Original Article

Endothelium-derived Hyperpolarizing Factor (EDHF) Mediates Endothelium-dependent Vasodilator Effects of Aqueous Extracts from *Eucommia ulmoides* Oliv. Leaves in Rat Mesenteric Resistance Arteries

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The vascular effects of an aqueous extract prepared from the leaves of *Eucommia ulmoides* Oliv. (ELE), a medicinal herb commonly used in antihypertensive herbal prescriptions in China, were investigated in rat mesenteric resistance arteries. The mesenteric vascular bed was perfused with Krebs solution and the perfusion pressure was measured with a pressure transducer. In preparations with an intact endothelium and precontracted with 7μM methoxamine, perfusion of ELE ($10^{-7}-10^{-2}$mg/ml for 15 min) caused a concentration-dependent vasodilatation, which was abolished by chemical removal of the endothelium. The ELE-induced vasodilatation was inhibited by neither indomethacin (INDO, a cyclooxygenase inhibitor) nor N^G-nitro-L-arginine-methyl ester (L-NAME, a nitric oxide inhibitor). The ELE-induced vasodilatation was significantly inhibited by tetraethylammonium (TEA, a K^+ channel blocker) and 18α-glycyrrhetinic acid (18α-GA, a gap-junction inhibitor), and abolished by high K^+-containing Krebs' solution. Atropine (a muscarinic acetylcholine receptor antagonist) significantly inhibited the vasodilatation induced by ELE at high concentrations. These results suggest that the ELE-induced vasodilatation is endothelium-dependent but nitric oxide (NO)- and prostaglandin I_2 (PGI_2)-independent, and is mainly mediated by the endothelium-derived hyperpolarizing factor (EDHF) in the mesenteric resistance arteries. Furthermore, the ELE-induced EDHF-mediated response involves the activation of K^+-channels and gap junctions.

Key words: *Eucommia ulmoides* Oliv. leaf extract, endothelium-dependent vasodilation, endothelium-derived hyperpolarizing factor, mesenteric resistance artery

*E*ucommia ulmoides Oliv. (also termed Duzhong or Tuzhong in Chinese) has been used as a traditional medicine to treat hypertension, pain, and stress. *Eucommia ulmoides* Oliv. is also used as a tonic for the liver and kidney, to improve detoxification and circulation, respectively. Pharmacological studies of *Eucommia ulmoides* Oliv. extract have revealed antihypertensive effects. Kwan *et al.* reported that the aqueous extracts isolated from both leaves and bark of *Eucommia ulmoides* Oliv. induced endothelium-depen-

dent relaxation in the rat thoracic aorta in a concentration-dependent manner [1]. Another report demonstrated that the endothelium-dependent vascular relaxation induced by the bark extract is mediated by endothelium-derived relaxing factor (EDRF) nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) in small vessels [2]. However, the mechanism underlying the antihypertensive effect of the *Eucommia ulmoides* Oliv. leaves extract (ELE) has been unclear. Also, as blood vessels become smaller, EDHF becomes functionally more active interdependent with NO in endothelium-dependent relaxant events [3, 4].

Therefore, we examined the vasodilator mechanisms of ELE in the rat mesenteric vascular bed, which includes the resistance arteries and plays an important role in the regulation of blood pressure. In this report, we provide evidence that ELE induces endothelium-dependent vasodilation of the rat mesenteric resistance arteries, which is mainly mediated by EDHF and involves the activation of K^+-channels and gap junctions.

Materials and Methods

Animals.   Male Wistar rats weighing 280–350 g were used. All animals were given food and water *ad libitum*. They were housed in the Animal Research Center of Okayama University under a controlled ambient temperature of 22 ± 2℃ with $50 \pm 10\%$ relative humidity and a 12h light/12h dark cycle (lights on 08:00 h). This study was carried out in accordance with the Guidelines for Animal Experiments at the Okayama University Advanced Science Research Center, Japanese Government Animal Protection and Management Law (No. 105), and the Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6). Every effort was made to minimize the number of animals used and their suffering.

