and Environmental Sciences
University of Tsukuba
Tsukuba City, 305-8572, Japan

DOI: 10.1002/mnfr.201701043

considered to be an ergogenic supplementation for athletes or in sports.[12,13]

Normally, physiological NO is derived from L-arginine. However, absorption of orally administered L-arginine is hampered by first-pass and systemic metabolism. L-citrulline, on the other hand, is only subject to systemic metabolism. L-citrulline, a nonessential amino acid, has recently been recognized as an effective alternative source of NO, and therefore can be taken as a dietary supplement to increase intracellular NO production.[14] Oral supplementation with L-citrulline increases NO levels by increasing endothelial NO synthase (eNOS) expression that results in improved endothelial function.[15,16] Neuronal NOS (nNOS) and eNOS are also expressed in the skeletal muscle.[11] L-citrulline is a precursor of L-arginine but has several functional advantages over L-arginine.[14,15] L-citrulline is superior to L-arginine in terms of ease of handling and palatability since it is tasteless, odorless, and non-hygroscopic, whereas L-arginine tends to be extremely bitter and highly water absorbent.[17] Therefore, L-citrulline is considered to be more effective in enhancing exercise performance by virtue of its effect on skeletal muscle regulation. Several studies have reported that L-citrulline supplementation enhances exercise performance[18–20]; however, the underlying mechanism of its effect on skeletal muscles and exercise performance, has yet to be elucidated. In this study, the effect of L-citrulline supplementation on exercise and the underlying cause of those effects were investigated in vivo, using exercised mice, and in vitro, using skeletal muscle cells.

2. Experimental Section

2.1. Chemicals

Rhodamine 123, L-NAME, and sodium dodecyl sulfate (SDS) were purchased from Wako (Tokyo, Japan). ISOGEN was purchased from Nippongene (Tokyo, Japan). DMEM, RIPA buffer, protease inhibitor cocktail, and β-Actin antibody were purchased from Sigma (MO, USA). Fetal bovine serum (FBS) and house serum (HS) were purchased from Gibco (NY, USA). MTT or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and L-NAME were purchased from Dojindo (Kumamoto, Japan). L-citrulline was supplied by Kyowa Hakko Bio Co., Ltd., Tokyo, Japan) while PGC-1α (3G6) antibody was purchased from Cell Signaling Technology (Hertfordshire, UK). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (6C5) was purchased from Santa Cruz Biotechnology (CA, USA).

2.2. Animal Experiments

Five-week-old male ICR mice were obtained from Charles River Laboratories (Kanagawa, Japan) and maintained under a 12-h light/dark cycle, with free access to water and a normal diet (MF, Oriental Yeast Co., Ltd., Japan). Body weight was measured daily while food intake was measured once a week. After 1 week of acclimatization, the mice were divided into three groups. Group 1 ("no exercise" group) was orally administered with distilled water (D.W.) without swimming exercise ($n = 6$). Group 2 (control group) was orally administered with water. Group 3 (L-citrulline group) was orally administered with 250 mg kg^{-1} day of L-citrulline. The L-citrulline dose used in this study was based on Takeda et al.'s[21] study. Both Groups 2 and 3 performed swimming exercises ($n = 7$ per group). Oral administration of 100 μL sample solution was done using animal-feeding needles (Group 1 and 2: D.W. or Group 3: 250 mg kg^{-1} L-citrulline dissolved in water) every day for 15 days, 1 h before the swimming exercise, and at the same time on "no exercise" days. The exercise protocol was adapted from Takeda et al.[21] with some modifications. Briefly, mice with a load corresponding to 5% of their body weight attached to their tails, were trained to perform the swimming exercise for 10 min, in a tank (30 × 30 × 40 cm) filled with water to a depth of 25 cm., with water temperature kept at 30 ± 1 °C. The swimming exercise was performed every other day for 14 days. On day 15, the mice were made to swim to exhaustion with a load corresponding to 10% of their body weight. Each mouse was considered to have reached its point of exhaustion when it failed to raise its face from the water surface to breathe within a period of 5 s. Blood lactate levels were measured before and after exercise (0 and 60 min) using Lactate Pro 2 (Arkrey, Japan). Blood glucose levels were measured after exercise (0 min) using Glucose Pilot system (Iwai Chemicals Company, Japan). The mice were sacrificed and blood and tissues from the liver, gastrocnemius, and biceps femoris were collected. The serum was separated from the blood by centrifugation at 3000 × g for 10 min and the serum biochemical parameters (BUN, creatinine, total ketone bodies, AST, ALT, ALP, nonessential fatty acid [NEFA]) were analyzed by Oriental Yeast Co., Ltd., (Japan) using test kits obtained from Wako Pure Chemical Industries (Osaka, Japan). All animal experiments performed are in compliance with the guidelines and regulations for Animal Experiments of the University of Tsukuba (No. 16-044), and were approved by the International Animal Care and Use Committee of the University of Tsukuba.

2.3. Real-Time PCR Analysis

Total RNA was isolated from tissue samples (50 mg) and C2C12 myotubes using ISOGEN. For C2C12 myotubes, cells were treated with or without L-citrulline or NOS inhibitor L-NAME. Total RNA isolation and TaqMan real-time PCR amplification reactions were performed as previously reported.[22] For the quantification of the gene expression in muscle tissues and C2C12 myotubes, the following specific TaqMan probes purchased from Applied Biosystems (CA, USA) were used: *β-actin* (Mm00607939_s1), *PGC-1α* (Mm01208835_m1), *LDHa* (Mm01612132_g1), *LDHb* (Mm01267402_m1), *MCT1* (Mm01306379_m1), *CPT-1β* (Mm00487191_g1), *TFAM* (Mm00447485_m1), vascular endothelial growth factor α (*VEGFα*) (Mm00437306_m1), and insulin-like growth factor 1 (*IGF-1*) (Mm00439560_m1). The mRNA levels of all genes were normalized to *β-actin* mRNA levels (internal control).

