*Full Paper***Characterization of the Inhibitory Effect of Vascular Endothelium on Agonist-Induced Vasoconstriction in Rat Mesenteric Resistance Arteries**

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Abstract. Vascular endothelium regulates vascular tone by releasing endothelium-derived vasoactive substances. We performed this study to characterize the inhibitory effect of the endothelium on vasoconstrictor stimuli in rat mesenteric vascular beds. Changes in perfusion pressure induced by continuous perfusion of Krebs solution containing methoxamine (α_1 -adrenoceptor agonist) or high KCl were measured over 180 min. In preparations with intact endothelium, methoxamine-induced vasoconstriction was time-dependently decreased to cause 60% – 80% reduction of the initial vasoconstriction level, while no reduction was observed in high-KCl-induced vasoconstriction. Endothelium removal significantly blunted the time-dependent reduction of methoxamine-induced vasoconstriction without affecting high-KCl-induced vasoconstriction. Neither a nitric oxide synthase inhibitor (L-NAME) nor indomethacin (cyclooxygenase inhibitor) altered the time-dependent reduction of vasoconstriction. High KCl, K^+ -channel inhibitors tetraethylammonium and apamin plus charybdotoxin, and 18α -glycyrrhetic acid (18α -GA, a gap-junction inhibitor) significantly inhibited the time-dependent reduction of methoxamine-induced vasoconstriction. In precontracted preparations, bolus injection of acetylcholine and Ca^{2+} -ionophore A23187 (A23187) evoked a sharp vasodilation, which was inhibited by endothelium removal, high KCl and tetraethylammonium, but not indomethacin, L-NAME, or 18α -GA. However, 18α -GA plus L-NAME inhibited vasodilation induced by A23187, but not acetylcholine. These findings suggest that endothelium-derived hyperpolarizing factor (EDHF) via gap junctions mainly counteracts vasoconstriction induced by methoxamine in mesenteric resistance arteries.

Keywords: methoxamine-induced vasoconstriction, endothelium-derived hyperpolarizing factor, rat mesenteric resistance artery, gap junction

Introduction

Vascular smooth muscles in physiological conditions are always exposed to vasoconstrictor stimuli from the outside adventitia via perivascular sympathetic adrenergic nerves, which release the neurotransmitter noradrenaline to produce active tone by activating α_1 -adrenoceptors on smooth muscle cells (1). It is widely accepted that the endothelium at the luminal surface of

blood vessels is an important regulator of vascular tone via release of various endothelium-derived endogenous substances (2, 3). Endothelial cells release endothelium-derived factors such as relaxing factors (EDRF) and contracting factors (EDCF) (4 – 6). EDCF and EDRF include endothelin, prostaglandin $F_{2\alpha}$, and thromboxane A_2 and include nitric oxide (NO), prostaglandin I_2 (PGI_2), and endothelium-derived hyperpolarizing factor(s) (EDHF), respectively. The removal and dysfunction of the vascular endothelium have been recognized to augment vasoconstriction induced by various stimulus and vasoactive agonists such as adrenergic nerve stimulation, noradrenaline, and serotonin, suggesting that the

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endothelium regulates vasoconstriction by releasing EDRF including NO and PGI₂ in the aorta (7–9). Our recent study demonstrated that the endothelium also regulates vasodilation by releasing EDCF, including vasoconstrictor prostanoids and thromboxane A₂ (10).

Evidence has accumulated that NO and PGI₂ of EDRF play a prominent role in the tone control of large conduit arteries, whereas EDHF plays a major role in the distal mesenteric arteries in response to vasoactive substances or physical stimuli such as shear stress and pulsatile stretch (11). Therefore, it has been assumed that in the rat mesenteric circulation, as the vessel size decreases the role of EDHF in the endothelium-dependent relaxation increases, and there is a significant interdependent interaction between NO, PGI₂, and EDHF (12, 13). EDHF, which has been described as an endothelium-derived non-NO and non-PGI₂ factor, induces hyperpolarization of vascular smooth muscle by opening K⁺-channels (5, 14, 15) in response to endothelial agonists such as acetylcholine and bradykinin and various physical stimuli. Additionally, gap junctions between endothelial cells and smooth muscle cells have been shown to be involved in EDHF-mediated relaxation (16). However, the role of EDHF in the modulation of the agonist-induced vasoconstrictor response of vascular smooth muscle in peripheral resistance arteries has not been extensively studied.

