



Research article

L-Citrulline ameliorates the attenuation of acetylcholine-induced vasodilation of retinal arterioles in diabetic rats

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ARTICLE INFO

Keywords:

Acetylcholine

L-citrulline

Hyperglycemia

Large-conductance Ca²⁺-activated K⁺ channels

Retinal circulation

ABSTRACT

In our previous study, we found that the vasodilation of retinal arterioles induced by acetylcholine and BMS-191011, a large-conductance Ca²⁺-activated K⁺ (BK_{Ca}) channel opener, were diminished in diabetic rats. Currently, few agents ameliorate the impaired vasodilator responses of retinal blood vessels. Our recent finding that the intravenous infusion of L-citrulline dilated retinal arterioles, suggests that L-citrulline could be a potential therapeutic agent for circulatory disorders of the retina. In this study, we determined the effect of an oral L-citrulline treatment on impaired acetylcholine- and BMS-191011-induced vasodilation in the retinal arterioles of diabetic rats. To induce diabetes, rats were administered an intravenous dose of streptozotocin (65 mg/kg) and a 5% D-glucose solution as drinking water. The L-citrulline (2 g/kg/day) and L-arginine (2 g/kg/day) treatments commenced either 15 days before or just after the streptozotocin injection and continued throughout the experimental period. A 29-day treatment with L-citrulline, but not L-arginine, significantly ameliorated the impaired acetylcholine- and BMS-191011-induced retinal vasodilation in diabetic rats without affecting their plasma glucose levels. The 2-week L-citrulline treatment tended to ameliorate the dysfunction of the acetylcholine-induced retinal vasodilation in diabetic rats. In conclusion, these results showed that the retinal blood vessel dysfunction induced by diabetes mellitus could be prevented by the long-term administration of L-citrulline and suggest that the latter could play a potentially prophylactic role in diabetic retinopathy.

1. Introduction

Diabetic retinopathy is one of major causes of blindness, and retinal circulatory disorders induced by hyperglycemia are known to be involved in the development and progression of diabetic retinopathy. Therefore, the amelioration of abnormal retinal circulatory responses may be useful in preventing and treating diabetic retinopathy. However, there are currently only a few drugs available to treat impaired retinal circulatory responses.

L-Citrulline stimulates nitric oxide (NO) biosynthesis via the recycling of L-citrulline to L-arginine (Babri et al., 2013; Allerton et al., 2018) and induces endothelium-dependent relaxation by enhancing the NO release in isolated rat aortas (Raghavan and Dikshit, 2001). We recently reported that the intravenous infusion of L-citrulline dilates the retinal arterioles via the NO- and prostaglandin-dependent pathways in rats, and that the

recycling of L-citrulline to L-arginine is involved in the regulation of the vascular tone in retinal blood vessels (Mori et al., 2015a). Although the acute or short-term administration of L-citrulline has not been reported to improve the endothelial function in healthy subjects despite increasing the bioavailability of NO (Schwedhelm et al., 2008; Kim et al., 2015), other researchers have reported that the long-term supplementation of L-citrulline has several beneficial effects on the cardiovascular system, such as the amelioration of chronic hypoxia-induced pulmonary hypertension, ventricular function in heart failure patients, and acute arteriogenic erectile dysfunction (Ananthkrishnan et al., 2009; Orozco-Gutiérrez et al., 2010; Balderas-Munoz et al., 2012; Shiota et al., 2013). Although these previous reports suggest that L-citrulline has beneficial effects on vascular dysfunction, it is still not clear whether a long-term oral treatment with L-citrulline ameliorates retinal circulatory dysfunction in the context of pathological conditions, such as diabetes.

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We previously demonstrated that the intravenous infusion of acetylcholine results in the dilation of the retinal arterioles via the opening of large-conductance Ca^{2+} -activated K^+ (BK_{Ca}) channels (Mori et al., 2011a), and that retinal vasodilatory responses mediated by acetylcholine and BMS-191011, a BK_{Ca} channel opener, are reduced at the early stage of diabetes in rats (Mori et al., 2009, 2011b). In these studies, we induced severe diabetes by injecting rats with streptozotocin (65 mg/kg) intravenously and providing them with a 5% D-glucose solution as drinking water; our results showed that the BK_{Ca} channel-dependent component of the vasodilator response of retinal arterioles to acetylcholine was attenuated 2 weeks after streptozotocin and D-glucose treatment. This protocol enables us to accelerate the onset of the development of retinal vascular dysfunction; therefore, these severely diabetic rats constitute a suitable model to screen drugs within a short period.

In the present study, we investigated whether the long-term oral administration of L-citrulline, which commenced 15 days prior to the induction of diabetes, would reduce the dysfunction of vasodilation in

the retinal arterioles of diabetic rats. Given our initial findings that the long-term administration of L-citrulline significantly ameliorated the dysfunction of the acetylcholine-induced retinal vasodilation, we also examined the effects of the short-term administration of L-citrulline, which commenced immediately after the induction of diabetes, as well as of the long-term administration of L-arginine on this dysfunction.

2. Materials and methods

2.1. Animals and induction of diabetes

The experimental procedures follow the Association for Research in Vision and Ophthalmology Statement Regarding the Use of Animals in Ophthalmic and Vision Research and Regulations for the Care and Use of Laboratory Animals in Kitasato University and are approved by Ethics Committee for Animal Care and Use in Kitasato University (approval number: T04-1).

