J Pharmacol Sci **118**, 373 – 381 (2012)

Journal of Pharmacological Sciences

Full Paper

Endothelium-Derived Relaxing Factor–Mediated Vasodilation in Mouse Mesenteric Vascular Beds

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Received October 20, 2011; Accepted January 17, 2012

Abstract. The endothelium in rat mesenteric vascular beds has been demonstrated to regulate vascular tone by releasing mainly endothelium-derived hyperpolarizing factor (EDHF), which is involved in the activation of K^+ channels and gap-junctions. However, it is unclear whether the endothelial system in mouse resistance arteries contributes to regulation of the vascular tone. The present study was designed to investigate the role of the endothelium using acetylcholine and A23187 (Ca^{2+} ionophore) in mesenteric vascular beds isolated from male C57BL/6 mice and perfused with Krebs solution to measure perfusion pressure. In preparations with active tone produced by methoxamine in the presence of guanethidine, injections of acetylcholine, A23187, and sodium nitroprusside (SNP) caused a concentration-dependent decrease in perfusion pressure due to vasodilation. The vasodilator responses to acetylcholine and A23187, but not SNP, were abolished by endothelium dysfunction and significantly inhibited by N^{ω}-nitro-L-arginine methyl ester (nitric oxide synthase inhibitor) and tetraethylammonium (K^+-channel inhibitor) but not glibenclamide (ATP-sensitive K^+-channel inhibitor). Indomethacin (cyclooxygenase inhibitor) significantly blunted only A23187-induced vasodilation, while 18α-glycyrrhetinic acid (gap-junction inhibitor) attenuated only acetylcholine-induced vasodilation. These results suggest that the endothelium in mouse mesenteric arteries regulates vascular tone by prostanoids, EDHF, and partially by nitric oxide, different from the endothelium of rat mesenteric arteries.

Keywords: endothelium-dependent vasodilation, acetylcholine, Ca^{2+} ionophore, mesenteric vascular bed

Introduction

It is widely accepted that the vascular endothelium generates and releases a vasodilator, endothelium-derived relaxing factor (EDRF), and a vasoconstrictor, endothelium-derived constricting factor, including endothelin, prostaglandin $F_{2\alpha}$, and thromboxane A_2. These factors have been shown to regulate vascular tone as hormonal factors along with neuronal factors in perivascular nerves. It is well established that endothelium-dependent vasodilator responses are mediated by EDRF, which is produced and released in response to physical and chemical stimuli such as bloodstream shear stress and various endogenous and exogenous agonists. EDRF is now indentified as three major vasodilator signals, nitric oxide (NO) (1, 2), prostaglandin I_2 (PGI_2) (3), and endothelium-derived hyperpolarizing factor (EDHF) (4, 5). However, it is generally accepted that the contribution of EDRF to endothelium-mediated vasodilation depends on animal species and vessel size. NO is the main EDRF in aorta and large conduit arteries, while EDHF plays a critical role in the endothelium-dependent relaxation of small and resistance arteries, especially mesenteric arteries. PGI_2 has been shown to mediate the endothelium-dependent relaxation in both aorta and resistance arteries and contribute to the regulation of vascular tone (6). EDHF

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Published online in J-STAGE
doi: 10.1254/jphs.11197FP

is released from the endothelium upon physical and chemical stimulation, resulting in membrane hyperpolarization and relaxation of the artery, which were observed even in the presence of a NO synthase (NOS) inhibitor and cyclooxygenase inhibitor (7). To date, several candidates for EDHF have been reported, including epoxyeicosatrienoic acids (8), potassium ions (9), hydrogen peroxide (H_2O_2) (10), and gap-junctions (11, 12). However, the nature of EDHF remains unknown.

In the rat mesenteric vascular bed, we have proposed that different EDRFs, especially EDHF, are involved in endothelium-dependent vasodilation in response to histamine, bradykinin, acetylcholine (muscarinic acetylcholine receptor agonist), and A23187 (Ca^{2+} ionophore) (13 – 16). Previous reports showed vascular responses in mouse aorta and large arteries including thoracic and abdominal arterial ring preparations (17, 18). However, the role of EDRF in vascular responses of mouse mesenteric resistance arteries is still unclear. Since it is easier to produce models through genetic engineering in mice than rats, the functions of endothelium in pathological states are often analyzed using gene-knockout and gene-over-expressing mice. However, there is little reported on the functions of endothelium in the mouse mesenteric vascular bed.

