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RESEARCH PAPER

Endothelium removal augments endothelium-independent vasodilatation in rat mesenteric vascular bed

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Background and purpose: The vascular endothelium regulates vascular tone by releasing various endothelium-derived vasoactive substances to counteract excess vascular response. We investigated whether the vascular endothelium regulates vasodilatation via released endothelium-derived contracting factors (EDCFs), by examining the effect of endothelium removal on responses to periarterial nerve stimulation (PNS) and various vasodilator agents.
Experimental approach: The rat mesenteric vascular bed was perfused with Krebs solution. Vasodilator responses to PNS and 5 min perfusion of vasodilator agents in preparations with endothelium were compared with those in the same preparations without endothelium. The endothelium was removed by 30 s perfusion with sodium deoxycholate.
Key results: Endothelium removal significantly augmented vasodilator responses to PNS and calcitonin gene-related peptide (CGRP), isoprenaline (β-adrenoceptor agonist), SNP and 8-bromo-cGMP (8-Br-cGMP; cGMP analogue) but not BAY41-2272 (soluble guanylate cyclase activator). The augmentation of SNP-induced vasodilatation after denudation was much greater than that of CGRP- or isoprenaline-induced vasodilatation. In the preparations with an intact endothelium, L-NAME (nitric oxide synthase inhibitor) significantly augmented vasodilator responses to PNS and CGRP, isoprenaline, SNP and 8-Br-cGMP, but not BAY41-2272. Indomethacin (cyclooxygenase inhibitor) and seratrodast (thromboxane A_2 receptor antagonist), but not phosphoramidon (endothelin-1-converting enzyme inhibitor) or BQ-123 (selective endothelin type A receptor antagonists), significantly augmented vasodilator responses to PNS and CGRP, isoprenaline, SNP and BAY41-2272.
Conclusion and implication: These results suggest that the endothelium in rat mesenteric arteries regulates and maintains vascular tone via counteracting not only vasoconstriction through releasing endothelium-derived relaxing factors, but also vasodilatation, in part by releasing an EDCF, thromboxane A_2.
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Keywords: vascular endothelium removal; vasodilatation; endothelium-derived relaxing factor; endothelium-derived contracting factor; periarterial nerve stimulation; calcitonin gene-related peptide; sodium nitroprusside; isoprenaline

Abbreviations: 8-Br-cGMP, 8-bromo-cGMP; CGRP, calcitonin gene-related peptide; EDCF, endothelium-derived contracting factor; EDRF, endothelium-derived relaxing factor; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; PNS, periarterial nerve stimulation; sGC, soluble guanylate cyclase; SNP, sodium nitroprusside; TXA_2, thromboxane A_2

Introduction

The endothelium at the luminal surface of blood vessels is an important regulator of blood vessel tone via release of various endothelium-derived endogenous substances (Furchgott and Zawadzki, 1980; Ress *et al.*, 1986). Endothelial cells have been shown to release endothelium-derived factors such as relaxing factors (EDRFs; nitric oxide (NO) and prostaglandin I_2) and contracting factors (EDCFs; endothelin, prostaglandin $F_2\alpha$ and thromboxane A_2 (TXA_2) (Moncada *et al.*, 1991a; Vanhoutte and Mombouli, 1996). It is widely recognized that endothelium removal and dysfunction result in enhancement of contractile responses to vasoconstrictor agents (Moncada *et al.*, 1991b; Urabe *et al.*, 1991; Dora *et al.*, 2000). This is considered to be due to lack of, or deficient release of EDRF, which counteracts

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vasoconstriction. Additionally, removal of the endothelium from rat aortic rings has been shown to increase exogenous NO (NO donor)-mediated vasodilatation, but, in contrast, the vasodilator response to isoprenaline was only slightly increased. This implies that the removal of the basal NO-mediated vasodilator tone leads to a specific supersensitivity to nitrovasodilators associated with the upregulation of soluble guanylate cyclase (sGC) (Moncada *et al*., 1991b). However, little is known about whether endothelium removal in small arteries affects vasodilator responses to various vasodilator agents that act by stimulating adenylate or guanylate cyclase.

The perivascular nerves distributed in the adventitia layer of blood vessels are also an important regulator of blood vessel tone, which is mainly maintained by perivascular sympathetic adrenergic nerves that release vasoconstrictor transmitters such as noradrenaline, neuropeptide Y and ATP (Lundberg *et al*., 1982). The rat mesenteric resistance arteries have been shown to be innervated not only by adrenergic nerves, but also by nonadrenergic, noncholinergic nerves (Bevan and Brayden, 1987; Kawasaki *et al*., 1988). Previously, we demonstrated that nonadrenergic, noncholinergic nerves in which CGRP, a potent vasodilator neuropeptide, acts as a neurotransmitter, innervate the rat mesenteric artery and regulate the vascular tone along with adrenergic nerves (Kawasaki *et al*., 1988). The endothelium has been shown to modify the function of perivascular nerves (Burnstock and Ralevic, 1994). Ralevic (2002) showed that endothelium removal augments the perivascular nerve stimulation (PNS)- and NO donor (sodium nitroprusside; SNP)-induced vasodilatation, but not CGRP-induced vasodilatation, in the mesenteric vascular beds.

Therefore, the present study was undertaken to investigate the effect of endothelium removal on vasodilator responses to stimulation of CGRP-containing (CGRPergic) nerves and to various vasodilator agents including CGRP, which activates adenylate cyclase via CGRP receptors to increase cAMP production (Kubota *et al*., 1985); isoprenaline, which activates adenylate cyclase via β-adrenoceptors to increase cAMP production (Grace *et al*., 1988); SNP, which activates sGC via NO to increase cGMP production (Ralevic and Burnstock, 1991); and PNS, which induces CGRP release (Kawasaki *et al*., 1988). Moreover, we found that removal of the endothelium augmented vasodilator responses to not only PNS, but also vasodilator agents that stimulate guanylate cyclase or adenylate cyclase.