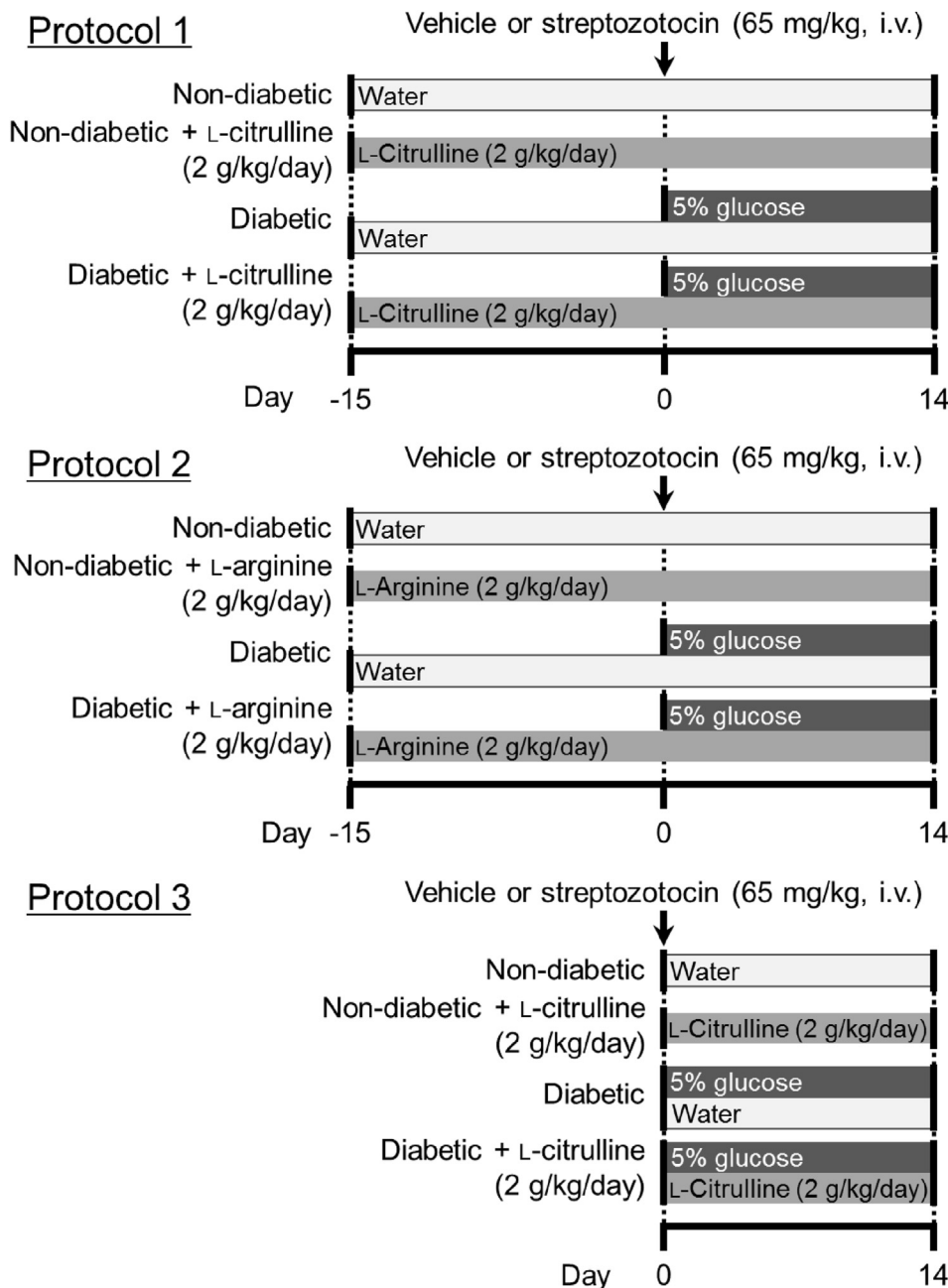


Figure 1. Experimental protocol. In all of the protocols, the streptozotocin-treated animals were given a 5% D-glucose solution as drinking water for 2 weeks to accelerate the onset of the development of retinal vascular dysfunction. The experiments were performed two weeks after the streptozotocin treatment. Protocol 1 (long-term administration of L-citrulline): the rats in the L-citrulline-treated group were given an L-citrulline solution from day -15 to day 14. Streptozotocin or its vehicle were administered on day 0. Protocol 2 (long-term administration of L-arginine): the rats in the L-arginine-treated group were given an L-arginine solution from day -15 to day 14. Streptozotocin or its vehicle were administered on day 0. Protocol 3 (short-term administration of L-citrulline): the rats in the L-citrulline-treated group were given an L-citrulline solution from day 0 to day 14. Streptozotocin or its vehicle were administered on day 0.

Male Wistar rats (3 or 5 weeks old) were reared for at least 1 week with *ad libitum* access to standard rat chow and tap water under a 12:12-h light:dark cycle. Diabetes was induced in these 6-week-old rats with a single intravenous injection of streptozotocin (65 mg/kg) via the tail vein. An equal volume of sodium citrate buffer (pH 4.5), the vehicle used for streptozotocin, was injected to age-matched control rats. Then, a 5% D-glucose solution was provided to the streptozotocin-treated animals as drinking water for 2 weeks to accelerate the development of retinal vessel dysfunction (Mori et al., 2009). Commercially available kits (Glucose CII Test Wako; FUJIFILM Wako Pure Chemical Industries, Osaka, Japan) were used to measure the plasma glucose levels.

2.2. Reagents

Acetylcholine, fluorescein sodium salt, and methoxamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). BMS-191011 was purchased from Tocris Bioscience (Bristol, United Kingdom). Pontamine sky blue 6B was purchased from Tokyo Chemical Industry (Tokyo, Japan). L-Arginine, pentobarbital sodium, streptozotocin, and tetrodotoxin were purchased from Nacalai Tesque (Kyoto, Japan). Hydroxyethyl cellulose (Scopisol 15®) was purchased from Senju Pharmaceutical (Osaka, Japan). L-Citrulline was provided by KYOWA HAKKO BIO CO., LTD (Tokyo, Japan).

2.3. Experimental protocols

The experimental protocols are shown in Figure 1.

Protocol 1 (long-term administration of L-citrulline): the administration of L-citrulline (2 g/kg/day) commenced 15 days prior to the streptozotocin injection and continued for 29 days. Our previous study revealed that the 2-week feeding of D-glucose to non-diabetic rats does not change blood glucose levels and the vascular responses to acetylcholine (Mori et al., 2009). Therefore, the rats were divided into four groups: non-diabetic rats ($n = 9$), non-diabetic rats treated with L-citrulline (non-diabetic + L-citrulline; $n = 11$), diabetic rats ($n = 10$), and diabetic rats treated with L-citrulline (diabetic + L-citrulline; $n = 12$). For the non-diabetic + L-citrulline and diabetic + L-citrulline groups prior to the administration of streptozotocin, L-citrulline was dissolved in tap water so that the final concentration is 8 mg/mL. For the diabetic + L-citrulline group after the administration of streptozotocin, L-citrulline was dissolved in 5% D-glucose solution so that the final concentration is 1.25 mg/mL. Drinking water containing L-citrulline was provided *ad libitum*. The water intake and body weight of each rat were measured every other day. Based on the water intake and the body weight, the estimated mean daily L-citrulline dose was determined. The final dose of L-citrulline (2 g/kg/day) was almost the same for the non-diabetic + L-citrulline and the diabetic + L-citrulline groups.

Protocol 2 (long-term administration of L-arginine): the administration of L-arginine (2 g/kg/day) commenced 15 days prior to the streptozotocin injection and continued for 29 days. The rats were divided into four groups: non-diabetic rats ($n = 5$), non-diabetic rats treated with L-arginine (non-diabetic + L-arginine; $n = 4$), diabetic rats ($n = 6$), and diabetic rats treated with L-arginine (diabetic + L-arginine; $n = 6$). L-Arginine was dissolved in drinking water and administered in the same manner as L-citrulline was administered (protocol 1).