Perfusion of the mesenteric vascular beds.
The animals were anesthetized with pentobarbital-Na (50 mg/kg, intraperitoneally) and the mesenteric vascular beds were isolated and prepared for perfusion as described previously [5, 6]. The superior mesenteric artery was cannulated and flushed gently with Krebs-Ringer bicarbonate solution (Krebs solution) to eliminate blood in the vascular bed. After removal of the

entire intestine and associated vascular bed, the mesenteric vascular bed was separated from the intestine by cutting close to the intestinal wall. Only 4 main arterial branches from the superior mesenteric trunk running to the terminal ileum were perfused. All other branches of the superior mesenteric vascular bed were tied off. The isolated mesenteric vascular bed was then placed in a water-jacketed organ bath maintained at 37℃ and perfused with a modified Krebs solution at a constant flow rate of 5 ml/min with a peristaltic pump (model AC-2120; ATTO Co., Tokyo, Japan). Preparations were also superfused with the same solution at a rate of 0.5 mL/min to prevent drying. The Krebs solution was bubbled with a mixture of 95% O_2–5% CO_2 before passage through a warming coil maintained at 37℃. The modified Krebs solution was of the following composition: 119.0 mM NaCl, 4.7 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 0.03 mM disodium EDTA, and 11.1 mM dextrose (pH 7.4). Changes in the perfusion pressure were measured with a pressure transducer (model TP-400T; Nihon Kohden, Tokyo, Japan) and recorded using a pen recorder (model U-228; Nippon Denshi Kagaku, Tokyo, Japan).

Chemical removal of the vascular endothelium.   To remove the vascular endothelium, preparations with resting tone were perfused with a 1.80 mg/ml solution of sodium deoxycholate (SD) in saline for 30 sec, as described previously [7, 8]. This caused a transient increase (20–30 mmHg) in perfusion pressure. Then, the preparations were rinsed with SD-free Krebs solution for 40 min. After the preparations were contracted by perfusion with Krebs solution containing 2 μM methoxamine, chemical removal of the endothelium was assessed by the lack of a relaxant effect after a bolus injection of 1 nmol acetylcholine (ACh), which was injected directly into the perfusate proximal to the arterial cannula with an injection pump (model 975; Harvard Apparatus, Holliston, MA, USA). The injection volumes were 100 μl for 12 sec.

Experimental protocols.   After the basal perfusion pressure had stabilized, the mesenteric vascular beds with an intact endothelium were perfused with Krebs solution containing 7 μM methoxamine (an α_1-adrenoceptor agonist). After the elevated perfusion pressure stabilized, Krebs solution contain-

ing methoxamine and ELE at a final concentration of $10^{-7} \sim 10^{-2}$ mg/ml was perfused for 15 min. After the effect reached a maximum, the next concentration of ELE was perfused. In preparations without an endothelium, the experimental protocol was the same, but the concentration of methoxamine required to raise the active tone was reduced to 2μM after denudation, since chemical denudation of the vascular endothelium increased the vasoconstriction induced by methoxamine.

To assess the underlying mechanisms of the vasodilator effect of ELE, the effects of 100μM N^{G}-L-nitro arginine methyl ether (L-NAME; an NO synthase inhibitor), 1μM indomethacin (a cyclo-oxygenase inhibitor), 60 mM KCl (a nonselective K^+ channel inhibitor), 5 mM tetraethylammonium (TEA; a nonselective Ca^{2+} activated K^+ channel inhibitor), 10μM 18α-glycyrrhetinic acid (18α-GA; a gap junction inhibitor) and 1μM atropine (a muscarinic acetylcholine receptor antagonist) on the vasodilator response to ELE were examined in preparations with an endothelium.

At the end of each experiment, 100μM papaverine was perfused to produce complete relaxation. Vasodilation was expressed as a percentage of the perfusion pressure at the maximum relaxation induced by papaverine.

