Role of Endothelium-Derived Hyperpolarizing Factor in Human Forearm Circulation

Kosuke Inokuchi, Yoshitaka Hirooka, Hiroaki Shimokawa, Koji Sakai, Takuya Kishi, Koji Ito, Yoshikuni Kimura, Akira Takeshita

Abstract—Endothelium-derived hyperpolarizing factor (EDHF) contributes to endothelium-dependent relaxation of isolated arteries, but it is not known whether this also occurs in the case of humans in vivo. The present study examined the role of EDHF in human forearm circulation. Forearm blood flow (FBF) was measured by strain-gauge plethysmography in 31 healthy, normal subjects (mean±SE age, 23±2 years; 24 men and 7 women). After oral administration of aspirin (486 mg), we infused N\textsuperscript{6}-monomethyl-L-arginine (8 μmol/min for 5 minutes) into the brachial artery. We used tetraethylammonium chloride (TEA, 1 mg/min for 20 minutes), a K\textsubscript{Ca} channel blocker, as an EDHF inhibitor, and nicorandil as a direct K\textsuperscript{+} channel opener. TEA significantly reduced FBF (P<0.05) but did not change systemic arterial blood pressure. Furthermore, TEA significantly inhibited the FBF increase in response to substance P (0.8, 1.6, 3.2, and 6.4 ng/min, n=8) and bradykinin (12.5, 25, 50, and 100 ng/min, n=8; both P<0.001), whereas it did not affect the FBF increase in response to acetylcholine (4, 8, 16, and 32 μg/min, n=8), sodium nitroprusside (0.4, 0.8, 1.6, and 3.2 μg/min, n=8), or nicorandil (0.128, 0.256, 0.512, and 1.024 mg/min, n=8). These results suggest that EDHF contributes substantially to basal forearm vascular resistance, as well as to forearm vasodilation evoked by substance P and bradykinin in humans in vivo. (Hypertension. 2003;42:919-924.)

Key Words: endothelium ▪ vasodilation ▪ nitric oxide ▪ prostaglandins ▪ bradykinin

The vascular endothelium plays an important role in modulating vascular tone.\textsuperscript{1,2} The major vasorelaxive factors involved in this role are nitric oxide (NO), prostacyclin (PGI\textsubscript{2}), and endothelium-derived hyperpolarizing factor (EDHF).\textsuperscript{1,2} Compared with the well-documented roles of NO and PGI\textsubscript{2}, the role of EDHF in modulating vascular smooth muscle contraction is not fully understood, although >10 years have passed since the first reports of EDHF.\textsuperscript{3,4} This is partly because the exact nature of EDHF remains to be identified. The EDHF candidates include epoxyeicosatrienoic acids, which are metabolites of cytochrome P-450 monoxygenase\textsuperscript{5,6}; K\textsuperscript{+}; gap junctions\textsuperscript{8}; and hydrogen peroxide.\textsuperscript{9,10} Thus, more than one EDHF might exist, and the contribution of each EDHF to endothelium-dependent relaxation might vary, depending on the species tested and the vessels used.\textsuperscript{1,2} In general, the hyperpolarizing mechanism of EDHF is considered to be mediated by Ca\textsuperscript{2+}-activated K\textsuperscript{+} (K\textsubscript{Ca}) channels on vascular smooth muscle.\textsuperscript{11-14}

Recent animal studies from our laboratories indicate that the role of EDHF in small vessels is important and is impaired with aging.\textsuperscript{15,16} These results suggest the physiologic importance of EDHF for modulating vascular smooth muscle tone. Furthermore, animal studies indicate that EDHF-mediated relaxation is impaired in hypertension,\textsuperscript{17,18} hypercholesterolemia,\textsuperscript{19} and diabetes mellitus.\textsuperscript{20} The existence and importance of EDHF have been demonstrated in various isolated human arteries.\textsuperscript{10,16,21} For example, EDHF-mediated vascular responses are observed in gastroepiploic and distal mesenteric arteries.\textsuperscript{16} Bradykinin-induced vasodilation of human coronary microvessels is largely due to EDHF.\textsuperscript{21} It remains to be elucidated, however, whether EDHF plays an important role in maintaining basal vascular tone and agonist-induced vasodilatation in humans in vivo. Furthermore, the relative contribution of EDHF to endothelium-dependent relaxation varies, depending on the agonist used.\textsuperscript{1,2,22}

