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Ferulic acid enhances the vasorelaxant effect of epigallocatechin gallate in tumor necrosis factor-alpha-induced inflammatory rat aorta

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Abstract

Previously, we demonstrated synergistic enhancement of vasorelaxation by combination treatment with Trp-His and epigallocatechin gallate (EGCg) in intact rat aorta. The aim of the present study was to determine whether this vasorelaxant synergy could be recapitulated in tumor necrosis factor-alpha (TNF-α)-induced inflammatory rat aorta, and to determine the extent of its modulation by anti-inflammatory phenolic acids. Synergistic enhancement of vasorelaxation in rat aorta by Trp-His and EGCg was significantly attenuated in the presence of TNF-α, an effect that was reversed by the addition of ferulic acid (FA, 250 μM). Moreover, FA markedly enhanced EGCg-induced vasorelaxation, but not Trp-His-induced vasorelaxation, in TNF-α-treated aorta. Structure-activity analysis showed that the unsaturated 2-propenoic moiety and the methoxy group of FA were important for the enhancement of vasorelaxation by EGCg. The stimulation of EGCg-induced vasorelaxation by FA was antagonized by the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine acetate, while FA enhanced vasorelaxant properties of the endothelial nitric oxide (NO) synthase activator acetylcholine in TNF-α-treated inflammatory aorta. Moreover, the EGCg-stimulated NO production was also enhanced by FA in TNF-α-treated aorta. These data indicate that stimulation of NO production by FA enhances the vasorelaxant properties of EGCg in TNF-α-induced inflammatory aorta.

Keywords: Vasorelaxation; Ferulic acid; Inflammation; Tumor necrosis factor-alpha; Epigallocatechin gallate

1. Introduction

Mild chronic inflammation is thought to be associated with endothelial dysfunction, leading to progression of cardiovascular disease [1,2]. The pro-inflammatory cytokine tumor necrosis factor (TNF)-α has been reported to play a crucial role in the development of endothelial dysfunction [3]. In the vasculature, TNF-α attenuates endothelial nitric oxide synthase (eNOS) activity and induces reactive oxygen species (ROS) generation, resulting in reducing vascular NO availability and impairment of endothelium-dependent vasorelaxation [4,5]. Inflammatory factors, and TNF-α in particular, are therefore considered to be important components in the development of cardiovascular diseases [6,7].

Functional food compounds have recently been receiving more attention as potential beneficial agents for cardiovascular diseases. Many studies have shown that functional food compounds such as peptides and polyphenols can regulate vascular tone, improve endothelial function, and decrease blood pressure in experimental models of hypertension [8–11]. In addition, because functional food compounds are commonly present and/or consumed as a mixture with a variety of other food components, interactions between these components are emerging as the focus of current investigations. Several studies have shown that combinations of certain food components can enhance their individual physiological properties such as antioxidative effects [12–14]. In a previous study, we demonstrated that the vasorelaxant effect of the dipeptide Trp-His was synergistically enhanced in the presence of the vasorelaxant flavonoid epigallocatechin gallate (EGCg) in isolated rat aorta [15]. In addition, since most of functional food compounds like EGCg and small peptides showed poor absorption properties (e.g. Cmax of EGCg (100 mg/kg-B.W., *p.o.*)=~3 μM [16] and Cmax of di-peptide=nM level [17]), the combination strategy for these functional food compounds must be efficient to express their physiological benefit *in vivo*.

Although the beneficial arterial effects of food compounds have been well investigated in vascular models such as Sprague-Dawley (SD) rats, Wistar Kyoto (WKY) rats, and spontaneously hypertensive rats (SHR), relatively few studies have been carried out under inflammatory conditions induced by TNF-α. The aim of the present study therefore was to characterize the synergistic vasorelaxant effect of EGCg and Trp-His in TNF-α-induced inflammatory aorta. Ferulic acid (FA), which is a common phenolic acid abundantly present in various fruits and vegetables and can be absorbed as intact form (Cmax (14 mg/kg-B.W., *p.o.*)=25.3 μM in Wister rat [18]), has been shown to possess anti-inflammatory [19], anti-hypertensive

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properties [20,21], and exerts protective effects in vascular tissues in the SHR model and in endothelial cells under oxidative stress [22,23]. Accordingly, we also aimed to investigate whether further combination of FA could recover or improve the vasorelaxant effect of EGCg and Trp-His in TNF-α-treated aorta rings in the present study.