Preparation of Eucommia ulmoides Oliv. leaves extract (ELE). ELE supplied by Kobayashi Pharmaceutical Co. (Osaka, Japan) was used in the present study. Leaves (2 tons) of *Eucommia ulmoides* Oliv. from the Schizuan District of China were boiled in 10 tons of water at $90\,°C$ for 1 h, and then the water was filtered off and the filtrate left standing. The filtrate was again filtered on cooling, and subjected to further concentration before drying under a vacuum to yield a powder (yield: 18%). The ELE ($10^{-7} \sim 10^{-2}$ mg/ml) was dissolved in Krebs solution containing $2-7 \mu$M methoxamine when perfused.

Statistical analysis. Data are shown as the mean $\pm$ S.E.M. from *n* number of experiments. Statistical significance was estimated with Student's *t*-test for unpaired observations between 2 groups. A *p*-value of less than 0.05 was regarded to be significant.

Drugs. The following drugs were used in this study: ACh chloride (Daiichi-Sankyo Pharmaceutical Co., Tokyo, Japan), 18α-GA (Wako Pure Chemical Ind. Ltd., Osaka, Japan), indomethacin (Sigma Aldrich Japan Co., Tokyo, Japan), methoxamine hydrochloride (Nihon Shinyaku, Kyoto, Japan), L-NAME (Sigma), papaverine hydrochloride (Dainippon-Sumitomo Pharmaceutical Co., Osaka, Japan), sodium deoxycholate (Ishizu Seiyaku Co., Tokyo, Japan) and TEA (Sigma). Sodium deoxycholate was dissolved in 0.9% saline. All other drugs except 18α-GA, which was dissolved in dimethylsulfoxide (DMSO), were dissolved in distilled water and diluted with Krebs solution containing $2-7 \mu$M methoxamine, when perfused or injected directly.

Results

Vasodilatation induced by the perfusion of ELE. In the preparation with an endothelium and with an active tone produced by methoxamine (7μM), the injection of a bolus of ACh (1 nmol) produced a rapid drop in perfusion pressure due to endothelium-dependent vasodilatation. In this preparation, perfusion of ELE decreased the vasodilatation-induced perfusion pressure in a concentration-dependent manner (Fig. 1A and 1C). In the preparation without endothelium, the vasodilatation induced by the perfusion of ELE was markedly attenuated (Fig. 1B and 1C).

Effects of L-NAME and indomethacin on the vasodilatation induced by ELE. To evaluate the involvement of endothelium-derived relaxation factors in the vasodilatation induced by ELE, the effects of L-NAME (an NO synthase inhibitor) and indomethacin (a cyclooxygenase inhibitor) were examined. As shown in Fig. 2, the vasodilatation induced by ELE was significantly augmented in the presence of 100μM L-NAME. However, in the presence of 1μM indomethacin, the vasodilatation induced by the ELE was not significantly inhibited (Fig. 2).

Effects of K^+ channel inhibitors and a gap junction inhibitor on the vasodilatation induced by ELE. To assess the possible mechanisms underlying the vasodilatation induced by the perfusion of ELE, the effects of 60 mM KCl, 5 mM TEA, and 10 μM 18α-GA were examined. As shown in Fig. 3A, the vasodilatation was significantly reduced by the TEA and almost abolished by the KCl.

