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Epigallocatechin Gallate Promotes the Vasorelaxation Power of the Antiatherosclerotic Dipeptide Trp-His in Contracted Rat Aorta

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ABSTRACT: The aim of this study was to demonstrate the enhancement of the vasorelaxation power of the antiatherosclerotic voltage-dependent L-type Ca^{2+} channel (VDCC)-blocking peptide Trp-His by epigallocatechin gallate (EGCg). We found that 300 μM EGCg dramatically enhanced the magnitude of Trp-His-induced vasorelaxation by a factor of >6 (EC_{50} of Trp-His: EGCg(−), 2.80 ± 0.05 mM; EGCg(+), 0.45 ± 0.04 mM) in phenylephrine-contracted rat aorta. The enhancing effect of EGCg was completely abolished in endothelium-removed aorta and high K^+-contracted aorta. The enhancement of Trp-His-induced vasorelaxation by EGCg was significantly diminished by either N^G-monomethyl-L-arginine acetate (NO synthase (NOS) inhibitor) or 1-*H*-[1,2,4]oxadiazolo[4,3]quinoxalin-1-one (soluble guanylyl cyclase inhibitor), together with the enhancement of NOS activity by EGCg. These results indicate that the enhancing effect of EGCg in Trp-His-induced vasorelaxation may be involved in the activation of NO/cGMP pathway.

KEYWORDS: *vasorelaxation, peptides, epigallocatechin gallate, synergistic effect*

INTRODUCTION

Hypertension and related cardiovascular diseases are considered one of the major causes of disability and premature death worldwide and are responsible for increased costs of medical treatment.[1] Therefore, preventive treatments for cardiovascular disease have become the foci of intensive research. To date, studies have found multiple functional compounds that can ameliorate hypertension and improve vascular health, including peptides[2−4] and polyphenols.[5,6] Several clinical studies have demonstrated the antihypertensive effect of small peptides. One such peptide, Val-Tyr, has been shown to contribute to lower blood pressure not only by inhibiting angiotensin I-converting enzyme (ACE)[7] but also by inducing vasorelaxation.[8] Research done in our laboratory has found that the peptide Trp-His can cause vasorelaxation in an endothelium-independent manner by blocking voltage-dependent L-type Ca^{2+} channels (VDCC) at an extracellular site on vascular smooth muscle cells in rat thoracic aorta.[9,10] In addition, in vivo administration of Trp-His (10 and 100 $mg \cdot kg^{-1} \cdot day^{-1}$) to apolipoprotein E-deficient mice ameliorated atherosclerotic lesion area.[11]

Recently, to enhance the benefit of antihypertensive drugs and reduce their side effects, combination drug therapy studies have been performed,[12] revealing some synergistic physiological effects. For example, one large-scale clinical trial has shown that angiotensin II receptor blocker (ARB) combined with dihydropyridine (DHP)-type Ca^{2+} channel blocker exerted an antihypertensive effect exceeding that of either drug alone.[12] Kubota et al.[13] have also demonstrated that the coadministration of DHP-type Ca^{2+} channel blocker and ARB produced a synergistic improvement in vascular function and inflammation in a porcine model. Ex vivo, the combination of insulin and troglitazone, a class of insulin-sensitizing agents, has also shown synergistic vasorelaxation via inhibition of VDCC.[14]

Interestingly, Van Hove et al.[15] demonstrated that a reduction in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) potentiates nitric oxide (NO)-dependent vasorelaxation capacity in the aortic tissue, because a DHP-type Ca^{2+} channel blocker, nifedipine, augmented the vasorelaxation induced by NO donor. Because Trp-His also reduces $[Ca^{2+}]_i$ by blocking VDCC,[9] this peptide is a likely candidate for improving vascular function in synergy with other components that can produce the vasorelaxant NO.

