Role of Endothelium-derived Hyperpolarizing Factor in Phenylephrine-induced Oscillatory Vasomotion in Rat Small Mesenteric Artery

Kayoko Okazaki, M.D.,* Sumihiko Seki, M.D.,† Noriaki Kanaya, M.D.,‡ Jun-ichi Hattori, M.D.,* Noritsugu Tohse, M.D., Ph.D.,§ Akiyoshi Namiki, M.D., Ph.D.¶

Background: In small mesenteric arteries, endothelium-derived hyperpolarizing factor (EDHF) in addition to endothelium-derived relaxing factors (EDRFs) including NO plays an important role in acetylcholine-induced vasodilation. It has been reported that EDRFs play an important role in α1-adrenoceptor agonist-induced oscillatory vasomotion and in limiting vasoconstrictor response to the agonists; however, contribution of EDHF to the α1-agonist-induced oscillation is unknown.

Methods: Rat small mesenteric arteries were isolated and cannulated at each end with a glass micropipette. The vessels were immersed in a bath (37°C) containing physiologic saline solution. Changes in vessel diameter were measured using an optical density video detection system.

Results: Denudation of the endothelium and inhibition of NO synthesis caused a leftward shift in the concentration-response relation for phenylephrine in the mesenteric arteries, whereas inhibition of cyclooxygenase by indomethacin had no effect. Blockade of Ca2+-activated K+ (KCa) channels by charybdotoxin and apamin caused a further leftward shift in the concentration-response relation in the vessels pretreated with Nω-nitro-L-arginine methyl ester and indomethacin. Phenylephrine at concentrations higher than 10−6 M caused endothelium-dependent oscillatory vasomotion, which was reduced but not abolished after combined inhibition of the cyclooxygenase and NO synthase pathways. However, the KCa channel blockers completely abolished the remaining component of oscillation.

Conclusions: Endothelium-derived NO is an important modulator of sustained agonist-induced vasoconstriction. NO, as well as endothelially-derived cyclooxygenase products and EDHF, also contribute significantly to phenylephrine-induced oscillatory vasomotion.

Vasoconstrictor response of small mesenteric arteries to α1-adrenoceptor stimulation results in reduction in intestinal microcirculation. However, endothelium-derived relaxing factors (EDRFs) such as NO have been shown to counterbalance or attenuate the α1-adrenoceptor-mediated constriction. In rat small mesenteric arteries, the contractile responses to sympathetic nerve stimulation and α1-adrenoceptor agonists are augmented by denudation of the endothelium or inhibition of NO synthase. The role of NO as an important modulator of α1-adrenergic constriction has also been demonstrated in the rat muscle and canine pulmonary arteries. Therefore, NO may contribute to maintenance of an adequate blood supply to organs during stimulation of the sympathetic nervous system. The α1-adrenoceptor agonists induce rhythmic oscillatory vasomotion in small mesenteric arteries. This oscillation may also provide advantages in the local flexible control of tissue perfusion during sympathetic activation. Gustafsson et al. reported that α1-agonist-induced oscillation was mediated by endothelium-derived NO, since norepinephrine-induced oscillatory vasomotion was abolished by inhibition of the endothelium-dependent relaxation system.

Endothelium-derived hyperpolarizing factor (EDHF) is known as another mediator of endothelium-dependent vasodilation. In acetylcholine-induced vasodilation, EDHF in addition to NO plays an important role in small mesenteric arteries. Recent study illustrates that phenylephrine, a selective α1-adrenoceptor agonist, induced oscillatory vasomotion, which remained after inhibition of NO synthase. This finding suggests that EDHF plays a role in the α1-agonist-induced oscillation. However, little is known about the attenuating effect of EDHF on the vasoconstrictor response to α1-adrenoceptor agonists.

The purpose of this study was to compare the influence of endothelially-released NO, cyclooxygenase products and EDHF on modulating phenylephrine-induced vasoconstriction and oscillatory vasomotion in vitro perfused mesenteric arteries.

Materials and Methods

Vessel Preparation

With approval from the Sapporo Medical University Animal Care and Use Committee, male Sprague-Dawley rats (6–9 weeks old) were anesthetized with ether and exsanguinated by hemorrhage. A section of the mesentry 5–10 cm distal to the pylorus was rapidly removed and placed in oxygenated cold physiologic saline solution (PSS). Segments of third or fourth branches of mesenteric arteries (~200 μm maximum diameter) were carefully isolated and removed the connective tissues.