As shown in Fig. 3B, 18α-GA markedly inhibited the ELE-induced vasodilatation.
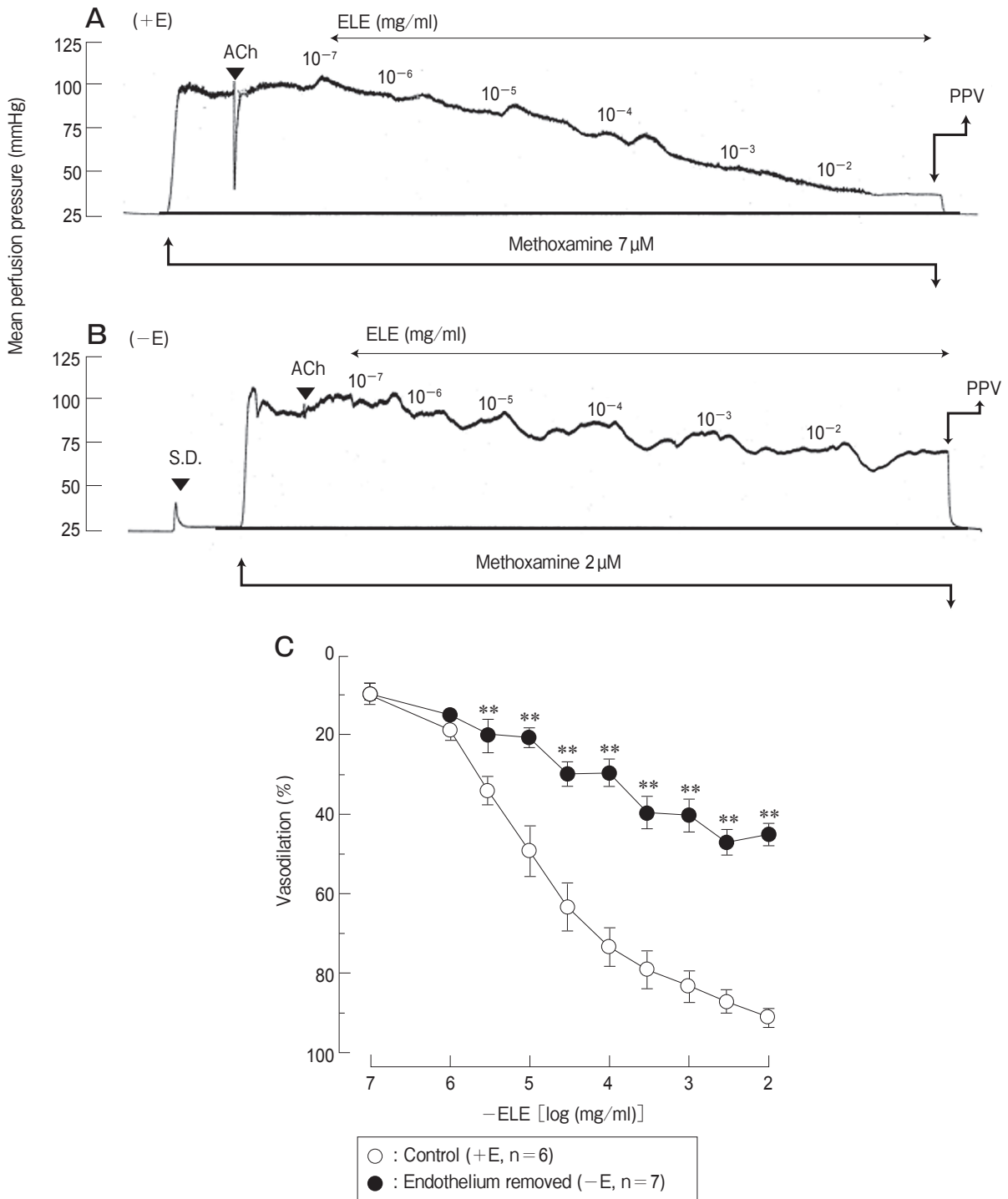
Fig. 1   Typical records (upper trace) and a line graph (lower graph) showing the vasodilator response to perfusion of ELE in rat perfused mesenteric vascular beds with an intact endothelium (**A**) and the endothelium removed (**B**). The active tone was produced by perfusion of methoxamine ($2\sim7\,\mu M$). ACh, bolus injection of acetylcholine (1 nmol). Each concentration of ELE ($10^{-7}\sim10^{-2}$mg/ml) was perfused for 15 min. SD, sodium deoxycholate perfusion for 30 s. PPV, perfusion of papaverine ($100\,\mu M$). In **C**, each point represents the mean $\pm$ S.E.M. from 5–7 experiments. $^{*}P<0.05$, $^{**}P<0.01$ compared with the responses in preparations with an intact endothelium ($+E$).