This strategy of combining drugs with different mechanisms to obtain a synergistic vasorelaxation effect is quite useful for functional food components such as peptides and polyphenols, because these compounds have lower bioactivities and lower bioavailability (C_{max} was pmol/mL-plasma order[16]) compared to the traditional drugs (C_{max} was nmol/mL-plasma order[17]). Thus, the aim of this study was to demonstrate the synergistic vasorelaxation of an antiatherosclerotic Ca^{2+} channel-blocking peptide, Trp-His, with other vasorelaxant natural compounds and to determine the mechanism of action. In this study, we used several major catechins that are widely consumed and have already been reported to possess antihypertensive effects in stroke-prone spontaneously hypertensive rats.[18] Among these catechins, (−)-epigallocatechin gallate (EGCg) is a well-studied bioactive flavonoid that reduces hypertension by inducing vasorelaxation. Some studies[19−21] showed that the mechanism of EGCg-induced vasorelaxation might be involved in an endothelium-dependent NO/cyclic guanosine monophosphate (cGMP) pathway. These findings led us to speculate that EGCg can enhance the vasorelaxation effect of Trp-His.

MATERIALS AND METHODS

Materials. Phenylephrine (PE) and acetylcholine (ACh) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Catechins ((−)-epicatechin (EC), (−)-epicatechin gallate (ECg),

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(−)-epigallocatechin (EGC), and EGCg) and 1-*H*-[1,2,4]oxadiazolo-[4,3]quinoxalin-1-one (ODQ) were from Sigma Chemical (St. Louis, MO, USA). N^G-Monomethyl-L-arginine acetate (L-NMMA) was a product of Dojindo (Kumamoto, Japan). Nifedipine was obtained from Nacalai Tesque (Kyoto, Japan). Trp-His used in this study was synthesized by Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan). All other chemicals were of analytical reagent grade and were used without further purification.

Animals and Preparation of Aorta Rings. Male 8−11-week-old Sprague−Dawley (SD) rats (SPF/VAF Crj:SD; Charles River Japan, Kanagawa, Japan) were used in this study. All animal experiments were carried out under 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. After rats were killed by exsanguination from the abdominal aorta, the thoracic aorta was removed within 5 min. The adhering fat and connective tissues were removed within 10 min after the removal of aorta. A ring segment (2−3 mm) from one aorta was used for each set of individual triplicate experiments. The segment was mounted between two stainless steel wires in a 5-mL organ bath filled 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)]. The ring was maintained at 37 °C for 45 min in bubbling PSS buffer with 95% O_2/5% CO_2 gas. The ring was then progressively stretched to a preloaded tension of 20 mN followed by an equilibration for another 45 min until stabilized. The 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 (Bridge8 Modules Low Noise Transducer Amplifier; World Precision Instruments, Sarasota, FL, 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.

Measurement of Vasorelaxation Effect. The vasorelaxation experiment in this study was performed according to our previous study.[10] To evaluate the vasorelaxation activities of vasorelaxant agents in the contracted aorta rings, experiments were primarily performed using aorta rings with intact endothelium. The vasorelaxation of the intact endothelium in the rat aorta rings was confirmed by a significant vasorelaxation effect induced by 100 μM ACh in 1.0 μM PE-contracted aorta rings. After a 45-min equilibration in PSS buffer, the ring was contracted with 1.0 μM PE or 70 mM KCl. For combination experiments, catechins (10−300 μM) were added to the bath, followed by the addition of Trp-His or nifedipine, once the plateau isometric tension was attained. The concentration of combination compounds added before the Trp-His or nifedipine addition was set up to the concentration inducing ∼20% vasorelaxation in order to obtain reasonable dose−response curves for Trp-His and nifedipine. To assess the vasorelaxation effect in a cumulative manner, EGCg was added to the bath, followed by the addition of varying concentrations of Trp-His (0.01−4.7 mM) or nifedipine (1 pM−100 nM). Each addition of Trp-His or nifedipine was performed within a 15-min interval in the cumulative experiments. For endothelium-removed vascular tension measurements, the endothelial layer was mechanically removed from intact aorta rings by gentle scraping with a stainless-steel wire. The removal was verified by the absence of a vasorelaxation response to 100 μM ACh in 1.0 μM PE-contracted aorta rings. Vasorelaxation activity was evaluated using an EC_{50} value, the effective concentration producing 50% vasorelaxation of the maximal contractile response.