Therefore, the present study was designed to investigate the role of EDRF, especially EDHF, in vasodilator responses to acetylcholine and a Ca^{2+} ionophore, A23187, in mouse mesenteric vascular beds.

Materials and Methods

Experimental animals

8- and 9-week-old male C57/BL mice (purchased from Shimizu Experimental Animals, Shizuoka) were used. The animals were given food and water ad libitum. They were housed in the Animal Research Center of Okayama University of Science at a controlled ambient temperature of 22°C with 50% ± 10% relative humidity and with a 12-h light / 12-h dark cycle (lights on at 8:00 AM). All experiments were approved by the Animal Care and Use Committee of the Okayama University of Science, 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 bed

The animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), and the mesenteric vascular beds were isolated and prepared for perfusion. A cannula (polyethylene tubing) was inserted into the superior mesenteric artery via the aorta and gently flushed with Krebs-Ringer bicarbonate solution (Krebs solution) to eliminate blood from 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 four main arterial branches from the superior mesenteric trunk running to the terminal ileum were perfused. All other branches of the superior mesenteric artery were tied off. The isolated mesenteric vascular bed was then placed in a water-jacketed organ bath, maintained at 37°C, and perfused with a modified (see below) Krebs solution, at a constant flow rate of 3 mL/min with a peristaltic pump (model AC-2120; Atto, Tokyo) and 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 plus 5% CO_2 before passage through a warming coil maintained at 37°C. The Krebs solution had 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 glucose (pH 7.4). Changes in the perfusion pressure were measured with a pressure transducer (model TP-400T; Nihon Kohden, Tokyo) and recorded using a pen recorder (model U-228; Nippon Denshi Kagaku, Tokyo).

Injection of acetylcholine, sodium nitroprusside (SNP), or A23187

Acetylcholine, SNP (a NO donor), or A23187 was directly injected into the perfusate proximal to the arterial cannula with an infusion pump (model 975; Harvard Apparatus Inc., Hollison, MA, USA). A volume of 60 μL was injected during a period of 12 s.

Chemical dysfunction of the vascular endothelium

The preparation with resting tone was perfused with a 1.80 mg/mL solution of sodium deoxycholate in saline for 30 s to eliminate the function of vascular endothelium, as described previously (19). Then, the preparation was rinsed with sodium deoxycholate-free Krebs solution for 30 min and examined for vascular responses.

Experimental protocol for preparations with active tone

To increase the perfusion pressure to approximately 100 mmHg, active tone was induced by perfusion with Krebs solution containing methoxamine (3 – 20 μM) (α_1-adrenoceptor agonist) and guanethidine (adrenergic neuron blocker) (5 μM), which was added to block adrenergic neurotransmission. After the elevated perfusion pressure had stabilized, acetylcholine (0.01, 0.1, and 1 nmol), SNP (1 and 10 nmol), and A23187 (0.1 and 1 nmol) were directly injected into the perfusate with the infusion pump.

To assess the mechanisms underlying the vascular effect of acetylcholine, SNP, or A23187, injections of these agents were performed in preparations with an intact endothelium during perfusion of N^{ω}-nitro-L-arginine methyl ester (NOS inhibitor; L-NAME, 100 or 500 μM), indomethacin (cyclooxygenase inhibitor, 1 μM), tetraethylammonium (K^+-channel inhibitor, 5 mM), glibenclamide (ATP-sensitive K^+-channel inhibitor, 10 μM), and 18α-glycyrrhetinic acid (gap-junction inhibitor, 100 nM).

To examine the involvement of the endothelium, injections of acetylcholine, SNP, and A23187 were made in preparations in which the endothelium function was chemically eliminated with sodium deoxycholate. After the perfusion of sodium deoxycholate, active tone was produced by perfusion with Krebs solution containing methoxamine (10 μM) and guanethidine (5 μM), and injections of the three agents were given.

At the end of each experiment, the preparations were perfused with 100 μM papaverine to cause complete relaxation. Vasodilator responses were expressed as a percentage of the perfusion pressure at maximum relaxation induced by papaverine.

Statistical analyses

Experimental results were presented as the mean ± S.E.M. The statistical analysis was done with Student's unpaired *t*-test or one-way analysis of variance followed by Tukey's test. A value of $P < 0.05$ was considered statistically significant.

