Oral administration of L-citrulline alters the vascular delivery of substances to rat skeletal muscles

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ABSTRACT

Vascular endothelial function deteriorates with age and disease, and the production of vasodilator factors like nitric oxide (NO) decreases. The free amino acid L-citrulline increases vasodilation and blood flow through increased NO production. We examined the effects of oral L-citrulline administration on vascular delivery of substances to skeletal muscles. In Experiment 1, following oral L-citrulline administration and subsequent intravenous Evans blue dye (EBD) administration to rats, EBD levels delivered to skeletal muscles were measured after 60 min. In Experiment 2, plasma concentrations of amino acids and NOx, an indicator of vasodilation, were measured over time after oral L-citrulline administration. In Experiment 3, we measured EBD levels in skeletal muscles of streptozotocin-induced type 1 diabetic rats following L-citrulline administration. In these experiments, EBD levels in the soleus muscle were higher in the L-citrulline group than in the control group (19.9 ± 0.7 vs. 22.5 ± 1.9 µg/g tissue, $p < 0.05$). Plasma L-arginine, L-citrulline, and NOx levels were increased within 30 min after L-citrulline administration. EBD levels in the soleus and gastrocnemius muscles were higher in diabetic rats with L-citrulline administration (18.7 ± 2.2 vs. 25.0 ± 4.3 µg/g tissue, $p < 0.05$ and 8.0 ± 0.5 vs. 9.2 ± 0.8 µg/g tissue, $p = 0.05$, respectively). These data suggest that oral L-citrulline administration may increase the level of substances delivered to skeletal muscles by increasing the NO production in both normal and vascular endothelial dysfunction models.

1. Introduction

The arteries branch from the aorta and further divide into the arterioles, which supply blood to the skeletal muscle capillary network. Capillaries are highly branched and tortuous around skeletal muscle cells. Pre-capillary sphincters were previously thought to regulate the flow of erythrocytes, but in recent years, arterioles have been shown to play a major role in blood flow regulation of skeletal muscles [1,2]. Vascular endothelial cells are present in the innermost layer of arterioles, while vascular smooth muscle cells are found outside the layer. The capillary network in skeletal muscles is essential for the delivery of oxygen and nutrients to skeletal muscle cells. Blood flow to the skeletal muscle is determined by the balance between vasoconstrictor and vasodilator signaling pathways in vascular smooth muscle cells. In diabetes, hypertension, dyslipidemia, aging, and obesity, vascular endothelial function is impaired [3–5] because the expression level of endothelial nitric oxide synthase (eNOS) in vascular endothelial cells decreases [6]. This can reduce the production of nitric oxide (NO), a

vasodilator, as well as the blood flow to organs and skeletal muscles. Particularly in diabetes, blood vessel counts, blood vessel density, blood flow, and angiogenesis in skeletal muscles are reportedly decreased [7–9]. It has been suggested that reduced delivery of nutrients to skeletal muscles due to decreased vascular endothelial function reduces the amino acid availability in skeletal muscle cells [10]. By contrast, NO-dependent vasodilation induced by insulin administration increases glucose uptake in rat and human skeletal muscles [11,12]. Furthermore, in young and elderly people, the synthesis of skeletal muscle proteins is upregulated when insulin-induced increases in blood flow improve vascular endothelial function [13,14]. However, it remains elusive whether nutrient delivery to skeletal muscles is increased when blood flow and vasodilation are improved by the intake of dietary-derived components.

L-citrulline is a nonessential alpha-amino acid initially isolated from watermelon (*Citrullus vulgaris*) [15]. This amino acid is an important component of the urea cycle in the liver and kidneys, and it is in vivo mainly present in the blood, urine, and cells as free L-citrulline [16].

L-citrulline is converted into L-arginine by argininosuccinate synthase and argininosuccinate lyase, resulting in increased blood L-arginine levels [17]. L-arginine is the precursor for eNOS-dependent NO synthesis in vascular endothelial cells, and NO induces vasorelaxation in smooth muscle cells [16,18]. Oral L-citrulline supplementation increases blood NO levels and improves vascular endothelial function in animals and humans [19–21]. L-arginine ingestion increases the blood flow in the lower limbs and blood NO levels in patients with type 1 and type 2 diabetes [22,23]. However, the vasodilatory effect of oral L-citrulline administration on skeletal muscles in diabetes has not been fully investigated.