Calculation of Combination Index. According to the report by Chou et al.,[22] the dose-reduction index (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 Trp-His and a compound (expressed as A) combined with Trp-His was calculated at 50% relaxation effect. To define the combination effect of the 2-drug interaction, the combination index (CI) should be calculated as

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 = 1/DRI_{Trp\text{-}His} + 1/DRI_A$$

where $(D)_1$ and $(D)_2$ indicate the dose of Trp-His and A, respectively, in combination use and $(D_x)_1$ and $(D_x)_2$ indicate the dose of Trp-His and A, respectively, in single use of each drug. Chou and Talalay[23] have proposed that CI < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively.

Measurement of NO Synthase Activity in Aorta Rings. To evaluate the NO synthase (NOS) activity in aorta rings, NO production from the aorta rings was measured. As an indicator of NO production, nitrite and nitrate (after enzymatic reduction) were determined simultaneously in 1 mM Arg-containing PSS buffer treated, using the 2,3-Diaminonaphthalene Kit (NO_2/NO_3 Assay Kit-FX(Fluorometric), Dojindo Laboratories) according to the manufacturer's procedures. Aorta rings were pretreated by the PSS buffer with or without EGCg for 10 min. For the combination experiment, Trp-His (2.8 mM) was subsequently added to each well followed by incubation for 60 min. The solutions incubated with aorta rings for 70 min were mixed with enzyme-containing reagent to convert nitrate to nitrite for 30 min at 37 °C. After the converting reaction, the 2,3-Diaminonaphthalene Kit reagent was added to each well. The fluorescence was measured using a Wallac 1420 microplate reader (Perkin-Elmer Lifescience) at an excitation wavelength of 360 nm and emission wavelength of 460 nm to detect nitrite. NOS activity was expressed as nitrite/nitrate production per milligram of dry weight of aorta rings per minute. Standard curves (0−10 μM) were prepared from serial dilutions of $NaNO_3$.

Statistical Analyses. Results are expressed as the mean ± SEM values. Vasorelaxation was expressed as a percentage change of maximal contraction. Statistical difference between groups was analyzed using a one-way analysis of variance (ANOVA), followed by Dunnett's or Tukey−Kramer's *t*-test for post hoc analysis. Value of $p < 0.05$ was considered statistically significant. All analyses were performed with Stat View J 5.0 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

Trp-His Induced Vasorelaxation in the Presence of EGCg in PE-Contracted Aorta. The effect of EGCg on the vasorelaxation induced by Trp-His was primarily examined in 1.0 μM PE-contracted aorta rings from SD rats. As shown in Figure 1, EGCg exerted 18.6 ± 2.9% vasorelaxation at 300 μM. Much higher concentration, e.g., 500 μM EGCg, also exerted ∼40% vasorelaxation and induced 68% vasorelaxation at 10 μM

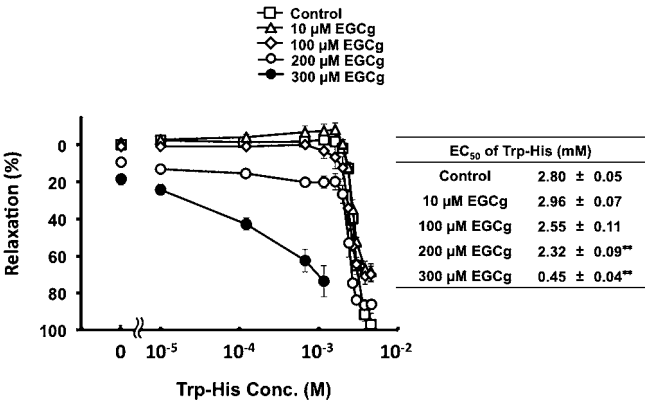

Figure 1. Dose−response curves of Trp-His-induced vasorelaxation in the absence (control) or presence of EGCg (10−300 μM) on 1.0 μM PE-contracted aorta rings from SD rats. The inset table shows EC_{50} values of Trp-His. Results are expressed as the mean ± SEM values (n = 3−6). Significant differences between EC_{50} values were analyzed by a one-way ANOVA followed by Dunnett's *t*-test for post hoc analysis. **$p < 0.01$ vs Control.