2. Methods and materials

2.1. Chemicals

Phenylephrine (PE), acetylcholine (ACh) and *p*-coumaric acid (COA) were obtained from Wako Pure Chemical Industries (Osaka, Japan). FA, caffeic acid (CAA), and 3-(4-hydroxy-3-methoxyphenyl) propionic acid (HMPA) were purchased from Tokyo Kasei Kogyo Co. Ltd (Tokyo, Japan). TNF-α was purchased from PeproTech (Rocky Hill, NJ, USA). N^G-Monomethyl-L-arginine acetate (L-NMMA) was a product of Dojindo (Kumamoto, Japan). EGCg, 3,4-dimethoxy cinnamic acid (DMCA) and diethylenetriamine/nitric oxide adduct (DETA-NO) were from Sigma Chemical (St. Louis, MO, USA). 3-Hydroxy-4-methoxy cinnamic acid (3H4MCA) was obtained from Alfa Aesar (Ward Hill, MA, USA). Trp-His was synthesized by Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan). All other chemicals were of analytical reagent grade and were used without further purification.

2.2. Animals and preparation of aorta rings

Seven- to nine-week-old male SD rats (SPF/VAF Crj:SD; Charles River Japan, Kanagawa, Japan) were used for this study. All animal experiments were in accordance with the Guidance for Animal Experiments in Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105, 1973) and Notification (No. 6, 1980 of the Prime Minister's Office) of the Japanese Government. All experiments were reviewed and approved by the Animal Care and Use Committee of Kyushu University (Permit Number: A24-051). Rats were killed by exsanguination from the abdominal aorta under the anesthesia with volatile anesthetics sevoflurane, because Nakamura et al. [24] have reported that the influence of sevoflurane to the vasorelaxation of ACh in aorta rings was removed by washing of aorta tissue with fresh buffer (without sevoflurane). After the exsanguination, the thoracic aorta was isolated within 5 min. The thoracic aorta was washed with modified PSS buffer (NaCl 145 mM, KCl 5 mM, Na_2HPO_4 1 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 0.5 mM, glucose 10 mM, and HEPES 5 mM [pH 7.4]), placed in the buffer, maintained at 37°C for 45 min and gassed with 95% O_2/5% CO_2. In the case of TNF-α treated aorta, the isolated thoracic aorta was placed into PSS buffer containing 1.0 ng/mL TNF-α, which was present during all vascular tension measurement procedures referred to below. The adhering fat and connective tissues were removed from the thoracic aorta. Each ring segment (2–3 mm) from an aorta was prepared and used for each tested group in an experimental item. The ring was then mounted between two stainless steel wires in a 5-mL organ bath filled with PSS buffer with or without TNF-α and progressively stretched to a preloaded tension of 20 mN followed by equilibration for another 45 min until stabilization. Contractile responses (isometric tension, in mN) were measured by a force transducer (Micro Tissue Organ Bath, Model MTOB-1Z; Labo Support, Osaka, Japan) coupled to a data acquisition system (EMKA Technologies Inc., Falls Church, VA, USA). To verify the viability of aorta rings, a 1.0 μM PE-contracted response (more than 3 mN in isometric tension) was confirmed before sample-induced vasorelaxation experiments.

2.3. Measurement of the vasorelaxant effect

The vasorelaxation experiment was performed according to our previous study [8]. The viability of the intact endothelium in the rat aorta rings was confirmed by a significant vasorelaxant effect induced by 100 μM ACh in 1.0 μM PE-contracted aorta rings. After the 45-min equilibration in an organ bath with PSS buffer, the ring was contracted with 1.0 μM PE. When the contraction reached a plateau, the vasorelaxant effects of combination addition of Trp-His (700 μM) and EGCg (300 μM) with or without FA (250 μM) were evaluated in intact or TNF-α-treated aorta rings. To examine the effect of FA, the vasorelaxation induced by cumulative addition of EGCg (1-300 μM), Trp-His (10-4700 μM), ACh (0.01-2 μM) or DETA-NO (0.1-64 μM) was measured in the presence or absence of FA (250 μM) in the aorta rings. Each addition was conducted within a 10-min interval in the cumulative experiments. To characterize the importance of specific structural groups, we examined the effects on EGCg-induced vasorelaxation in intact or TNF-α-treated aorta rings of selected phenolic acids and FA analogues (250 μM), namely, HMPA, COA, CAA, 3H4MCA and DMCA. Vasorelaxant activity was evaluated using an EC_{50} value, the effective concentration producing 50% vasorelaxation of the maximal contractile response.