We hypothesized that oral L-citrulline administration would increase Evans blue dye (EBD) delivery to skeletal muscles through NO increase in normal rats and diabetic rats, a rat model of vascular endothelial dysfunction. For this model, we used a streptozotocin (STZ)-induced type 1 diabetes model, which has been reported to show decreased vascular endothelial function [7,9]. Since eNOS expression and activity in skeletal muscles are decreased in type 1 diabetes [24], this model is suitable for evaluating NO-dependent vasodilatory L-citrulline effects. In this model, EBD was intravenously administered immediately after oral L-citrulline administration in normal or diabetic rats. The soleus and gastrocnemius muscles, which have different vascular densities [25], were sampled. Following EBD delivery, we assessed the EBD levels in the skeletal muscles and examined the effects of oral L-citrulline administration on the delivery of substances to the skeletal muscles.

2. Materials and methods

2.1. Ethical considerations

This study was carried out in accordance with the Institutional Committee on Care and Use of Laboratory Animals of Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan) and was approved by the Otsuka Pharmaceutical Animal Experiment Committee.

2.2. Experiment 1

2.2.1. Animals

Nine-week-old male Crl:CD(SD) rats (Charles River Japan, Inc., Yokohama, Japan) were housed in a room maintained at $23 \pm 3\ °C$ with a 12-h light/dark cycle (light: 7:00 a.m.–7:00 p.m.) and $55 \pm 15\%$ humidity. The rats were allowed free access to a standard diet (AIN-93G; Oriental Yeast, Osaka, Japan) and water during the experimental period. After a 1-wk acclimation period, 300 mg/kg L-arginine (Kyowa Hakko Bio Co. Ltd., Tokyo, Japan) was intravenously administered to some rats (ARG, n = 6) in accordance with the study by Ohta et al. showing improved blood flow by intravenous L-arginine administration [26]. Other rats received orally administered water (CON, n = 5), or 500 mg/kg (CIT500, n = 6) or 1000 mg/kg (CIT1000, n = 5) L-citrulline (Kyowa Hakko Bio Co., Ltd.) for three consecutive days.

2.2.2. EBD assay

All rats underwent a 1-cm neck incision and a 1-cm dorsal skin incision under sodium pentobarbital anesthesia. A silicon catheter was placed in the jugular vein of each animal. After the operation, all rats were placed in individual metabolic cages for 3 d. All animals in each group were fasted overnight before sacrifice. EBD levels in the muscles were evaluated using modified versions of the methods reported by Xu et al. and Wang et al. [27,28]. EBD solution was prepared by dissolving dye powder (Wako Pure Chemical Industries, Osaka, Japan) in 0.9% saline. Immediately after the last administration of L-citrulline, 75 mg/kg EBD was injected into the jugular vein for over 10 s, and the catheter was flushed with 0.4 mL 0.9% saline. Following EBD infusion, the rats turned visibly blue, confirming the uptake and distribution of the dye. Fifty mg/kg sodium pentobarbital anesthesia was intraperitoneally administered 30 min after EBD infusion to exclude the effect of

activity on the results. The soleus and gastrocnemius muscles were carefully collected under anesthesia 60 min after EBD infusion. After measurement of the muscles wet weight, EBD was extracted by incubating each muscle in 1.5 mL (soleus) or 7.0 mL (whole gastrocnemius) formamide at 37 °C for 20 h. There were no differences in the concentration of EBD between 12 h and 20 h incubation time, therefore, it was thought that 20 h was enough period to extract EBD. The extract was centrifuged for 15 min at 12,000 rpm, and then 100 μL of the supernatant was used to measure the absorbance at 620 nm, the maximum absorption EBD in formamide, using a standard plate reader (DS Pharma Biomedical Co. Ltd., Osaka, Japan). The concentration of EBD in the muscles was calculated from a standard curve of EBD in formamide (0, 1, 2, 4, 8, 16 and 32 μg/mL). Results are expressed in micrograms of EBD per gram of tissues.

2.3. Experiment 2

2.3.1. Animals

Nine-week-old male Crl: CD (SD) rats were fed a standard diet and water ad libitum during the experimental period. After a 1-wk acclimation period, the rats were orally administered water (CON) or 1000 mg/kg L-citrulline (CIT) for three consecutive days. Following overnight fasting, blood samples were obtained from the abdominal aorta under anesthesia at 10, 20, and 30 min after the last oral L-citrulline administration.