Diameter Measurement Experiment

Using an optical density video detection, we measured changes in internal diameters of vessels with controlled intraluminal pressure. This method may be more suitable than other in vitro methods for investigation of physiologic vasomotion.
After each dissected vessel (~5 mm in length) was placed in a microvessel chamber filled with PSS, both the proximal and distal ends were cannulated with a glass micropipette and secured with 10-0 nylon sutures. After securing the proximal end, the lumen was gently perfused to flush and clear the vessel of clotted blood before securing the distal end. Then the PSS in the chamber was continually circulated from a reservoir in which the solution was aerated with a 95% O₂ - 5% CO₂ mixture. The volume of the chamber and reservoir was 100 ml, and the rate of flow of the suffusing solution was 15 ml/min. The PSS was heated before passing into the chamber in order to maintain the bath temperature at 37°C using an automatic temperature controller (TC-324B, Warner Instrument, Hamden, CT). The chamber was placed on the stage of an inverted microscope (IX70, Olympus, Tokyo, Japan) connected to a video camera (WAT-308A, WATEC, Yamagata, Japan), and the vessel image was projected onto a television screen (Sony, Tokyo, Japan). The changes in vessel internal diameters were measured using a video dimension analyzer system (Living Systems Instrumentation, Burlington, VT). Measurements of diameters were recorded using a personal computer connected to the analyzer.

Experimental Protocols

Intraluminal pressure was kept constant at 50 mmHg by a pressure servo-control pump in a no-flow state to exclude the influence of shear stress. After a 30-min equilibration period, each vessel was initially constricted with 60 mM potassium solution (equimolar substitution of KCl by NaCl in PSS). After removal of the high-potassium solution and return of the diameters to prestimulation values, phenylephrine concentration-response relationships under various conditions were obtained for each vessel by increasing the concentration of phenylephrine in half-log increments (10⁻⁸ to 3 × 10⁻³ M) after the response to each preceding concentration had reached a steady state.

Protocol 1: Effects of Endothelium Denudation on Phenylephrine-induced Vasocostriction and Oscillatory Vasomotion. First, we compared the responses to phenylephrine in vessels with and without endothelium to assess the roles of endothelium function in the modulation of phenylephrine-induced vasoconstriction and oscillatory vasomotion. The endothelium was removed by inserting a hair into the lumen before the cannulation with micropipettes. Removal of the endothelium was verified by loss of vasodilator response to 10⁻⁶ M of acetylcholine after administration of the highest concentration of phenylephrine.

Protocol 2: Effects of α₁-Adrenoceptor Antagonist on Phenylephrine-induced Vasocostriction and Oscillatory Vasomotion. To confirm that the phenylephrine-induced constriction is mediated by α₁-adrenoceptor activation, endothelium-intact vessels were incubated with an α₁-adrenoceptor antagonist, prazosin (10⁻⁶ M).

Protocol 3: Effects of Inhibitors of Endothelium-derived Mediators on Phenylephrine-induced Vasocostriction and Oscillatory Vasomotion. A third series of experiments was performed to identify the specific endothelium-derived mediators involved in the modulation of vasoconstrictor response and the oscillatory vasomotion. For 30 min before the administration of phenylephrine, endothelium-intact vessels were incubated with one or both of the following pharmacologic inhibitors: Nω-nitro-L-arginine methylester (L-NAME; 10⁻⁴ M), an inhibitor of NO synthase, and indomethacin (3 × 10⁻⁵ M), an inhibitor of cyclooxygenase.

Protocol 4: Effects of EDHF Inhibition on Phenylephrine-induced Vasocostriction and Oscillatory Vasomotion. To assess the role of EDHF in phenylephrine-induced oscillatory vasomotion, a fourth series of experiments was performed using endothelium-intact vessels in the presence of indomethacin (3 × 10⁻⁵ M) and L-NAME (10⁻⁷ M).