Therefore, the present study was designed to investigate the role of the vascular endothelium in the regulation of methoxamine (α_1 -adrenoceptor agonist)-induced vasoconstriction in rat mesenteric vascular beds. We here show evidence that EDHF via gap junctions mainly counteracts continuous vasoconstriction induced by methoxamine in the mesenteric resistance arteries of the rat.

Materials and Methods

Animals

Male Wistar rats weighing 250–280 g (8-week-old) were used in this study. The animals were given food and water ad libitum. They were housed in the Animal Research Center of Okayama University at a controlled ambient temperature of 22°C ± 2 with 50 ± 10% relative humidity and a 12-h light/12-h dark cycle (lights on 0800 hours). This study was carried out in accordance with the Guidelines for Animal Experiments at Okayama University Advanced Science Research Center, Japanese Government Animal Protection and Management Law (No. 105), and Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6). Every effort was made to minimize the number of animals used and suffering.

Perfusion of the mesenteric vascular bed

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 (17, 18). The isolated mesenteric vascular bed was placed in a water-jacketed organ bath maintained at 37°C 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). 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% O₂–5% CO₂ before passage through a warming coil maintained at 37°C. Modified Krebs solution was of the following composition: 119.0 mM NaCl, 4.7 mM KCl, 2.4 mM CaCl₂, 1.2 mM MgSO₄, 25.0 mM NaHCO₃, 1.2 mM KH₂PO₄, 0.03 mM disodium ethylenediaminetetraacetic acid, and 11.1 mM dextrose (pH 7.4). High KCl solution was prepared by equimolar replacement of NaCl.

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).

Chemical removal of the vascular endothelium

To remove the vascular endothelium, preparations with resting tone were perfused with 1.80 mg/ml of sodium deoxycholate in saline for 30 s, as described previously (19, 20). This caused a transient increase (20 to 30 mmHg) in perfusion pressure. Then, the preparations were rinsed with sodium deoxycholate-free Krebs solution for 60 min and subjected to perfusion with various agents. After the preparation was contracted by perfusion with Krebs solution containing methoxamine (2 μ M), successful removal of the endothelium was assessed by the lack of a relaxant effect to a bolus injection of 1 nmol acetylcholine, which was injected directly into the perfusate proximal to the arterial cannula by an infusion pump (model 975; Harvard Apparatus, S Natick, MA, USA). Volumes were 100 μ l over 12 s.

Bolus injection of acetylcholine and A23187

After equilibration for 30 min, the preparation was perfused with Krebs solution containing methoxamine (2–7 μ M) to induce submaximal contraction. After stabilization of the elevated perfusion pressure, the preparation was subjected to bolus injections of 1 nmol acetylcholine (muscarinic acetylcholine-receptor agonist) and 10 μ mol A23187 (Ca²⁺-ionophore), which were infused directly into the perfusate proximal to the arterial cannula with an injection pump. Injection volumes were 100 μ l over 12 s.

Experimental protocols

After the basal perfusion pressure had stabilized, each preparation with or without the endothelium was perfused with Krebs solution containing methoxamine (3, 7, or 10 μ M) or high KCl medium (high KCl; 30, 60, or 80 mM) to induce an increase in perfusion pressure due to vasoconstriction, and changes in perfusion pressure were observed for over 180 min.

To assess the underlying mechanisms, changes in perfusion pressure induced by methoxamine and high KCl were observed in the preparation without an endothelium. In the experiment using the endothelium-denuded preparation, the concentration of methoxamine was reduced to 2 μ M, which induced an increase in perfusion pressure to a level similar to that in preparations with intact endothelium, since endothelium removal enhanced the vasoconstrictor response to methoxamine (10).

In another series of experiments, combined perfusion of N^G -L-nitro arginine methyl ether (NO synthase inhibitor; L-NAME, 100 μ M), indomethacin (cyclooxygenase inhibitor, 1 μ M), high KCl (30 mM), tetraethylammonium (Ca^{2+} -activated K^+ -channel blocker, 5 mM), apamin (a small conductance Ca^{2+} -activated K^+ -channel blocker; APA, 10 nM) plus charybdotoxin (a middle and large conductance Ca^{2+} -activated K^+ -channel blocker; ChTX, 10 nM) or 18 α -glycyrrhetic acid (gap-junction inhibitor; 18 α -GA, 10 μ M), and methoxamine at 2–7 μ M were examined in preparations with intact endothelium.