Methods

Animals

One hundred and fifty-one male Wistar rats (purchased from Shimizu Experimental Animals, Shizuoka, Japan), weighing 300–400 g, were used in this study. 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 $22 \pm 2\,^{\circ}C$ with $50 \pm 10\%$ relative humidity and with a 12-h light/12-h dark cycle (lights on at 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. 115) and 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. All experiments conformed to international guidelines on the ethical use of animals.

Perfusion of the mesenteric vascular bed

The animals were anaesthetized with sodium pentobarbital ($50\,mg\,kg^{-1}$, i.p.), and the mesenteric vascular beds were isolated and prepared for perfusion as described previously (Kawasaki *et al*., 1988, 1991). The superior mesenteric artery was cannulated and flushed gently 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 $37\,^{\circ}C$ and perfused with a modified (see below) Krebs solution, at a constant flow rate of $5\,ml\,min^{-1}$ with a peristaltic pump (model AC-2120; ATTO, Tokyo, Japan). The preparation was also superfused with the same solution at a rate of $0.5\,ml\,min^{-1}$ 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 $37\,^{\circ}C$. The modified Krebs solution had the following composition (mM): NaCl, 119.0; KCl, 4.7; $CaCl_2$, 2.4; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; disodium EDTA, 0.03; and dextrose, 11.1 (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).

PNS

After the basal perfusion pressure had been allowed to stabilize, the preparation was perfused with Krebs solution containing methoxamine (α_1-adrenoceptor agonist) at concentrations of 2–7 μM to induce submaximal vasoconstriction. After stabilization of the elevated perfusion pressure, the preparation was subjected to periarterial nerve stimulation (PNS). The PNS was applied for 30 s using bipolar platinum ring electrodes placed around the superior mesenteric artery. Rectangular pulses of 1 ms and a supramaximal voltage (50 V) were applied at 0.5, 1 and 2 Hz using an electronic stimulator (model SEN 3301; Nihon Kohden).

Chemical removal of the vascular endothelium

The preparation with resting tone was perfused with a $1.80\,mg\,ml^{-1}$ solution of sodium deoxycholate in saline for 30 s to remove the vascular endothelium, as described previously (Takenaga *et al*., 1995). Then, the preparation

was rinsed with sodium deoxycholate-free Krebs solution for 60 min. After the preparation was contracted by perfusion with Krebs solution containing methoxamine (1–2 μM), chemical 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 μl for 10 s.

Experimental protocols

To increase the perfusion pressure to approximately 100 mm Hg, active tone of isolated mesenteric vascular beds with intact endothelium was induced by perfusion with Krebs solution containing methoxamine (7 μM) and guanethidine (5 μM, added to block adrenergic neurotransmission). After the elevated perfusion pressure had stabilized, PNS (0.5–2 Hz) or perfusion of CGRP (0.01–10 nM), isoprenaline (a nonselective β-adrenoceptor agonist) (1 nM–10 μM), SNP (a NO donor) (0.1 nM–1 μM), BAY41-2272 (a selective sGC activator; Stasch *et al*., 2001) (1 nM–10 μM) and 8-bromo-cGMP (8-Br-cGMP; a cGMP analogue) (0.1–100 μM) was applied to induce a control response. The Krebs solution containing methoxamine and guanethidine and the final concentration of CGRP, isoprenaline, SNP, BAY41-2272 or 8-Br-cGMP was perfused for 5 min to cause a decrease in perfusion pressure, vasodilator response. After the perfusion pressure had returned to the preperfusion level, the Krebs solution was switched to Krebs solution containing a higher concentration of the vasodilator agent. After the control response in the presence of the intact endothelium had been obtained, the elevated perfusion pressure was returned to the resting level by switching to normal Krebs solution without methoxamine and the vascular endothelium was removed by perfusion with sodium deoxycholate. To observe reproducibility of the vasodilator response, the vascular endothelium was left intact by perfusing with Krebs solution without sodium deoxycholate ($n=3$ for each vasodilator agent). After the preparation had been washed with normal Krebs solution for 60 min, the active tone of the preparation was again induced by perfusing with Krebs solution containing guanethidine and methoxamine. The preconstriction level in the second perfusion study was designed to become similar to that of the first control perfusion study, approximately 100 mm Hg. To obtain this level, the concentration of methoxamine in the second perfusion study had to be changed accordingly. In the experiments where the effect of endothelium removal on responses to N^{ω}-nitro-L-arginine methyl ester (L-NAME) was investigated, the concentration of methoxamine was reduced to 1–2 μM as these treatments produced greater vasoconstriction. After the elevated perfusion pressure had stabilized, PNS or 5-min perfusions of CGRP, isoprenaline, SNP, BAY41-2272 or 8-Br-cGMP were applied using the same PNS or perfusion protocols ($n=5$–7).

In another series of experiments, to assess the involvement of NO, perfusion of various vasodilator agents was carried out during the perfusion of the NO synthase inhibitor L-NAME (100 μM), in preparations with an intact endothelium ($n=3$–5).

In another series of experiments, to assess the influence of EDCF, the vascular responses to the various vasodilator agents were determined in preparations with an intact endothelium during perfusion of EDCF inhibitors, including indomethacin (cyclooxygenase inhibitor), seratrodast (TXA_2 receptor antagonist), phosphoramidon (a neutral endopeptidase inhibitor and endothelin-1-converting enzyme inhibitor; Matsumura *et al*., 1990; Xu *et al*., 1994) and BQ-123 (a selective ET_A (endothelin type A) antagonist; Ihara *et al*., 1992) ($n=4$–5). In the experiment with indomethacin, the concentration of methoxamine was increased to 8–10 μM, as indomethacin inhibits methoxamine-induced vasoconstriction.