2.3.2. Blood analysis

Plasma was drawn into a blood collection tube containing ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA) and then immediately cooled on ice. Serum was dispensed into a blood collection tube and allowed to stand at room temperature. Blood samples were centrifuged for 10 min at 4 °C and 3000 rpm, after which the supernatants were collected. Plasma amino acid levels were determined using liquid chromatography-mass spectrometry (LC-MS2020; Shimadzu Co., Kyoto, Japan). The serum NOx (nitrite and nitrate) levels were measured using an NO detector-based high-performance liquid chromatography system (ENO20; Eicom, Kyoto, Japan).

2.4. Experiment 3

2.4.1. Animals

Eight-week-old male Crl:CD(SD) rats were fed a standard diet and water ad libitum during the experimental period. After a 1-wk acclimation period, type 1 diabetes mellitus was induced by a single STZ (Sigma-Aldrich, St. Louis, MO, USA) injection into the lateral tail vein. STZ (dissolved in 0.1 M sodium citrate buffer, pH 4.5) was administered at a dosage of 50 mg/kg. Diabetes was defined as a tail vein blood glucose level of >300 mg/dL 2 wk after STZ injection. Control rats orally received water (CON, n = 5), and diabetic rats received water (STZ, n = 5) or 500 mg/kg L-citrulline (STZ + CIT, n = 8) for 3 wk.

2.4.2. EBD assay

The EBD assay was performed as described in Experiment 1. Briefly, 75 mg/kg EBD was administered intravenously immediately after the last oral administration of L-citrulline. The soleus and gastrocnemius muscles were sampled after 60 min under anesthesia, and the level of EBD per tissue weight was calculated.

2.5. Statistical analysis

Data are presented as mean $\pm$ standard deviation. In Experiment 1, the Mann–Whitney *U* test was used to assess differences between CON and ARG groups. Differences among CON, CIT500, and CIT1000 groups were assessed by a nonparametric analysis of variance (ANOVA) (Kruskal–Wallis test), followed by Steel's test. In Experiment 2, one-way ANOVA was used to evaluate differences among all groups compared
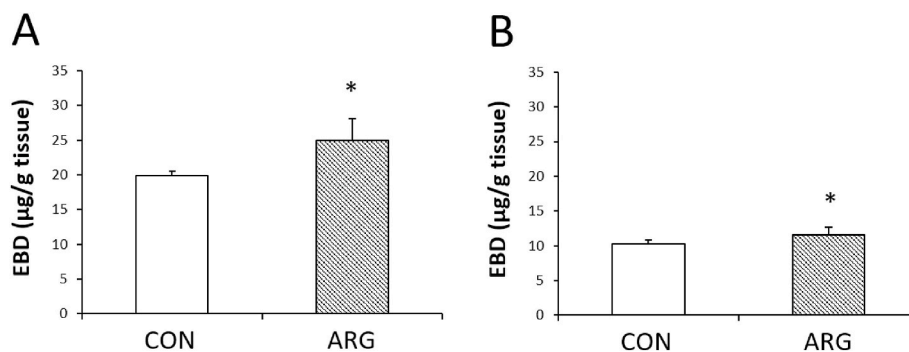
Fig. 1. Effects of ʟ-arginine administration on the vascular delivery of Evans blue dye (EBD) to skeletal muscles. EBD levels after ʟ-arginine administration in soleus (**A**) and gastrocnemius (**B**) muscles. CON, control rats; ARG, rats administered ʟ-arginine (300 mg/kg, i.v.). Data are presented as mean ± standard deviation (n = 5–6). *p < 0.05 by Mann–Whitney *U* test, compared with CON.
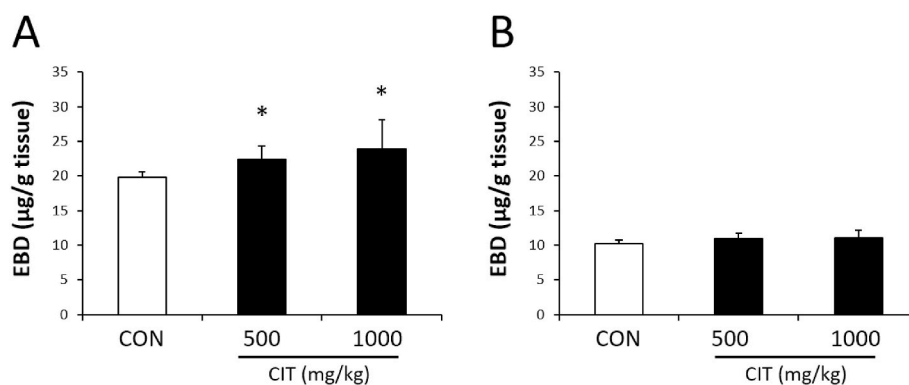