Although the nature of EDHF is still unresolved, recent evidences indicate that activation of the small and intermediate conductance Ca²⁺-activated K⁺ (KCa) channels on endothelial cells is one of the proposed mechanisms regulating EDHF release. 4,12-17 Therefore, the intralumens of endothelium-intact vessels were incubated with a combination of apamin (5 × 10⁻⁷ M, an inhibitor of small conductance KCa channels) and charybotoxin (10⁻⁷ M, an inhibitor of intermediate conductance KCa channels) for 30 min before the administration of phenylephrine. In this experiment, apamin and charybotoxin were perfused intraluminally to apply to the endothelium, and intraluminal pressure was maintained at 45 mmHg by hydrostatic pressure.

Solutions and Drugs

The PSS comprised the following: NaCl, 119 mM; KCl, 4.7 mM; NaHCO₃, 25 mM; CaCl₂, 2.5 mM; KH₂PO₄, 1.18 mM; MgSO₄, 1.17 mM; glucose, 5.5 mM; EDTA, 0.026 mM. The following drugs and chemicals were used: phenylephrine, acetylcholine chloride, indomethacin, L-NAME, prazosin, apamin and charybotoxin (Sigma Chemical, St. Louis, MO). All drug concentrations are expressed as final molar concentrations in the vessel chamber. Indomethacin was dissolved in ethanol (100%) and then diluted in distilled water to obtain the desired concentrations (final vessel chamber ethanol concentration was 0.075%). All other drugs were dissolved in distilled water.

Data Analysis

Values are expressed as the mean ± SD. Responses to phenylephrine are expressed as a percentage of the
vasoconstrictor response to 60 mm potassium solution. When endothelium-intact vessels exhibited oscillatory vasomotion, both maximum and minimum values were plotted. Amplitude of the phenylephrine-induced oscillatory vasomotion was calculated by subtracting the minimum value from the maximum value. The effects of denudation and the inhibitors on the concentration-response curves to phenylephrine were assessed by calculating the concentration of phenylephrine causing 50% of the maximal response (ED$_{50}$). This value was interpolated from the linear portion of the concentration-response curve by regression analysis and is presented as log ED$_{50}$. Statistical analyses of the data were performed using the two-tailed Student $t$ test for unpaired comparison. When more than two mean values were compared, analysis of variance and the Tukey-Kramer test were used. Values were considered to be significant when $P$ was less than 0.05.

**Results**

*Effects of Endothelium Denudation on Phenylephrine-induced Vasoconstriction and Oscillatory Vasomotion*

The mean baseline diameter of endothelium-intact vessels was not significantly different from the mean baseline diameter of endothelium-denuded vessels. Phenylephrine caused vasoconstriction in a concentration-dependent manner in both endothelium-intact and endothelium-denuded vessels (figs. 1 and 2). However, denudation of the endothelium significantly ($P < 0.05$) decreased the ED$_{50}$ for phenylephrine (log ED$_{50}$ = $-6.46 \pm 0.03$, $n = 6$) compared to that in endothelium-intact vessels (log ED$_{50}$ = $-6.07 \pm 0.03$, $n = 6$), indicating that the endothelium functioned as a modulator of phenylephrine-induced vasoconstriction. As shown in figure 1A, endothelium-intact vessels exhibited oscillatory vasoconstriction at concentrations of phenylephrine higher than 10$^{-6}$ M. In contrast, endothelium-denuded vessels showed a steady response to phenylephrine, without any rhythmic activity (fig. 1B). Figure 2B illustrates the amplitude of the oscillation at 10$^{-5}$ M phenylephrine. The endothelium denudation completely abolished the phenylephrine-induced oscillation. The peak of oscillatory vasomotion at 10$^{-5}$ M phenylephrine in endothelium-intact vessels (105 ± 8%) was equal to tonic vasoconstriction in endothelium-denuded vessels (105 ± 3%). Therefore, it is suggested that this oscillatory vasomotion is due to endothelium-dependent vasodilator mechanisms.