2.4. Western Blotting

Total protein was isolated from tissue samples (10 mg) using RIPA buffer containing a protease inhibitor cocktail according to

the manufacturer's instructions. Protein samples (15 μg) were separated using 10% SDS-PAGE and transferred to a PVDF membrane (Merck Millipore, USA). Membranes were incubated with primary antibody at 4 °C overnight, then washed, and incubated with secondary antibodies (IRDye 800CW donkey antirabbit IgG or IRDye 680LT goat antimouse [LI-COR, Inc., NE, USA]) at room temperature for 30 min. The signal was detected using the Odyssey Fc Imaging System (LI-COR, Inc., NE, USA).

2.5. Cell Culture and Differentiation

The mouse C2C12 myoblasts (ATCC, USA) were cultured in DMEM supplemented with 10% FBS and 1% penicillin (5000 μg mL^{-1})–streptomycin (5000 IU mL^{-1}) (Lonza, Tokyo, Japan). To induce C2C12 myoblasts to differentiate into C2C12 myotubes, C2C12 cells were cultured until confluent and then transferred to DMEM containing 2% horse serum, and incubated further for 5 days with the growth medium changed every other day.

2.6. MTT Assay

Following treatment and incubation with L-citrulline at different concentrations, MTT solution (5 mg mL^{-1}) was added to the C2C12 myotubes culture and incubated further for 3 h until formazan crystals were formed. Formazan crystals were then dissolved by adding 10% SDS and the plates incubated further for 16 h. The absorbance at 570 nm was measured using a Powerscan HT plate reader (Dainippon Sumitomo Pharma Co, Ltd., Japan).

2.7. Statistical Analysis

All the results are expressed as the mean ± standard deviation, and statistical evaluation was performed using the Student's *t*-test when two value sets were compared. Analysis that includes multiple comparisons were carried out using one-way analysis of variance or ANOVA, followed by Tukey's multiple comparison test using SPSS (IBM Statistics for Windows, version 22.0. IBM Corp., Armonk, NY). $p \leq 0.05$ was considered to be statistically significant.

3. Results

3.1. L-Citrulline Supplementation before Exercise Prevented Exercise-Induced Elevation of Blood Lactate and Decrease in Glucose Levels

To examine the effect of L-citrulline supplementation on blood lactate and glucose levels during exercise, mice were orally administered with L-citrulline or water (control), 1 h before exercise, for 15 days. On day 13, mice were made to perform weight-loaded forced swimming test for 10 min, and 1 h later, were given L-citrulline supplementation (**Figure 1**). Changes in the blood lactate levels during exercise are associated with exercise performance. Lactate that accumulates in the muscles produces H^+ ions that cause fatigue, impairing muscle function and performance.[23] As shown in **Figure 2**A, before exercise, the blood lactate levels between the control and L-citrulline groups were not significantly different. However, after exercise, the lactate level of the L-citrulline group was lowered (9.7 ± 2.0 mM vs. 7.3 ± 0.5 mM, $p = 0.055$). The difference in the lactate levels before and after exercise however, was bigger in the control group. Lactate is produced when glucose or glycogen is used as a fuel source, so that an increase in the glucose levels would lead to higher lactate levels. Additionally, the L-citrulline group had significantly higher glucose levels after exercise on day 13 compared to the control (142 ± 26.8 mg dL^{-1} vs. 174 ± 21.5 mg dL^{-1}, $p = 0.04$) (Figure 2B).

3.2. L-Citrulline Supplementation before Exercise Increased Muscle Weight and Increased Time to Exhaustion in Swimming

To evaluate the effect of L-citrulline supplementation on exercise performance, mice were made to perform a weight-loaded forced swimming test until exhaustion, with a load corresponding to 10% of their body weight, 1 h after L-citrulline supplementation (Figure 1). As shown in **Figure 3**A, the L-citrulline group had longer time to exhaustion, though it was not statistically significant, compared to that of the control groups (270 ± 64.4 s vs. 537 ± 285.2 s, $p = 0.07$). Although the mice in the L-citrulline supplementation group swam for a longer period of time than the control group, the lactate levels between these groups were not significantly different (12.6 ± 0.7 mM vs. 14.0 ± 2.6 mM, $p = 0.27$) (Figure 3B). Also, compared to the control, the L-citrulline group had significantly lower blood glucose levels (153 ± 5.4 mg dL^{-1} vs. 119 ± 7.9 mg dL^{-1}, $p < 0.01$) (Figure 3C). Additionally, L-citrulline supplementation showed an increase in the weight of the gastrocnemius (0.23 ± 0.02 g vs. 0.28 ± 0.02 g, $p = 0.02$) and biceps femoris muscles (0.34 ± 0.01 g vs. 0.52 ± 0.07 g, $p < 0.01$) compared to the control, even though the body weights of both groups were not significantly different (**Table 1**). The food intake, including the amino acids from feeds, was also not significantly different between the control and the L-citrulline-administered groups (data not shown). Although other studies have reported that L-citrulline elevates the levels of urea nitrogen, creatinine, and total ketone bodies in the blood after exercise,[24,25] this study shows that L-citrulline supplementation lowered the creatinine level (0.20 ± 0.05 mg dL^{-1} vs. 0.14 ± 0.02 mg dL^{-1}, $p = 0.06$) and total ketone bodies (1163.3 ± 245.7 μM vs. 948.4 ± 206.6 μM, $p = 0.15$) over time (**Table 2**).