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

Statistical analysis

Data are presented as the mean ± s.e.mean. Statistical analysis was performed by use of Student's paired *t*-test. A *P*-value less than 0.05 was considered significant.

For calculation of EC_{50} values for CGRP, isoprenaline, SNP, BAY41-2272 and 8-Br-cGMP vasodilator responses were expressed as a percentage of the maximal response and a probit analysis was performed. Statistical analysis was performed on the negative logarithm of the EC_{50} values. EC_{50} values are presented as the geometric mean and the 95% CL (confidence limit). $P<0.05$ was considered statistically significant and the χ^2 test was used for comparisons.

Drugs

The following drugs were used: acetylcholine chloride (Daiichi-Sankyo Pharmaceutical Co., Tokyo, Japan), methoxamine hydrochloride (Nihon Shinyaku, Kyoto, Japan), papaverine hydrochloride (Dainippon-Sumitomo Pharmaceutical Co. Ltd, Osaka, Japan), phosphoramidon (Peptide Institute, Osaka, Japan), rat CGRP (Peptide Institute), sodium deoxycholate (Ishizu Seiyaku Co., Tokyo, Japan) and seratrodast (Takeda Chemical Industries Ltd., Osaka, Japan). BAY41-2272 (5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1*H*-pyrazolo[3,4-*b*]pyridin-3-yl]-pyrimidin-4-ylamine), BQ-123(cyclo(D-α-aspartyl-L-propyl-D-valyl-L-leucyl-D-tryptophyl)sodium), 8-Br-cGMP, guanethidine sulphate, indomethacin, isoprenaline hydrochloride, L-NAME and SNP were obtained from Sigma Aldrich Japan Co. (Tokyo, Japan). All drugs, except indomethacin and sodium deoxycholate, were dissolved in distilled water and diluted with Krebs solution containing methoxamine and guanethidine, when perfused or injected directly. Indomethacin was dissolved in 50% ethanol and diluted with Krebs solution. Sodium deoxycholate was dissolved in 0.9% saline.

Results

Effect of endothelium removal on vasodilatation induced by PNS

In preparations with an intact endothelium and active tone, PNS at 0.5–2 Hz caused a frequency-dependent decrease in

Table 1 Effects of various treatments on vasodilatation induced by acetylcholine injection in rat perfused mesenteric vascular beds with active tone

Treatments (μM)	n	*Vasodilatation (%) induced by acetylcholine (0.5 nmol)*	
		Control	*Treatment*
Endothelium removal	20	88.8 ± 1.2	9.4 ± 2.9*
L-NAME (100)	17	88.8 ± 1.1	54.0 ± 4.6*
Phosphoramidon (10)	19	85.4 ± 1.7	79.4 ± 2.3
BQ-123 (1)	19	90.8 ± 1.0	90.4 ± 1.1
Indomethacin (0.5)	20	85.4 ± 1.3	83.3 ± 2.7
Seratrodast (1)	20	85.9 ± 1.6	87.7 ± 1.6

Abbreviation: L-NAME, N^{ω}-nitro-L-arginine methyl ester.
*$P<0.01$ vs control.

perfusion pressure due to vasodilatation. The vasodilator response to PNS has been shown to be mediated by CGRPergic nerves, as the response was blocked by CGRP (8–37) and capsaicin (a CGRP depleter) (Han *et al.*, 1990; Kawasaki *et al.*, 1991).

Table 1 shows that the acetylcholine-induced vasodilatation was abolished by perfusion of sodium deoxycholate, indicating that the vascular endothelium was effectively removed. As shown in Figure 1, the PNS (0.5, 1 and 2 Hz)-induced vasodilatation was significantly augmented by endothelium removal. Significant differences were found between the PNS-induced vasodilatation in the preparations with an intact endothelium and in denuded preparations.

Effect of endothelium removal on vasodilatation induced by perfusion of SNP, isoprenaline, CGRP and 8-Br-cGMP

As shown in Figures 2a and b, perfusion of SNP (0.1 nM–1 μM) for 5 min in preparations with an intact endothelium caused a concentration-dependent vasodilatation. The SNP-induced vasodilatation was significantly augmented by endothelium removal (Table 2) and the duration of the response was markedly prolonged.

Perfusion of isoprenaline (1 nM–10 μM) for 5 min caused concentration-dependent vasodilatation, which was blocked by propranolol (a β-adrenoceptor antagonist) (data not shown), indicating that the response was mediated by stimulation of β-adrenoceptors. As shown in Figure 2c, endothelium removal significantly augmented the isoproterenol-induced vasodilation (Table 2), and the duration of the response was markedly prolonged. After endothelium removal, vasoconstriction followed by a vasodilatation was observed in response to isoprenaline.

Perfusion of CGRP (0.01–10 nM) for 5 min caused sustained vasodilatation in a concentration-dependent manner (Figure 2d). The CGRP-induced vasodilatation has been shown to be mediated by postsynaptic CGRP receptors, as CGRP (8–37) blocked the CGRP-induced vasodilatation (Han *et al.*, 1990; Kawasaki *et al.*, 1991). In the preparations denuded chemically with sodium deoxycholate, the CGRP-induced vasodilatation was significantly augmented (Table 2) and the duration of the response was markedly prolonged.

Perfusion of 8-Br-cGMP (0.1–100 μM) for 5 min in preparations with an intact endothelium caused a concentration-dependent vasodilatation, which was smaller than the response to the other vasodilators used (Table 2). The 8Br-cGMP-induced vasodilator response was significantly augmented by endothelium removal (Table 2) and its duration was markedly prolonged.