*Effects of $\alpha_1$-Adrenoceptor Antagonist on Phenylephrine-induced Vasoconstriction and Oscillatory Vasomotion*

Baseline diameters were not significantly different before and after incubation of the vessels with prazosin, an $\alpha_1$-adrenoceptor antagonist. Prazosin caused a parallel rightward shift in the phenylephrine concentration-response curve (fig. 3A) and significantly ($P < 0.05$) increased the ED$_{50}$ (log ED$_{50}$ = $-5.82 \pm 0.16$, $n = 5$) compared to the control curve and ED$_{50}$ (log ED$_{50}$ = $-6.12 \pm 0.03$, $n = 5$). This finding indicates that the vasoconstrictor response to phenylephrine is selectively mediated by $\alpha_1$-adrenoceptor activation. As shown in figure 3B, prazosin also shifted the concentration of phenylephrine at which the oscillatory vasomotion was initiated (10$^{-6}$ M in control vessels and 3 $\times$ 10$^{-6}$ M in prazosin-incubated vessels). At concentrations of phenylephrine higher than 3 $\times$ 10$^{-6}$ M, there were no significant differences between the amplitudes of oscillation in control and prazosin-treated vessels (fig. 3B). Therefore, it is suggested that the phenylephrine-induced oscillation is mediated by $\alpha_1$-adrenergic receptors.
The effects of the endothelial inhibitors on phenylephrine-induced oscillatory vasomotion are summarized in figure 5. Either indomethacin alone or L-NAME alone significantly decreased the amplitude of the oscillatory vasomotion at $10^{-5}$ M phenylephrine (fig. 5). A combination of both inhibitors also decreased, but did not abolish the oscillatory vasomotion (fig. 5).

These results suggest that endothelial modulation of phenylephrine-induced vasoconstriction is mediated by NO but not by cyclooxygenase products. On the other hand, the phenylephrine-induced oscillatory vasomotion may involve a cyclooxygenase-dependent, NO-dependent, and other mechanisms.
Effects of EDHF Inhibition on Phenylephrine-induced Oscillatory Vasomotion

In the vessels incubated with a combination of indomethacin and l-NAME, it is expected that EDHF is responsible for the remaining component of phenylephrine-induced oscillatory vasomotion. Since recent studies indicate that EDHF-mediated vasodilation depends on activation of $K_{Ca}$ channels having small and intermediate conductance on endothelial cells,$^{12,17}$ a combination of apamin and charybdotoxin was applied into the lumens of endothelium-intact vessels. Consistent with previous reports,$^{14,18}$ this treatment did not alter the base-

Fig. 4. Effects of (A) cyclooxygenase inhibition by indomethacin (INDO, $3 \times 10^{-5}$ M), (B) NO synthase inhibition by l-NAME ($10^{-4}$ M) and (C) combined inhibition on vasoconstrictor response to phenylephrine ($n = 5$). Vasoconstrictions are expressed as percentages of $60 \text{mM KCl}$ constriction and are presented as the mean ± SD. When the vessels exhibited oscillation, both maximum (squares and solid line) and minimum (circles and dotted line) values were plotted.

Effects of EDHF Inhibition on Phenylephrine-induced Vasoconstriction and Oscillatory Vasomotion

In the vessels incubated with a combination of indomethacin and l-NAME, it is expected that EDHF is responsible for the remaining component of phenylephrine-induced oscillatory vasomotion. Since recent studies indicate that EDHF-mediated vasodilation depends on activation of $K_{Ca}$ channels having small and intermediate conductance on endothelial cells,$^{12,17}$ a combination of apamin and charybdotoxin was applied into the lumens of endothelium-intact vessels. Consistent with previous reports,$^{14,18}$ this treatment did not alter the base-

Fig. 5. (A) Typical records of oscillatory vasomotion at $10^{-5}$ M phenylephrine in vessels pretreated with no inhibitor (control), l-NAME ($10^{-4}$ M) alone, indomethacin (INDO, $3 \times 10^{-5}$ M) alone, and a combination of l-NAME and indomethacin. Arrows indicate amplitudes of oscillatory vasomotion. (B) Amplitude of $10^{-5}$ M phenylephrine-induced oscillation are summarized ($n = 5$). *Significantly different from control ($P < 0.05$).
line diameter. In these conditions, a greater constriction was observed for the same dose of phenylephrine (fig. 6A) and the ED$_{50}$ was significantly ($P < 0.05$) decreased (log ED$_{50}$ = −6.46 ± 0.10, n = 5) compared to the control value (log ED$_{50}$ = −6.19 ± 0.10, n = 5). The phenylephrine-induced oscillation was abolished completely by the K$_{Ca}$ blockers (figs. 6B and 6C), indicating that the remaining component of oscillation in the present study was mediated by EDHF.