3.3. L-Citrulline Supplementation Upregulated the PGC-1α Expression in Gastrocnemius and Biceps Femoris

PGC-1α in the skeletal muscle has significant regulatory role in the muscles' several adaptations to exercise such as lactate metabolism, angiogenesis, and muscle growth.[1,5] Since the gastrocnemius and biceps femoris are extensively used during swimming, we specifically evaluated the effects of L-citrulline supplementation on the gastrocnemius and biceps femoris. Supplementation with L-citrulline greatly increased *PGC-1α*

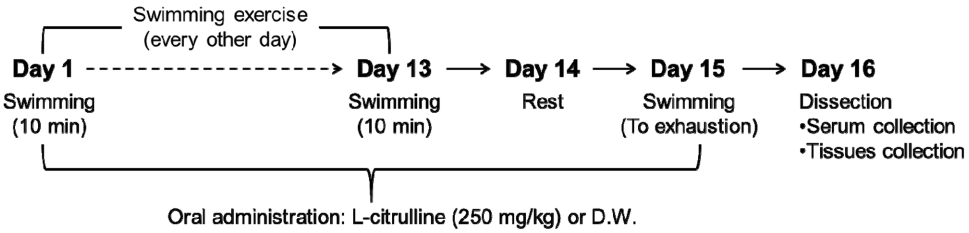


Figure 1. Study design. Mice were made to perform a swimming exercise every other day for 14 days. A swimming-until-exhaustion test was carried out on day 15. During the experimental period, mice were orally administrated with L-citrulline (250 mg kg^{-1}) or distilled water (D.W.) every day.

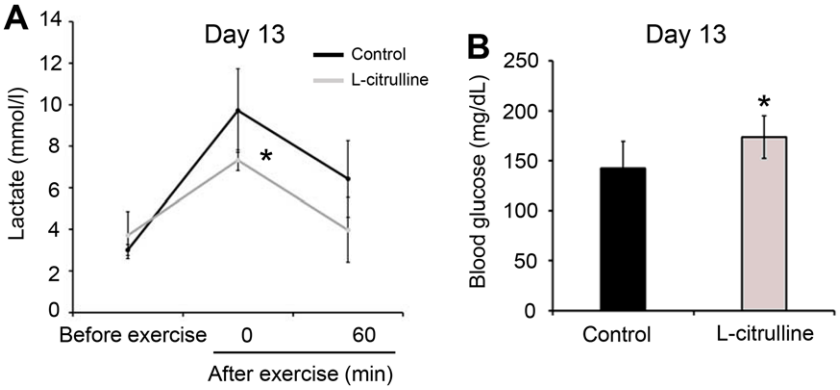


Figure 2. Effect of L-citrulline supplementation on the blood lactate and glucose levels after weight-loaded exercise performance. Mice orally administrated with L-citrulline (250 mg kg^{-1}) or distilled water (D.W.) were made to perform a weight-loaded forced swimming test for 10 min on day 13. A) Blood lactate levels before and after exercise (0 and 60 min) and B) blood glucose levels after exercise (0 min) were evaluated. Values are expressed as the mean ± standard deviation. $*p \leq 0.05$ and $**p \leq 0.01$ indicate a significant difference compared to the control group.

expression in the gastrocnemius (**Figure 4**A; 3.6 ± 0.5-fold, $p < 0.01$) and biceps femoris (Figure 4B; 2.8 ± 0.6-fold, $p < 0.01$). As shown in **Figure 5**, the protein expression level of PGC-1α in the gastrocnemius and biceps femoris (2.6 ± 0.7-fold, $p < 0.01$ and 2.3 ± 0.8-fold, $p = 0.02$, respectively) was also increased with L-citrulline supplementation. VEGFα and *IGF-1*, cytokines released from skeletal muscle are important factors for angiogenesis and muscle growth, respectively, and are under the regulation of PGC-1α.[5] As shown in Figure 4, L-citrulline-supplemented mice had significantly higher levels of *VEGFα* and *IGF-1* in their gastrocnemius (1.8 ± 0.3-fold, $p < 0.01$ and 1.3 ± 0.1-fold, $p < 0.01$, respectively) and biceps femoris (1.4 ± 0.2-fold, $p < 0.01$ and 1.5 ± 0.2-fold, $p < 0.01$, respectively) compared to the control group. The role of PGC-1α in the promotion of lactate metabolism through increased lactate dehydrogenase (LDH) B and monocarboxylate transporter 1 (MCT1) expression in the skeletal muscle has already been established.[26] L-citrulline supplementation also increased the *MCT1* expression in the gastrocnemius (1.5 ± 0.4-fold, $p = 0.05$) and biceps femoris (1.3 ± 0.2-fold, $p < 0.05$). The upregulation of *LDH B*, however, was only observed in the gastrocnemius (1.6 ± 0.3-fold, $p < 0.01$) (Figure 4).