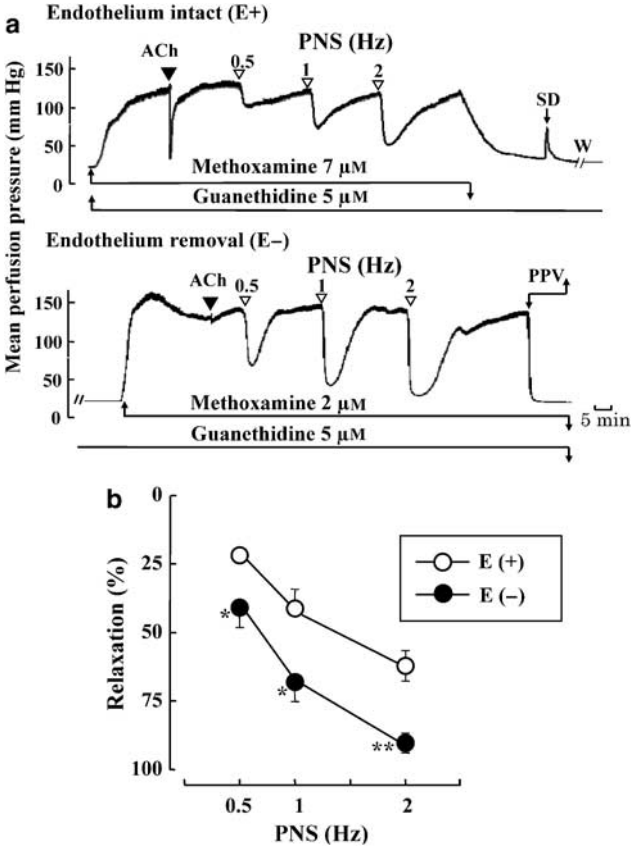

Figure 1 A typical record (**a**) and a line graph (**b**) showing the effect of endothelium removal on vasodilator responses to periarterial nerve stimulation (PNS) in rat perfused mesenteric vascular beds. ACh, bolus injection of acetylcholine (0.5 nmol; closed inverted circles). SD, perfusion of sodium deoxycholate for 30 s. W, washing with Krebs solution without SD. PPV, perfusion of papaverine (100 μM). E(+) and E(−) indicate endothelium intact and endothelium removal, respectively. Note that endothelium removal augmented the vasodilatation induced by PNS. Each point represents the mean ± s.e.mean of five experiments. *$P<0.05$, **$P<0.01$, compared with endothelium intact.

As shown in Table 2, the vasodilator responses to the second perfusion of CGRP, isoprenaline, SNP and 8-Br-cAMP in the preparations with an intact endothelium were similar to those of the initial responses observed under control conditions.

Effect of L-NAME on vasodilatation induced by PNS and perfusion of isoprenaline, CGRP, SNP and 8-Br-cGMP

As shown in Table 1, L-NAME significantly inhibited the acetylcholine-induced vasodilatation, but did not abolish the response.

In preparations with an intact endothelium, frequency-dependent vasodilator responses to PNS (0.5–2 Hz) and concentration-dependent vasodilator responses to CGRP