In another series of experiments, the vasodilator response to a bolus injection of acetylcholine or A23187 was examined in preparations with or without the endothelium. After equilibration, the preparation was perfused with Krebs' solution containing methoxamine (2 to 7 μ M) or methoxamine plus one of various inhibitors (L-NAME, indomethacin, tetraethylammonium, APA plus ChTX, high KCL, or 18 α -GA) to induce submaximal contraction. After stabilization of the elevated perfusion pressure, acetylcholine (1 nmol) and A23187 (10 μ mol) diluted with Krebs solution containing methoxamine and various inhibitors were infused directly into the perfusate proximal to the arterial cannula with an infusion pump.

At the end of each experiment, 100 μ M papaverine was perfused to produce complete relaxation. In experiments using acetylcholine and A23187, vasodilator activity was expressed as a percent of the perfusion pressure at maximum relaxation induced by papaverine. The maximum increase in perfusion pressure induced by methoxamine and high KCl was taken as the maximum vasoconstrictor response and 100%, which was set as time 0, and the time-dependent changes in perfusion

pressure were observed for 180 min and expressed as a percent of the maximum vasoconstriction (peak amplitude). Changes in the peak amplitude at 60, 120 and 180 min were also expressed as percent change of maximum vasoconstriction.

Statistical analysis

Data are each shown as the mean $\pm$ S.E.M. Statistical analysis was performed by Student's *t*-test for unpaired observations between two groups and one-way analysis of variance followed by Tukey's test for multiple comparisons. $P < 0.05$ was considered statistically significant.

Drugs

The following drugs were used in this study: A23187 (5-methylamino-2-[[[(2*S*,3*R*,5*R*,6*S*,8*R*,9*R*)-3,5,9-trimethyl-2-[(2*S*)-1-oxo-1-(1*H*-pyrrol-2-yl)propan-2-yl]-1,7-dioxaspiro[5.5]undecan-8-yl]methyl]benzoxazole-4-carboxylic acid) (Sigma Aldrich Japan, Tokyo), acetylcholine chloride (Daiichi-Sankyo Pharmaceutical Co., Tokyo), APA (Sigma Aldrich), 18 α -GA (Wako Pure Chemical Ind., Ltd., Osaka), ChTX (Sigma Aldrich), indomethacin (Sigma Aldrich), methoxamine hydrochloride (Nihon Shinyaku, Kyoto), KCl (Wako), L-NAME (Sigma Aldrich), papaverine hydrochloride (Dainippon-Sumitomo Pharmaceutical Co., Osaka), sodium deoxycholate (Ishizu Seiyaku Co., Tokyo), and tetraethylammonium (Sigma Aldrich). 18 α -GA was dissolved in dimethylsulfoxide, and sodium deoxycholate was dissolved in 0.9% saline. All other drugs were dissolved in distilled water and diluted with Krebs solution containing 1–10 μ M methoxamine when perfused or injected directly.

Results

Vasoconstrictor responses to continuous perfusion of methoxamine and high KCl

As shown in Fig. 1, in the preparations with intact endothelium, continuous perfusion of methoxamine at concentrations of 3, 7, and 10 μ M induced an initial sharp increase in perfusion pressure (phasic constriction) followed by a continuous increase in perfusion pressure (tonic constriction) in a concentration-dependent manner. However, the amplitude of sustained contraction induced by methoxamine was gradually reduced during 180-min perfusion in a time-dependent manner. As shown in Fig. 2A, the initial maximum vasoconstriction in responses to methoxamine at concentrations of 3, 7, and 10 μ M were decreased to 50%, 20%, and 60% of the initial level at 180 min, respectively. The time-dependent reduction of contractile response to methoxamine perfusion at a concentration of 7 μ M was greater than those to

Fig. 1. Typical records showing changes in perfusion pressure following perfusion of methoxamine at concentrations of 3 (A), 7 (B), and 10 (C) μ M for 180 min in rat-perfused mesenteric vascular beds with intact endothelium. Time 0 indicates the maximum increase in perfusion pressure after starting continuous perfusion of methoxamine. PPV, perfusion of 100 μ M papaverine.

other concentrations (Fig. 2B).