Trp-His (data not shown). By considering the dynamic range of enhancing vasorelaxation response of Trp-His by EGCg, EGCg used in combination experiments with Trp-His was set at 300 μM, so as not to exceed 20% vasorelaxation. As a result, vasorelaxation power by Trp-His was greatly enhanced at 300 μM EGCg, by a factor of >6; the curve showing Trp-His-induced vasorelaxation shifted significantly to the left at this concentration of EGCg (EC_{50} of Trp-His: EGCg(−), 2.80 ± 0.05 mM; EGCg (+), 0.45 ± 0.04 mM, $p < 0.01$).

According to the theoretical basis of drug combination by Chou et al.,[22] the DRI of Trp-His and EGCg at 50% relaxation effect were calculated as 6.22 and 2.87, respectively, because the EC_{50} value of EGCg on PE-contracted aorta was 860 μM in the present experimental conditions (data not shown). To define the combination effect of Trp-His and EGCg, CI was calculated as described in Materials and Methods. From the relaxation effect obtained by Trp-His and EGCg used in combination (Figure 1), the CI was calculated as 0.51 at 50% vasorelaxation, which clearly demonstrated the "synergistic effect".

Combinatorial Vasorelaxation Effect of Trp-His with Major Catechins. In the next experiment, the vasorelaxation effect of the major catechins, namely, EC, ECg, EGC, and EGCg, was examined in 1.0 μM PE-contracted aorta rings from SD rats. As summarized in Table 1, among all tested catechins, only EGCg exerted vasorelaxation at a concentration of 300 μM. At 1.2 mM, Trp-His alone does not induce vasorelaxation; however, the addition of 300 μM EGCg resulted in a great enhancement of Trp-His-induced vasorelaxation (73.8% ± 6.4%). Interestingly, the other catechins (EC, ECg, and EGC) did not enhance Trp-His-induced vasorelaxation.

Table 1. Vasorelaxation Effect of Catechins in the Absence or Presence of Trp-His

	vasorelaxation	
catechin	catechin[a] (%)	catechin + Trp-His[b] (%)
EC	no relaxation	no relaxation
ECg	no relaxation	no relaxation
EGC	no relaxation	no relaxation
EGCg	18.6 ± 2.9	73.8 ± 6.4

[a]Vasorelaxation effect induced by 300 μM catechin. [b]Vasorelaxation effect induced by 1.2 mM Trp-His plus 300 μM catechin.

Involvement of Endothelium on the Combinatorial Vasorelaxation Effect of Trp-His with EGCg. Figure 2 shows the Trp-His-induced vasorelaxation in the presence or absence of EGCg on 1.0 μM PE-contracted aorta with or without endothelium. No vascular response to the addition of 100 μM ACh was observed on the endothelium-removed aorta rings. The removal of endothelium resulted in complete abolition of the enhancement of Trp-His-induced vasorelaxation by 300 μM EGCg, showing that EGCg potentiated the vasorelaxation power of Trp-His in endothelium-dependent manner.

Combinatorial Vasorelaxation Effect of Trp-His with EGCg on High K^+-Contracted (Depolarized) Aorta. Next, the vasorelaxation of Trp-His with EGCg was examined on 70 mM KCl-contracted (depolarized) aorta rings. The addition of 300 μM EGCg did not enhance the vasorelaxation power of Trp-His in 70 mM KCl-contracted aorta rings as shown in Figure 3 (EC_{50} of Trp-His: EGCg(−), 3.89 ± 0.34 mM; EGCg(+), 4.56 ± 0.16 mM).

Involvement of NO/cGMP Pathway on the Combinatorial Vasorelaxation Effect of Trp-His with EGCg. We then performed the vasorelaxation experiments of Trp-His plus EGCg in the presence of L-NMMA (100 μM) as an inhibitor of NOS or ODQ (10 μM) as an inhibitor of soluble guanylyl cyclase (sGC), which produces the vasorelaxant cGMP. The results shown in Figure 4A,B demonstrate that Trp-His-induced vasorelaxation was not enhanced by 300 μM EGCg in the presence of L-NMMA (EC_{50} of Trp-His: EGCg(−), 2.52 ± 0.06 mM; EGCg(+), 2.40 ± 0.15 mM) or ODQ (EC_{50} of Trp-His: EGCg(−), 2.37 ± 0.04 mM, EGCg(+), 2.35 ± 0.07 mM). Taking into consideration that NOS activity in aorta rings treated with EGCg was significantly ($p < 0.05$) increased compared to control rings (Figure 5), enhanced NO/cGMP pathway by EGCg may be involved in the synergistic vasorelaxation effect.