Fig. 2 Line graph showing the effects of N^G-nitro-L-arginine-methyl ester (L-NAME, 100 μM) and indomethacin (INDO, 1 μM) on the ELE-induced vasodilatation in rat perfused mesenteric vascular beds with active tone. Each point represents the mean ± S.E.M. from 5–7 experiments. *$P < 0.05$, **$P < 0.01$ compared with the responses in preparations with an intact endothelium (+E).
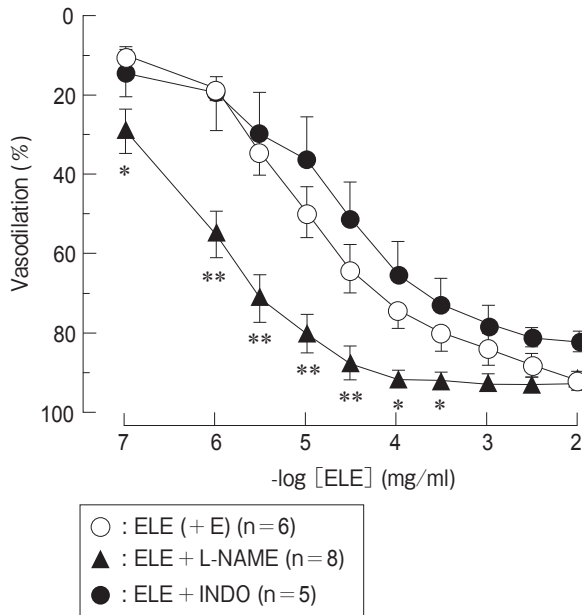
Effect of atropine on the vasodilatation induced by ELE. As shown in Fig. 4, in the presence of atropine, the vasodilatation induced by high concentrations of ELE was significantly inhibited.

Effects of various agents on the vasodilatation induced by acetylcholine. Fig. 5 shows a comparison of the effects of various inhibitors on ACh-induced vasodilation and ELE (10^{-3} mg/ml)-induced vasodilation. In preparations with an intact endothelium and active tone, a bolus of 1 nmol acetylcholine produced a sharp and transient decrease in perfusion pressure due to vasodilation, which was abolished by removal of the endothelium. As shown in Fig. 5, the acetylcholine-induced vasodilation was not affected by treatment with L-NAME, indomethacin, or 18α-GA, while it was significantly reduced by TEA (5 mM) and KCl (60 mM).

Discussion

The present study demonstrated that, in the rat mesenteric artery with an intact endothelium and active tone produced by methoxamine, perfusion of
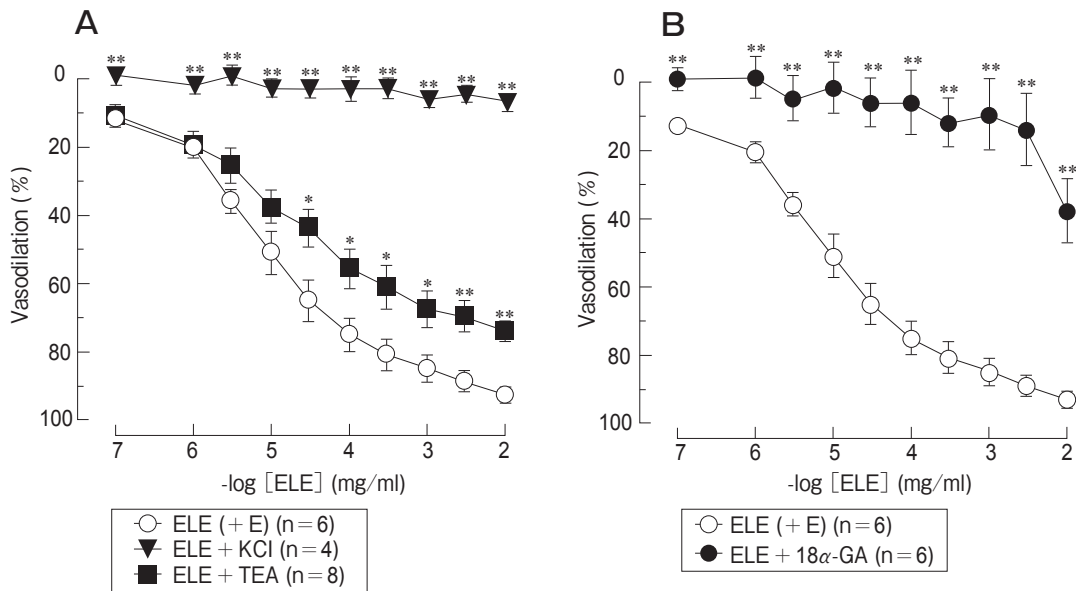