Perfusion of Krebs solution with high KCl (30, 60, and 80 mM) induced vasoconstriction in a concentration-dependent manner (Fig. 3). However, initial levels of increased perfusion pressure induced by perfusion of high KCl at concentrations of 30, 60, and 80 mM, which produced perfusion pressure increases similar to methoxamine at 3, 7, and 10 μ M (Table 1), respectively, were maintained over 180 min throughout the experiment and no time-dependent reduction was observed (Figs. 3 and 4).

Effects of endothelium removal on vasoconstriction induced by methoxamine and high KCl

Vasoconstriction induced by methoxamine, but not high KCl, was augmented by chemical removal of the vascular endothelium. Therefore, to obtain a perfusion pressure increase similar to that induced by 7 μ M methoxamine in the preparation with endothelium, the concentration of methoxamine was reduced to 2 μ M in the preparation without endothelium. In this preparation, the time-dependent reduction of methoxamine-induced vasoconstriction was significantly attenuated as shown in Fig. 5, A, C, and D. However, no significant change in

Fig. 2. A line graph (A) showing time-course changes (%) in maximum increase in perfusion pressure induced by continuous perfusion of 3, 7, and 10 μ M methoxamine for 180 min and a bar graph (B) showing percent changes in methoxamine-induced maximum perfusion pressure increase at 60, 120, and 180 min in rat perfused mesenteric vascular beds with intact endothelium. Time 0 indicates the maximum increase in perfusion pressure after starting continuous perfusion of methoxamine and was taken as 100%. MTX, methoxamine. Data each indicate the mean $\pm$ S.E.M. * P < 0.05, ** P < 0.01, compared with 7 μ M MTX.

vasoconstrictor responses induced by high KCl (60 mM) was observed after endothelium removal (Fig. 5: B, C, and D).

Effects of L-NAME and indomethacin on vasoconstriction induced by methoxamine

In preparations with intact endothelium, combined perfusion of 100 μ M L-NAME, but not indomethacin, augmented the methoxamine-induced vasoconstriction as observed in endothelium removal. Therefore, the concentration of methoxamine in combination with L-NAME was reduced to 2 μ M, which induced a perfusion pressure increase similar to that induced by 7 μ M methoxamine without L-NAME. As shown in Fig. 6, neither L-NAME nor indomethacin had a significant effect on the time-dependent reduction of methoxamine-induced vasoconstriction.

Effects of K⁺-channel inhibitors and a gap-junction inhibitor on vasoconstriction induced by methoxamine

As shown in Fig. 7A, in preparations with intact endothelium, combined perfusion of high KCl (30 mM), 5 mM tetraethylammonium or 10 nM APA plus 10 nM ChTX and methoxamine (7 μ M) significantly inhibited

Fig. 3. Typical records showing changes in perfusion pressure following perfusion of Krebs solution containing high KCl at 30 (A), 60 (B), and 80 (C) mM KCl for 180 min in rat-perfused mesenteric vascular beds with intact endothelium. Time 0 indicates the maximum increase in perfusion pressure after starting continuous perfusion of high KCl. PPV, perfusion of 100 μ M papaverine.

the time-dependent reduction of methoxamine-induced vasoconstriction. In the presence of high KCl or tetraethylammonium, the initial vasoconstriction (phasic phase) induced by methoxamine was sustained over 180 min, while a small time-dependent reduction in vasoconstriction was observed in combination with APA and ChTX (Fig. 7C).

As shown Fig. 7B, 18 α -GA, a gap-junction inhibitor,

Fig. 4. A line graph (A) showing time-course changes (%) in maximum increase in perfusion pressure induced by continuous perfusion of Krebs solution containing high KCl at 30, 60, and 80 mM KCl for 180 min and a bar graph (B) showing percent changes of high KCl-induced perfusion pressure increase at 60, 120, and 180 min in rat-perfused mesenteric vascular beds with intact endothelium. Time 0 indicates the maximum increase in perfusion pressure after starting continuous perfusion of high KCl and was taken as 100%. Data each indicate the mean $\pm$ S.E.M.

significantly inhibited the time-dependent reduction of methoxamine-induced vasoconstriction.