Drugs

The following drugs were used: acetylcholine chloride (Daiichi-Sankyo Pharmaceutical, Tokyo); A23187, 18α-glycyrrhetinic acid, glibenclamide, indomethacin, L-NAME, methoxamine hydrochloride, sodium deoxycholate, sodium nitroprusside, tetraethylammonium (Sigma-Aldrich Japan, Tokyo); guanethidine sulfate (Tokyo Kasei, Tokyo); and papaverine hydrochloride (Dainippon-Sumitomo Pharmaceutical, Osaka). A23187, 18α-glycyrrhetinic acid, and glibenclamide were dissolved in 100% dimethyl sulfoxide. Indomethacin was dissolved in 100% ethanol. These agents were diluted with Krebs solution containing methoxamine (3 – 20 μM) and guanethidine (5 μM) when perfused. Sodium deoxycholate was dissolved in 0.9% saline. Acetylcholine, SNP, and A23187 were diluted with Krebs solution containing methoxamine (3 – 20 μM) and guanethidine (5 μM) when injected directly.

Results

Vasodilator responses to acetylcholine, SNP, and A23187 in mouse mesenteric vascular beds with active tone

To maintain active tone in the mesenteric artery, each preparation was contracted by continuous perfusion of 3 – 20 μM methoxamine in the presence of 5 μM guanethidine. As shown in Fig. 1A, in preparations with an intact endothelium and active tone, injections of acetylcholine, SNP, and A23187 caused a concentration-dependent decrease in perfusion pressure due to vasodilation. The injection of acetylcholine induced a sharp and transient vasodilation, while SNP and A23187 induced an initial fast vasodilation followed by a second long-lasting vasodilation (Fig. 1A).

As shown in Fig. 1, B and C, in the preparations inactivated endothelium, injections of acetylcholine did not cause vasodilator responses. Additionally, A23187 injections at low concentrations did not induce vasodilation, while high concentrations caused minor vasodilation (Fig. 1: B and C). However, SNP caused a concentration-dependent vasodilation similar to those in preparations with an intact endothelium, indicating that the vasodilator function of vascular smooth muscle cells was normally maintained after sodium deoxycholate treatment (Fig. 1: B and C).

Effect of L-NAME on vasodilator responses to acetylcholine, A23187, and SNP

As shown in Fig. 2, in preparations with an intact endothelium and active tone, L-NAME at 100 μM augmented SNP-induced vasodilation (Fig. 2B), but did not significantly affect vasodilation in response to acetylcholine and A23187 (Fig. 2: A and C). However, 500 μM of L-NAME significantly inhibited vasodilator responses to acetylcholine and A23187, but not SNP (Fig. 2: A and C).

Effect of indomethacin on vasodilator responses to acetylcholine, A2318, and SNP

As shown in Fig. 3, A and B, treatment with indomethacin (1 μM) did not affect vasodilator responses to acetylcholine and SNP. However, indomethacin significantly inhibited the A23187-induced vasodilation (Fig. 3C).

Effects of tetraethylammonium and glibenclamide on vasodilator responses to acetylcholine, A23187, and SNP

In preparations with an intact endothelium and active tone, the non-selective K^+-channel inhibitor tetraethylammonium significantly inhibited vasodilator responses to acetylcholine and A23187, as shown in Fig. 4, A and C.