Fig. 2. Effects of ʟ-citrulline administration on the vascular delivery of Evans blue dye (EBD) to skeletal muscles. EBD in soleus (**A**) and gastrocnemius (**B**) muscles. EBD levels were determined 60 min after the last oral ʟ-citrulline administration. CON, control rats; CIT500 and CIT1000, rats administered ʟ-citrulline (500 mg/kg and 1000 mg/kg, p.o., respectively). Data are presented as mean ± standard deviation (n = 5–6). *p < 0.05 by Steel's test, compared with CON.

Table 1
Blood amino acid concentrations.

	CON	CIT		
		10 min	20 min	30 min
Arginine (nmol/ mL)	169.2 ± 12.1	325.9 ± 33.0 *	389.5 ± 35.5 *	455.4 ± 18.3 *
Citrulline (nmol/ mL)	64.6 ± 7.8	1089.0 ± 98.9 *	1599.3 ± 178.3 *	2230.0 ± 192.3 *
Isoleucine (nmol/mL)	105.6 ± 12.2	87.0 ± 12.7 *	83.2 ± 9.9 *	89.4 ± 6.8 *
Leucine (nmol/ mL)	155.7 ± 15.0	127.8 ± 18.6 *	122.9 ± 14.7 *	124.9 ± 11.8 *
Ornithine (nmol/ mL)	55.2 ± 7.1	81.8 ± 8.4 *	120.3 ± 26.3 *	132.6 ± 16.0 *
Valine (nmol/ mL)	201.2 ± 24.0	159.0 ± 23.2 *	164.7 ± 23.6 *	168.5 ± 11.9 *
BCAA (nmol/mL)	462.5 ± 50.6	373.8 ± 53.9 *	370.7 ± 47.5 *	382.7 ± 30.3 *

CON, control rats; CIT, rats 10, 20, and 30 min after ʟ-citrulline (1000 mg/kg, p. o.) administration; BCAA, branched-chain amino acids. Data are presented as the mean ± standard deviation, n = 6. *p < 0.05 by Dunnett's test, compared with CON.

with the CON group, followed by Dunnett's test. In Experiment 3, the statistical significance among the three groups was assessed using one-way ANOVA, followed by Tukey-Kramer's test. Results with p < 0.05 were considered statistically significant. Statistics were processed using the EXSUS software (version 7.7, CAC Croit Corporation, Tokyo, Japan).

3. Results

3.1. Experiment 1

3.1.1. EBD assay

The levels of EBD delivered to skeletal muscles after ʟ-arginine administration are shown in Fig. 1; the ratios of EBD to muscle mass were in both soleus and gastrocnemius muscles significantly higher in the ARG group than in the CON group (p < 0.05). Fig. 2 shows the EBD delivery to the muscles after oral ʟ-citrulline administration. EBD levels in the soleus muscle were significantly higher in the CIT500 and CIT1000 groups compared to the CON group (p < 0.05, Fig. 2A). By contrast, no differences among groups were observed in the gastrocnemius muscle (Fig. 2B).

3.2. Experiment 2

3.2.1. Plasma amino acid levels

Table 1 lists the measured plasma amino acid levels. Plasma ʟ-arginine, ʟ-citrulline, and ornithine levels were significantly higher in the CIT group than in the CON group at 10, 20, and 30 min after oral ʟ-citrulline administration (p < 0.05). However, plasma isoleucine, leucine, valine, and branched-chain amino acid (BCAA) levels were significantly lower in the CON group at all time points after ʟ-citrulline administration (p < 0.05).