Therefore, we investigated the inhibitory effect of tetraethylammonium chloride (TEA), a K\textsubscript{Ca} channel blocker, as a tool to inhibit the EDHF-mediated component of endothelium-dependent, forearm vasodilatation in humans in vivo.\textsuperscript{13,14} For this purpose, we examined forearm vasodilator responses to substance P, bradykinin, and acetylcholine after inhibition of NO and PGI\textsubscript{2} synthesis. TEA has been previously used in human studies.\textsuperscript{23} The results indicate that EDHF plays an important role in human forearm circulation in vivo in an agonist-specific manner.
Methods

Subjects
All subjects were young, healthy volunteers (n=31; 24 men and 7 women; mean±SE age, 23±2 years) at our university. The protocol was explained, and written, informed consent was obtained from each subject. This study was approved by the Ethics Committee for Human Research at our institute.

General Procedures
The study was conducted with the participants in a supine position and in a postabsorptive state in an air-conditioned room at 25°C to 26°C. Under local anesthesia with 2% procaine, the left brachial artery was cannulated with a 20-gauge cannula for drug infusion and direct measurement of arterial pressure. Forearm blood flow (FBF) was measured by strain-gauge plethysmography with the venous occlusion technique, as described previously. Forearm vascular resistance (FVR) was calculated by dividing the mean arterial pressure by FBF. These values are expressed as units throughout this report. After placement of the cannula and strain-gauge plethysmography, the subject was allowed to establish a stable baseline before data collection. Before beginning a protocol, we obtained blood samples from the antecubital vein for analysis of serum chemistry.

Protocols

Protocol 1
TEA (Sigma Chemical Co) was infused intra-arterially in graded doses (0.25, 0.5, and 1.0 mg/min for 20 minutes each), and FBF was measured in 6 normal subjects.

Protocol 2
Forearm vasodilating responses to substance P, bradykinin, acetylcholine, sodium nitroprusside, and nicorandil were examined after inhibition of NO and PGI2 synthesis (n=18). Each subject was given aspirin (486 mg) orally 30 minutes before starting measurement. Previously, 600 mg aspirin was reported to block cyclooxygenase activity by at least 85%, with recovery occurring during the following 6 hours. We considered the dosage used in the present study suitable after comparing the average body mass of Japanese to that of Western people. After baseline FBF was measured, N^ε-monomethyl-L-arginine (L-NMMA, Clinalfa), an NO synthase inhibitor, was infused into the brachial artery (8 μmol/min for 5 minutes). This dose of L-NMMA blocks the release of NO under both basal conditions and during stimulation with acetylecholine. Forearm vascular responses to the intra-arterial infusion of substance P (0.8, 1.6, 3.2, and 6.4 ng/min; Sigma), bradykinin (12.5, 25, 50, and 100 ng/min; Sigma), acetylcholine (4, 8, 16, and 32 μg/min; Ovisot, Daiichi Pharmaceutical Co, Ltd), and sodium nitroprusside (0.4, 0.8, 1.6, and 3.2 μg/min; Nitrop inj; Maruishi Pharmaceutical Co, Ltd) were measured. Each agonist was infused for 2 minutes, as described previously. These measurements were repeated after inhibition of KCa channels with TEA. FBF was measured at a dose of 1 mg/min for 20 minutes. We used TEA at this dose because TEA selectively blocks single KCa channels in arteriolar smooth muscle cells at concentrations of <1 mmol/L, and from our initial protocol that monitored FBF. Based on an estimated forearm volume of 800 mL, 1 mg/min TEA should achieve a local plasma concentration of ~0.6 mmol/L. Subsequently, cumulative doses of substance P, bradykinin, acetylcholine, and sodium nitroprusside were infused with TEA. Two or 3 of the agonists were infused into each subject. In addition, we infused nicorandil (Sigmart, Chugai Pharmaceutical Co, Ltd), an ATP-sensitive K+ channel (KATP) opener, intra-arterially before and after TEA administration.