3.4. L-Citrulline-Induced *PGC-1α* Upregulation in C2C12 Myotubes was Suppressed by NO Synthesis Inhibition

To investigate the effect of L-citrulline on the expression of *PGC-1α* in the skeletal muscle cells, the effective and non-cytotoxic concentrations of citrulline were first determined by performing MTT assay using C2C12 myotubes, a cellular model of skeletal muscle. L-citrulline treatment significantly increased the proliferation of C2C12 myotubes but a gradual decrease was observed starting at concentrations of more than 500 μM L-citrulline (**Figure 6**A). Considering the MTT assay results, 100 μM L-citrulline was chosen as the best concentration to use in the succeeding experiments. Treatment with 10, 50, and 100 μM L-citrulline for 1 h increased the *PGC-1α* expression in C2C12 myotubes by 1.2 ± 0.1-fold, 1.4 ± 0.1-fold, and 1.5 ± 0.1-fold, respectively ($p < 0.01$) (Figure 6B). L-citrulline is known to elevate intracellular NO levels by promoting NOS activity.[14–16] Consequently, an elevation of intracellular NO production increases muscle cell's *PGC-1α* expression.[27] In the current study, the upregulation of *PGC-1α* expression in C2C12 myotubes by L-citrulline treatment was suppressed in the presence of NOS inhibitor L-NAME (Figure 6C).

4. Discussion

In the field of sports physiology, nitrate supplementation is believed to be an effective strategy for the enhancement of exercise performance. Increased intracellular NO production increases PGC-1α expression via phosphorylation of CREB.[11] The transcriptional activator PGC-1α has an important role in the regulation of genes associated with adaptation to exercise such as oxidative metabolism, angiogenesis, and muscle growth.[1] It has been reported that non-protein amino acids, (e.g., ornithine, citrulline,

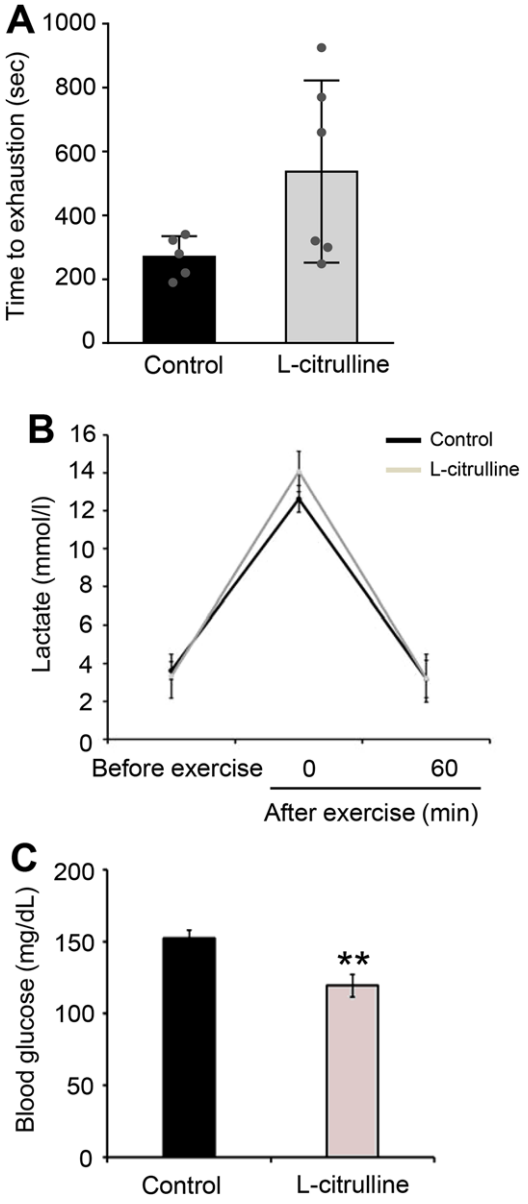


Figure 3. Effect of L-citrulline supplementation on swimming endurance. Mice were trained to perform swimming exercise every other day for 14 days, then a swimming-until-exhaustion test was carried out on day 15. A) Swimming time to exhaustion, B) blood lactate levels before and after exercise (0 and 60 min), and C) blood glucose levels after exercise (0 min) were evaluated. Values are expressed as the mean ± standard deviation. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ indicate a significant difference compared to the control group. The individual raw data was plotted over the bar graph.

Table 1. Mice body weight, food intake, and tissue weight.

		Swimming	
	No exercise	Control	L-citrulline
Initial body weight [g]	28.7 ± 1.3	28.2 ± 1.4	28.5 ± 0.8
Final body weight [g]	30.7 ± 1.2	31.6 ± 2.0	32.5 ± 1.7
Food intake [g day^{-1}]	3.67 ± 0.12	3.64 ± 0.23	3.92 ± 0.32$^{\#}$
Kidney [g]	0.43 ± 0.01	0.50 ± 0.03**	0.45 ± 0.02$^{\#}$
Liver [g]	1.19 ± 0.04	1.18 ± 0.04	1.21 ± 0.06
Gastrocnemius [g]	0.24 ± 0.03	0.23 ± 0.02	0.28 ± 0.02$^{*,\#\#}$
Biceps femoris [g]	0.26 ± 0.03	0.34 ± 0.01**	0.52 ± 0.07$^{**,\#}$

Values are expressed as the mean ± standard deviation. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ indicate a significant difference from the "no exercise" group. $^{\#}p \leq 0.05$ and $^{\#\#}p \leq 0.01$ indicate a significant difference compared to the control group.

Table 2. Level of serum biochemical parameters at the end of the study.