Protocol 3 (short-term administration of L-citrulline): the administration of L-citrulline (2 g/kg/day) commenced immediately after the streptozotocin injection and continued for 2 weeks. The rats were divided into four groups: non-diabetic rats ($n = 5$), non-diabetic rats treated with L-citrulline (non-diabetic + L-citrulline; $n = 4$), diabetic rats ($n = 5$), and diabetic rats treated with L-citrulline (diabetic + L-citrulline; $n = 5$). For the non-diabetic + L-citrulline group, L-citrulline was dissolved in tap water so that the final concentration was 8 mg/mL. For the diabetic + L-citrulline group, L-citrulline was dissolved in 5% D-glucose solution so

that the final concentration was 1.25 mg/mL. The drinking water containing L-citrulline was provided *ad libitum*.

2.4. Experimental procedures

The experimental procedures were described previously (Mori et al., 2009, 2011b). Briefly, arterial pressure and heart rate were monitored from the cannula inserted into the left femoral artery with a pressure transducer (DX-360, Nihon Kohden, Tokyo, Japan), a preamplifier (AP-610G, Nihon Kohden), and a cardiometer (AT-601G, Nihon Kohden). To take fundus photographs at the same angle during the experiment, tetrodotoxin (50 µg/kg) was administered intravenously. The decrease in the arterial pressure induced by tetrodotoxin was compensated by the infusion of methoxamine at a constant rate. The dose of methoxamine needed to restore the arterial pressure to the value before treatment with tetrodotoxin was varied in each experiment. In addition, the contractile response induced by the stimulation of α -adrenoceptor in peripheral resistant vessels, but not in retinal blood vessels is attenuated in diabetic rats (Nakazawa et al., 2008). In the present study, methoxamine was infused at a rate of 15–50 µg/kg/min for non-diabetic rats and 25–60 µg/kg/min for diabetic rats, so that the mean arterial pressure was restored to the value before treatment with tetrodotoxin.

Each dose of acetylcholine (0.3–10 µg/kg/min) or BMS-191011 (10–100 µg/kg/min) was infused stepwise into the femoral vein by using a syringe pump (Harvard Apparatus, South Natick, MA, USA). After all of the experimental procedures were completed, the rats were euthanized by an overdose of pentobarbital sodium.

2.5. Fundus photography and measurement of the retinal arteriole diameter

The fundus photography and measurement of the retinal arteriole diameter procedures were carried out as described previously (Mori et al., 2007, 2015b). Fundus photographs were taken with a digital camera (EOS7D; Canon, Tokyo, Japan) and a borescope-type objective lens (Model 01; Scalar, Tokyo, Japan). The region including a retinal arteriole (138 × 276 pixels, 138 × 276 µm) was clipped from the original fundus photograph (5184 × 3456 pixels, 5184 × 3456 µm) to measure the diameter of the retinal arteriole. The diameter of the retinal blood vessel focused on clearly throughout the experiment was measured in rats. The basal diameters of most retinal arterioles chosen for measurement of the diameter were approximately 40 µm in all the groups.

2.6. Measurement of the plasma concentrations of L-citrulline, L-arginine, and L-ornithine

Blood samples were collected from the rats of the Protocol 1 and 2 groups at day 14. The plasma was separated by centrifugation, and then deproteinized with an equal volume of 3% (w/v) sulfosalicylic acid. The L-citrulline, L-arginine, and L-ornithine concentrations were measured with an amino acid analyzer (JLC-500/V; JEOL, Tokyo, Japan) as described elsewhere (Moinard et al., 2008; Morita et al., 2014; Sakamoto et al., 2015).

2.7. Statistical analysis

The data were expressed as mean ± standard error of the mean (S.E.M.). Student's *t*-test was used to compare the difference between two means. The vasodilator responses of the different groups were compared using a two-way analysis of variance followed by the Tukey-Kramer test. Differences were considered to be statistically significant if $P < 0.05$. All the statistical analyses were carried out on Prism 6 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Plasma glucose levels and body weight

The changes in the plasma glucose levels and body weight during the experimental period are summarized in Tables 1 and 2, respectively. At day 14, we confirmed a significant elevation of the plasma glucose levels in diabetic rats. Although the body weights of rats in all groups increased significantly on day 14 compared with day -15, the weight gain rate was significantly lower in the streptozotocin-treated rats than in the non-diabetic rats. Neither the plasma glucose levels nor the body weights were affected either by the long- or short-term L-citrulline treatment or the long-term L-arginine treatment.

3.2. Baseline retinal arteriolar diameter, mean arterial pressure, and heart rate values

The baseline retinal arteriolar diameter, mean arterial pressure, and heart rate values were adjusted to be almost similar among the experimental groups (Table 3). The baseline heart rate values in the diabetic groups were lower than those in the non-diabetic groups, which tended to be ameliorated by the long-term administration of L-citrulline.

3.3. Effect of the long-term administration of L-citrulline on the dysfunction of acetylcholine-induced retinal vasodilatory responses in diabetic rats

Figure 2 shows the representative fundus images before and 0.5 min after initiating intravenous infusion of acetylcholine (10 μ g/kg/min). The 4-week L-citrulline treatment (2 g/kg/day) did not affect the acetylcholine-induced dilatation in the retinal arterioles of non-diabetic rats (Figure 3A). The acetylcholine-induced vasodilatory responses in the retinal arterioles of diabetic rats were impaired (Figure 3A). L-Citrulline played a significant role in preventing this impairment in diabetic rats (Figure 3A) and did not affect the changes in the mean arterial pressure and heart rate during acetylcholine infusion (Figures 3B and 3C).

3.4. Effect of the long-term administration of L-arginine on the dysfunction of the acetylcholine-induced retinal vasodilatory response in diabetic rats

The 4-week L-arginine treatment (2 g/kg/day) did not affect the retinal vasodilatory response and the changes in the mean arterial

pressure and heart rate evoked by acetylcholine infusion in either the non-diabetic or the diabetic rats (Figure 4).

3.5. Effect of the long-term administration of L-citrulline on the dysfunction of the BMS-191011-induced retinal vasodilatory response in diabetic rats

The 4-week L-citrulline treatment (2 g/kg/day) did not affect the BMS-191011-induced dilatation in the retinal arterioles of non-diabetic rats (Figure 5A). The BMS-191011-induced vasodilation responses in the retinal arterioles of diabetic rats were impaired (Figure 5A). L-Citrulline played a significant role in preventing this impairment in diabetic rats (Figure 5A) and it did not have an effect on the changes in the mean arterial pressure and heart rate during the BMS-191011 infusion (Figures 5B and 5C).