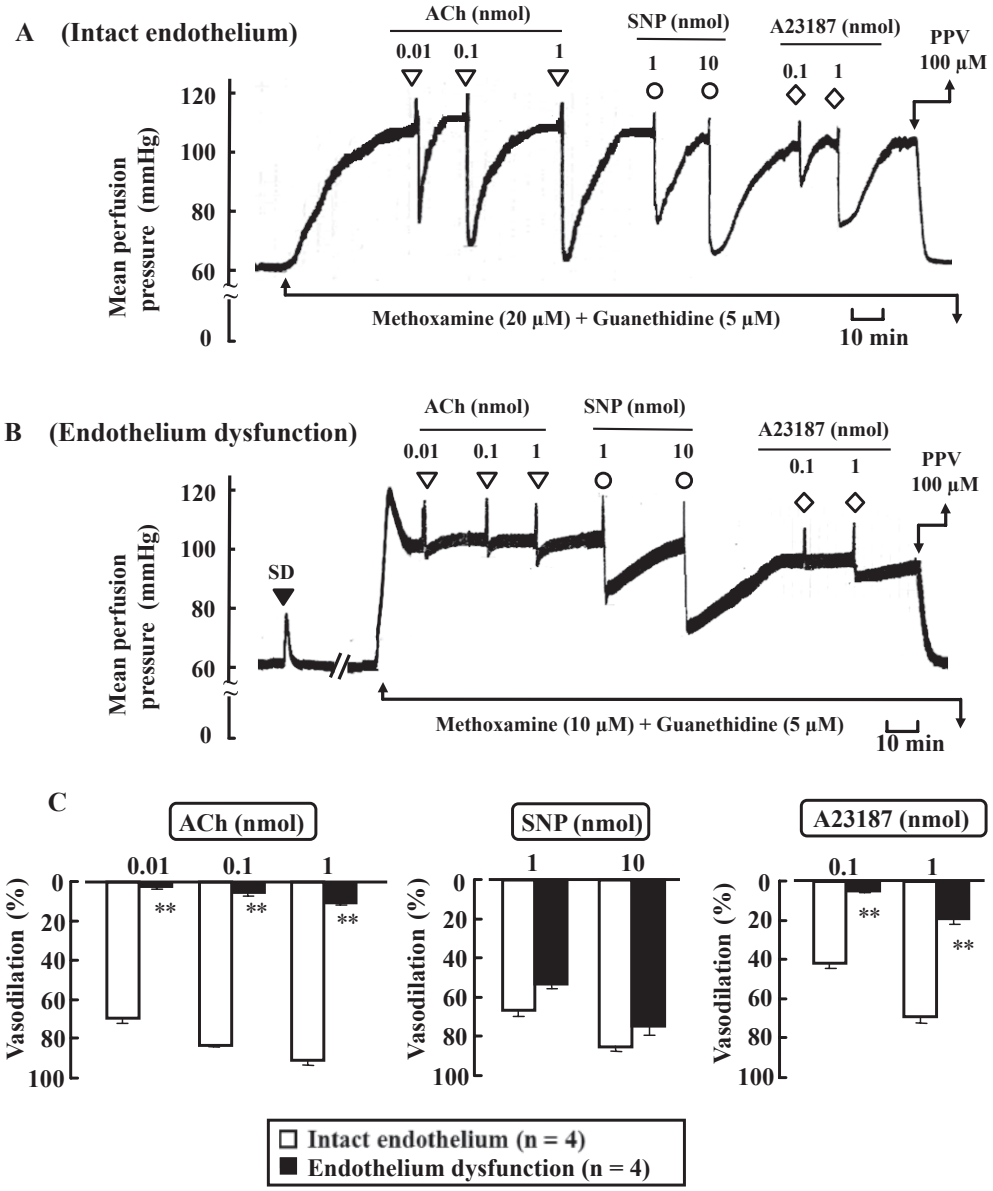

Fig. 1. Typical recordings showing vascular responses to injections of acetylcholine (ACh: 0.01, 0.1, and 1 nmol), sodium nitroprusside (SNP: 1 and 10 nmol), and Ca^{2+} ionophore (A23187: 0.1 and 1 nmol) in mouse mesenteric vascular beds with an intact endothelium (A) and inactivated endothelium (B). Active tone of the preparation was produced by perfusion of methoxamine. Papaverine (PPV: 100 μM) was perfused at the end of the experiment to induce complete relaxation. In panel B, sodium deoxycholate was perfused for 30 s to inactivate the vascular endothelium. C) Bar graphs showing the effect of endothelium dysfunction on vascular responses to injections of acetylcholine, sodium nitroprusside, and calcium ionophore in mouse perfused mesenteric vascular beds. Each bar indicates the mean ± S.E.M. $^{**}P < 0.01$, compared with an inactivated endothelium.

However, tetraethylammonium did not affect vasodilation in response to SNP (Fig. 4B).

As shown in Fig. 5, glibenclamide (10 μM) did not affect vasodilation in response to acetylcholine, SNP, and A23187.

Effect of 18α-glycyrrhetinic acid on vasodilator responses to acetylcholine, A23187, and SNP

In preparations with an intact endothelium and active tone, 18α-glycyrrhetinic acid (100 nM) significantly inhibited acetylcholine-induced vasodilation, as shown in Fig. 6A. However, neither acetylcholine- nor SNP-induced vasodilations were significantly affected by 18α-glycyrrhetinic acid (Fig. 6: B and C).