3.2.2. Blood NOx levels

Fig. 3 shows the blood levels of the final NO metabolites nitrite and nitrate. Although no significant differences were observed in nitrite levels among the groups (Fig. 3A), nitrate levels were significantly
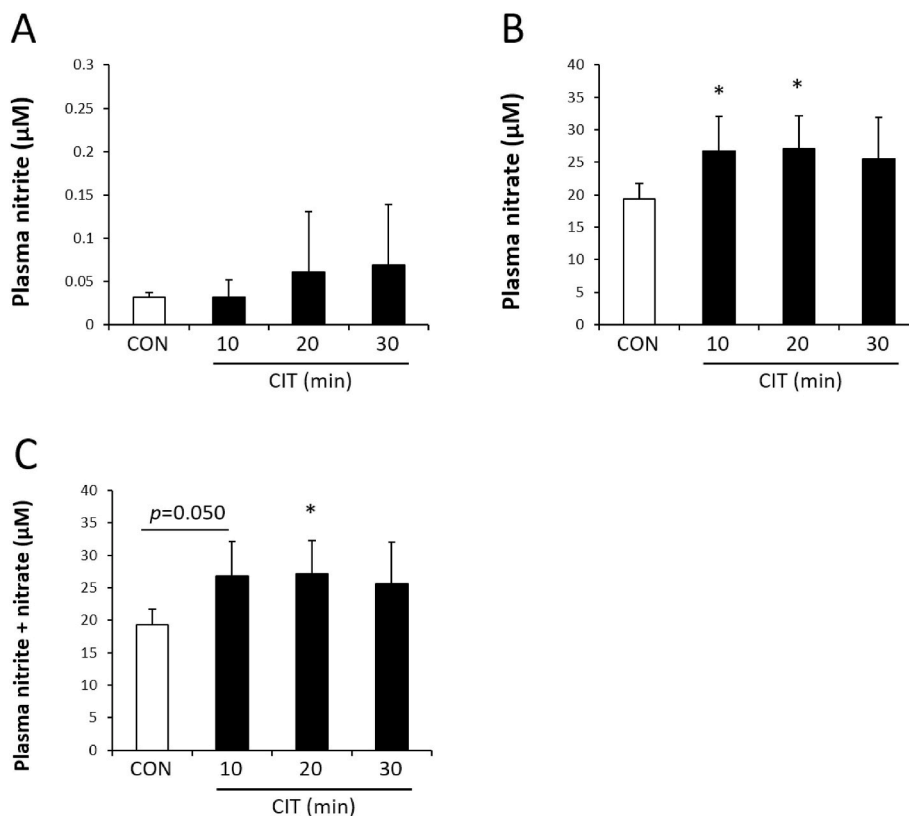
A — Plasma nitrite (μM) vs CON, CIT (min) 10, 20, 30

B — Plasma nitrate (μM) vs CON, CIT (min) 10, 20, 30

C — Plasma nitrite + nitrate (μM) vs CON, CIT (min) 10, 20, 30

Fig. 3. Blood nitrite and nitrate levels. CON, control rats; CIT, rats 10, 20, and 30 min after L-citrulline (1000 mg/kg, p.o.) administration. Data are presented as mean ± standard deviation, n = 6. *p < 0.05 by Dunnett's test, compared with CON.

Table 2
Body weight, absolute and relative skeletal muscle mass and blood glucose level.

	CON	STZ	STZ + CIT
Body weight (g)	459.3 ± 39.2[a]	374.0 ± 42.9 [b]	383.1 ± 40.3 [b]
Soleus muscle (mg)	184.5 ± 9.4	157.4 ± 23.5	163.7 ± 23.4
Soleus muscle/body weight (mg/g)	0.40 ± 0.03	0.42 ± 0.06	0.43 ± 0.05
Gastrocnemius muscle (mg)	2346.1 ± 171.8[a]	1913.4 ± 313.5 [b]	2006.3 ± 158.3 [b]
Gastrocnemius muscle/body weight (mg/g)	5.1 ± 0.1	5.1 ± 0.3	5.3 ± 0.4
Blood glucose levels (mg/dL)	125.0 ± 11.8[a]	675.4 ± 277.8 [b]	585.9 ± 169.0 [b]

CON, control rats administered water; STZ, streptozotocin (STZ) rats administered water; STZ + CIT, STZ rats administered L-citrulline (500 mg/kg, p.o.). Data are presented as mean ± standard deviation (n = 5–8). Different letters indicate significant differences between groups (p < 0.05 by Tukey-Kramer's test).

higher at 10 and 20 min after oral L-citrulline administration compared to the CON group (p < 0.05, Fig. 3B). The NOx level tended to be higher at 10 min after L-citrulline administration than that in the CON group (p = 0.050) and was significantly higher at 20 min (p < 0.05, Fig. 3C).