Vasodilator responses to acetylcholine and A23187

In preparations with intact endothelium and active tone, bolus injection of acetylcholine (1 nmol) or A23187 (10 μ M) produced a sharp and transient decrease in perfusion pressure due to vasodilation. As

Table 1. The perfusion pressure at resting tension, maximum increase in perfusion pressure with perfusion of methoxamine and high KCl, and perfusion pressure after perfusion of papaverine (PPV)

	Resting tension (mmHg)	Maximum increase in perfusion pressure (mmHg)	After PPV perfusion (mmHg)
Methoxamine			
3 μ M (n = 6)	27.1 $\pm$ 2.0	58.6 $\pm$ 5.1**	27.9 $\pm$ 1.8
7 μ M (n = 6)	28.5 $\pm$ 2.0	114.2 $\pm$ 4.5**	31.8 $\pm$ 1.8
10 μ M (n = 6)	31.3 $\pm$ 1.6	149.1 $\pm$ 9.5**	34.9 $\pm$ 1.2
KCl			
30 mM (n = 5)	29.4 $\pm$ 1.5	47.7 $\pm$ 5.4**	31.5 $\pm$ 2.4
60 mM (n = 6)	31.1 $\pm$ 0.9	117.8 $\pm$ 7.2**	33.9 $\pm$ 2.4
80 mM (n = 6)	28.6 $\pm$ 0.7	142.2 $\pm$ 4.3**	31.9 $\pm$ 1.1

Data each indicate the mean $\pm$ S.E.M. ** $P < 0.01$, compared with resting tone. n, number of rats.

Fig. 5. Effect of endothelium removal on vasoconstrictor responses to continuous perfusion of 2 μ M methoxamine and high KCl (60 mM) for 180 min in rat perfused mesenteric vascular beds. Upper charts are typical records of methoxamine (A) and high KCl (B). A line graph (C) and a bar graph (D) show time-course changes in maximum increase in perfusion pressure induced by 2 μ M methoxamine and high KCl (60 mM) and percent changes in methoxamine- and high-KCl-induced maximum perfusion pressure increase at 60, 120, and 180 min, respectively. Time 0 indicates the maximum increase in perfusion pressure after starting continuous perfusion of methoxamine and high KCl and was taken as 100%. MTX, methoxamine; PPV, perfusion of 100 μ M papaverine; (+E), with intact endothelium; (-E), endothelium removal. Data each indicate the mean $\pm$ S.E.M. ** $P < 0.01$, compared with methoxamine (+E).

shown in Table 2, the relaxations induced by both acetylcholine and A23187 were almost abolished by removal of the endothelium, and significantly inhibited by high KCl (30 mM) and tetraethylammonium (5 mM), while L-NAME (100 μ M) or L-NAME (100 μ M) plus indomethacin (1 μ M) had no effect on the acetylcholine- and A23187-induced vasodilation. Furthermore, 18 α -GA (10 μ M) alone had no significant effect on the acetylcholine- and A23187-induced vasodilation. However, the combined presence of L-NAME (100 μ M) and 18 α -GA (10 μ M) significantly inhibited the vasodilation induced by A23187, but not acetylcholine.

Fig. 6. A line graph (A) showing the effects of *N*^G-nitro-L-arginine-methyl ester (L-NAME, 100 μ M) and indomethacin (INDO, 1 μ M) on time-course changes (%) in maximum increase in perfusion pressure induced by continuous perfusion of methoxamine (7 μ M) and a bar graph (B) showing percent changes in methoxamine-induced maximum perfusion pressure increase at 60, 120, and 180 min in rat perfused mesenteric vascular beds with intact endothelium. Time 0 indicates the maximum increase in perfusion pressure after starting continuous perfusion of methoxamine and was taken as 100%. Data each indicate the mean $\pm$ S.E.M. CON, control.