Discussion

In the present study, the major findings are that exogenously applied acetylcholine and A23187 in the mouse perfused mesenteric vascular bed caused a concentration-dependent vasodilation, which is well consistent with the response in the rat perfused mesenteric vascular bed (13, 14). Since responses to both acetylcholine and A23187 were abolished by endothelium dysfunction, they appear to be dependent on an intact endothelium, although the underlying signaling pathways differ between acetylcholine and A23187. Acetylcholine (1 nmol)- and A23187 (1 nmol)-induced vasodilation partially remained in preparations with endothelium dysfunction. In rat mes-

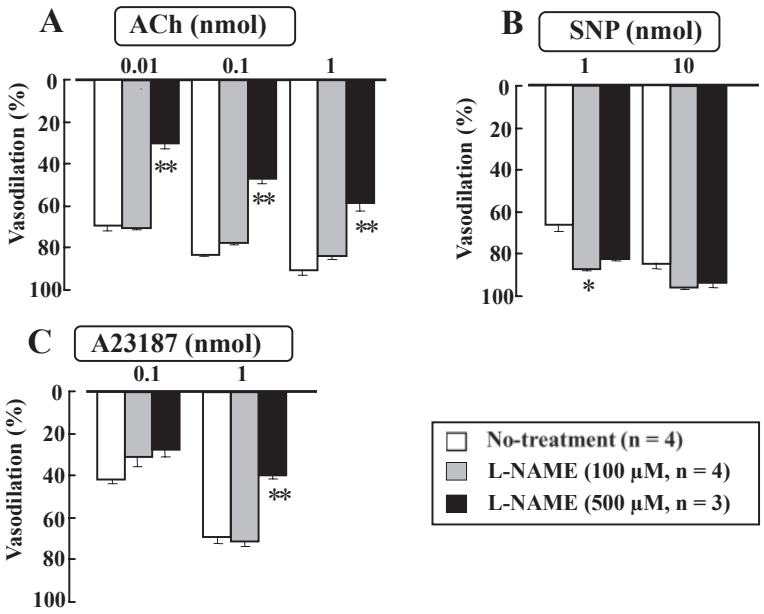

Fig. 2. Effect of the nitric oxide synthase inhibitor L-NAME (100 or 500 μM) on vasodilator responses to injections of acetylcholine (ACh: A), sodium nitroprusside (SNP: B), and Ca^{2+} ionophore (A23187: C) in mouse perfused mesenteric vascular beds with an intact endothelium and active tone. Each bar indicates the mean ± S.E.M. $*P < 0.05$, $**P < 0.01$, compared with the no-treatment group.

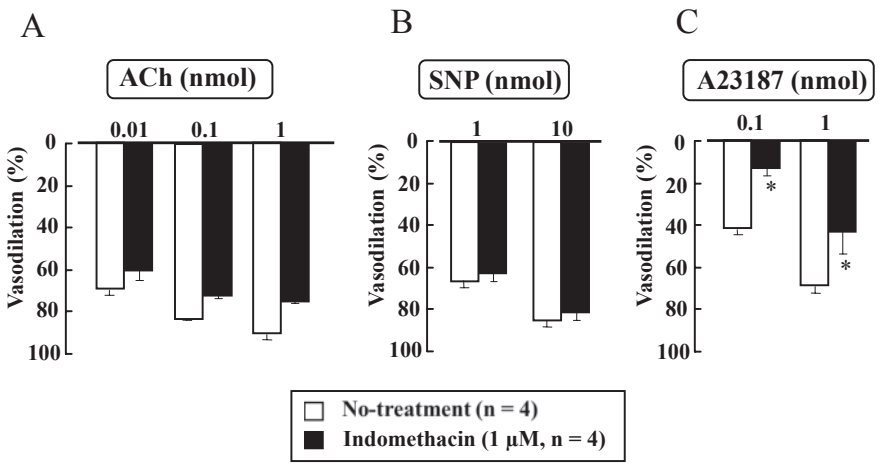

Fig. 3. Effect of the cyclooxygenase inhibitor indomethacin (1 μM) on vasodilator responses to injections of acetylcholine (ACh: A), sodium nitroprusside (SNP: B), and Ca^{2+} ionophore (A23187: C) in mouse perfused mesenteric vascular beds with intact endothelium and active tone. Each bar indicates the mean ± S.E.M. $*P < 0.05$, compared with the no-treatment group.