3.6. Effect of the short-term administration of L-citrulline on the dysfunction of the acetylcholine-induced retinal vasodilatory response in diabetic rats

The 15-day L-citrulline treatment (2 g/kg/day) did not affect the acetylcholine-induced dilatation in the retinal arterioles of non-diabetic rats (Figure 6A). The short-term L-citrulline treatment tended to improve the acetylcholine-induced retinal vasodilation in diabetic rats, although the recovery was not complete or to the same extent as with the long-term L-citrulline treatment (Figure 6A). L-Citrulline did not affect changes in the mean arterial pressure and heart rate during acetylcholine infusion (Figures 6B and 6C).

3.7. Changes in L-citrulline, L-arginine, and L-ornithine plasma levels induced by the long-term administration of L-citrulline and L-arginine

The plasma levels of L-citrulline, L-arginine, and L-ornithine 2 weeks after the streptozotocin injection in the long-term administration groups are summarized in Table 4. The plasma L-citrulline level was not affected by diabetes. L-Citrulline treatment increased the plasma L-citrulline levels of the diabetic more than those of the non-diabetic rats. L-Arginine treatment did not affect the plasma L-citrulline levels in either the non-diabetic or the diabetic rats. The plasma L-arginine levels in both the non-diabetic and the diabetic rats tended to increase with the L-citrulline and L-arginine treatments. L-Arginine treatment increased the plasma L-ornithine level more in the diabetic than in the non-diabetic rats.

Table 1. Plasma glucose concentration 15 days before (day -15), just before (day 0), and 2 weeks after (day 14) the streptozotocin or the vehicle injection.

	Plasma glucose (mg/dL)		
	Day -15	Day 0	Day 14
<i>Protocol 1 (long-term administration of L-citrulline: Figures 3 and 5)</i>			
Non-diabetic (n = 9)	131 \pm 5	138 \pm 6	138 \pm 5
Non-diabetic + L-citrulline (n = 11)	125 \pm 5	138 \pm 6	134 \pm 5
Diabetic (n = 10)	122 \pm 3	136 \pm 3	871 \pm 44 ^{a,b}
Diabetic + L-citrulline (n = 10)	125 \pm 3	128 \pm 4	883 \pm 53 ^{a,c}
<i>Protocol 2 (long-term administration of L-arginine: Figure 4)</i>			
Non-diabetic (n = 5)	122 \pm 9	143 \pm 3	128 \pm 4
Non-diabetic + L-arginine (n = 4)	122 \pm 5	133 \pm 1	126 \pm 3
Diabetic (n = 6)	133 \pm 6	134 \pm 6	787 \pm 53 ^{a,b}
Diabetic + L-arginine (n = 6)	132 \pm 7	143 \pm 3	778 \pm 54 ^{a,d}
<i>Protocol 3 (short-term administration of L-citrulline: Figure 6)</i>			
Non-diabetic (n = 5)		121 \pm 5	133 \pm 7
Non-diabetic + L-citrulline (n = 4)		106 \pm 1	126 \pm 4
Diabetic (n = 5)		125 \pm 6	721 \pm 86 ^{a,b}
Diabetic + L-citrulline (n = 5)		125 \pm 9	804 \pm 52 ^{a,c}

The values are expressed as means \pm S.E.M. ^a $P < 0.05$ vs. the corresponding values of day -15. ^b $P < 0.05$ vs. the non-diabetic group. ^c $P < 0.05$ vs. the non-diabetic + L-citrulline group. ^d $P < 0.05$ vs. the non-diabetic + L-arginine group.

Table 2. Body weight measured 15 days before (day -15), just before (day 0), and 2 weeks after (day 14) the streptozotocin or the vehicle injection.

	Body weight (g)		
	Day -15	Day 0	Day 14
Protocol 1 (long-term administration of L-citrulline: Figures 3 and 5)			
Non-diabetic (n = 9)	77 ± 2	175 ± 4 ^a	283 ± 6 ^a
Non-diabetic + L-citrulline (n = 11)	81 ± 1	181 ± 4 ^a	300 ± 6 ^a
Diabetic (n = 10)	80 ± 2	194 ± 4 ^a	239 ± 5 ^{a,b}
Diabetic + L-citrulline (n = 10)	79 ± 1	187 ± 5 ^a	234 ± 4 ^{a,c}
Protocol 2 (long-term administration of L-arginine: Figure 4)			
Non-diabetic (n = 5)	75 ± 1	199 ± 4 ^a	297 ± 13 ^a
Non-diabetic + L-arginine (n = 4)	76 ± 1	202 ± 7 ^a	292 ± 8 ^a
Diabetic (n = 6)	76 ± 1	200 ± 5 ^a	238 ± 8 ^{a,b}
Diabetic + L-arginine (n = 6)	74 ± 2	201 ± 7 ^a	259 ± 8 ^a
Protocol 3 (short-term administration of L-citrulline: Figure 6)			
Non-diabetic (n = 5)		173 ± 7	243 ± 7 ^a
Non-diabetic + L-citrulline (n = 4)		161 ± 2	229 ± 9
Diabetic (n = 5)		157 ± 3	209 ± 3 ^{a,b}
Diabetic + L-citrulline (n = 5)		161 ± 2	216 ± 4 ^a

The values are expressed as means ± S.E.M. ^a*P* < 0.05 vs. the corresponding values of day -15. ^b*P* < 0.05 vs. the non-diabetic group. ^c*P* < 0.05 vs. the non-diabetic + L-citrulline group.

Table 3. The baseline retinal arteriolar diameter, mean arterial pressure, and heart rate values.

Treatments	Retinal arteriolar diameter (μm)	Mean arterial pressure (mmHg)	Heart rate (beats/min)
Protocol 1 (long-term administration of L-citrulline: Figures 3 and 5)			
Non-diabetic (n = 9)	36 ± 1	113 ± 1	357 ± 7
Non-diabetic + L-citrulline (n = 11)	35 ± 1	111 ± 1	362 ± 3
Diabetic (n = 10)	40 ± 2	109 ± 2	330 ± 10 ^a
Diabetic + L-citrulline (n = 12)	37 ± 1	110 ± 1	342 ± 4
Protocol 2 (long-term administration of L-arginine: Figure 4)			
Non-diabetic (n = 5)	38 ± 2	115 ± 2	353 ± 11
Non-diabetic + L-arginine (n = 4)	38 ± 1	112 ± 1	326 ± 12
Diabetic (n = 6)	39 ± 2	111 ± 2	340 ± 9
Diabetic + L-arginine (n = 6)	37 ± 1	118 ± 2	326 ± 10
Protocol 3 (short-term administration of L-citrulline: Figure 6)			
Non-diabetic (n = 5)	40 ± 1	112 ± 2	340 ± 6
Non-diabetic + L-citrulline (n = 4)	42 ± 0	109 ± 0	344 ± 9
Diabetic (n = 5)	43 ± 3	107 ± 2	327 ± 5
Diabetic + L-citrulline (n = 5)	41 ± 3	106 ± 3	333 ± 4

The values are expressed as means ± S.E.M. These values were measured just before the acetylcholine or BMS-191011 infusion under infusion with methoxamine following bolus injection of tetrodotoxin. ^a*P* < 0.05 vs. the non-diabetic group.