**Discussion**

Vascular endothelium has been implicated as an important modulator of $\alpha$-adrenergic constriction. The specific mechanisms through which the endothelium exerts this modulation may involve the release of NO, cyclooxygenase products, and EDHF. In the present study, NO synthase inhibition, but not cyclooxygenase inhibition, caused a leftward shift in the phenylephrine concentration-response curve. This indicates that NO is an important modulator of phenylephrine-induced constriction in the rat mesenteric artery. In contrast to the abundance of information on NO and cyclooxygenase products, little is known about the role of EDHF during $\alpha_1$-adrenoceptor-mediated vasoconstriction. Tuttle et al. reported that the presence of tetrabutylammonium, which has been thought to block K$_{Ca}$ channels, did not alter the $\alpha$-adrenergic vasoconstriction in rat skeletal muscle arteries. In the rat small mesenteric artery, Dora et al. reported that blockade of K$_{Ca}$ channels by charybdotoxin and apamin increased the constrictor response to phenylephrine. Consistent with the latter report, we observed that a combination of charybdotoxin and apamin caused a leftward shift in the phenylephrine concentration-response curve. The present results suggest that not only NO but also EDHF is involved in the reduction of $\alpha_1$-adrenergic-stimulated tone in the rat small mesenteric artery.

Agonist-induced oscillatory vasomotion in small arteries is observed in most tissues and species and can be produced via both endothelium-independent and dependent mechanisms. We demonstrated that phenylephrine at concentrations higher than 10$^{-6}$ M induced spontaneous oscillatory vasomotion in an endothelium-dependent manner. The peak constriction during oscillation in the endothelium-intact vessels was equal to tonic constriction in the endothelium-denuded vessels.
suggesting that the oscillation is due to rhythmic vaso-
dilatation mediated by an endothelium-dependent mecha-
nism. Gustafsson et al.\(^9\) reported that norepinephrine-
induced tension oscillation was abolished by NO
synthesis inhibition in the rat small mesenteric artery,
indicating that the oscillation is mediated mainly by
release of NO from the endothelium. In the present
study, however, we found that not only NO synthesis
inhibition but also cyclooxygenase inhibition attenuated
the oscillation. Furthermore, the oscillation still re-
mained even after the combined inhibition of the NO
synthase and cyclooxygenase pathways. The oscillation
that remained was abolished by additional blockade of
K\(_{Ca}\) channels by charybotoxin and apamin. Therefore,
besides the role of NO, these results indicate important
roles of cyclooxygenase products and EDHF in the en-
dothelium-dependent oscillation. Consistent with our
findings, Dora et al.\(^4\) observed that the phenylephrine-
induced oscillation that remained after NO synthase
inhibition was abolished by K\(_{Ca}\) channel blockers in the rat
small mesenteric artery, although they did not describe
about that in detail. Furthermore, in the rabbit ear artery,
phenylephrine-induced oscillation was inhibited by
charybotoxin but not by NO synthesis inhibition.\(^25\)
These studies support our speculation that EDHF is in-
volved in the mechanisms of phenylephrine-induced os-
cillatory vasomotion.