3.3. Experiment 3

3.3.1. Body weight, muscle mass, and blood glucose levels

The body weight, skeletal muscle mass, and blood glucose levels of the rats in each group are shown in Table 2. Although body weight and absolute gastrocnemius muscle mass were significantly lower in the STZ group than in the CON group (p < 0.05), no significant differences were observed between the STZ and STZ + CIT groups. Blood glucose levels

were significantly higher in the STZ group than in the CON group (p < 0.05), whereas no significant difference was observed between the STZ and STZ + CIT groups.

3.3.2. EBD assay

Fig. 4 shows the EBD delivery to skeletal muscles after oral L-citrulline administration. The EBD level per skeletal muscle mass was significantly higher in the STZ + CIT group than in the STZ group in the soleus muscle (p < 0.05, Fig. 4A) and tended to be higher in the STZ + CIT group than in the STZ group in the gastrocnemius muscle (p = 0.052, Fig. 4B).

4. Discussion

In this study, we investigated the effects of oral L-citrulline administration on the level of substances delivered to skeletal muscles in normal and STZ-induced type 1 diabetes model rats. Our data suggest that acute oral administration of L-citrulline increased EBD delivery to the skeletal muscles in these rats. These effects may have resulted from NO-induced vasodilation, following early increase in NOx levels after oral L-citrulline administration.

In Experiment 1, the EBD level normalized with soleus and gastrocnemius muscles wet weight was increased by intravenously administering L-arginine (positive control) in normal rats. It has been suggested that L-arginine as a substrate of eNOS in vascular endothelial cells promotes NO production, resulting in vasodilation [16,18]. Our data suggest that the level of EBD delivered to the soleus muscle was increased by oral L-citrulline administration. However, a similar phenomenon was not observed in the gastrocnemius muscle. The soleus muscle, which has a high proportion of slow muscle fibers with a high capillary density [25], may be more susceptible to the vasodilatory effect of L-citrulline administration than the gastrocnemius muscle, which contains predominantly fast muscle fibers. Arterioles are mainly
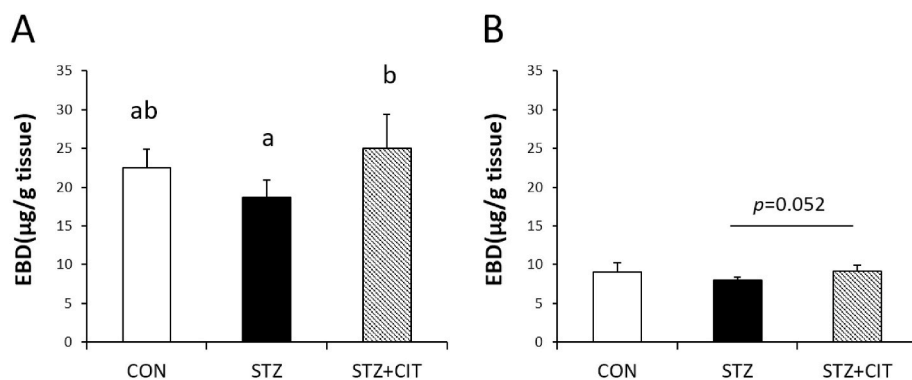
Fig. 4. Effects of L-citrulline administration on the vascular delivery of Evans blue dye (EBD) to muscles in diabetic rats. EBD in soleus (**A**) and gastrocnemius muscles (**B**) muscles. EBD levels were determined 60 min after the last oral L-citrulline administration. CON, rats administered water; streptozotocin (STZ), STZ rats administered water; STZ + CIT, STZ rats administered L-citrulline (500 mg/kg, p.o.). Data are presented as mean ± standard deviation (n = 5–8). Different letters indicate significant differences between groups (p < 0.05 by Tukey-Kramer's test).

involved in blood volume regulation of skeletal muscle [1,2]. In the present study, increased NO by L-citrulline oral administration relaxed smooth muscles in arterioles, and EBD levels in the skeletal muscles might have increased through capillary vasodilation.