Synergy of EGCg and the VDCC Blocker Nifedipine on PE-Contracted Aorta. To further examine the enhanced vasorelaxation effect of EGCg on VDCC blocking by Trp-His, we performed vasorelaxation experiments using nifedipine, which is a VDCC blocker that binds at similar site on the channel protein as Trp-His.[9] As shown in Figure 6, vasorelaxation was induced by nifedipine in a dose-dependent manner (EC_{50} = 19.5 ± 9.3 nM). A much greater enhancement of the nifedipine-induced vasorelaxation was observed in the presence of 300 μM EGCg. The EC_{50} value of nifedipine in combination with 300 μM EGCg (EC_{50} = 3.91 ± 2.90 pM) was ~5000-fold stronger than that in the absence of EGCg.

DISCUSSION

In this study, the vasorelaxation induced by an antiatherosclerotic VDCC-blocking peptide, Trp-His, was synergistically enhanced in the presence of EGCg (CI = 0.51; synergistic), but not in the presence of other catechins (EC, ECg, and EGC). Interestingly, among the tested catechins, EGCg was the only one that independently showed a relaxation effect—18.6% relaxation at 300 μM—leading to the speculation that the EGCg-induced vasorelaxation signaling could be involved in the enhancement of Trp-His-induced vasorelaxation.

Trp-His-induced vasorelaxation was not altered by EGCg in the endothelium-removed aorta ring (Figure 2), suggesting that the enhanced vasorelaxation power of Trp-His plus EGCg is involved in the endothelium-derived vasorelaxation signal(s) induced by EGCg. Previous studies have shown that EGCg-induced vasorelaxation is endothelium dependent in nature and is related to the activation of the NO/cGMP pathway;[19,20] this is consistent with our results.

Using high K^+-contracted aorta rings, we found that the enhancing effect of EGCg in Trp-His-induced vasorelaxation is abolished under depolarizing conditions. According to Magnon et al.,[24] the vasorelaxation effect of K^+ channel openers, such as aprikalim, bimakalim, levcromakalim, and minoxidil sulfate, decreased strongly with an increase in the contracting K^+ concentration and was abolished in >50 mM KCl contracted aorta rings from SD rats, indicating that membrane depolarization significantly decreased the vasorelaxation due to K^+ channel opening. Thus, the present finding suggests that the vasorelaxation power of Trp-His might be potentiated by EGCg-induced vasorelaxant signaling related to hyperpolarization, such as K^+ channel opening. Romano et al.[21] have also demonstrated that K^+ channel and endothelial NO are important mediators for EGCg-exerting vasorelaxation in bovine ophthalmic artery, in which a selective blocker of