The mechanisms of the release of endothelial vasodi-
lators that contribute to phenylephrine-induced oscill-
a are still unknown. Since the release of endothelium-
derived factors depends on the increase in endothelial
[Ca\(_{2+}\)]\(_i\), it is postulated that phenylephrine-induced e-
volution of endothelial [Ca\(_{2+}\)]\(_i\), and consequent release of
endothelial vasodilators may contribute to the oscillation.
In fact, it has been reported that \(\alpha_1\)-adrenergic agonists, including norepinephrine\(^6\) and phenyleph-
Mine, increase endothelial [Ca\(_{2+}\)]\(_i\) in isolated vessels.
Although there is no evidence of the oscillatory release of
endothelium-derived factors, tension oscillation ac-
companied by endothelial [Ca\(_{2+}\)]\(_i\) oscillation was ob-
served in the rat tail artery.\(^24\) This may support the
concept that a rise in endothelial [Ca\(_{2+}\)]\(_i\) is key event for
phenylephrine-induced oscillation. However, phenyl-
ephrine failed to directly increase [Ca\(_{2+}\)]\(_i\) in endothelial
cells freshly isolated from small mesenteric arteries.\(^4\)
Therefore, it is suggested that the increase in [Ca\(_{2+}\)]\(_i\) is
due to indirect action of phenylephrine on endothelial
cells. Recently, it has been suggested that a rise in vas-
cular smooth muscle [Ca\(_{2+}\)]\(_i\), stimulated by phenyleph-
rine may diffuse to underlying endothelial cells through
myoendothelial gap junctions.\(^5,18\) Tuttle et al.\(^6\) dem-
onstrated that the vasoconstrictor prostaglandin F\(_{2\alpha}\) did not
increase endothelial [Ca\(_{2+}\)]\(_i\) in rat muscle arteries. In this
study, norepinephrine produced a large transient peak in
vascular smooth muscle [Ca\(_{2+}\)]\(_i\), whereas the vasocon-
strictor prostaglandin F\(_{2\alpha}\) produced a rise in vascular
smooth muscle [Ca\(_{2+}\)]\(_i\), without a peak. In our prelimi-
nary experiments, prostaglandin F\(_{2\alpha}\) induced a tonic
vasoconstriction with only a small oscillation in rat small
mesenteric arteries. Therefore, it is predicted that a large
transient increase in vascular smooth muscle [Ca\(_{2+}\)]\(_i\) will
be followed by an elevation of endothelial [Ca\(_{2+}\)]\(_i\) and
consequent release of vasodilators, which may contrib-
tute to the oscillatory vasomotion. Many previous studies
have indicated that \(\alpha_1\) and \(\beta\)-adreceptors on endothe-

cial cells may play a physiologic role in the regulation of
vasomotor tone.\(^25\) However, activation of these endothe-

dial adrenoreceptors by phenylephrine may not contribute
to the oscillation mainly, because phenylephrine does
not directly increase endothelial [Ca\(_{2+}\)]\(_i\), in endothelial
cells freshly isolated from small mesenteric arteries.\(^4\)

The precise nature of EDHF is still a matter of great
debate and may involve more than one factor. Cyto-
chrome P450 metabolites (EETs) have been considered
as potential candidates for EDHF.\(^20,27\) However, cyto-
chrome P450 inhibition by SKF525A did not alter the am-
plitude of phenylephrine-induced oscillation in our pre-
liminary experiments. Recently, many studies\(^12,15,16,17,28\) have
suggested that a rise in endothelial [Ca\(_{2+}\)]\(_i\) elicit opening
of endothelial K\(_{Ca}\) channels and that the consequent
hyperpolarization is conducted to smooth muscle via
myoendothelial gap junctions. We speculate therefore
that the EDHF-mediated component of phenylephrine-
induced oscillation is secondary to increase in vascular
smooth muscle [Ca\(_{2+}\)]\(_i\). The Ca\(_{2+}\) diffusion from smooth
muscle through myoendothelial gap junctions may elicit
opening of endothelial K\(_{Ca}\) channels, and the con-
sequent hyperpolarization conducted to smooth muscle
via myoendothelial gap junctions may contribute to the
EDHF-mediated component of oscillation.

To the extent that our findings in isolated small mes-
enteric arteries may apply in the intact intestinal circu-
lation, the oscillatory vasomotion may provide advan-
tages in the regional control of intestinal perfusion\(^8,29\) in
patients with circulatory shock treated with a high dose
of \(\alpha_1\)-adrenergic agonists. In addition, our finding of the
EDHF-mediated oscillation may provide a suitable model
for studying the effects of anesthetics on physiologic
function of EDHF. In many previous studies, acetyl-
choline has been used to stimulate EDHF release; however,
itis not a physiologic mediator of release of EDHF.

In conclusion, the endothelium plays an important role
in modulation of phenylephrine-induced vasoconstric-
tion in the rat mesenteric artery. Phenylephrine at con-
centrations higher than 10\(^{-6}\) m produces endothelium-
dependent oscillatory vasomotion, which is partly
mediated by EDHF.

References

1. Nase GP, Boegehold MA: Nitric oxide modulates arteriolar responses to