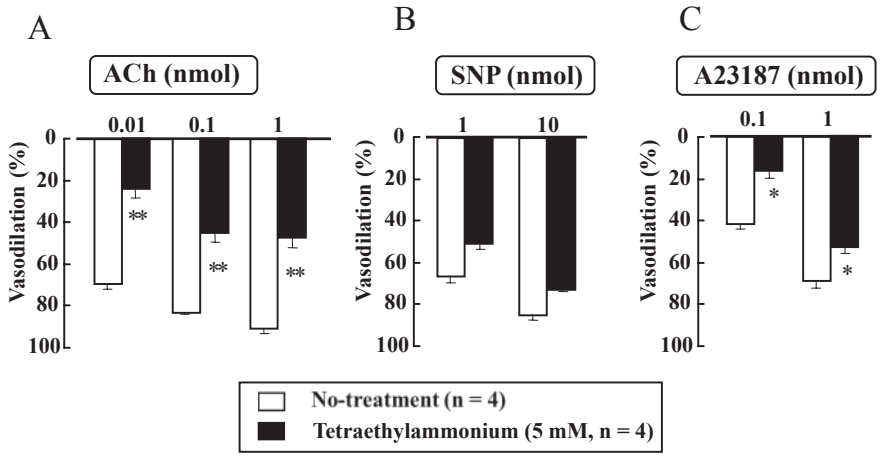

Fig. 4. Effect of the K^+-channel inhibitor tetraethylammonium (5 mM) on vasodilator responses to injections of acetylcholine (ACh: A), sodium nitroprusside (SNP: B), and Ca^{2+} ionophore (A23187: C) in mouse perfused mesenteric vascular beds with an intact endothelium and active tone. Each bar indicates the mean ± S.E.M. $*P < 0.05$, $**P < 0.01$, compared with the no-treatment group.

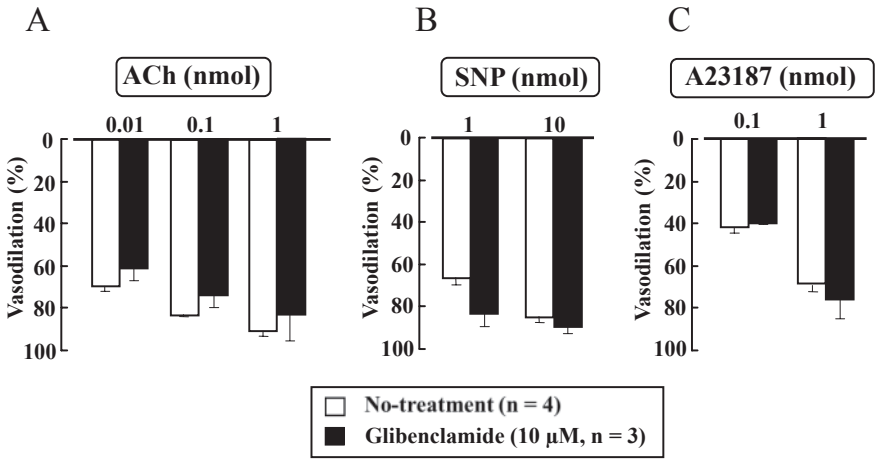

Fig. 5. Effect of the ATP-sensitive K^+-channel inhibitor glibenclamide (10 μM) on vasodilator responses to injections of acetylcholine (ACh: A), sodium nitroprusside (SNP: B), and Ca^{2+} ionophore (A23187: C) in mouse perfused mesenteric vascular beds with an intact endothelium and active tone. Each bar indicates the mean ± S.E.M.