4. Discussion

We have found that the acetylcholine-induced dilation in the retinal blood vessels is at least partially mediated by the opening of the BK_{Ca} channels, and that the retinal vasodilatory responses induced by BMS-191011, an activator of BK_{Ca} channels, are attenuated in diabetic rats (Mori et al., 2011a, 2011b) (Figure 7A). The results of the present study demonstrated that a long-term L-citrulline treatment almost completely prevented the dysfunction of the vasodilatory responses evoked by BMS-191011 in diabetic rats. Therefore, the prevention of the dysfunction of the BK_{Ca} channels could be one of the mechanisms underlying the protective effects of L-citrulline on the hyperglycemia-induced impairment of the retinal vasodilatory responses in rats (Figure 7B).

L-Arginine is metabolized to NO and L-citrulline by NO synthases, and to L-ornithine and urea by arginase (Romero et al., 2008). In this study, the plasma concentrations of L-arginine and L-ornithine in the diabetic rats tended to decrease compared with those in the non-diabetic rats. The decrease of the plasma L-ornithine levels in diabetic rats could have been

caused by a decrease in the plasma levels of L-arginine, a precursor of L-ornithine. The L-citrulline treatment tended to increase the plasma levels of L-citrulline and L-arginine in both the non-diabetic and the diabetic rats. On the contrary, L-arginine treatment tended to increase the plasma L-arginine levels but not the plasma L-citrulline levels. In addition, the plasma L-ornithine levels tended to be higher in the L-arginine-treated diabetic rats than in the L-citrulline-treated diabetic rats, whereas the plasma L-arginine levels in both groups were almost identical. These results suggest that L-citrulline inhibits arginase activity, which was suggested to be the case in the coronary artery of the diabetic rats in a previous study (Romero et al., 2008).

Both the activity and the protein level of arginase I are higher in diabetic murine retinas than in control murine retinas, while both groups lack one copy of the arginase I gene, and treatment with an arginase I inhibitor prevents the dysfunction of the retinal vasculature in diabetic mice (Elms et al., 2013). These results suggest that the inhibition of arginase I can suppress the dysfunction of retinal vasculature in diabetes. It is possible that L-citrulline ameliorates the diabetes-induced

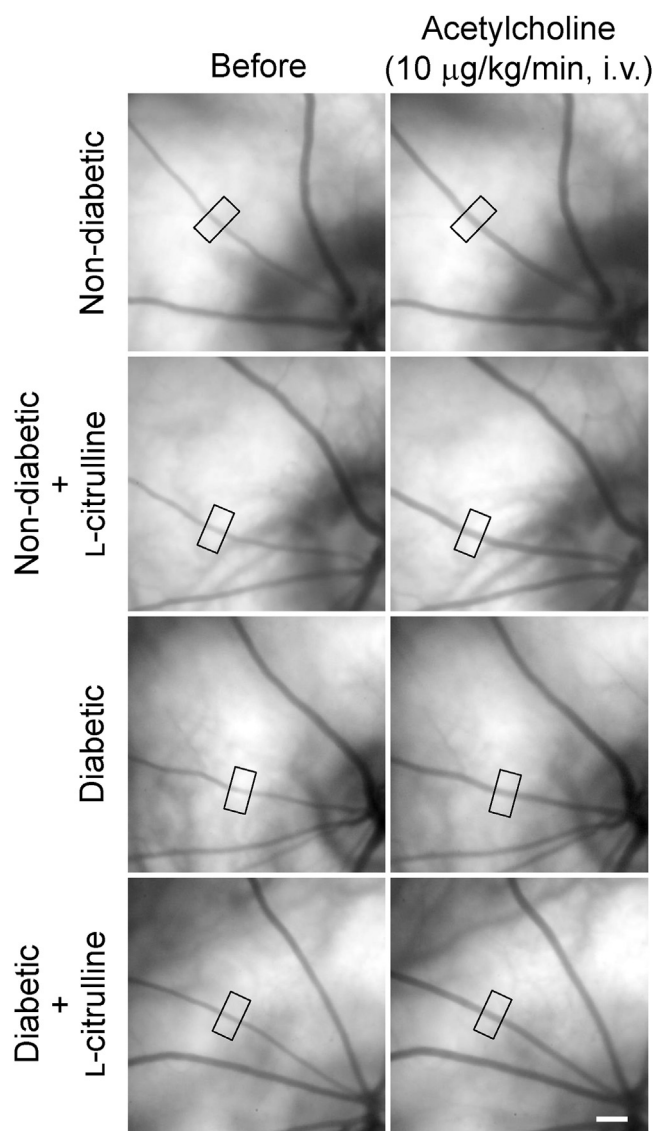


Figure 2. Representative fundus images before and 0.5 min after initiating intravenous infusion of acetylcholine (10 µg/kg/min) in non-diabetic, non-diabetic + L-citrulline, diabetic and diabetic + L-citrulline group. The diameter of the retinal arteriole in the selected region is expressed as a percentage of the baseline value obtained before acetylcholine infusion. Changes in the diameter of the retinal arteriole in non-diabetic, non-diabetic + L-citrulline, diabetic and diabetic + L-citrulline group were 31%, 33%, 23% and 33%, respectively. Scale bar is 200 µm.

dysfunction of the retinal blood vessels via inhibiting the activity of arginase I and preserving the L-arginine levels in the retina. Although the plasma L-arginine concentrations in the L-citrulline- and L-arginine-treated diabetic rats were identical, we cannot exclude the possibility that the concentration in the retinas of both groups may be different. If L-arginine is distributed to the retinal tissue in a similar manner, the tissue concentrations of L-arginine should be identical in both groups. Further experiments measuring the L-arginine levels, the arginase activity, and the arginase levels in the retina are required to prove our hypothesis.