Discussion

The present study demonstrated that, in the rat mesenteric artery with intact endothelium, continuous perfusion of an α_1 -adrenoceptor agonist, methoxamine, concentration-dependently caused a long-lasting increase in perfusion pressure due to vasoconstriction, which consisted of an initial fast constriction (phasic phase) followed by sustained constriction (tonic phase). However, the tonic phase of methoxamine-induced vasoconstriction gradually decreased during 180-min perfusion, causing 80% reduction in the initial level at 180 min, indicating that the tonic phase is time-dependent. In contrast, continuous perfusion of Krebs solution with high KCl induced a K⁺-concentration-dependent increase in perfusion pressure that was sustained over 180-min perfusion without reduction. This finding suggests that the contractile ability of smooth muscle cells in the rat mesenteric artery is maintained over a 180-min perfusion. It is unlikely that the time-dependent reduction of methoxamine-induced vasoconstriction is due to decreased constrictive activity of arterial smooth muscles. Furthermore, since high KCl blunts K⁺-channels activity, K⁺-channels are likely to be involved in the time-dependent reduction of methoxamine-induced vasoconstriction.

Fig. 7. Line graphs showing the effects of high KCl (30 mM) (A), tetraethylammonium (TEA, 5 mM) (A), 10 nM apamin plus 10 nM charybdotoxin (APA + ChTX) (A), and 18 α -glycyrrhetinic acid (18 α -GA, 10 μ M) (B) on time-course changes (%) in maximum increase in perfusion pressure induced by continuous perfusion of methoxamine (7 μ M). A bar graph (C) showing the effects of KCl, TEA, APA + ChTX, and 18 α -GA on percent changes in methoxamine-induced maximum perfusion pressure increase at 60, 120, and 180 min in rat perfused mesenteric vascular beds with intact endothelium. Time 0 indicates the maximum increase in perfusion pressure after starting continuous perfusion of methoxamine and was taken as 100%. Data each indicate the mean $\pm$ S.E.M. * P <0.05, ** P <0.01, compared with control (CON).

induced by methoxamine. Furthermore, indomethacin, a prostanoid synthase cyclooxygenase inhibitor, did not alter the phasic or tonic constriction in response to methoxamine, while NO synthase inhibition by L-NAME augmented the methoxamine-induced phasic constriction to a similar extent to that of endothelium removal, but it did not alter the time-dependent reduction of tonic phase constriction. Thus, it is suggested that NO released from the endothelium is responsible for suppression of methoxamine-induced phasic constriction. It is very likely that non-NO and non-prostacyclin factors released from the endothelium are involved in time-dependent reduction of tonic phase constriction.

Methoxamine selectively stimulates α_1 -adrenoceptors to induce direct constriction of vascular smooth muscle in rat mesenteric arteries. However, methoxamine has been shown to have no direct action on the endothelial cells (21), implying that it does not directly stimulate endothelial cells to release EDRF. The most likely explanation is that methoxamine-induced vasoconstriction in isolated mesenteric resistance arteries results in

In the present study, removal of the endothelium augmented the phasic phase of the methoxamine-induced vasoconstriction and markedly attenuated the time-dependent reduction of tonic phase constriction. This finding strongly suggests that the endothelium plays an important inhibitory role in vasoconstriction

Table 2. Effects of various treatments on vasodilation induced by acetylcholine (ACh) and Ca^{2+} ionophore A23187 (A23187) in rat perfused mesenteric vascular beds with active tone

Treatments	Vasodilation (%)	
	1 nmol ACh (n)	10 μ mol A23187 (n)
Control	81.7 $\pm$ 3.1 (6)	71.7 $\pm$ 4.0 (6)
Endothelium removal	9.4 $\pm$ 2.1** (6)	11.8 $\pm$ 5.9** (5)
L-NAME (100 μ M)	71.3 $\pm$ 1.9 (6)	68.7 $\pm$ 4.2 (6)
L-NAME (100 μ M) + INDO (1 μ M)	75.2 $\pm$ 2.2 (5)	68.0 $\pm$ 5.4 (5)
High KCl (30 mM)	24.7 $\pm$ 1.4** (6)	21.5 $\pm$ 6.5** (5)
Tetraethylammonium (5 mM)	49.3 $\pm$ 8.9* (5)	56.2 $\pm$ 6.9* (5)
18 α -GA (10 μ M)	82.4 $\pm$ 6.6 (5)	76.2 $\pm$ 2.0 (5)
L-NAME (100 μ M) + 18 α -GA (10 μ M)	70.7 $\pm$ 4.0 (5)	41.3 $\pm$ 8.0* [†] (5)

Data each indicate the mean $\pm$ S.E.M. (n), number of rats. L-NAME, N^G -L-nitro arginine methyl ether; INDO, indomethacin; 18 α -GA, 18 α -glycyrrhetinic acid. * P <0.05, ** P <0.01, compared with the control. [†] P <0.05, compared with L-NAME alone.

increased endothelial mechanical stimuli, which stimulate the endothelium and elicit the release of EDRF.