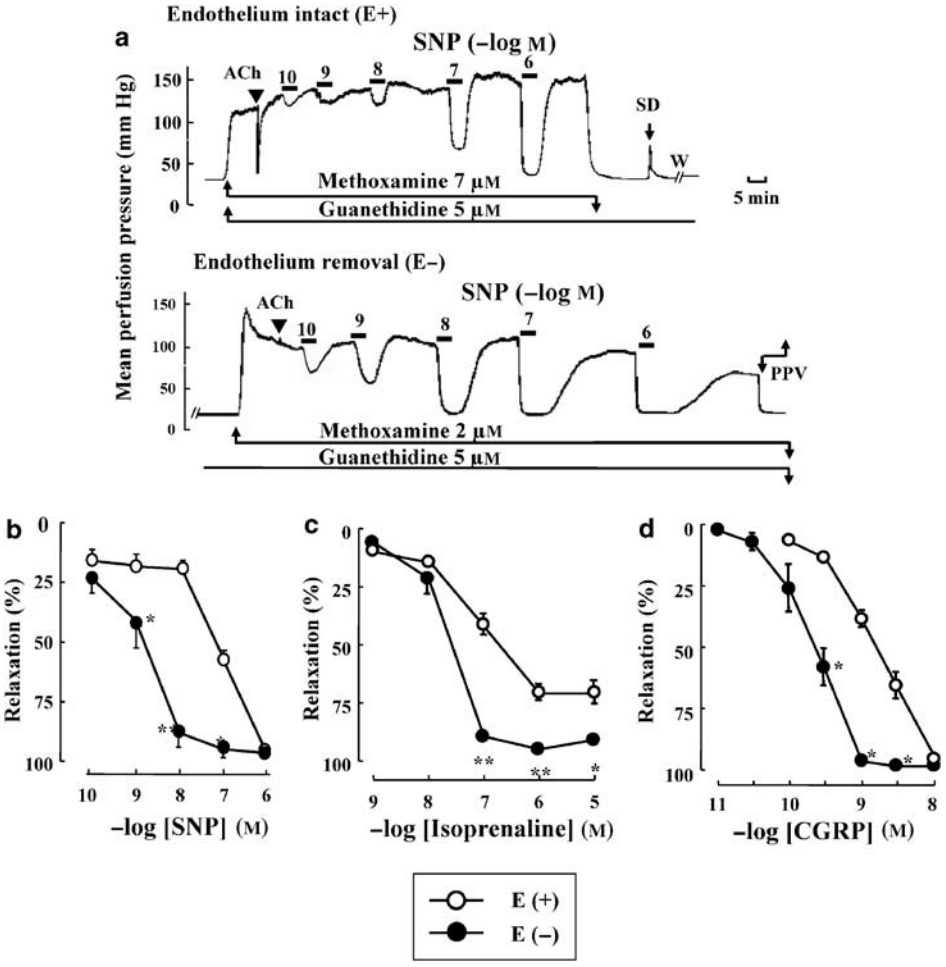

Figure 2 A typical record (**a**) and line graphs showing the effect of endothelium removal on vasodilator responses to perfusion of sodium nitroprusside (SNP) (**a** and **b**), isoprenaline (**c**) and calcitonin gene-related peptide (CGRP) (**d**) in rat perfused mesenteric vascular beds. SNP was perfused for 5 min at the times indicated by horizontal bars. ACh, bolus injection of acetylcholine (0.5 nmol); SD, perfusion of sodium deoxycholate for 30 s; W, washing with Krebs solution without SD; PPV, perfusion of papaverine (100 μM). E(+) and E(−) indicate endothelium intact and endothelium removal, respectively. Note that endothelium removal augmented the vasodilatation induced by SNP, isoprenaline and CGRP. Each point represents the mean ± s.e.mean of five experiments. *$P<0.05$, **$P<0.01$, compared with endothelium intact.

(0.01–10 nM), isoprenaline (1 nM–10 μM), SNP (0.1 nM–1 μM) and 8-Br-cGMP (0.1–100 μM) were significantly increased in the presence of L-NAME (100 μM), as shown in Figure 3 and Table 2. The vasodilator response to perfusion of SNP was augmented significantly by L-NAME, and to a greater extent than the responses induced by PNS, CGRP, isoprenaline or 8-Br-cGMP. The augmentation of SNP-induced vasodilatation in the presence of L-NAME was similar to that observed in the endothelium-denuded preparations.

Effects of endothelium removal and L-NAME on vasodilatation induced by BAY41-2272

Perfusion of BAY41-2272 (1 nM–10 μM) for 5 min in preparations with an intact endothelium caused concentration-dependent vasodilatation (Table 2). BAY41-2272-induced vasodilatation was not altered by endothelium removal (Table 2). In addition, BAY41-2272-induced vasodilatation was not affected by L-NAME (100 μM) (Table 2).

Effects of phosphoramidon and BQ-123 on vasodilatation induced by PNS and perfusion of isoprenaline, CGRP and SNP

In endothelium-intact preparations treated with phosphoramidon (10 μM) or BQ-123 (1 μM), PNS (0.5–2 Hz) or perfusion of CGRP (0.01–10 nM), isoprenaline (1 nM–10 μM) or SNP (0.1 nM–1 μM) caused frequency-dependent or concentration-dependent vasodilatation, respectively. Neither phosphoramidon (Table 2) nor BQ-123 (Table 2) affected the PNS-, CGRP-, isoprenaline- or SNP-induced vasodilator responses (Table 2).

Effects of indomethacin and seratrodast on vasodilatation induced by PNS and perfusion of isoprenaline, CGRP, SNP and BAY41-2272

In endothelium-intact preparations treated with indomethacin (0.5 μM) or seratrodast (1 μM), PNS (0.5–2 Hz) and perfusions of CGRP (0.01–10 nM), isoprenaline (1 nM–10 μM), SNP (0.1 nM–1 μM) or BAY41-2272 (1 nM–10 μM)

Table 2 Effect of endothelium removal or treatment with L-NAME, phosphoramidone, BQ-123, indomethacin or seratrodast on the potency of CGRP, isoprenaline, SNP, BAY41-2272 and 8-Br-cGMP

Treatment	*CGRP (nM)*	*Isoprenaline(nM)*	*SNP (nM)*	*BAY41-2272 (nM)*	*8-Br-cGMP (μM)*
Endothelium (+)					
First perfusion	1.63 (1.39–2.26)	190 (132–300)	47.9 (24.1–114.5)	5.32 (2.33–18.1)	425 (204–3398)
Second perfusion	1.28 (1.08–1.94) ($n=3$)	135 (80–262) ($n=3$)	38.6 (17.7–107.5) ($n=3$)	4.71 (2.21–12.9) ($n=3$)	358 (255–5576) ($n=3$)
Endothelium (+) control	1.22 (1.00–1.51)	350 (202–665)	48.0 (21.3–105)	3.57 (2.27–5.70)	300 (473–7264)
Endothelium (–)	0.22* (0.12–0.32) ($n=5$)	38.1* (16.4–76.0) ($n=5$)	0.54* (0.12–1.28) ($n=5$)	2.39 (1.52–3.67) ($n=5$)	0.78* (0.42–1.71) ($n=7$)
Endothelium (+)					
Control	1.27 (0.96–1.71)	208 (105–655)	38.8 (20.1–89.6)	8.19 (0.53–14.2)	563 (658–3173)
+L-NAME	0.63* (0.46–0.82) ($n=4$)	50.3* (24.4–134) ($n=4$)	0.99* (0.41–1.88) ($n=5$)	8.36 (5.27–15.0) ($n=5$)	0.65* (0.27–2.07) ($n=4$)
Control	0.76 (0.60–0.96)	132 (68.5–337)	56.8 (32.0–115)	ND	ND
+Phosphoramidon	0.63 (0.50–0.78) ($n=5$)	72.5 (39.7–158) ($n=5$)	24.7 (15.2–42.3) ($n=5$)	ND	ND
Control	1.21 (0.95–1.55)	97.0 (52.7–213)	25.4 (13.3–54.3)	ND	ND
+BQ-123	0.86 (0.63–1.17) ($n=5$)	23.7 (12.5–49.1) ($n=4$)	26.5 (14.2–54.9) ($n=5$)	ND	ND
Control	1.32 (1.05–1.68)	144 (75.9–370)	34.6 (17.5–82.8)	7.18 (4.19–13.7)	ND
+Indomethacin	0.24* (0.14–0.34) ($n=6$)	4.08* (2.66–6.08) ($n=6$)	4.70* (2.79–7.61) ($n=5$)	1.57* (0.82–2.66) ($n=4$)	ND
Control	1.38 (0.97–2.10)	263 (128–977)	54.6 (30.7–111)	14.6 (8.22–35.7)	ND
+Seratrodast	0.66* (0.45–0.92) ($n=5$)	66.5* (41.4–116) ($n=5$)	10.3* (5.48–19.5) ($n=5$)	1.16* (0.59–1.96) ($n=3$)	ND

