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Oral supplementation with a combination of L-citrulline and L-arginine rapidly increases plasma L-arginine concentration and enhances NO bioavailability

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ABSTRACT

Background: Chronic supplementation with L-citrulline plus L-arginine has been shown to exhibit anti-atherosclerotic effects. However, the short-term action of this combination on the nitric oxide (NO)–cGMP pathway remains to be elucidated. The objective of the present study was to investigate the acute effects of a combination of oral L-citrulline and L-arginine on plasma L-arginine and NO levels, as well as on blood circulation.

Methods: Rats or New Zealand white rabbits were treated orally with L-citrulline, or L-arginine, or a combination of each at half dosage. Following supplementation, plasma levels of L-arginine, NO_x, cGMP and changes in blood circulation were determined sequentially.

Results: L-Citrulline plus L-arginine supplementation caused a more rapid increase in plasma L-arginine levels and marked enhancement of NO bioavailability, including plasma cGMP concentrations, than with dosage with the single amino acids. Blood flow in the central ear artery in rabbits was also significantly increased by L-citrulline plus L-arginine administration as compared with the control.

Conclusion: Our data show for the first time that a combination of oral L-citrulline and L-arginine effectively and rapidly augments NO-dependent responses at the acute stage. This approach may have clinical utility for the regulation of cardiovascular function in humans.

1. Introduction

Nitric oxide (NO) is a widespread signaling molecule in the cardiovascular system as well as in various cell types, and also protects in multiple ways against the initiation and progression of atherosclerosis [1,2]. NO produced from L-arginine by endothelial NO synthase (eNOS) plays an important role in regulating endothelium-dependent vasodilatation [1–3], preventing the adhesion of blood cells and platelets along the endothelial cell layer of blood vessels [4], and inhibiting vascular smooth muscle cell proliferation [5]. NO also shows scavenging effects against oxygen radical species, including the prevention of oxidation of LDL-cholesterol [6]. Thus, reduced NO bioavailability caused by endothelial impairment is associated with atherosclerotic coronary artery disease [7,8].

To augment NO-dependent responses, oral treatment with L-arginine of animals [9–11] and humans [12–14] has been studied extensively, with the aim of suppressing the progression of atherosclerosis or its component processes by restoring physiological levels of NO. One of the principal explanations of the therapeutic function of L-arginine is increased availability of substrate for eNOS, for example, by competing with asymmetric dimethylarginine which is elevated in states of atherosclerosis as an endogenous competitive inhibitor of eNOS [15]. However, relatively large doses of 5–15 g/day would be required to improve endothelial function in humans.

L-Citrulline is a colorless, water-soluble α-amino acid that is a potent endogenous precursor of L-arginine. In a recent clinical study, L-citrulline supplementation dose-dependently increases plasma L-arginine levels in healthy human volunteers more effectively than equivalent doses of L-arginine itself [16]. Furthermore,

Abbreviations: NO, nitric oxide; NO_x, $NO_2^- + NO_3^-$; cGMP, cyclic GMP; eNOS, endothelial nitric oxide synthase.

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we and other researchers have demonstrated in clinical trials that L-citrulline supplementation functionally improves arterial stiffness [17], decreases the state of lipoprotein oxidation [18], reduces ankle blood pressure and carotid wave reflection [19], and causes a reduction in the heart rate-corrected QT interval as a marker of sudden cardiac death [20]. Thus, L-citrulline supplementation has been shown to exhibit several beneficial effects on the cardiovascular system.

In the present study, we focused on a potential strategy for promoting the L-citrulline-to-L-arginine recycling pathway, which is the principal mechanism for sustaining localized L-arginine availability for eNOS-catalyzed NO production, by simultaneous application of L-citrulline and L-arginine. Our previous study revealed that chronic administration of a combination of L-citrulline and L-arginine has a better therapeutic effect on high-cholesterol induced atherosclerosis in rabbits [21]. However, the short-term actions of this combination have as yet not been investigated. Therefore, the purpose of this study was to evaluate the acute effects of simultaneous administration with L-citrulline plus L-arginine on plasma L-arginine, NO bioavailability and blood circulation associated with the NO-cGMP pathway.

2. Methods

2.1. Animals

Animal studies were conducted in accordance with the guidelines for the Institutional Animal Care and Use Committee of Nagoya University and KYOWA HAKKO BIO CO., LTD.

Fifteen male Sprague–Dawley rats (9 weeks old; Japan SLC Inc., Hamamatsu, Japan) were given free access to standard rat chow (CE-2, CLEA JAPAN Inc., Tokyo, Japan) and tap water in a room with controlled temperature (22 ± 2 °C), humidity (55 ± 5 °C) and a 12-h light/dark cycle. After the rats had been anesthetized with pentobarbital sodium (30 mg/kg, i.p.), a catheter was inserted into the jugular vein. Following three days of acclimation, the rats were used for the following experiments.