It should be noted that the time-dependent reduction of methoxamine (3, 7, and 10 μ M)-induced tonic constriction was not concentration-dependent, and reduction of vasoconstriction induced by the concentration of 7 μ M (middle) was greater than that induced by the other two concentrations (low and high). The explanation for this phenomenon is that vasoconstriction induced by a low concentration is an insufficient stimulus to promote the EDRF release process, while the endothelium is unable to overwhelm higher concentration-induced vasoconstriction. This notion is supported by the report by Gündüz et al. (22) that high intravascular pressure impairs both endothelium-dependent and independent dilation of rat small mesenteric arteries.

EDHF, which has been described as an endothelium-derived non-NO and non-PGI₂ factor, induces hyperpolarization of vascular smooth muscle by opening K⁺ channels. Furthermore, several studies showed evidence that gap junctions also play an important role in EDHF-mediated relaxation (16, 23, 24). In the present study, the time-dependent reduction of methoxamine-induced vasoconstriction was abolished by nonselective K⁺-channel blockade by 30 mM high KCl medium and significantly inhibited by K⁺-channel blockers, including tetraethylammonium and APA plus ChTX; this combination of APA and ChTX has been reported to attenuate the action of EDHF (25, 26). Furthermore, the inhibition of gap junctions by 18 α -GA resulted in marked inhibition of time-dependent reduction of methoxamine-induced vasoconstriction. It has been reported that 18 α -GA inhibits endothelium-dependent EDHF-type relaxation mediated via myo-endothelial gap junctions (27, 28). Therefore, it seems likely that myo-endothelial gap junctions are mainly responsible for the time-dependent reduction, but involvement of inter-endothelial and inter-smooth muscle junctions may also be possible. Taken together, these findings suggest that the endothelium acts to depress methoxamine-induced tonic phase vasoconstriction by the EDHF-mediated response, which is associated with activation of multiple K⁺-channels and myo-endothelium gap-junction involvement in mesenteric resistance arteries. Several studies suggested that NO and EDHF have an interdependent action on endothelium function. Therefore, it is assumed that in the present study the endothelium released NO to suppress the initial phase of vasoconstriction, followed by the EDHF-mediated response to compensate for the insufficient inhibitory effect of NO, resulting in a time-dependent reduction of tonic phase vasoconstriction.

Acetylcholine evokes endothelium-dependent relaxation through activation of muscarinic acetylcholine

receptors, which increase the Ca²⁺ levels of endothelial cells from intracellular stores and Ca²⁺-influx. However, A23187 increases Ca²⁺ concentrations by directly increasing intracellular Ca²⁺-levels from internal stores without stimulating any receptors. Here, we demonstrated that both acetylcholine- and A23187-induced vasodilation were significantly attenuated by endothelium removal, tetraethylammonium, and high KCl and slightly inhibited by 18 α -GA, but neither were affected by L-NAME or indomethacin, suggesting that the responses to both A23187 and acetylcholine are endothelium-dependent and mainly mediated by non-NO and non-prostanoid EDHF. Furthermore, the response to A23187, but not acetylcholine, was significantly inhibited by the combined presence of 18 α -GA and L-NAME. These findings suggest that gap junctions are responsible for A23187-induced EDHF-mediated response as involved in the time-dependent reduction of methoxamine-induced vasoconstriction, whereas gap junctions do not contribute to the acetylcholine-induced EDHF-mediated response. Therefore, it is possible that the endothelium releases different types of EDHF to regulate vascular tone in response to various stimuli in mesenteric resistance arteries.

In conclusion, the present results suggest that the endothelium in mesenteric resistance arteries acts to depress methoxamine-induced tonic phase vasoconstriction by the EDHF-mediated response, which is associated with activation of multiple K⁺-channels and gap-junction involvement.

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