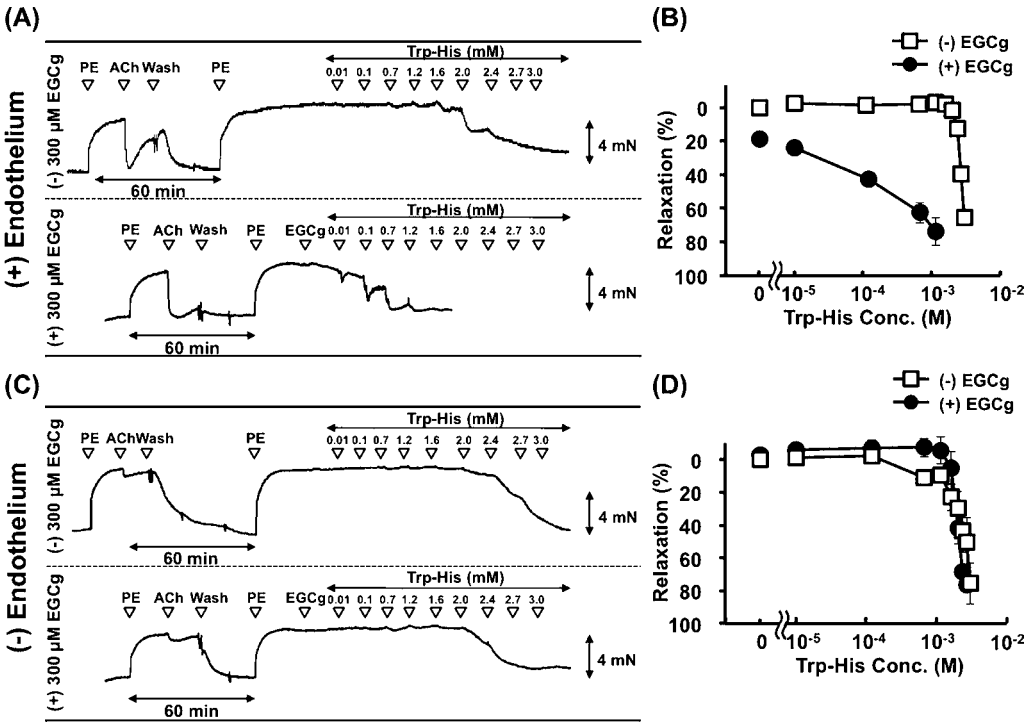

Figure 2. Traces (A, C) and dose−response curves (B, D) of Trp-His-induced vasorelaxation in the absence (−) or presence (+) of 300 μM EGCg on 1.0 μM PE-contracted aorta rings with (+; A, B) or without (−; C, D) endothelium. Traces of vasorelaxation induced by Trp-His (A) were recorded in the absence ((−) 300 μM EGCg) or presence of 300 μM EGCg ((+) 300 μM EGCg) in endothelium-intact ((+) endothelium) aorta rings contracted by 1.0 μM PE. Traces of vasorelaxation induced by Trp-His (C) were recorded in the absence ((−) 300 μM EGCg) or presence of 300 μM EGCg ((+) 300 μM EGCg) in endothelium-removed ((−) endothelium) aorta rings contracted by 1.0 μM PE. Results are expressed as the mean ± SEM values (n = 3−6).

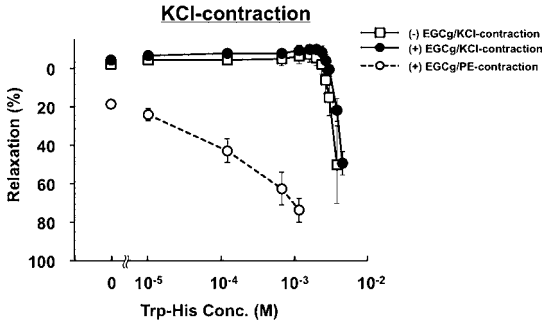

Figure 3. Dose−response curves of Trp-His-induced vasorelaxation in the absence (−) or presence (+) of 300 μM EGCg on 70 mM KCl-contracted (shown in solid line) or 1.0 μM PE-contracted (shown in dashed line) aorta rings. Results are expressed as the mean ± SEM values (n = 3−6).

large conductance Ca^{2+}-dependent K^+ (BK_{Ca}) channels significantly reduced the vasorelaxation of EGCg. This finding partly supports our result, and led us to further investigate the involvement of the NO/cGMP pathway in enhanced vasorelaxation. As shown in Figure 4, we demonstrated that the enhanced NO/cGMP pathway by EGCg via NOS activation (Figure 5) is a key factor for the enhancement of Trp-His-induced vasorelaxation power, because 300 μM EGCg did not alter the vasorelaxation of Trp-His in the presence of L-NMMA (NOS inhibitor). Moreover, the treatment with ODQ (an inhibitor of sGC, which is activated by NO) also abolished the EGCg-induced enhancement of the vasorelaxation power of Trp-His. It was reported that vasorelaxation was caused by activated cGMP-dependent protein kinase I via NO/cGMP, followed by the activation of BK_{Ca} channel in VSMC.[25,26] The finding that the synergistic vasorelaxation of Trp-His with EGCg was abolished in high K^+-contracted aorta (Figure 3) may, thus, provide useful information that activated BK_{Ca} channel via NO/cGMP pathway might be a candidate for the synergistic effect of EGCg in Trp-His-induced vasorelaxation.