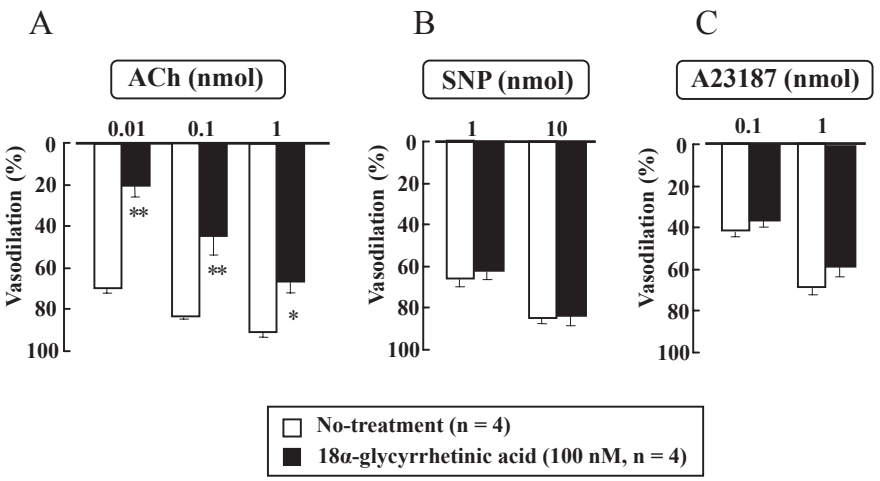

Fig. 6. Effect of the gap-junction inhibitor 18α-glycyrrhetinic acid (100 nM) on vasodilator responses to injections of acetylcholine (ACh: A), sodium nitroprusside (SNP: B), and Ca^{2+} ionophore (A23187: C) in mouse perfused mesenteric vascular beds with an intact endothelium and active tone. Each bar indicates the mean ± S.E.M. $*P < 0.05$, $**P < 0.01$, compared with the no-treatment group.

enteric vascular beds without endothelium, Takenaga et al. (19) reported that acetylcholine induces endothelium-independent vasodilation, which is mediated by perivascular CGRPergic nerves. Therefore, it seems likely that perivascular CGRPergic nerves in the mouse mesenteric artery are involved in acetylcholine- and A23187-induced endothelium-independent vasodilation. However, further study needs to clarify the actual mechanism. In the rat perfused mesenteric vascular bed, acetylcholine- and A23187-, but not SNP-, mediated vasodilator responses were abolished by endothelium dysfunction with sodium deoxycholate (13, 20). Methods for denudation of the endothelium in arteries and vascular beds include mechanical rubbing (21), air bubbling treatment (22), and sodium deoxycholate perfusion (19). In this study, we used sodium deoxycholate because Cusma-Pelógia et al. (23) and Takenaga et al. (19) reported that it has no effect on the responsiveness of vascular smooth muscle, abolishes endothelium-dependent responses, and induces inactivation of endothelium without morphological changes. Furthermore, in this study, the vasodilator response to SNP was not affected by endothelium dysfunction, indicating that treatment with sodium deoxycholate effectively caused dysfunction of the endothelium of mouse mesenteric vascular beds without affecting the function of smooth muscle cells. As shown in Fig. 1, A and B, an initial phase of methoxamine-induced vasoconstriction was augmented by endothelium dysfunction. A previous report has demonstrated that in rat mesenteric arteries, endothelium acts to depress methoxamine-induced vasoconstriction by releasing EDHF (14, 24). Therefore, EDRF and/or EDHF in the endothelium of mouse mesenteric arteries is likely to suppress methoxamine-induced vasoconstrictions.

In the mouse mesenteric vascular bed, L-NAME, an arginine analog commonly used as a reversible inhibitor of NOS, at high concentration (500 μM), but not low concentration (100 μM), significantly inhibited acetyl-

choline- and A23187-induced vasodilation. In the rat mesenteric vascular bed, L-NAME at 100 μM has been shown to have weak inhibitory effects on acetylcholine- and A23187-induced vasodilation (13, 14). Furthermore, we have reported that L-NAME could inhibit NOS at concentrations lower than 100 μM in rat mesenteric arteries (25). Therefore, in mouse mesenteric arteries, it might be enough to inhibit NOS activity by L-NAME at 100 μM and suggest that NO is not the main EDRF. It is assumed that high concentration of L-NAME had a non-specific inhibitory effect on acetylcholine- and A23187-induced vasodilation. To clarify the contribution of NO in regulation of vascular tone in mouse mesenteric arteries, further studies are needed. Our results demonstrated that SNP-induced vasodilation was augmented by L-NAME treatment. We have reported that SNP-induced vasodilation was markedly augmented by L-NAME in the rat mesenteric vascular beds (26). This is due to elimination of NO by L-NAME, leading to increased sensitivity to soluble guanylate cyclase. It is assumed that this action occurs in the mouse mesenteric artery. Additionally, high concentration of L-NAME caused no further augmentation of SNP-induced vasodilation, supporting that 500 μM L-NAME have non-specific action.