Nine male New Zealand White rabbits (8 weeks old; Kitayama Labes Co., Ltd., Nagano, Japan) were housed individually in a room with controlled temperature (20 ± 3 °C) and free access to water. Feeding of standard feed was limited to 120 g per day per rabbit.

2.2. Experimental designs

After a 16-h fast, the rats and the rabbits were each randomly assigned to three separate groups respectively, and received single oral gavage of either L-citrulline (2.85 mmol/kg), L-arginine (2.85 mmol/kg) or a combination of each at half dosage (1.43 mmol/kg). L-citrulline and L-arginine were obtained from KYOWA HAKKO BIO CO., LTD. (Tokyo, Japan). Blood samples were collected from the catheter (rats) or the ear vein (rabbits) at 0 (pre-value), 0.5, 1, 2, and 4 h after the administration. We used the rat plasma for analyzing L-arginine levels only, and the rabbit plasma for all the measurements. The blood flow level in the central ear artery in rabbits was measured at 40 min after supplementation.

2.3. Measurements of plasma L-arginine, NO_x and cGMP

Blood samples were collected into heparinized tubes for L-arginine assay, or vacuum tubes containing sodium EDTA for NO_x (nitrite plus nitrate) and cGMP. They were immediately centrifuged to obtain the supernatant. Analytical procedures and methods were basically the same as described previously. Briefly, the concentration of L-arginine in plasma was determined using an amino acid analyzer (JLC-500/V; JEOL, Tokyo, Japan) [22]. Plasma NO_x was measured using an NO detector-HPLC system (ENO10; Eicom, Kyoto, Japan) as described [23]. Plasma cGMP concentration was determined by specific radioimmunoassay (RPN226, Amersham, Buckinghamshire, England) [24].

2.4. Determination of tissue blood flow

To measure the blood flow in a 3 cm-length from the proximal region of the right central ear artery, we used a laser Doppler imaging system (LDPI: Laser Doppler Perfusion Imager PIMII, PERIMED AB, Linköping, Sweden), as we reported previously [21]. This method provides 2-dimensional mapping of blood flow in tissue and is based on the principles of laser Doppler flowmetry. Rabbits treated orally with purified water were used as controls.

2.5. Data analysis

Results were expressed as mean ± SD, and represent unpaired data. Following a comparison of the data by non-repeated analysis of variance (*ANOVA*), the Bonferroni correction for multiple tests or Scheffe's test for multiple comparisons was used to identify the differences among treatments. A p-value of less than 0.05 was considered to be statistically significant. Statistical analysis was performed using Statcel software for Windows (Version 2, OMS Publishing, Inc., Saitama, Japan) and the ystat 2000 Statistical Program File (Igaku Tosho Shuppan, Tokyo, Japan).

3. Results

Fig. 1 shows the time-course of changes in plasma L-arginine concentrations. Compared with the single amino acid, simultaneous administration with L-citrulline and L-arginine to rabbits

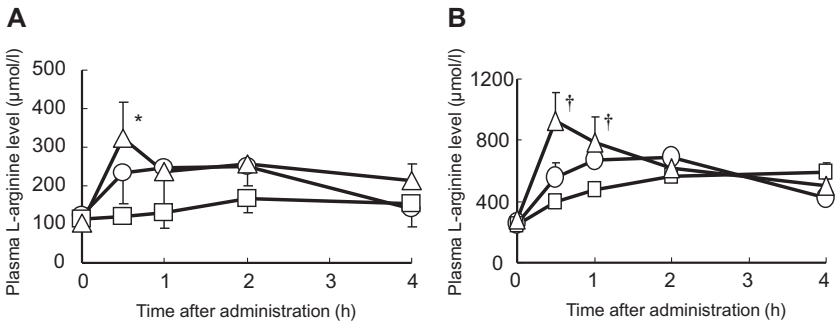

Fig. 1. Time course of changes in plasma L-arginine concentrations. Rabbits (A) or rats (B) were administered either L-citrulline (□), L-arginine (○) or a combination of each at half dosage (△) by oral gavage. Blood samples were collected sequentially before and after administration to analyze plasma L-arginine concentrations. Values are expressed as mean with SD. Significant difference from the L-citrulline group (*$p < 0.05$), or the L-citrulline group, L-arginine group (†$p < 0.05$). $n = 3$ (A), $n = 5$ (B).