Both genistein and kaempferol potentiate the vasorelaxation effect of sodium nitroprusside (SNP), an NO donor,[27,28] although their underlying mechanisms are unknown. These findings imply that the NO-related vasorelaxation signal might be responsible for the synergistic effect. Chan et al.[29] have also demonstrated that the vasorelaxant butylidenephthalide (BDPH), which is derived from *Ligusticum chuanxiong*, induced synergistic vasorelaxation with SNP. Since BDPH-induced vasorelaxation might partly comprise endothelium-independent vasorelaxation signal, such as Ca^{2+} channels regulation,[30] the synergistic vasorelaxation of BDPH with SNP suggests that the interaction between NO/cGMP pathway and Ca^{2+} channels regulation might be important in exerting an enhanced vasorelaxation effect. Goud et al.[14] have also reported that troglitazone, an insulin-sensitizing agent, synergistically enhanced insulin-induced vasorelaxation (which was involved in both activation of the NO/cGMP pathway and inhibition of VDCC) via upregulation of VDCC inhibition in the thoracic aorta from SD rats. These findings suggest that the activation of NO/cGMP pathway might enhance vasorelaxation induced by $[Ca^{2+}]_i$ regulation via Ca^{2+} channels, especially VDCC, and seem to be consistent with the present mechanism of the enhanced vasorelaxation effect of Trp-His or nifedipine by EGCg.

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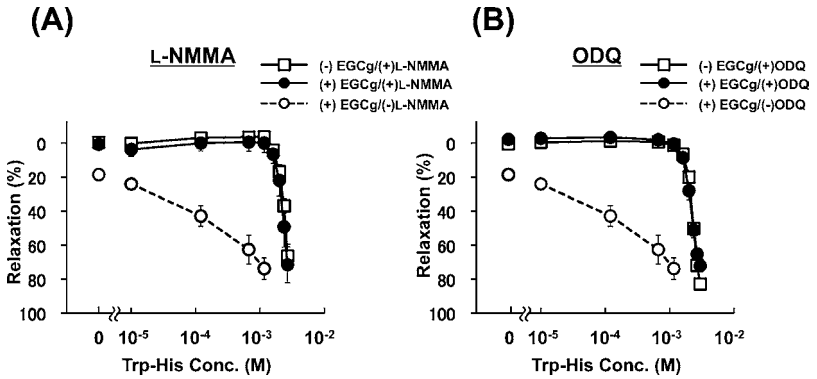

Figure 4. Dose−response curves of Trp-His-induced vasorelaxation in the absence (−; shown in dashed line) or presence (+; shown in solid line) of 100 μM L-NMMA, an NOS inhibitor (A), or 10 μM ODQ, a sGC inhibitor (B). Vasorelaxation of Trp-His was examined in the presence (+) or absence (−) of 300 μM EGCg on 1.0 μM PE-contracted aorta rings treated with 100 μM L-NMMA (A) or 10 μM ODQ (B). Results are expressed as the mean ± SEM values (n = 3−6).

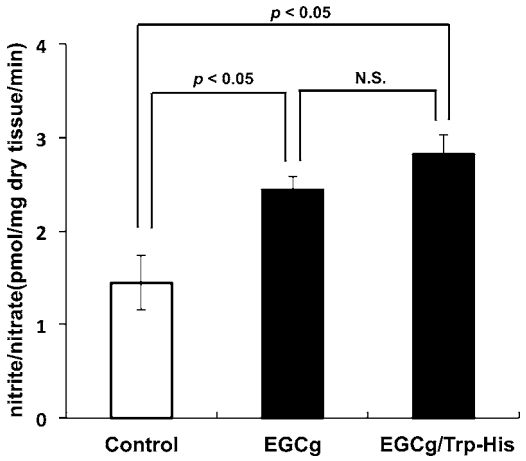

Figure 5. The activity of NOS in aorta rings incubated with or without (control) 300 μM EGCg in the presence or absence of 2.8 mM Trp-His. Results are expressed as the mean ± SEM values (n = 3). Significant differences between NO activities were analyzed by a one-way ANOVA followed by Turkey−Kramer's t-test for post hoc analysis. N.S., not significant.