It should be noted that indomethacin, a nonselective cyclooxygenase inhibitor, inhibited the vasodilator response to A23187, but not acetylcholine, in mouse mesenteric vascular beds, suggesting that prostanoids were involved in Ca^{2+} ionophore–mediated vasodilation. Indomethacin, which inhibits the production of prostaglandin from arachidonic acid, has been reported to attenuate endothelium-dependent vasodilation by inhibiting production of EDRF prostanoids (27). In the present study, treatment with indomethacin inhibited significantly the vasodilator response to A23187-, but not acetylcholine. This finding is consistent with a previous report that vasodilation in response to Ca^{2+} ionophore, but not acetylcholine, is cyclooxygenase-dependent in mouse brain arterioles (28). Therefore, the present result strongly suggests that Ca^{2+} ionophore–released EDRF in the mouse mesenteric artery is not only NO but also prostanoids to produce vasodilation.

In rat mesenteric arteries, EDHF is responsible for acetylcholine- and A23187-induced vasodilation, since both responses were inhibited by K^+ channel inhibitors including tetraethylammonium, high K^+ medium, and apamin plus charybdotoxin, but not an ATP-sensitive K^+ channel inhibitor (13, 14). Additionally, the involvement of gap-junctions in acetylcholine-mediated vasodilation in the rat mesenteric vascular bed has been demonstrated by inhibition of the response with 18α-glycyrrhetinic acid (gap-junction inhibitor) (13). Potassium channels, which are widely distributed in vascular smooth muscle cells, are classified into four types; Ca^{2+}-activated K^+ channels, inwardly rectifying K^+ channels, tandem pore domain K^+ channels, and voltage-gated K^+ channels. The Ca^{2+}-activated K^+ channel or ATP-sensitive inwardly rectifying K^+ channel contributes to EDHF-induced hyperpolarization (7, 29). In the present study, the K^+-channel blocker tetraethylammonium caused inhibition of acetylcholine- and A23187-mediated vasodilation, whereas glibenclamide, a selective ATP-sensitive K^+-channel blocker, had no effect on vasodilator responses to acetylcholine, A23187, and SNP. These results suggest that EDHF is responsible for acetylcholine-and A23187-mediated vasodilation in mouse mesenteric arteries, with the probable involvement of Ca^{2+}-activated K^+ channels.

EDHF-mediated relaxation evoked by acetylcholine and A23187 has been proposed to involve signaling via endothelial gap-junctions and the extracellular space (11). EDHF induces the electronic spread of endothelial hyperpolarization to smooth muscle through myoendothelial gap-junctions (12). Hutcheson et al. (30) reported that acetylcholine-induced relaxation requires endothelium-derived factors provided to smooth muscle via gap-junctions, whereas A23187 releases EDHF directly into the extracellular space, suggesting that Ca^{2+} ionophore–induced endothelium-dependent hyperpolarization is not the same as the acetylcholine-induced response. According to a previous report (31), 18α-glycyrrhetinic acid induced dephosphorylation of connexin protein and selectively inhibited gap-junctions, which are composed of two connexins, connecting across the intracellular space (12). To investigate whether gap junction is involved in acetylcholine or A23187-induced vasodilation in mice mesenteric artery, we used 18α-glycyrrhetinic acid. The present study demonstrated that 18α-glycyrrhetinic acid significantly reduced the vasodilator response to acetylcholine, but not A23187, suggesting that EDHF released by acetylcholine is involved in myoendothelium gap-junctions and different from EDHF released by A23187 in mouse mesenteric vascular beds.

In experiments with basal tone, L-NAME, indomethacin, TEA, glibenclamide, and 18α-glycyrrhetinic acid without methoxamine and guanethidine did not affect the basal tone (data not shown). These observations suggest that the effect of EDRF or EDHF might be masked by adrenergic nerve vascular tone regulation in the basal condition.

In the present study, we used a non-selective NOS inhibitor, L-NAME, to clarify the NO contribution to endothelium-related vasodilation. Therefore, what type of NOS isoform participates in EDHF responses remains unknown. Our previous study suggested that in rat mes-

enteric arteries, nNOS plays an important role to regulate vascular tone (25). Recent evidence in NOSs-knockout mice demonstrated that the endothelial NOSs system mainly contributes to the EDHF responses (32). Thus, the contributions of the NOSs isoform to the endothelium-dependent responses need to be further studied.

In conclusion, the present study suggests that the endothelium in the mouse mesenteric artery releases different EDRFs responsive to various stimuli. It is also suggested that the endothelium regulates vascular tone by releasing NO, prostanoids, and EDHF. EDHF-mediated vasodilations are associated with not only K^+ channels but also the gap-junction, whereas in rat mesenteric arteries, EDHF-mediated vasodilations involve K^+ channels (33). Thus, there seems to be a difference in EDHF between mouse and rat mesenteric arteries.

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