		Swimming	
	No exercise	Control	L-citrulline
BUN [mg dL^{-1}]	32.3 ± 0.9	23.9 ± 2.1**	23.3 ± 2.7**
Creatinine [mg dL^{-1}]	0.15 ± 0.01	0.20 ± 0.05	0.14 ± 0.02
Total ketone bodies [μM]	593.3 ± 53.3	1163.3 ± 245.7**	948.4 ± 206.6*
AST [IU L^{-1}]	1782.5 ± 280.9	1922.0 ± 461.2	1750.4 ± 238.1
ALT [IU L^{-1}]	226.3 ± 3.5	226.8 ± 40.4	201.7 ± 23.7
ALP [IU L^{-1}]	345.0 ± 17.9	433.7 ± 37.6**	369.4 ± 40.9$^{\#}$
NEFA [μEq L^{-1}]	1125.8 ± 140.9	1298.2 ± 129.8	1180.3 ± 158.1

Values are expressed as the mean ± standard deviation. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ indicate a significant difference compared to the "no exercise" group. $^{\#}p \leq 0.05$ indicates a significant difference compared to the control group. BUN, urea nitrogen; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; NEFA, non-esterified fatty acid.

and homocysteine) which are not used for protein synthesis, play an important role in cell metabolism.[28]

L-citrulline, a non-essential amino acid, has recently been demonstrated to be effective in increasing of intracellular NO production by increasing NOS expression.[14–16] The major findings of this study are that the upregulation of PGC-1α in the skeletal muscle by L-citrulline is associated with increased exercise performance and muscles weight. L-citrulline is a precursor of L-arginine that is considered to be preferable than L-arginine in terms of solubility and taste, as well as several functional advantages.[14,15,17] Moreover, L-arginine can enhance NO production but it is rapidly metabolized in the small intestine and liver when administered orally.[14] Therefore, when compared to L-citrulline, L-citrulline comes out as a more effective sports supplement for enhancing sports performance compared to L-arginine. Some studies, have reported that single supplementation of L-citrulline does not help improve exercise performance[21,29] suggesting the need for long-term supplementation of L-citrulline (more than 1 week) to effectively enhance exercise tolerance.[20,21] The current study also provides data to validate assumptions of past researches that L-citrulline supplementation for 15 days can enhance exercise performance and increase muscle mass. The results of this study therefore suggest that long-term intake of L-citrulline has a positive impact on exercise performance.

Lactate is produced during intense exercise when the supply of O_2 is insufficient. The blood lactate level during exercise is dependent on the ratio of lactate production to lactate clearance.[23,30] Hydrogen ions (H^+) dissociate from lactic acid and accumulate in the muscles, causing fatigue and depressing muscle function and contraction.[23] In addition, decreased blood glucose levels

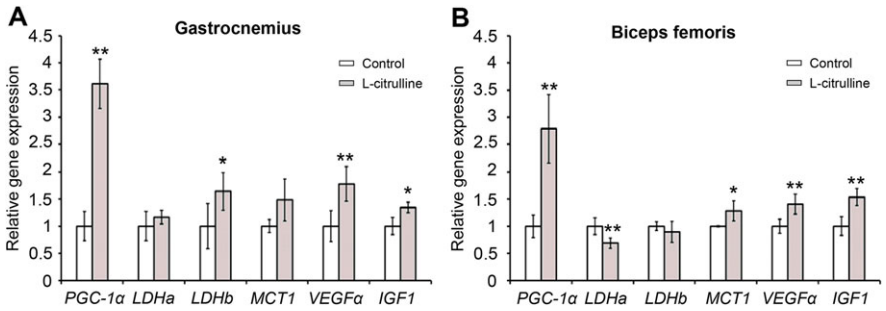


Figure 4. Effect of L-citrulline supplementation on the expression of *PGC-1α* and PGC-1α-related genes in the gastrocnemius and biceps femoris. Expression levels of *PGC-1α* and PGC-1α-targeted genes in the A) gastrocnemius and B) biceps femoris were evaluated. Expression levels of mRNA were normalized to the *β-actin* expression level. Values are expressed as the mean ± standard deviation and relative to the "no exercise" group. $*p \leq 0.05$ and $**p \leq 0.01$ indicate a significant difference compared to the control group.

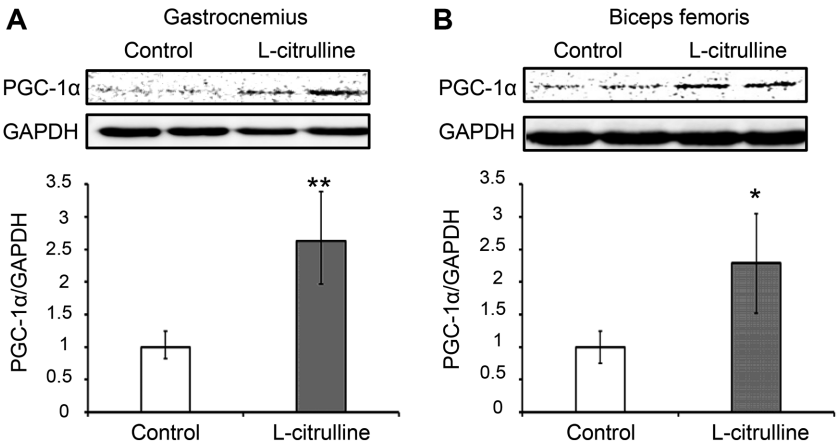


Figure 5. Effect of L-citrulline supplementation on the protein expression of PGC-1α in the gastrocnemius and biceps femoris. Protein expression levels of PGC-1α in the A) gastrocnemius and B) biceps femoris were evaluated. All gels were run under the same experimental conditions and the representative blots were shown. The protein expression levels were normalized to GAPDH expression. Values are expressed as the mean ± standard deviation and relative to the unexercised group. $*p \leq 0.05$ and $**p \leq 0.01$ indicate a significant difference from the control group.