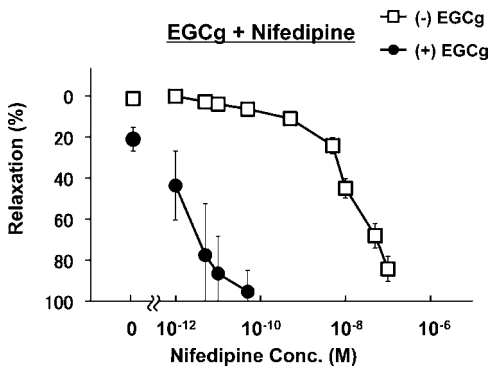

Figure 6. Dose−response curves of nifedipine (a DHP-type Ca^{2+} channel blocker)-induced vasorelaxation in the absence (−) or presence (+) of 300 μM EGCg on 1.0 μM PE-contracted aorta rings. Results are expressed as the mean ± SEM values (n = 3).

Thus, this study is the first to demonstrate that the synergistic vasorelaxation effect of the VDCC blocker Trp-His and EGCg is likely due to activation of the NO/cGMP pathway, which is induced by the reduction in $[Ca^{2+}]_i$. Moreover, as shown in Figure 6, vasorelaxation by a DHP-type VDCC blocker, nifedipine, was greatly enhanced by the addition of 300 μM EGCg, indicating that the decreased $[Ca^{2+}]_i$ by Trp-His is likely contribute to the EGCg-enhanced vasorelaxation by Trp-His through the activation of NO/cGMP pathway. The differing extent of EGCg-enhanced vasorelaxation by Trp-His and nifedipine seems to be caused by their activity on VDCC blocking, which also suggests the involvement of $[Ca^{2+}]_i$ regulation in the enhanced vasorelaxation. However, the critical regulating molecule(s) in cellular vasorelaxation signaling is still unclear in the synergistic vasorelaxation of Trp-His with EGCg. Considering our previous findings[9,31] that Trp-His regulated VDCC action by both blocking[9] and inhibiting phosphorylation of VDCC,[31] further investigations to elucidate how the enhanced NO/cGMP pathway affects various Trp-His-induced vasorelaxation signalings must be performed to clarify the underlying mechanism of synergistic effect of Trp-His with EGCg.

The present study demonstrated that the vasorelaxation power of the antiatherosclerotic VDCC blocking peptide Trp-His was synergistically enhanced by 300 μM EGCg by a factor of >6. The activation of the NO/cGMP pathway by EGCg was essential for the enhancement of Trp-His-induced vasorelaxation. Vasorelaxation of a DHP-type VDCC blocker, nifedipine, was also enhanced by the addition of 300 μM EGCg, which strongly suggests that the synergistic effect of Trp-His plus EGCg may be due to the EGCg-induced activation of the NO/cGMP pathway and Trp-His-induced reduction in $[Ca^{2+}]_i$. This study demonstrates that the combination of functional food compounds can result in an enhancement of their individual physiological activities, which might provide a novel strategy for the development or improvement of functional foods with remarkable health benefits.

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Notes

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■ ABBREVIATIONS USED

ACE, angiotensin I-converting enzyme; VDCC, voltage-dependent L-type Ca^{2+} channel; ARB, angiotensin II receptor blocker; DHP, dihydropyridine; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; NO, nitric oxide; EGCg, (−)-epigallocatechin gallate; cGMP, cyclic guanosine monophosphate; PE, phenylephrine; ACh, acetylcholine; EC, (−)-epicatechin; ECg, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; ODQ, 1-*H*-[1,2,4]oxadiazolo[4,3]quinoxalin-1-one; L-NMMA, N^G-monomethyl-L-arginine acetate; SD rat, Sprague−Dawley rat; DRI, dose-reduction index; CI, combination index; NOS, nitric oxide synthase; sGC, soluble guanylyl cyclase; BK_{Ca}, large conductance Ca^{2+}-dependent K^+ channel; SNP, sodium nitroprusside; BDPH, butylidenephthalide; PI3K, phosphatidylinositol 3-kinase.

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