Fig. 3 Line graphs showing the effects of tetraethylanmonium (TEA; 5 mM), KCl (60 mM) (**A**), and 18α-glycyrrhetinic acid (18α-GA; 10 μM) (**B**) on the ELE-induced vasodilatation in rat perfused mesenteric vascular beds with active tone. Each point represents the mean ± S.E.M. from 5–7 experiments. *$P < 0.05$, **$P < 0.01$ compared with the responses in preparations with an intact endothelium (+E).

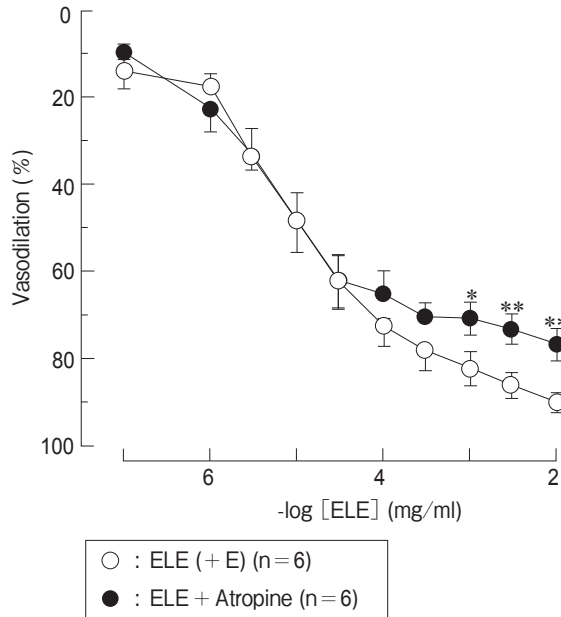ELE induced a concentration-dependent vasodilator

Fig. 4   Line graph showing the effect of atropine (1 μM) on the ELE-induced vasodilatation in rat perfused mesenteric vascular beds with active tone. Each point represents the mean ± S.E.M. from 5 -7 experiments. $*P < 0.05$, $**P < 0.01$ compared with the responses in preparations with an intact endothelium (+E).

response. The ELE-induced vasodilatation was inhibited by endothelium removal, suggesting it to be endothelium-dependent and mediated by EDRF. It is well known that EDRF is NO, or prostaglandin I_2 (prostacyclin), but EDHF has not been identified. In the present study, indomethacin, a prostanoid synthase cyclooxygenase inhibitor, did not affect the vasodilator response to ELE. Additionally, the NO synthase inhibition by L-NAME did not inhibit the ELE-induced vasodilatation but rather enhanced it. Thus, it is unlikely that NO and prostacyclin are involved in the ELE-induced endothelium-dependent vasodilation in rat mesenteric resistance arteries.

The major finding of this study is that the ELE-induced vasodilatation was markedly inhibited by the blockade of Ca^{2+}-activated K^+ channels by TEA and abolished by high KCl medium. Furthermore, the inhibition of gap junctions by 18α-GA resulted in a marked decrease in the ELE-induced vasodilatation. Taken together, these findings strongly suggest that ELE-induced vasodilatation is involved in the activation of K^+ channels and mediated by gap junctions, which are associated with the release of EDHF in the rat mesenteric arteries. Since atropine did not inhibit the vasodilation induced by ELE, the active components of ELE may cause EDHF release from the
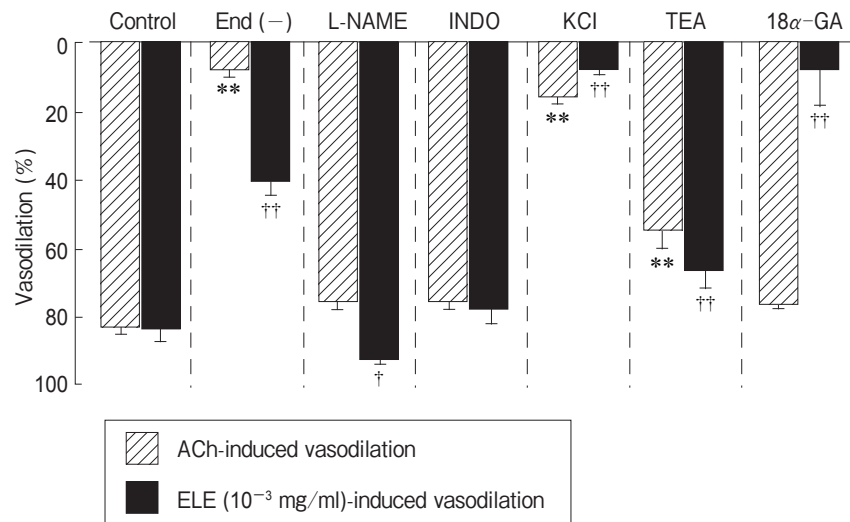