Endothelial NO synthase (eNOS) is an enzyme producing NO from L-arginine and plays an important role in endothelium-dependent vasodilation. A decrease in eNOS expression or NO bioavailability or both is related to endothelial dysfunction (Sena et al., 2013). In diabetes, the attenuation of endothelium-dependent vasodilatory response induces microvascular dysfunction (Johnstone et al., 1993). Supplemental oral

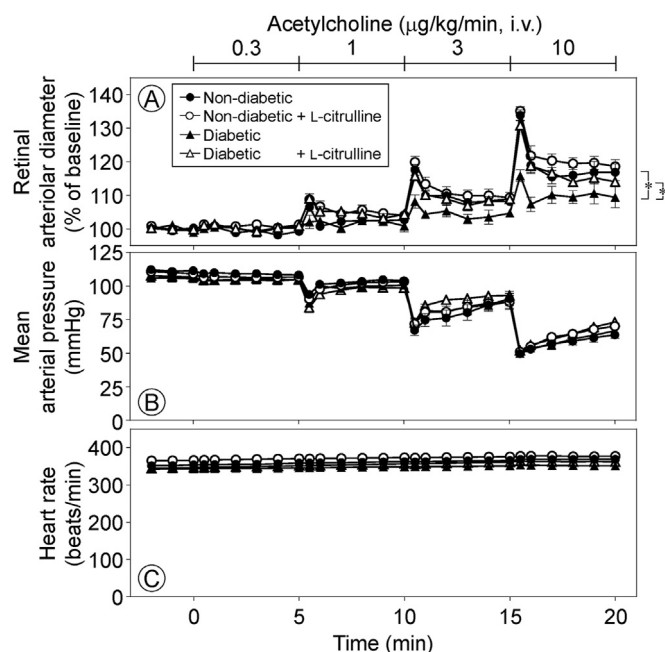


Figure 3. The effects of the long-term administration of L-citrulline on the retinal arteriolar diameter (A), mean arterial pressure (B), and heart rate (C) changes induced by the intravenous infusion of acetylcholine (0.3–10 µg/kg/min) in diabetic and the age-matched control (non-diabetic) rats. The data are expressed as the percentages (%) of the baseline values (the values measured before the acetylcholine infusion). Each point with a vertical bar represents the mean ± S.E.M. of 5–7 animals. **P* < 0.05.

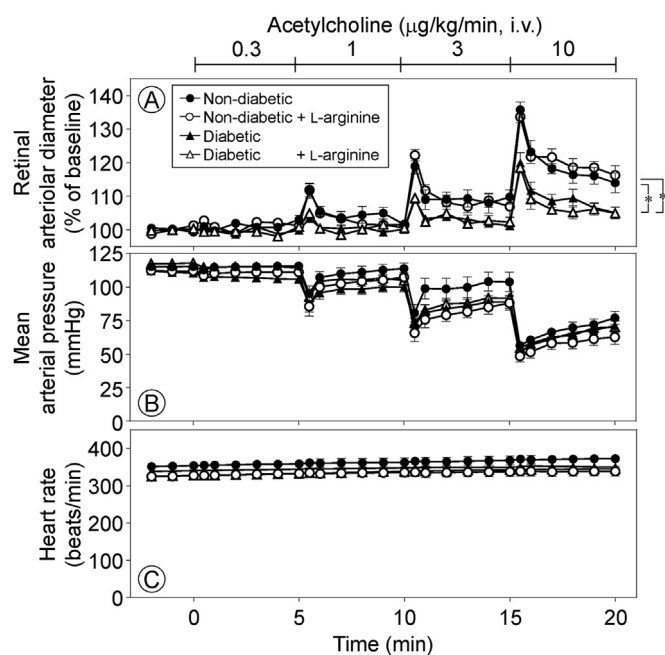


Figure 4. The effects of the long-term administration of L-arginine on the retinal arteriolar diameter (A), mean arterial pressure (B), and heart rate (C) changes induced by the intravenous infusion of acetylcholine (0.3–10 µg/kg/min) in diabetic and the age-matched control (non-diabetic) rats. The data are expressed as the percentages (%) of the baseline values (the values measured before the acetylcholine infusion under infusion with methoxamine following bolus injection of tetrodotoxin). Each point with a vertical bar represents the mean ± S.E.M. of 4–6 animals. **P* < 0.05.

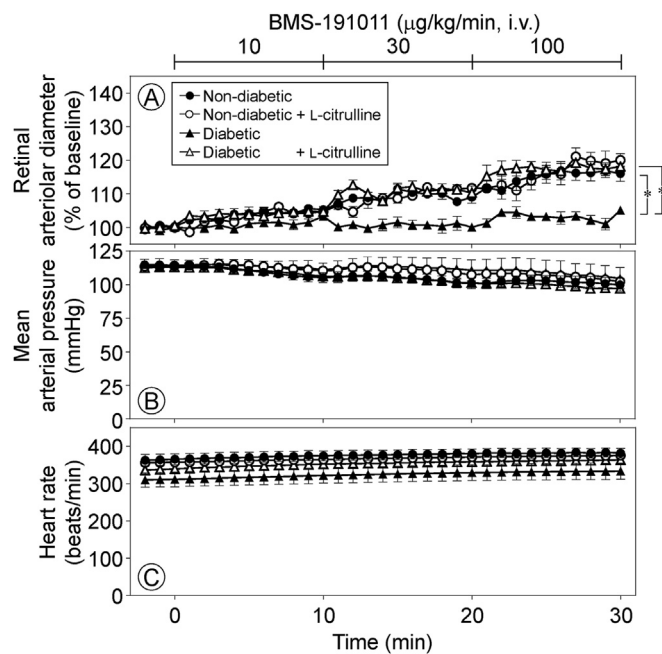


Figure 5. The effects of the long-term administration of L-citrulline on the retinal arteriolar diameter (A), mean arterial pressure (B), and heart rate (C) changes induced by the intravenous infusion of BMS-191011 (10–100 µg/kg/min) in diabetic and the age-matched control (non-diabetic) rats. The data are expressed as the percentages (%) of the baseline values (values measured before the BMS-191011 infusion under infusion with methoxamine following bolus injection of tetrodotoxin). Each point with a vertical bar represents the mean \pm S.E.M. of 4–5 animals. * $P < 0.05$.

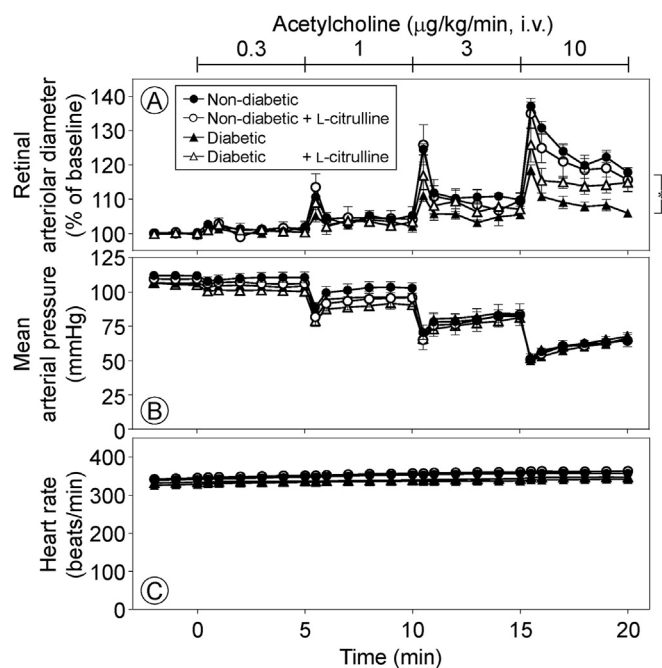


Figure 6. The effects of the short-term administration of L-citrulline on the retinal arteriolar diameter (A), mean arterial pressure (B), and heart rate (C) changes induced by the intravenous infusion of acetylcholine (0.3–10 µg/kg/min) in diabetic and the age-matched control (non-diabetic) rats. The data are expressed as the percentages (%) of the baseline values (the values measured before the acetylcholine infusion under infusion with methoxamine following bolus injection of tetrodotoxin). Each point with a vertical bar represents the mean \pm S.E.M. of 4–5 animals. * $P < 0.05$.