Protocol 3
To determine the physiologic role of EDHF, we examined forearm vasodilating responses to substance P, bradykinin, acetylcholine, and sodium nitroprusside before and after TEA in the absence of aspirin and L-NMMA (n=7). Two or 3 of the agonists were infused into each subject. TEA was infused in Protocol 2.

Statistical Analysis
All values are expressed as mean±SEM. Values at rest were compared with an unpaired t test. One-way ANOVA was used to compare the FBF before and after infusion of TEA alone. Post hoc comparisons between different doses were made with the Bonferroni correction for multiple comparisons. The regional vascular responses to drugs before and during TEA coinfusion were compared with a 2-way ANOVA. A value of P<0.05 was considered statistically significant.

Results

Baseline Characteristics of the Subjects
The Table shows the baseline characteristics of the subjects. None of the subjects had hypertension, hypercholesterolemia, or a smoking habit. In addition, the blood pressure or heart rate of each subject did not change during each protocol.

Responses to Intra-Arterial Infusion of TEA
Intra-arterial infusion of TEA at graded doses reduced FBF (Figure 1). The dose of 1 mg/min TEA (P<0.05) significantly decreased FBF. Furthermore, even after cyclooxygenase and NO synthase were blocked, infusion of TEA at 1 mg/min for 20 minutes significantly reduced FBF and FVR (Figure 2). Mean blood pressure or heart rate did not change (Figure 2).

Responses to Intra-Arterial Infusion of Substance P
After cyclooxygenase and NO synthase were blocked, intra-arterial infusion of substance P increased FBF in a dose-dependent manner (Figure 3; P<0.001). After infusion of

Subject Characteristics

<table>
<thead>
<tr>
<th>Male/female, n</th>
<th>24/7</th>
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<tr>
<td>Age, y</td>
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<tr>
<td>Height, cm</td>
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<td>Body weight, kg</td>
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<tr>
<td>Serum blood glucose, mg/dL</td>
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</tr>
</tbody>
</table>

Values are n or mean±SE.

Figure 1. FBF in response to intra-arterial infusion of graded doses of TEA (n=6). *P<0.05 vs the value at 0 mg/min.
TEA, however, the substance P–induced increase in FBF ($P<0.001$) was significantly attenuated (Figure 3). Similar results were obtained in the absence of aspirin and L-NMMA (Figure 4).

### Responses to Intra-Arterial Infusion of Bradykinin

After cyclooxygenase and NO synthase were blocked, intra-arterial infusion of bradykinin elicited an increase in FBF in a dose-dependent manner (Figure 3; $P<0.001$). After infusion of TEA, however, the bradykinin-induced increase in FBF ($P<0.001$) was significantly attenuated (Figure 3). Similar results were obtained in the absence of aspirin and L-NMMA (Figure 4).

### Responses to Intra-Arterial Infusion of Acetylcholine

After cyclooxygenase and NO synthase were blocked, intra-arterial infusion of acetylcholine elicited an increase in FBF (Figure 5; $P<0.001$). Because NO synthesis was blocked, the changes were small at doses of 4, 8, and 16 µg/min. Therefore, we also infused an additional dose of 32 µg/min acetylcholine. After infusion of TEA, the acetylcholine-induced increase in FBF was unaltered (Figure 5). Similar results were obtained in the absence of aspirin and L-NMMA (Figure 6).

### Responses to Intra-Arterial Infusion of Sodium Nitroprusside

After cyclooxygenase and NO synthase were blocked, intra-arterial infusion of sodium nitroprusside elicited a dose-dependent increase in FBF (Figure 5; $P<0.001$). After TEA infusion, the sodium nitroprusside–induced increase in FBF was unaltered (Figure 5). Similar results were obtained in the absence of aspirin and L-NMMA (Figure 6).