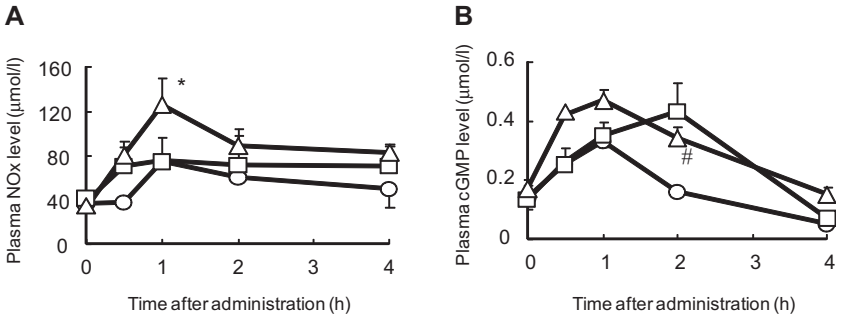

Fig. 2. Time course of changes in plasma NO_x (A) and cGMP (B) concentrations. Rabbits were administered either L-citrulline (□), L-arginine (○) or a combination of each at half dosage (△) by oral gavage. Blood samples were collected sequentially before and after administration to analyze plasma NO_x and cGMP concentrations. Values are expressed as mean with SD. Significant difference from L-citrulline group, L-arginine group ($^*p < 0.05$), or L-arginine group ($^\#p < 0.05$). $n = 3$.

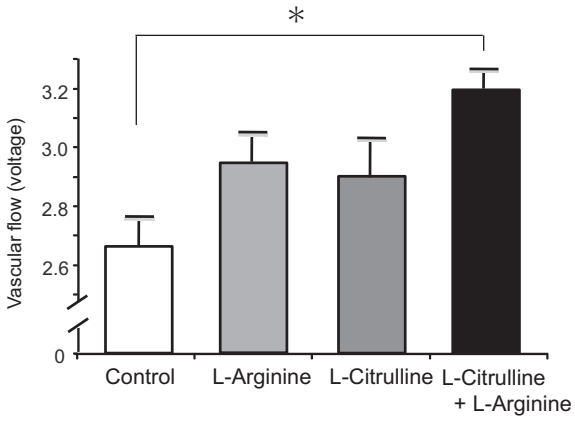

Fig. 3. Blood flow levels in the central ear artery after administration. Rabbits were administered either L-citrulline, L-arginine or a combination of each at half dosage by oral gavage. Blood flow in the central ear artery was measured at 40 min after administration. Values are expressed as mean with SD. Significant difference from control group ($^*p < 0.05$). $n = 3$.

rapidly raised plasma L-arginine levels, with time to reach peak concentration of less than an hour after supplementation (Fig. 1A). This rapid kinetics in plasma L-arginine was corroborated and reappeared clearly in healthy rats (Fig. 1B).

Fig. 2 shows the time-course of changes in plasma NO_x and cGMP concentrations. We observed an approximately twofold increase in plasma NO_x levels after L-citrulline or L-arginine administration as compared with 0 h (pre-value). Furthermore, a combination of each at half dosage caused a significantly greater increase in NO_x levels than a single amino acid (Fig. 2A). Plasma levels of cGMP, an important signaling molecule in the vasodilative pathway, also increased more rapidly in the 1 h following simultaneous administration with L-citrulline plus L-arginine when compared with a single amino acid (Fig. 2B).

The blood flow levels in the central ear artery at 40 min following supplementation are given in Fig. 3. Compared with the control, L-citrulline or L-arginine supplementation tends to increase blood flow, but not statistically significant. In contrast, simultaneous administration with both at half dosage results in a significant enhancement in the blood flow level as compared with the control.

4. Discussion

The primary objective of the present study was to determine the short-term action of oral L-citrulline plus L-arginine on plasma parameters for the NO-cGMP pathway. This is the first study on the rapid kinetics of plasma L-arginine, NO_x and cGMP levels after simultaneous administration in comparison with the single amino acids. The major findings of our study are that a combination of oral L-citrulline and L-arginine effectively and rapidly increases plasma L-arginine and augments NO-dependent responses, particularly within 1 h after supplementation.

NO, the endothelium-derived vasoactive factor, is produced from L-arginine by eNOS via the L-citrulline/L-arginine recycling pathway [1–3]. To investigate the effects of L-arginine, the substrate for NO synthesis, many researchers have carried out studies on vascular function. It has been reported that intervention with L-arginine can improve endothelial dysfunction in humans and animal models [9,10,12–14,25]. However, the recent literature has reported no effects of chronic administration of L-arginine [26–28], because orally administered L-arginine is strongly trapped in the gastrointestinal tract and the hepatic tissue, where it is extensively catabolized by arginase [27,29], suggesting it to have limited oral bioavailability as a substrate for eNOS. On the other hand, several studies have persuasively demonstrated that L-citrulline is an effective precursor of L-arginine, thus contributing to sustained L-arginine supply for eNOS activity [16,18,22]. As a noteworthy finding in this study, a rapid increase in plasma L-arginine levels was observed with a combination of oral L-citrulline plus L-arginine than for a single amino acid. Interestingly, previous researches *in vitro* and *in vivo* have demonstrated that L-citrulline suppresses arginase activity, acting as a strong allosteric inhibitor [30], and exerts an anti-hypertensive effect in animals via a mechanism involving arginase inhibition by L-citrulline [31]. This could provide one possible mechanism for the rapid and significant increase in plasma L-arginine levels, suggesting that L-arginine could pass through the gastrointestinal tract and liver without being influenced by intestinal and hepatic-first pass effects, probably due to inhibition of arginase activity by L-citrulline (Fig. 4). These findings suggest the role played by L-citrulline in upregulating L-arginine bioavailability, leading to enhancement of the NO-cGMP pathway.