L-arginine treatment has been reported to improve endothelial dysfunction (Romero et al., 2006; Allerton et al., 2018). However, a high dose of L-arginine is required to induce therapeutic effects, because a considerable amount of orally absorbed L-arginine is metabolized in the intestine and liver. Orally absorbed L-citrulline is effectively converted into L-arginine in the kidney, thereby increasing serum L-arginine levels more than the orally absorbed L-arginine. Therefore, the chronic administration of L-citrulline may ameliorate some cardiovascular diseases more effectively than that of L-arginine (Romero et al., 2006; Allerton et al., 2018). Exposure of human umbilical vein endothelial cells to high glucose increases senescence and reactive oxygen species, decreases NO production, and downregulates eNOS levels. Treatment with L-citrulline than with L-arginine has shown to more significantly reduce the above high glucose-induced endothelial damage responses (Tsuboi et al., 2018), suggesting that L-citrulline has protective effects against retinal endothelial cell dysfunction. Our results in the present study are consistent with these previous findings.

We have reported that the intravenous infusion of L-citrulline induced the dilation of the retinal arterioles via NO-dependent mechanisms in the non-diabetic rats (Mori et al., 2015a). Thus, the long-term oral treatment with L-citrulline may have resulted in the dilation of the retinal blood vessels via NO-dependent mechanisms compared to the results obtained without this treatment. Compared with the rats that were not treated with L-citrulline, the continuous oral administration of L-citrulline in both the non-diabetic and diabetic rats did not affect their basal retinal arteriolar diameter, despite the high concentration of plasma L-citrulline levels. The basal diameters of retinal arterioles may be affected by treatment with methoxamine and tetrodotoxin. Especially, methoxamine infusion may have caused the strong contraction of retinal arterioles in the present study. According to Spada et al. (2001), the methoxamine-induced retinal vasoconstrictive effect may be limited on rodent retinal vessels. However, tetrodotoxin-induced vasodilation may affect the vasoconstrictive effect of methoxamine. Therefore, it is unclear whether the vasoconstrictive effect of methoxamine in the rats was weak or not in the present study. Our previous study showed that the basal diameter of retinal arterioles under thiobutobarbital anesthesia not treated with tetrodotoxin and methoxamine was approximately 60 µm (Ogawa et al., 2009). In the present study, the basal diameter of retinal arterioles treated with tetrodotoxin and methoxamine was approximately 40 µm. These results suggest that the retinal arteriole in the present study was constricted by methoxamine, and that the constrictive effect may mask the vasodilatory effect of L-citrulline. Further experiments are needed to clarify whether orally administered L-citrulline induces retinal vasodilatory responses under general anesthesia in the absence of tetrodotoxin and methoxamine treatment.

As per our previous study, the vasodilatory effect of acetylcholine via endothelial NO was preserved 14 days after treatment with streptozotocin plus D-glucose (Mori et al., 2009). Therefore, endothelial NO does not seem to be involved in the protective mechanism of L-citrulline on dysfunction of acetylcholine-induced vasodilation in the present study, i.e., 14 days after treatment with streptozotocin plus D-glucose. Because L-citrulline restored the vasodilatory effect of BMS-191011, it has been strongly suggested that the prevention of the dysfunction of the BK_{Ca} channels is involved in the protective effects of L-citrulline in the present study. Further studies are needed to investigate both the molecular mechanisms underlying the BK_{Ca} channel dysfunction and the protective effects of L-citrulline on the function of the BK_{Ca} channel under high glucose conditions.

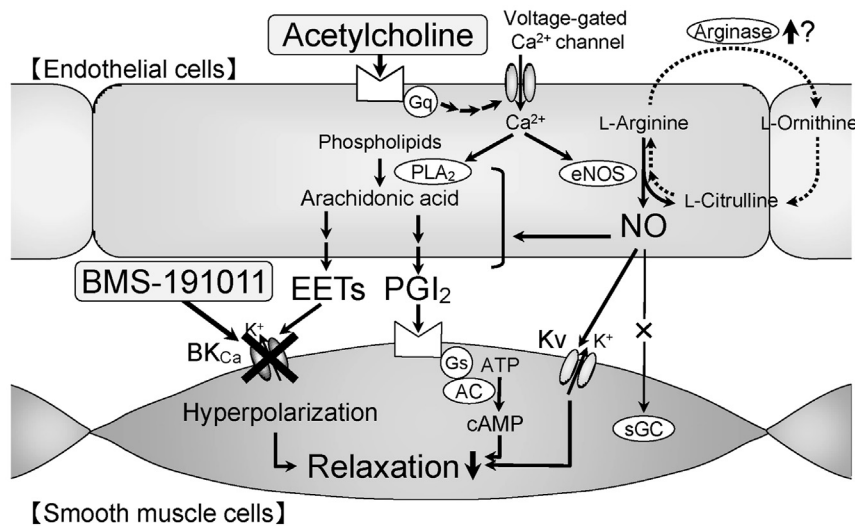
Presently, it is unclear whether the vasodilatory responses via endothelial NO is attenuated in longer-term severe diabetes. Because the formation of diabetic cataracts became severe, it was difficult to observe the fundus images and evaluate the response of retinal arterioles 14 days after treatment with streptozotocin plus D-glucose in our experimental system. To clarify whether the endothelial NO-induced vasodilatory responses are attenuated in longer-term severe diabetes and whether L-citrulline is protective against such endothelial dysfunction, we need to

Table 4. The plasma L-citrulline, L-arginine, and L-ornithine levels in the long-term treatment group.