(hypoglycemia) during exercise also causes fatigue and low energy, leading to exercise cessation.[31] The L-citrulline supplemented mice group had lower blood lactate levels and higher glucose levels immediately after exercising with the same load (Figure 2). These results therefore suggest that the observed inhibition of lactate production and/or increased lactate metabolism during exercise can be attributed to L-citrulline supplementation. However, the blood lactate levels after exercise were not different from the after the swimming-to-exhaustion test blood lactate levels (Figure 3). Following a swimming-to-exhaustion test, the blood lactate levels gradually increase depending on the duration of the exercise.[32] In this study, L-citrulline supplemented group had longer average swimming to exhaustion time compared to the control (270 s vs. 537 s, respectively) (Figure 3A). Therefore, it can be assumed that blood lactate levels before and after the swimming-to-exhaustion test remained the same. Skeletal muscle PGC-1α promotes lactate metabolism by increasing the expression of LDH B and MCT1, and conversely, prevents lactate production by suppressing the expression of LDH A that catalyzes the conversion of pyruvate to lactate.[1,26] A decrease in *LDH A* expression and increase in *MCT1* and *LDH B* levels were also observed following L-citrulline supplementation (Figure 3). Several studies reported have established that L-citrulline has antidiabetic and antiobesity effects[33–35] but does not affect the blood glucose level. So, in this study, it can then be assumed that L-citrulline did not affect the blood glucose levels and instead suggests that a decrease in lactate production and an increase in lactate metabolism are therefore involved in the regulation of blood lactate and glucose levels. PGC-1α mRNA level is elevated after performing exercise but the level returns back to its "before exercise level" during the rest period, specifically within 24 h after exercise.[36,37] Even though the muscle samples were collected 24 h after exercise, an increase in PGC-1α mRNA and protein expression due to L-citrulline supplementation was still observed (Figure 2). Therefore, it is clear that the increase in lactate metabolism can be attributed to the longitudinal PGC-1 α upregulation by L-citrulline rather than a transient upregulation by exercise.

Increased blood flow enhances not only exercise performance, by improving nutrient and oxygen delivery in muscle, but also by boosting protein synthesis and muscle fiber repair.[38] Several reports have suggested that L-citrulline supplementation can improve blood pressure, VO_2 kinetics, and exercise performance in healthy adults,[19,20] and believed to be due to PGC-1α upregulation in the muscles that promoted formation of new blood vessels (angiogenesis), and thus, integrating oxygen/nutrient

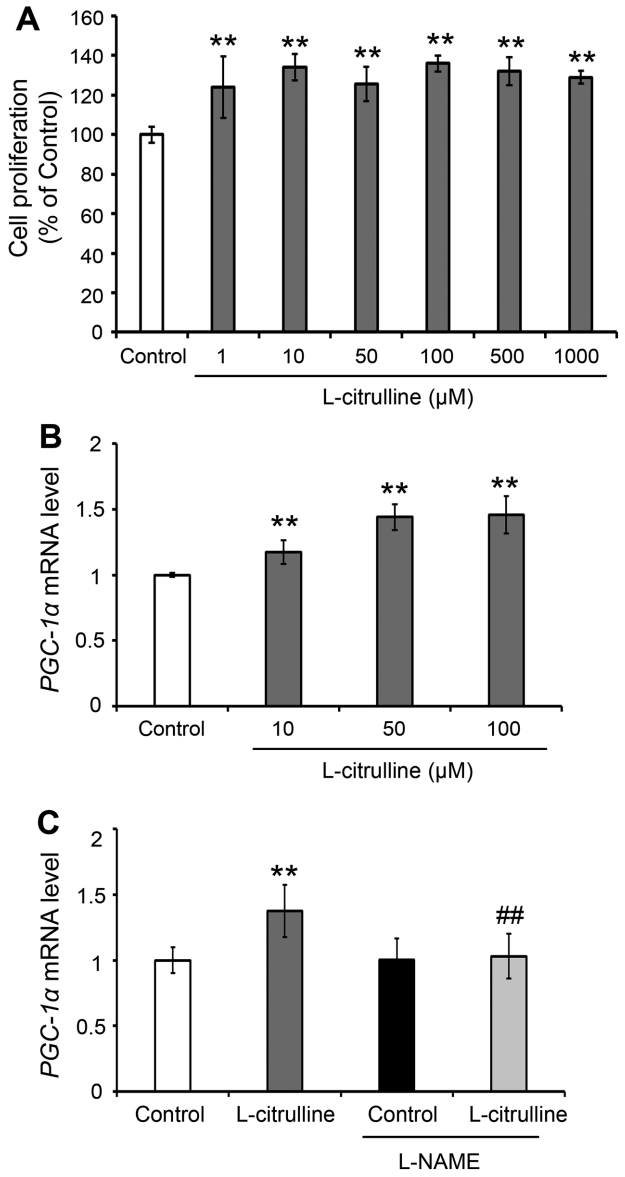


Figure 6. Effect of L-citrulline on the gene expression of *PGC-1α* in C2C12 myotubes. Differentiated C2C12 myotubes were treated with or without L-citrulline for 24 h. A) After that, cell viability was evaluated and value expressed as percentage (%) of control. B) C2C12 myotubes were treated with or without L-citrulline (10, 50, 100 μM) for 1 h. C) L-citrulline (50 μM) treatment was performed with or without 100 μM NG-nitro-L-arginine methyl ester hydrochloride (L-NAME) for 1 h. Following treatment, *PGC-1α* mRNA levels were quantified using real-time PCR and the values normalized to the expression level of *β-actin*. Values are expressed as the mean ± standard deviation of triplicate experiments. $^{**}p \leq 0.01$ indicates a significant difference from the control group. $^{\#\#}p \leq 0.01$ indicate a significant difference from the L-NAME-treated L-citrulline group.