Abbreviations: 8-Br-cGMP, 8-bromo-cGMP; CGRP, calcitonin gene-related peptide; *n*, number of animals used; L-NAME, N^{ω}-nitro-L-arginine methyl ester; ND, not determined; SNP, sodium nitroprusside.
The potency of CGRP, isoprenaline, SNP and BAY41-2272 is expressed as the EC_{50} (the concentration that produces 50% of the maximum response to each agonist) with a 95% CL (confidence limit) (lower CL–upper CL). *$P<0.01$ vs control.

caused frequency-dependent or concentration-dependent vasodilator responses, respectively.

The vasodilator response to PNS in the presence of indomethacin (Figure 4) or seratrodast (Figure 5) was significantly greater than that in the absence of each drug (Table 2). Similar results in the presence of indomethacin or seratrodast were observed with the responses to isoprenaline and BAY41-2272 (Figures 4 and 5 and Table 2). Additionally, the vasodilator response to CGRP was significantly augmented in the presence of indomethacin or seratrodast (Table 2).

Changes in vascular responses to an injection of acetylcholine

In preparations with an intact endothelium with active tone, a bolus injection of acetylcholine (1 nmol) induced a transient sharp decrease in perfusion pressure due to vasodilatation. As shown in Table 1, endothelium removal abolished acetylcholine-induced vasodilatation. Vasodilatation in response to acetylcholine injection was markedly reduced by L-NAME, but it was not significantly affected by phosphoramidone, BQ-123, indomethacin or seratrodast (Table 1).

Discussion

The present study demonstrated that perfusion of sodium deoxycholate in perfused mesenteric arteries with active tone abolished the rapid vasodilatation induced by acetylcholine, which has been shown to be dependent on an intact endothelium (Furchgott and Zawadzki, 1980), indicating that the function of the endothelium is effectively removed and/or eliminated by this method. In the mesenteric artery with an intact endothelium and with an active tone produced by methoxamine, PNS caused frequency-dependent vasodilatation and perfusion of various vasodilator agents induced concentration-dependent vasodilatation. However, PNS, CGRP, isoprenaline and SNP were still able to induce long-lasting vasodilatation after endothelium removal, suggesting that PNS or these agents induce endothelium-independent vasodilatation. In addition to these findings, the present study demonstrated that these vasodilator responses were markedly augmented by endothelium removal. As vascular endothelial cells release both EDRF and EDCF, it is very likely that these factors are responsible for augmentation of these vasodilator responses.

It is widely recognized that endothelium removal results in augmentation of vasoconstriction in response to vasoconstrictor agents (Moncada *et al*., 1991a; Urabe *et al*., 1991; Dora *et al*., 2000). This phenomenon has been explained as being due to the fact that endothelium removal eliminates EDRF, which counteracts the vasoconstriction induced by vasoconstrictor agents. In the present study, we used a neurogenic stimulus and various vasodilator agents that have different vasodilator mechanisms: that is, CGRP and isoprenaline, which activate adenylate cyclase and increase cAMP production; SNP, which activates sGC and increases cGMP production; and PNS, which induces neurogenic CGRP release. All vasodilator responses to PNS and vasodilator agents used in the present study were augmented by endothelium removal. Therefore, it is likely that the absence of endothelial cells, rather than some altered intracellular

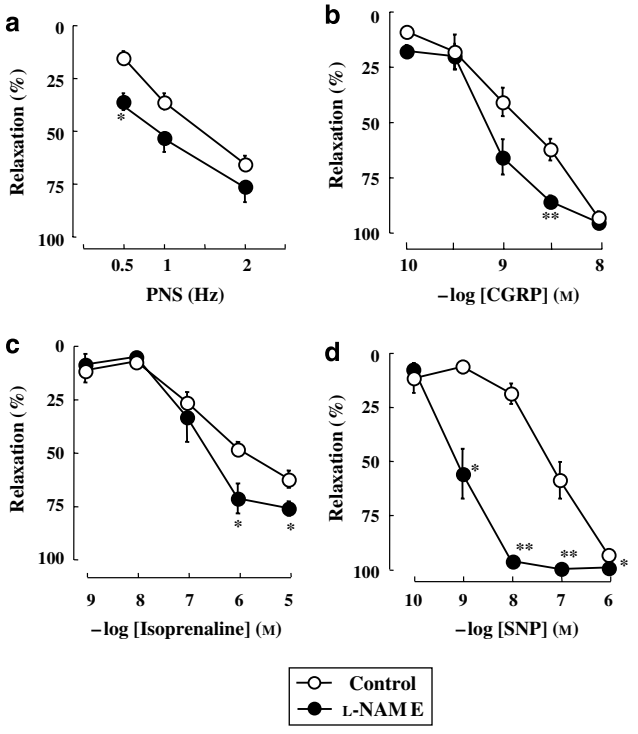

Figure 3 Effect of N^{ω}-nitro-L-arginine methyl ester (L-NAME; 100 μM) on vasodilator responses to periarterial nerve stimulation (PNS) (**a**) and perfusion of calcitonin gene-related peptide (CGRP) (**b**), isoprenaline (**c**) and sodium nitroprusside (SNP) (**d**) in rat perfused mesenteric vascular beds with an intact endothelium and active tone. The presence of L-NAME augmented the vasodilatation induced by PNS, CGRP, isoprenaline and SNP. Each point represents the mean ± s.e.mean of four to five experiments. *$P<0.05$, **$P<0.01$, compared with control.