Treatments	L-citrulline (μM)	L-arginine (μM)	L-ornithine (μM)
Non-diabetic (n = 5)	86.9 ± 7.9	232.0 ± 22.2	60.9 ± 3.3
Non-diabetic + L-citrulline (n = 3)	211.5 ± 38.7	339.6 ± 48.7	61.0 ± 5.6
Non-diabetic + L-arginine (n = 3)	81.0 ± 11.1	408.4 ± 64.9	64.6 ± 2.6
Diabetic (n = 6)	86.9 ± 9.5	174.1 ± 19.8	51.1 ± 6.1
Diabetic + L-citrulline (n = 4)	409.6 ± 49.4 ^a	298.4 ± 8.3	81.3 ± 2.8
Diabetic + L-arginine (n = 4)	102.3 ± 8.3	279.4 ± 10.3	96.8 ± 6.7 ^b

The values are expressed as means ± S.E.M. ^aP < 0.05 vs. the non-diabetic + L-citrulline group. ^bP < 0.05 vs. the non-diabetic + L-arginine group.

(A) Retinal blood vessels in diabetic rats



(B) Retinal blood vessels in diabetic rats treated with L-citrulline

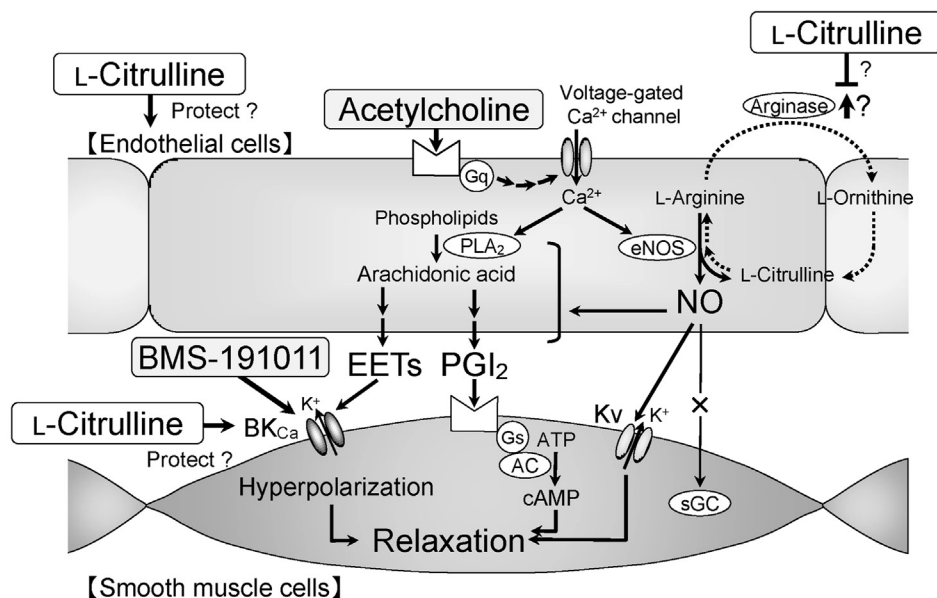


Figure 7. Schema of the proposed mechanisms by which acetylcholine dilates the retinal arteriole in diabetic rats (A) and in diabetic rats treated with L-citrulline (B). Acetylcholine dilates rat retinal arterioles through NO-dependent and NO-independent mechanisms. NO stimulates the arachidonic acid cascade/prostaglandin I₂/cAMP signaling pathway and activates 4-aminopyridine-sensitive K_v channels. Epoxyeicosatrienoic acids activates BK_{Ca} channels (Ogawa et al., 2009; Mori et al., 2011a, 2015b, 2020, 2021). These pathways contribute to the relaxation of vascular smooth muscle cells. The function of BK_{Ca} channels but not eNOS is impaired in the retinal arteriole of diabetic rat, so that acetylcholine and BMS-191011-induced retinal vasodilation responses in diabetic rats attenuated as compared with those in non-diabetic rats (Mori et al., 2009, 2011b). The activity and the expression of arginase, which metabolizes L-Arginine to L-ornithine, may be increased in diabetic rats, and the augmentation of arginase may be involved in dysfunction of retinal vasodilation in diabetes (Elms et al., 2013) (A). Long-term administration of L-citrulline suppresses the dysfunction of retinal vasodilation via activation of BK_{Ca} channels. L-Citrulline inhibits arginase activity (Romero et al., 2008). And L-citrulline protects the high glucose-induced endothelial damages in human umbilical vein endothelial cells (Tsuboi et al., 2018). Therefore, decrease in arginase activity or protection of endothelial cells by L-citrulline may inhibit attenuation of retinal vasodilation response through BK_{Ca} channels activation in diabetic rats. And L-citrulline may protect BK_{Ca} channels directly, however the mechanisms are still unknown (B). AC; adenyl cyclase, BK_{Ca}; BK_{Ca} channel, EETs; epoxyeicosatrienoic acids, eNOS; endothelial NO synthase, sGC; soluble guanylyl cyclase, K_v; 4-aminopyridine-sensitive voltage-gated K⁺ channel.

overcome the above-mentioned experimental difficulty. If L-citrulline is protective against endothelial dysfunction induced by longer-term severe diabetes, it is interesting to examine the effect of long-term treatment with L-citrulline on the changes in the NO emission activity in human retinal microvascular endothelial cells.

Interactions among neuronal, glial, and vascular cells in the retina are critical for the maintenance of an adequate retinal blood flow (Nakahara et al., 2013; Someya et al., 2019). We previously reported that the inner nuclear layer of the retina was thinned without retinal ganglion cell loss in diabetic rats (Mori et al., 2009). In the present study, we found that a

long-term treatment with L-citrulline could not ameliorate the thinning of the inner nuclear layer induced by diabetes (no data shown). This result suggests that the protective effects of L-citrulline on retinal neurons are weak, and that the neural effect is not involved in the mechanisms of the preventive effects of L-citrulline on diabetes-induced retinal vascular dysfunction.

In the present study, we reached the following conclusions: 1) the long-term oral administration of L-citrulline, but not of L-arginine, offered protection against hyperglycemia-induced retinal vasodilator dysfunction; 2) the oral pretreatment with L-citrulline that commenced before the induction of diabetes improved the vasodilation responses more than the short-term oral treatment with L-citrulline that commenced just after the induction of diabetes. The intake of L-citrulline on a regular basis may delay the onset of the damage of retinal blood vessels caused by diabetes, such as in the case of diabetic retinopathy.

Declarations

Author contribution statement

Asami Mori: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Toshiaki Takei: Performed the experiments; Analyzed and interpreted the data.

Namiko Suzuki; Tsutomu Nakahara: Analyzed and interpreted the data.

Kenji Sakamoto: Analyzed and interpreted the data; Wrote the paper.

Masahiko Morita: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Satoshi Nakagawa: Contributed reagents, materials, analysis tools or data.

Kunio Ishii: Conceived and designed the experiments.

Funding statement

This work was supported by Kitasato University Research Grant for Young Researchers (A.M.)

Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare the following conflict of interests: This study was funded by a research Grant from KYOWA HAKKO BIO CO., LTD (Tokyo, Japan).

Additional information

No additional information is available for this paper.

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