consumption and supply. PGC-1α expression in cultured muscle cells and in skeletal muscle promotes the expression of several angiogenic factors, including VEGF, which plays a crucial role in vascular development. At the same time, muscle vascularization by VEGFα increases blood supply and oxygen availability in the muscle, increasing exercise time and endurance.[39] In the current study, an L-citrulline-induced increase in skeletal muscle *VEGFα* expression was observed (Figure 4), suggesting that L-citrulline supplementation-induced angiogenesis in skeletal muscle can be associated with the observed increase in swimming time. It is a well-known fact that oral supplementation with L-citrulline elevates NO levels by increasing NOS expression, resulting in improved endothelial function.[15] On the other hand, NOS inhibition has been established to reduce maximal oxygen uptake during exercise in humans.[13] However, increased intracellular NO production induces PGC-1α expression.[11] In this study, L-citrulline-induced *PGC-1α* upregulation in C2C12 myotubes was suppressed by L-NAME, a NOS inhibitor (Figure 6), suggesting, therefore, that L-citrulline-increased skeletal muscle PGC-1α level was due to the rise in the intracellular NO production.

Several PGC-1α variants are expressed from alternative gene promoter, namely PGC-1α-b and PGC-1α4, and have been shown to induce VEGF expression in skeletal muscle and angiogenesis.[40] Transgenic expression of PGC-1α4 in skeletal muscle in mice induces angiogenesis in vivo.[40] PGC-1α4 also activates a hypertrophic gene program in skeletal muscle. In addition, PGC-1α4 also regulates skeletal muscle growth by inducing the anabolic hormone IGF-1 and repressing myostatin, a powerful inhibitor of muscle differentiation and growth.[7,41] In this study, skeletal muscle weight was increased corresponding to PGC-1α and IGF-1 upregulation by L-citrulline. It can therefore be inferred from these results that L-citrulline induces PGC-1α4 in association with L-citrulline-induced *VEGFα* and *IGF-1* upregulation. Elevated PGC-1α in muscle dramatically protects against the sarcopenia, obesity, and diabetes that normally accompanies aging.[42] Therefore, the use of L-citrulline to increase PGC-1α expression may be useful in the management of diseases such as obesity, diabetes, and sarcopenia, as well as in the enhancement of exercise performance.

5. Concluding Remarks

L-citrulline supplementation before exercise upregulates PGC-1α expression in the skeletal muscle, resulting in a significant increase in skeletal muscle weight. A longer average time before mice became exhausted during exercise was also observed in L-citrulline-supplemented animals, but the significance of this effect needs further verification in a clinical trial. Furthermore, inhibition of NOS expression suppresses the L-citrulline-induced *PGC-1α* upregulation. Further experiments would aim to compare the effect of other amino acids to the effect of L-citrulline on enhancing exercise performance and increasing muscle weight. This study has demonstrated that L-citrulline supplementation resulted in a significant improvement in exercise performance and increased skeletal muscle weight, and therefore may be used to enhance sports or exercise performance.

Acknowledgments

This work was supported by the Center of Innovation Program (COI) of the University of Tsukuba and financially supported by the Ministry of Education Culture and Sports (MEXT), Japan, and Japan Science and Technology

(JST). M.O.V., H.I., and T.M. designed the study; T.M. conducted the experiments, and analyzed the data with M.O.V. and H.I.; M.O.V. and T.M. wrote the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

exercise performance, L-citrulline, *PGC-1α*, skeletal muscle weight, supplementation