Upregulation of the L-citrulline/L-arginine recycling pathway and increased eNOS expression are accompanied by increased plasma NO_x and cGMP. Here, the intracellular L-arginine concentration is sufficient level for the km (2–15 μM) for L-arginine as a substrate for eNOS [27,32]. However, a previous report suggests that intracellular endothelial L-arginine may not in fact be available for NO production, since cytosolic L-arginine availability for eNOS may be limited by its sequestration in the plasmalemmal caveolae in which the eNOS and the L-citrulline to L-arginine recycling pathway are localized [33]. Moreover, recent study has suggested one possible mechanism in which the principal source of available L-arginine for NO production is uptake from extracellular, not cytosolic L-arginine [34]. These observations support the notion that a combination of L-citrulline plus L-arginine effectively increases

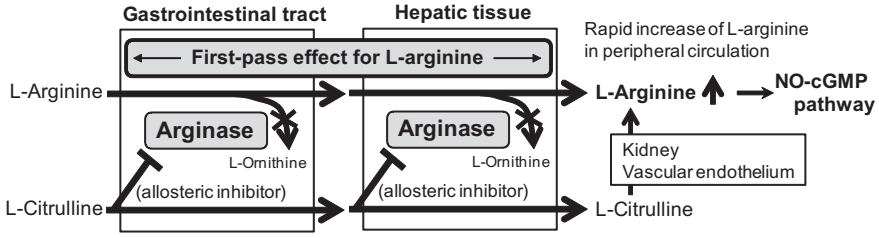

Fig. 4. A proposed mechanism for simultaneous administration with L-citrulline plus L-arginine leading to rapid raising of plasma L-arginine levels.

plasma L-arginine levels, leading to NO production. The results of the present study clearly indicate L-arginine-linked enhancement of plasma NO_x and cGMP levels following simultaneous administration with L-citrulline and L-arginine.

Interestingly, marked increases in plasma NO_x and cGMP levels were observed after L-citrulline supplementation, although the rises in plasma L-arginine after L-citrulline supplementation were a slight change in rabbits. It has been indicated that L-citrulline added to cultured endothelial cells sustains NOS activity and NO production under L-arginine-deficient conditions [27]. L-Citrulline itself can also be transported into endothelial cells by the neural amino acid system N transporter 1 (SN1) [35]. We therefore speculate that direct uptake of L-citrulline into the endothelial cells may provide a substrate for the formation of L-arginine via L-citrulline/L-arginine recycling pathway and contribute to upregulation of eNOS activity.

Here, time to reach peak concentration (T_{max}) of plasma L-arginine after oral L-citrulline is approximately 2 h in humans [16] and 4 h in animals (our unpublished data), which suggests that the combination of L-citrulline plus L-arginine is useful for short-acting effects, as shown in the present study, while L-citrulline alone is useful for long-acting enhancement of L-arginine availability. Indeed, this suggestion is further supported by our finding that L-citrulline administration to rats significantly increases the area under the concentration–time curve (AUC_{0-12h}) to a greater extent than L-arginine itself (data not shown).

Our previous study revealed that long-term L-citrulline plus L-arginine supplementation causes a significant improvement in endothelium-dependent vasorelaxation and dramatic regression in atheromatous lesions in experimentally-induced atherosclerosis in rabbits [21]. As one possible mechanism, we have also shown increased expression of eNOS protein by combination of these two amino acids [21]. However, the short-term action of the combination of L-citrulline plus L-arginine on the NO-cGMP pathway remains to be elucidated. Thus, our corroborative findings in this study propose a potentially novel strategy for rapidly potentiating NO-cGMP dependent reactions in the short-term, which is capable of increasing plasma L-arginine and NO_x levels, as well as blood circulation rapidly and effectively, likely due to upregulation of the L-citrulline/L-arginine recycling pathway and eNOS expression.

Growing evidences suggest endothelial dysfunction, which is strongly associated with reduced NO bioavailability, to be an initiating factor in cardiovascular diseases [7,8,36]. Our data suggests the clinical benefit on vascular function of L-citrulline plus L-arginine supplementation. However, we believe a further study in humans is needed to be able to present a therapeutic option with clinical utility.

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