The time course of blood NOx level changes in rats after oral L-citrulline administration has not been investigated sufficiently. There were no differences in blood NOx levels in 8-week-old male NZW rabbits at 30, 60, and 120 min after oral administration of 500 mg/kg L-citrulline [29]. In Experiment 2, oral L-citrulline administration increased blood NOx levels in rats after 10 and 20 min. Further studies are necessary to examine transitional changes in blood NOx levels after oral L-citrulline administration. In this study, plasma L-arginine and L-citrulline levels were increased at least up to 30 min after oral L-citrulline administration. Morita et al. reported that oral administration of 500 mg/kg L-citrulline in normal rats resulted in a gradual increase in plasma L-arginine levels up to approximately 120 min [29]. The plasma amino acid and NOx levels in this study suggest that blood NOx levels increase before the maximum levels of plasma L-arginine and L-citrulline are reached. Furthermore, plasma BCAA levels decreased after L-citrulline administration. Previous studies have reported a decrease in blood BCAA levels after oral L-citrulline intake in healthy individuals, as well as the possibility of an increase in amino acid utilization through the intake of L-citrulline [30]. However, the reason for the decrease in blood BCAA levels on L-citrulline intake remains unclear.

Under hyperglycemic conditions, an increase in the expression of vasoconstrictive peptides and a decrease in the production of vasodilators like NO are observed due to inflammation, free fatty acids, oxidative stress, and production of advanced glycation end products, resulting in vascular endothelial dysfunction [31]. Decreased skeletal muscle vessel counts, vessel diameter, capillary-to-fiber ratio, vessel density, and blood flow have been observed in type 1 diabetes [7–9,32,33]. Moreover, increased capillary orientation along the muscle fiber longitudinal axis and decreased oxygen delivery to the skeletal muscles were observed in STZ-induced type 1 diabetic rats [32,34], and these structural and functional changes have the potential to impact capillary blood flow and substrate delivery. The blood vessel volume in the soleus muscle, which is mainly composed of slow muscle fibers, is strongly affected by diabetes in rats and humans [35,36]. Although according to Experiment 1, increased levels of EBD per skeletal muscle mass following oral L-citrulline administration were only found in the soleus muscle, the results of Experiment 3 suggest that this effect can be observed in both soleus and gastrocnemius muscles of diabetic rats. The results of Experiment 3 suggest that oral L-citrulline intake increases the delivery of EBD to skeletal muscles in diabetic rats, regardless of the skeletal muscle fiber type. L-citrulline administration did not affect blood glucose levels in STZ rats (Table 2). A 9-wk administration of L-citrulline improved glucose metabolism by decreasing HOMA-R (homeostasis model assessment for insulin resistance) values and blood insulin levels in high-fat diet-fed SD rats and type 2 diabetic KK-Ay mice [37]. Furthermore, a 15-wk L-citrulline administration decreased blood

glucose levels through improved skeletal muscle mitochondrial function in obese mice fed a high-fat diet [38]. The distinct results of this and previous studies may be due to differences in animal models and L-citrulline administration periods.

Our study findings indicate that L-citrulline, as a food item and not as a drug, has the potential to improve vascular endothelial function. However, this study has some limitations. In some cases, we could not obtain sufficient number of animals because some rats were excluded, and some outcomes may have been limited by underpowered analyses. Substance delivery was evaluated using the EBD assay, and EBD, which is not metabolized in vivo, shows a high affinity for blood proteins, especially albumin, after intravenous administration [28]. Since albumin-bound EBD is localized in blood vessels, EBD is generally used to quantify the blood volume in tissues of humans and animals. Our data reflect the EBD level in blood vessels of skeletal muscles. It is necessary to examine the detailed time course of the changes in EBD levels. In addition, the effect of L-citrulline administration on substance delivery should be assessed in more detail using the highly sensitive and accurate microsphere method [39,40] and measuring arterial-venous pressure gradient. This study consisted of three independent experiments. Experiments 1 and 2 were separately performed to eliminate the possibility that blood sampling would affect the blood volume results in the EBD assay. The skeletal muscle capillary density decreases with age [25]. Furthermore, eNOS expression levels and blood flow decrease in disuse atrophy, i.e., skeletal muscle loss induced by inactivity, mainly in the soleus muscle [41]. Therefore, it is necessary to investigate the effects of oral L-citrulline administration on vascular endothelial function in these models.

Authorship

Research conception and design: SM, KH, GE, and SF; experiments: SM and KH; statistical analysis: SM; interpretation of experimental results: SM, KH, and SF; figure preparation: SM; manuscript drafting: SM and SF; manuscript editing and revision: SM, KH, GE, and SF. All authors read and agreed to the published version of the manuscript.

Declaration of competing interest

S.M., K.H., and G.E. are employed by Otsuka Pharmaceutical Factory, Inc. The other author declares no competing interests.

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