### Responses to Intra-Arterial Infusion of Nicorandil

To determine the specificity of the inhibitory effect of TEA, we examined forearm vascular responses to nicorandil, a K$_{ATP}$ channel opener, before and after TEA infusion. FBF responses to nicorandil did not differ before or after TEA infusion (Figure 5).

### Discussion

The present study demonstrates that substance P– and bradykinin-induced forearm vasodilatation is inhibited by the K$_{Ca}$ channel blocker TEA, whereas acetylcholine- and sodium nitroprusside–induced vasodilatation was not affected. Furthermore, forearm vasodilatation evoked by nicorandil, a K$_{ATP}$ channel opener, was unaffected. In addition, TEA alone elicited a small but significant decrease in FBF. These results
suggest that substance P– and bradykinin-induced forearm vasodilatation is substantially mediated by EDHF but that acetylcholine- and sodium nitroprusside–induced vasodilatation of the human forearm is not. Moreover, EDHF might contribute to the basal vascular smooth muscle tone in the human forearm circulation.

Previous studies demonstrated that substance P has an endothelium-dependent vasodilator effect in the human forearm and coronary circulation. In human forearm vessels, L-arginine augmented acetylcholine-induced vasodilatation but not that evoked by substance P. In contrast, L-NMMA markedly inhibited the vasodilatation evoked by acetylcholine but only minimally inhibited that evoked by substance P. Forearm vasodilatation in response to substance P is also resistant to indomethacin. In our previous study, we excluded the possibility that repeated infusion of substance P results in attenuated forearm vasodilatation owing to tachyphylaxis. These findings indicate that substance P–induced endothelium-dependent vasodilatation is mediated primarily by endothelial factor(s) other than NO or PGI2. In the present study, TEA attenuated the substance P–induced forearm vasodilatation, suggesting that substance P–induced forearm vasodilatation is mediated primarily by EDHF. Indeed, animal studies also demonstrated that substance P–induced vascular relaxation is mediated primarily by EDHF. Moreover, Panza et al reported that the forearm vasodilating response to substance P is mediated substantially by EDHF. Other studies have reported that the forearm vasodilating response to substance P is significantly inhibited by L-NMMA in humans, in contrast to our previous studies. In those studies, however, the possibility that the remaining forearm vasodilatation was mediated by EDHF was not excluded. We also demonstrated that the forearm vasodilator response to acetylcholine was markedly attenuated by L-NMMA but not that to substance P. In addition, we previously demonstrated that NO contributes differently to substance P–induced vasodilatation in the coronary artery and forearm vasculature beds in humans. The contribution of NO to substance P–induced vasodilatation is greater in the coronary artery than in the forearm circulation. Substance P–induced vasodilatation is more potent in distal than in proximal arterial segments, suggesting that the contribution of substance P–induced vasodilatation is important in vascular resistance. We do not deny the possibility that NO contributes to the forearm vasodilating response to substance P. On the basis of the results of the present study, however, and those from the aforementioned studies, we consider that the forearm vasodilating response to substance P is primarily mediated by EDHF, rather than NO or PGI2.

Bradykinin is a potent, endogenous vasodilator that stimulates endothelial B1 receptors, leading to the release of NO, PGI2, and EDHF. Bradykinin induces endothelium-derived hyperpolarization in many blood vessels. Bradykinin-induced forearm vasodilatation was markedly attenuated after TEA administration, suggesting that its activity is mediated by EDHF. This is consistent with the results of Honig et al., who reported that the increase in FBF was inhibited in response to lower doses of bradykinin with TEA but not the FBF response to the highest dose of bradykinin (200 ng/100 mL of forearm volume per minute). They suggested that other relaxing factors are released by high doses of bradykinin. Importantly, Honig et al. infused bradykinin before and after using the NO clamp technique and reported that the bradykinin-induced forearm vasodilatation did not change. This finding excludes the possibility of a tachyphylaxis effect from repeated infusions of bradykinin. As discussed in their report, however, other studies reported that bradykinin-induced forearm vasodilatation was inhibited by L-NMMA, suggesting that this vasodilatation is mediated by NO. We did not intend to exclude the involvement of NO in bradykinin-induced forearm vasodilatation, as in the case of substance P. We did examine the bradykinin-induced forearm vasodilatation after L-NMMA, however, and the remaining vasorelaxant effect of bradykinin was inhibited by TEA, strongly suggesting that bradykinin-induced forearm vasodilatation is mediated substantially by EDHF.