Fig. 5   Bar graph showing the effects of the endothelium removal (End (−)), L-NAME (100 μM), indomethacin (INDO, 1 μM), KCl (60 mM), tetraethylammonium (TEA, 5 mM), and 18α-glycyrrhetinic acid (18α-GA, 10 μM) on vasodilation induced by the infusion of ACh (1 nM) as a bolus and perfusion of ELE (10^{-3} mg/ml) in rat perfused mesenteric vascular beds with active tone. Each point represents the mean ± S.E.M. from 5-6 experiments. $*P < 0.05$, $**P < 0.01$ vs the ACh-infused control.
$^{†}P < 0.05$, $^{††}P < 0.01$ vs the ELE-infused control.

endothelial cells via a mechanism independent of the muscarinic ACh receptors, unlike ACh, which activates endothelial muscarinic ACh receptors and causes endothelial cells to release EDRF. However, atropine inhibited the vasodilation induced by the highest concentration of ELE (10^{-3} to 10^{-2} mg/ml). Therefore, it seems likely that ELE has agonistic components that stimulate muscarinic ACh receptors. Therefore, the muscarinic agonistic components contained in ELE may stimulate endothelial muscarinic ACh receptors to release EDHF from endothelial cells.

Kwan *et al.* [1] have reported that the aqueous extract of *Eucommia ulmoides* Oliv. leaves and bark induced endothelium-dependent NO-mediated relaxation in large arteries of rats and dogs, whereas in rat mesenteric arteries, the vasodilatation induced by the bark extract was mediated by both NO and EDHF. Furthermore, Kwan *et al.* [1, 2] provided pharmacological evidence that K$^+$-channels are also involved in *Eucommia*-induced relaxation, demonstrating that TEA as well as 4-aminopyridine (K$^+$-channel inhibitor) was able to inhibit the relaxation. By contrast, in the present study, we found that L-NAME markedly augmented the ELE-induced vasodilatation, the ELE-induced EDHF activity being enhanced in the absence of NO activity. This notion is likely supported by the finding that EDHF-mediated responses compensate for the absence of endothelial NO [9].

Little is known about the nature of EDHF, which has been described as an endothelium-derived non-NO and non-PGI$_2$ factor that induces hyperpolarization of vascular smooth muscle by opening K$^+$ channels [10, 11]. Furthermore, some studies suggest that gap junctions between endothelium and smooth muscle cells also play an important role in EDHF-mediated relaxation [12]. In the present study, we compared ELE-induced vasodilatation with ACh-induced vasodilatation, finding the former to be inhibited by either K$^+$ channel blockers or a gap junction inhibitor, and the latter to be inhibited by only the blockade of K$^+$ channels. Therefore, it is possible that a different EDHF is responsible for ELE and acetylcholine in mesenteric resistance arteries.

In conclusion, the present study demonstrates that ELE-induced vasodilatation is mainly mediated by EDHF, and that this mediation involves the activation of K$^+$ channels via gap junctions. In addition, ELE may have muscarinic agonistic components that stimulate muscarinic ACh receptors on the endothelium and release EDHF.

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