Received: December 18, 2017
Revised: May 8, 2018
Published online: May 24, 2018

[1] L. Chen, J. Pei, J. Kuang, H. Chen, Z. Chen, Z. Li, H. Yang, *Metab*. **2015**, *64*, 338.
[2] A. Safdar, J. P. Little, A. J. Stokl, B. P. Hettinga, M. Akhtar, M. A. Tarnopolsky, *J. Biol. Chem*. **2011**, *286*, 10605.
[3] D. E. Warburton, A. Charlesworth, A. Ivey, L. Nettlefold, S. S. Bredin, *Int. J. Behav. Nutr. Phys. Act*. **2010**, *7*, 39.
[4] H. Brønnum-Hansen, K. Juel, M. Davidsen, J. Sørensen, *Scand. J. Public Health*. **2007**, *35*, 510.
[5] A. Safdar, J. P. Little, A. J. Stokl, B. P. Hettinga, M. Akhtar, M. A. Tarnopolsky, *J. Biol. Chem*. **2011**, *286*, 10605.
[6] Z. Yan, M. Okutsu, Y. N. Akhtar, V. A. Lira, *J. Appl. Physiol*. **2011**, *110*, 264.
[7] M. Tadaishi, S. Miura, Y. Kai, Y. Kano, Y. Oishi, O. Ezaki, *PloS One* **2011**, *6*, e28290.
[8] C. Handschin, C. A. Choi, S. Chin, S. Kim, D. Kawamori, A. J. Kupad, N. Neubauer, J. Hu, V. K. Mootha, Y.-B. Kim, R. N. Kulkarni, G. I. Shulman, B. M. Spiegelman, *J. Clin. Invest*. **2007**, *117*, 3463.
[9] M. C. Chan, Z. Arany, *Metabolism* **2014**, *63*, 441.
[10] A. M. Jones, D. P. Wilkerson, I. T. Campbell, *J. Physiol*. **2004**, *560*, 329.
[11] S. Baldelli, D. Lettieri Barbato, G. Tatulli, K. Aquilano, M. R. Ciriolo, *J. Cell Sci*. **2014**, *127*, 4813.
[12] R. Bescos, A. Sureda, J. A. Tur, A. Pons, *Sports Med*. **2012**, *42*, 99.
[13] A. M. Jones, *Sports Med*. **2014**, *44*(Suppl 1), S35.
[14] E. Schwedhelm, R. Maas, R. Freese, D. Jung, Z. Lukacs, A. Jambrecina, W. Spickler, F. Schulze, R. H. Boger, *Br. J. Clin. Pharmacol*. **2008**, *65*, 51.
[15] T. Hayashi, P. A. Juliet, H. Matsui-Hirai, A. Miyazaki, A. Fukatsu, J. Funami, A. Iguchi, L. J. Ignarro, *Proc. Natl. Acad. Sci. U.S.A*. **2005**, *102*, 13681.
[16] G. Wu, J. K. Collins, P. Perkins-Veazie, M. Siddiq, K. D. Dolan, K. A. Kelly, C. L. Heaps, C. J. Meininger, *J. Nutr*. **2007**, *137*, 2680.
[17] K. Akashi, C. Miyake, A. Yokota, *FEBS Lett*. **2001**, *508*, 438.
[18] D. Bendahan, J. P. Mattei, B. Ghattas, S. Confort-Gouny, L. M. E. Guern, P. J. Cozzone, *Br. J. Sports Med*. **2002**, *36*, 282.
[19] S. J. Bailey, J. R. Blackwell, T. Lord, A. Vanhatalo, P. G. Winyard, A. M. Jones, *J. Appl. Physiol. (1985)*. **2015**, *19*, 385.
[20] T. Suzuki, M. Morita, Y. Kobayashi, A. Kamimura, *J. Int. Soc. Sports Nutr*. **2016**, *13*, 1.
[21] K. Takeda, M. Machida, A. Kohara, N. Omi, T. Takemasa, *J. Nutr. Sci. Vitaminol. (Tokyo)*. **2011**, *57*, 246.
[22] T. Matsukawa, T. Inaguma, J. Han, M. O. Villareal, H. Isoda, *J. Nutr. Biochem*. **2015**, *26*, 860.
[23] F. Aldeam, R. Irving, L. Dilworth, *AJSSM* **2013**, *1*, 42.
[24] S. Nozaki, M. Tanaka, K. Mizuno, S. Ataka, H. Mizuma, T. Tahara, T. Sugino, T. Shirai, A. Eguchi, K. Okuyama, K. Yoshida, Y. Kajimoto, H. Kuratsune, O. Kajimoto, Y. Watanabe, *Nutrition* **2009**, *25*, 51.
[25] S. Y. Wang, W. C. Huang, C. C. Liu, M. F. Wang, C. S. Ho, W. P. Huang, C. C. Hou, H. L. Chuang, C.-C. Huang, *Molecules* **2012**, *17*, 11864.
[26] S. Summermatter, G. Santos, J. Pérez-Schindler, C. Handschin, *Proc. Natl. Acad. Sci. U.S.A*. **2013**, *110*, 8738.
[27] V. A. Lira, D. L. Brown, A. K. Lira, A. N. Kavazis, Q. A. Soltow, E. H. Zeanah, D. S. Criswell, *J. Physiol*. **2010**, *588*, 3551.
[28] G. Wu, *Amino Acids* **2009**, *37*, 1.
[29] P. T. Cutrufello, S. J. Gadomski, G. S. Zavorsky, *J. Sports Sci*. **2015**, *33*, 1459.
[30] B. Stallknecht, J. Vissing, H. Galbo, *Scand. J. Med. Sci. Sports*. **1988**, *8*, 127.
[31] J. F. Brun, M. Dumortier, C. Fedou, L. Mercier, *Diabetes Metab*. **2001**, *27*, 92.
[32] S. E. Gaskill, B. C. Ruby, A. J. Walker, O. A. Sanchez, R. C. Serfass, A. S. Leon, *Med. Sci. Sports Exerc*. **2001**, *33*, 1841.
[33] P. Persson, A. Fasching, T. Teerlink, P. Hansell, F. Palm, *Hypertension* **2014**, *64*, 323.
[34] M. Kudo, H. Yashitomi, M. Momoo, S. Suguro, Y. Yamagishi, M. Gao, *Biol. Pharm. Bull*. **2017**, *40*, 524.
[35] S. Ikeda, T. Kizaki, S. Haga, H. Ohno, T. Takemasa, *Biochem. Biophys. Res. Comm*. **2008**, *368*, 323.
[36] H. Pilegaard, B. Saltin, P. D. Neufer, *J. Physiol*. **2003**, *546*, 851.
[37] A. S. Mathai, A. Bonen, C. R. Benton, D. L. Robinson, T. E. Graham, *J. Appl. Physiol (1985)*. **2008**, *105*, 1098.
[38] D. J. Weinert, *J. Can. Chiropr. Assoc*. **2009**, *53*, 186.
[39] H. Delavar, L. Noqueira, P. D. Wagner, M. C. Hoqan, D. Metzqer, E. C. Breen, *Am. J. Physiol. Regul. Integr. Comp. Physiol*. **2014**, *306*, 586.
[40] R. Thom, G. C. Rowe, C. Jang, A. Safdar, Z. Arany, *J. Biol. Chem*. **2014**, *289*, 8810.
[41] J. L. Ruas, J. P. White, R. R. Rao, S. Kleiner, K. T. Brannan, B. C. Harrison, N. P. Greene, J. Wu, J. L. Estall, B. A. Irving, I. R. Lanza, K. A. Rasbach, M. Okutsu, K. S. Nair, Z. Yan, L. A. Leinwand, B. M. Spiegelman, *Cell* **2012**, *151*, 1319.
[42] L. L. Ji, C. Kang, *Gerontology* **2015**, *61*, 139.