In contrast to substance P or bradykinin, acetylcholine-induced forearm vasodilatation was not attenuated. This suggests that the forearm vasodilating response to acetylcholine is mediated by NO, although acetylcholine-induced vascular relaxation was partly mediated by EDHF in animal studies. Consistent with the results of our study, the forearm vasodilating response to acetylcholine was unaffected by miconazole, a cytochrome P-450 enzyme inhibitor, although the inhibitory effect of miconazole might not be specific for inhibiting EDHF-mediated responses. The fact that sodium nitroprusside–induced forearm vasodilatation was not affected by TEA indicates that the effects of TEA are specific for each agonist. Indeed, sodium nitroprusside has a minimal effect on membrane potential despite significant vasodilatation.

Another important finding of the present study is that TEA did not alter the forearm vasodilating response evoked by nicorandil, which is a KATP channel opener. This is particularly important, because EDHF has been suggested to induce vasorelaxation in part through the opening of KATP channels. Although this hypothesis is controversial, our results indicate that the inhibitory effect of TEA on the forearm vasodilating response is unrelated to KATP channels. In support of our findings, glibenclamide, a selective KATP channel blocker, fails to inhibit bradykinin-induced vasodilatation of isolated human coronary arterioles, whereas TEA inhibits this response.
TEA infusion alone and TEA infusion after administration of aspirin and L-NMMA caused a significant decrease in FBF. These observations are in contrast to the results of other studies.\textsuperscript{37} We do not have a clear explanation for these differences; however, the NO clamp technique used in the previous study\textsuperscript{37} might have affected the results. It is also possible that a difference in the study population influenced the results. Interestingly, TEA enhances vasoconstrictor responses to norepinephrine and angiotensin II in cats.\textsuperscript{14} Thus, the resting vascular tone evoked by these endogenously released substances might be regulated by EDHF under normal conditions in vivo. In conclusion, our results strongly suggest that EDHF-mediated vasodilatation contributes to resting forearm vascular tone as well as to agonist-induced forearm vasodilatation induced by substance P and bradykinin in humans in vivo.

**Perspectives**

Our results indicate that substance P and bradykinin can be used as a tool to investigate the contribution of EDHF in the human forearm, in vivo, in disease states such as hypertension, diabetes mellitus, and heart failure. EDHF might play an important role in the vasodilatation response, as does NO. In fact, when there is a decrease in NO activity, EDHF release might be increased to maintain vascular smooth muscle tone.\textsuperscript{44} We previously demonstrated that forearm vasodilatation evoked by acetylcholine is impaired whereas that by substance P is preserved in patients with heart failure.\textsuperscript{31} Therefore, we suggest that EDHF might play an important compensatory role for the loss of NO-induced forearm vasodilatation in patients with heart failure. In addition, the compensatory role of EDHF in hypertension might be important.\textsuperscript{45} A recent study indicated that EDHF might compensate for the loss of NO and preserve the endothelium-dependent relaxation in mesenteric arteries of hypertensive rats induced by a high-salt diet.\textsuperscript{46} Furthermore, EDHF-mediated responses are improved by inhibition of the renin-angiotensin system.\textsuperscript{47} An increase in bradykinin levels caused by angiotensin-converting enzyme inhibitors is a possible explanation. An angiotensin receptor blocker, however, has also been reported to improve age-related EDHF-mediated endothelial dysfunction.\textsuperscript{47} Therefore, it is important to determine whether EDHF-mediated endothelial function is improved after treatment with such drugs. Finally, recent studies suggest that brain natriuretic peptide- and C-type natriuretic peptide-induced forearm vasodilatation is partly mediated by EDHF because these effects were blocked by TEA.\textsuperscript{48,49} The implication of these observations awaits further study.

**Acknowledgments**

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