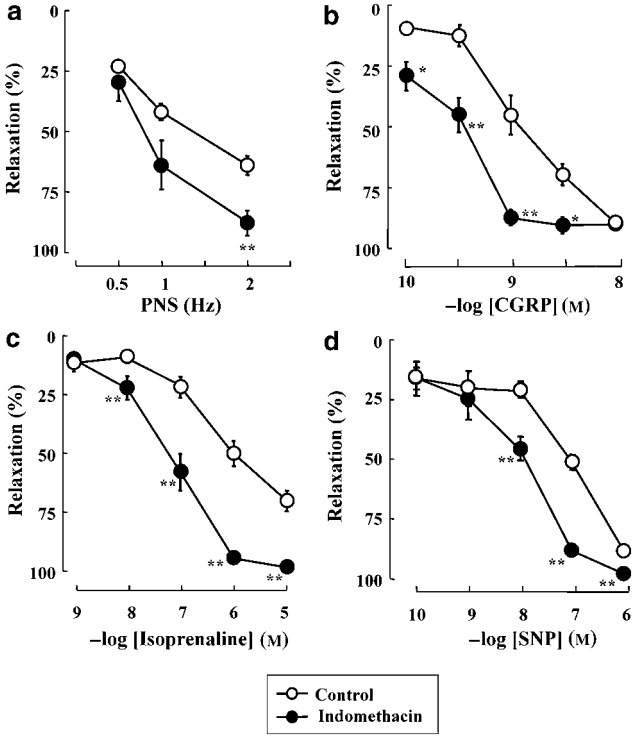

Figure 4 Effect of indomethacin (0.5 μM) on vasodilator responses to periarterial nerve stimulation (PNS) (**a**) and perfusion of calcitonin gene-related peptide (CGRP) (**b**), isoprenaline (**c**) and sodium nitroprusside (SNP) (**d**) in rat perfused mesenteric vascular beds with an intact endothelium and active tone. The presence of indomethacin augmented vasodilatation induced by PNS, CGRP, isoprenaline and SNP. Each point represents the mean ± s.e.mean of five to six experiments. *$P<0.05$, **$P<0.01$, compared with control.

mechanisms inside smooth muscle cells, is essential to the augmentation of these responses. It is conceivable that EDCF is involved in the augmentation of vasodilator responses seen after endothelium removal. As in the case of EDRF, it appears that endothelium removal eliminates EDCF, which counteracts vasodilatation in response to vasodilator agents, and, as a result, vasodilator agents cause excessive vasodilator responses due to the lack of EDCF.

It is well known that endothelial cells serve as a barrier that reduces the ability of vasoactive agents to reach smooth muscle cells (Husain and Moss, 1988). It is assumed that loss of the barrier by the removal or destruction of endothelial cells may result in a high concentration of vasoactive agents reaching the blood smooth muscle cells, and that these vasoactive agents in preparations without an endothelium can induce greater vascular responses compared with responses in preparations with an intact endothelium. However, this was ruled out by the present finding that endothelium removal augmented the vasodilator response to PNS, which is mediated by endogenous CGRP released from CGRPergic nerves. Results similar to the present findings were obtained by Ralevic (2002); endothelium removal was found to augment the PNS- and SNP-induced vasodilatation, but not CGRP-induced vasodilatation, in the mesenteric vascular beds. Because CGRPergic nerves innervate the adventitia layers outside the artery, CGRP released from CGRPergic nerves could reach blood smooth muscle cells without passing the endothelial cells inside the artery. The finding that endothelium removal augmented neuronally evoked vasodilatation indicates that the ease with which various agents can pass endothelial cells is not the main cause of the augmented vasodilator response in denuded preparations.

It should be noted that L-NAME, as observed in preparations with endothelium removal, markedly augmented the vasodilator response to the NO donor SNP even in preparations with an intact endothelium. Similar results were obtained by Moncada *et al*. (1991b); they showed that endothelium removal or inhibition of basal NO production by L-NAME in rat aortic rings resulted in enhanced vasodilator responses to nitrovasodilators, including SNP, but to a lesser extent to isoprenaline or 8-Br-cGMP. From these results, they suggested that the removal of NO in the vasculature leads to an increase in the sensitivity to vasodilators that act by stimulating sGC. On the basis of this notion, as perfused mesenteric arteries with an intact endothelium are constantly exposed to NO released by shear stress, it is likely that elimination of NO in the presence of L-NAME upregulates sGC activity in vascular smooth muscle cells and under this condition NO donated by SNP results in exaggerated vasodilator responses. However, in the present study the vasodilatation induced by BAY41-2272, which selectively activates sGC (Stasch *et al*., 2001), was not