2.4. Detection of vascular $O_2\cdot^-$ production

$O_2\cdot^-$ production in aortic segments was detected by BES-So, a highly specific fluorescent probe for $O_2\cdot^-$, as described in previous study [25]. Aorta rings were pretreated as in the vascular tension measurement, in which rings were incubated in PSS buffer with or without 1.0 ng/mL TNF-α at 37°C for 2 h. Aorta rings were then incubated with FA (250 μM) and/or EGCg (50–250 μM) in the presence of BES-So (10 μM) and NADPH (500 μM) for 60 min. Changes in the rate of $O_2\cdot^-$ production were determined by measuring fluorescence intensity with a Wallac 1420 microplate reader (Perkin Elmer Life Science, Waltham, MA, USA) at an excitation wavelength of 492 nm and an emission wavelength of 535 nm. $O_2\cdot^-$ production was expressed as relative fluorescence intensity per milligram of wet weight of aorta rings.

2.5. Measurement of nitrite/nitrate production in aorta rings

Nitrite/nitrate production from aorta rings was determined by monitoring the production of nitrite and nitrate by using the 2,3-Diaminonaphthalene Kit (NO_2/NO_3 Assay Kit-FX (Fluorometric), Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Aorta rings used for measurement of nitrite/nitrate production were prepared according to same procedures for vascular tension measurement and subjected to incubation in PSS buffer with or without 1.0 ng/mL TNF-α for 2 h. After the incubation, the aorta rings were further incubated with FA (250 μM) and/or EGCg (250 μM) for 60 min at 37°C in PSS buffer containing 1 mM L-arginine. Aliquots of the solutions incubated with the aorta rings were mixed with nitrate-reductase-containing reagent for 30 min at 37°C to convert nitrate to nitrite. After the conversion reaction, the 2,3-diaminonaphthalene kit reagent was added to each well. Fluorescence was measured using a Wallac 1420 microplate reader at an excitation wavelength of 360 nm and an emission wavelength of 460 nm to detect nitrite. Results were expressed as nitrite/nitrate production per milligram of wet weight of aorta rings. Standard curves (0–10 μM) were prepared from serial dilutions of $NaNO_3$.

2.6. Calculation of Combination Index (CI)

In order to evaluate the synergistic vasorelaxative effects of combinatorial treatments, a combination index (CI) was used, as described in our previous study [15]. According to a report by Chou et al., [26] the dose-reduction indices (DRI: the order of magnitude [fold] of dose reduction that was allowed in combination use of the drugs in which a given degree of effect was induced as compared with the dose of each drug alone) of EGCg and a compound (expressed as A) in combination use was calculated at 50% vasorelaxation effect. To define the combination effect of the interaction between the two drugs, the combination index (CI) is calculated as follows:

$$CI = (D)_1/(D_x)_1 + (D)_2/(Dx)_2 = 1/DRI_{EGCg} + 1/DRI_A$$

where $(D)_1$ and $(D)_2$ indicate the dose of EGCg and A, respectively, in combination use and $(D_x)_1$ and $(D_x)_2$ indicate the dose of EGCg and A, respectively, in single use of each drug, and DRI_{EGCg} and DRI_A were dose-reduction indices for EGCg ($1/DRI_{EGCg} = (D)_1/(D_x)_1$) and A ($1/DRI_A = (D)_2/(Dx)_2$), respectively. Chou and Talalay [27] have proposed that CI<1,CI=1, and CI>1 indicate synergistic, additive and antagonistic effects, respectively.

2.7. Statistical analyses

Results are expressed as the mean±S.E.M. Vasorelaxation is expressed as the percentage change of maximal contraction. Statistical differences between vasorelaxation dose-response curves were analyzed using a two-way analysis of variance (ANOVA). Statistical differences between two groups and more than two groups were analyzed using an unpaired Student's *t*-test and a one-way ANOVA followed by Tukey-Kramer's *t*-test for post-hoc analysis, respectively. A value of $P<.05$ was considered statistically significant. All analyses were performed with Graph Pad Prism version 5.0 for windows (Graph Pad Software, La Jolla, CA, USA).

3. Results

3.1. Trp-His and EGCg-induced vasorelaxation in intact and TNF-α-treated aorta rings and the effect of FA treatment

We first evaluated the synergistic vasorelaxant effect of Trp-His and EGCg in intact and TNF-α-treated aorta rings. The vasorelaxation induced by combination use of Trp-His and EGCg in aorta rings was significantly attenuated by treatment with TNF-α compared with intact aorta rings (TNF-α-treated aorta: 58.5%±6.7%, intact aorta: 83.0%±2.1%; Fig. 1). We next examined the effect of FA on the attenuation by TNF-α of the vasorelaxation induced by Trp-His and EGCg in aorta rings. Addition of 250 μM FA to the Trp-His/EGCg mixture reversed TNF-α-attenuated vasorelaxation in aorta rings by up to 88.3%±6.9%, but it had no effect in intact aorta rings. When used alone, 250 μM FA had no appreciable (<5%) vasorelaxant effects, either in intact aorta rings or in TNF-α-induced inflammatory aorta rings (data not shown).