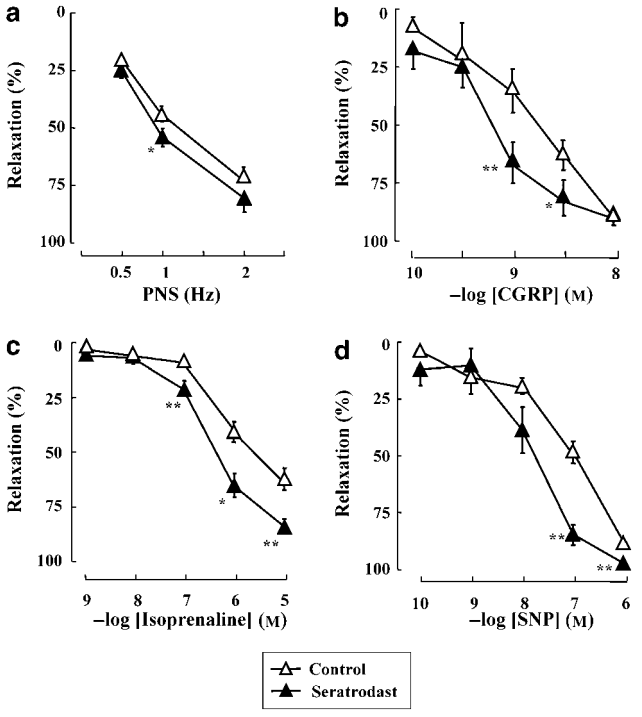

Figure 5 Effect of seratrodast (1 μM) on vasodilator responses to periarterial nerve stimulation (PNS) (**a**) and perfusion of calcitonin gene-related peptide (CGRP) (**b**), isoprenaline (**c**) and sodium nitroprusside (SNP) (**d**) in rat perfused mesenteric vascular beds with an intact endothelium and active tone. The presence of seratrodast augmented the vasodilatation induced by PNS, CGRP, isoprenaline and SNP. Each point represents the mean ± s.e.mean of five to six experiments. *$P<0.05$, **$P<0.01$, compared with control.

enhanced by endothelium removal or L-NAME. These findings suggest that the activity of sGC in the mesenteric resistance artery is not influenced by endothelium removal or the elimination of basal NO. It is likely that endothelium removal affects the activity of the cellular transduction system that acts downstream sGC. This notion is supported by the present findings that endothelium removal and L-NAME treatment resulted in augmented vasodilator response to 8-Br-cGMP, a substance that acts directly on protein kinase G downstream of sGC. However, increased activity downstream of sGC should have induced an augmented vasodilator response to BAY41-2272, but this was not the case in the present study. Therefore, it is assumed that BAY41-2272 may act on a different guanylate cyclase, which is activated by NO. However, BAY41-2272 has been shown to activate the same enzyme as NO (Dumitrascu *et al.*, 2006). Reports that BAY41-2272 has endothelium-dependent vasodilator activity and releases NO from endothelial cells (Priviero *et al.*, 2005) may explain the present findings showing no changes in BAY41-2272-induced vasodilatation after endothelium removal and treatment with L-NAME. From these findings, it is inferred that endothelium removal and L-NAME eliminate BAY41-2272-induced endothelium-dependent vasodilatation and decrease the vasodilator response, but the treatments augment the endothelium-independent vasodilatation induced by BAY41-2272 and so increase the response. Therefore, it is likely that NO elimination due to endothelium removal and L-NAME result in no change in the vasodilator response to BAY41-2272 due to the simultaneous occurrence of both inhibitory and augmenting effects, which are of equal potency.

It was interesting to find in the present study that endothelium removal enhanced the vasodilatation induced by isoprenaline and CGRP, which acts by stimulating adenylate cyclase. Therefore, another possible explanation for the results is that endothelium removal results in increased sensitivity to adenylate cyclase, probably due to the loss of EDCF. However, L-NAME treatment also enhanced vasodilatation induced by isoprenaline and CGRP; therefore, it is unlikely that L-NAME prevents EDCF release. It is more likely that protein kinase G, a downstream sGC that has been shown to have an important role in both cGMP and cAMP-mediated relaxation (Dhanakoti *et al.*, 2000), is responsible for the augmentation. NO elimination by L-NAME seems to produce altered protein kinase G activity of downstream sGC, and the alteration may induce increased sensitivity to adenylate cyclase leading to increased intracellular production of cAMP, resulting in augmented vasodilatation.

Vasodilatation induced by PNS, CGRP, isoprenaline and SNP in preparations with an intact endothelium was significantly augmented in the presence of indomethacin (an inhibitor of cyclooxygenase). It seems likely that arachidonic acid cascades, and especially prostaglandin I_2, which is metabolized through cyclooxygenase and is an EDRF, are involved in the enhanced vasodilator responses to various vasodilator agents following endothelium removal. Prostaglandin I_2 is produced in endothelial cells and causes a vasodilator response by increasing the level of intracellular cAMP in the vascular smooth muscle cells. Therefore, inhibition of prostaglandin I_2 production by indomethacin may lead to increased sensitivity to vasodilator agents (CGRP and isoprenaline), which act by stimulating adenylate cyclase. However, this notion cannot explain the finding that treatment with indomethacin augments SNP-induced vasodilatation, which is mediated by cGMP formation as a result of stimulating sGC. Treatment with seratrodast (TXA_2 receptor antagonist) augmented the vasodilatation induced by PNS, CGRP, isoprenaline and SNP in the mesenteric arteries with an intact endothelium, although the degree of augmentation was lower than that caused by indomethacin. This finding suggests that TXA_2, an EDCF, counteracts the vasodilatation induced by various vasodilator agents and that elimination of TXA_2, which induces potent vasoconstriction, by indomethacin and seratrodast results in increased vasodilator responses to these agents. This hypothesis is supported by the present finding that indomethacin and seratrodast augmented the vasodilatation induced by BAY41-2272, which resulted in no change after treatment with L-NAME.

In contrast, in the present study, it was found that phosphoramidon (an endothelin-1-converting enzyme inhibitor) and an endothelin receptor antagonist did not affect the vasodilatation induced by the various vasodilator agents studied or to PNS in mesenteric arteries with an intact endothelium. Therefore, it is unlikely that the potent EDCF endothelin is involved in the augmentation of vasodilatation observed after endothelium removal.

Conclusion

These results suggest that the endothelium in the mesenteric resistance artery regulates and maintains vascular tone by counteracting not only vasoconstriction through the release of EDRFs, but also vasodilatation in part through the releasing of an EDCF, TXA_2.

Acknowledgements

This paper is dedicated to the memory of Ms Yukiko Iwatani, who was working on the present study and passed away suddenly on 11 November 2007.

Conflict of interest

The authors state no conflict of interest.

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