Endothelium-Derived Hyperpolarizing Factor Determines Resting and Stimulated Forearm Vasodilator Tone in Health and in Disease

Muhiddin A. Ozkor, BSc, MD, MRCP; Jonathan R. Murrow, MD; Ayaz M. Rahman, MD; Nino Kavtaradze, MD; Ji Lin, PhD; Amita Manatunga, PhD; Arshed A. Quyyumi, MD, FRCP

Background—We assessed the contribution of endothelium-derived hyperpolarizing factors to resting and agonist-stimulated vasodilator tone in health and disease. Tetraethylammonium chloride (TEA) was used to inhibit K⁺/Ca channel activation and fluconazole was used to inhibit cytochrome P450 2C9–mediated epoxyeicosatrienoic acid synthesis. We hypothesized that endothelium-derived hyperpolarizing factors contribute to resting vascular tone by K⁺/Ca channel activation and epoxyeicosatrienoic acid release and that endothelium-derived hyperpolarizing factors compensate for reduced nitric oxide bioavailability at rest and with endothelium-dependent vasodilators.

Methods and Results—In 103 healthy subjects and 71 nonhypertensive subjects with multiple risk factors, we measured resting forearm blood flow (FBF) using venous occlusion plethysmography before and after intra-arterial infusions of N⁴G-monomethyl-L-arginine (L-NMMA), TEA, fluconazole, and their combination. The effects of these antagonists on resting FBF and on bradykinin- and acetylcholine-mediated vasodilation were studied. Resting FBF decreased with TEA and L-NMMA in all subjects (P<0.001); however, the vasoconstrictor response to L-NMMA was greater (P=0.04) and to TEA was lower (P=0.04) in healthy subjects compared with those with risk factors. Fluconazole decreased resting FBF in all subjects, and the addition of TEA further reduced FBF after fluconazole, suggesting that cytochrome P450 metabolites and other hyperpolarizing factor(s) activate K⁺/Ca channels. Both L-NMMA and TEA attenuated bradykinin-mediated vasodilation in healthy and hypercholesterolemic subjects (P<0.001). In contrast, acetylcholine-mediated vasodilation remained unchanged with TEA in healthy subjects but was significantly attenuated in hypercholesterolemia (P<0.04).

Conclusions—First, by activating TEA-inhibitable K⁺/Ca channels, endothelium-derived hyperpolarizing factors, together with nitric oxide, contribute to resting microvascular dilator tone. The contribution of K⁺/Ca channel activation compared with nitric oxide is greater in those with multiple risk factors compared with healthy subjects. Second, activation of K⁺/Ca channels is only partly through epoxyeicosatrienoic acid release, indicating the presence of other hyperpolarizing mechanisms. Third, bradykinin, but not acetylcholine, stimulates K⁺/Ca channel–mediated vasodilation in healthy subjects, whereas in hypercholesterolemia, K⁺/Ca channel-mediated vasodilation compensates for the reduced nitric oxide activity. Thus, enhanced endothelium-derived hyperpolarizing factor activity in conditions of nitric oxide deficiency contributes to maintenance of resting and agonist-stimulated vasodilation.

Clinical Trial Registration—URL: http://www.clinicaltrials.gov. Unique identifier: NCT00166166.
(Circulation. 2011;123:2244-2253.)

Key Words: endothelium ■ nitric oxide ■ vasodilation ■ endothelium-derived vasoconstrictor factors

The endothelium contributes to vasodilation by releasing a variety of paracrine factors. Persistent endothelium-dependent vasodilation after inhibition of both nitric oxide (NO) and prostacyclin has been attributed to endothelium-derived hyperpolarizing factor (EDHF) activity.1–4 Tonic release of NO contributes to resting vasodilator tone5–7 and to physiological vasodilation during exercise,2 and its activity is significantly impaired in individuals with cardiovascular risk factors.7–10 In contrast, the contribution of EDHF to vasodilator tone in the human circulation and the impact of risk factors that impair NO bioavailability on EDHF activity in vivo remain unknown.6,11,12

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In the human vasculature, endothelium-dependent hyperpolarization is at least partly attributed to the release of EDHF.
epoxyeicosatrienoic acids (EETs) from cytochrome P450–dependent metabolism of arachidonic acid. Epoxyeicosatrienoic acids promote vasodilation by stimulating small and large calcium-dependent potassium channels (K\(^{+}_{\text{Ca}}\)) on endothelial cells, with subsequent membrane hyperpolarization and vasodilation.\(^{1,13,21}\) Agonists such as bradykinin stimulate endothelial G-protein–coupled receptors, provoking an increase in intracellular calcium, leading to opening of endothelial K\(^{+}_{\text{Ca}}\) channels, and triggering the EDHF phenomena: synthesis of EETs, transmission of endothelial cell hyperpolarization to the vascular smooth muscle via gap junctions, and/or release of K\(^{+}\) from the endothelial cells, which in turn induces smooth muscle vasodilation by activating several other K\(^{+}\) channels.\(^{15–17}\) Experimentally, a combination of apamin and charybdotoxin is used to specifically inhibit EDHF responses caused by K\(^{+}_{\text{Ca}}\) activation, whereas in clinical studies, tetraethylammonium chloride (TEA) has been used as a K\(^{+}\) channel inhibitor.\(^{18–20}\) The role of EETs as potential EDHFs has been studied in humans using azoles such as miconazole, fluconazole, and sulfaphenazole, which selectively inhibit epoxidation (EET generation) of arachidonic acid.\(^{11,13,21}\)

In this study, we measured the contribution of EDHF to resting vasomotor tone in the human forearm microcirculation in health and disease in both the presence and absence of NO, with the hypothesis that activation of K\(^{+}_{\text{Ca}}\) channels and/or cytochrome P450 metabolites contributes to endothelium-dependent hyperpolarization, and that this contribution is altered in disease states with endothelial dysfunction. Second, we explored the contribution of EDHF to endothelium-dependent vasodilation in both the presence and absence of NO, with the hypothesis that activation of K\(^{+}_{\text{Ca}}\) channels in response to acetylcholine and bradykinin contributes to forearm microvascular vasodilation and that this contribution is altered in hypercholesterolemic compared with healthy circulation.

**Methods**

**Subjects**

Healthy and nonhypertensive subjects with multiple risk factors who were between 21 and 60 years of age were enrolled in 4 separate protocols that explored the contribution of EDHF to resting tone in healthy subjects and those with cardiovascular risk factors that included hypercholesterolemia and diabetes mellitus, the contribution of EETs to resting tone, the differential contribution of EETs and K\(^{+}_{\text{Ca}}\) channel activation to resting tone, and the contribution of EDHF to agonist-stimulated vasodilation in healthy and hypercholesterolemic subjects. All subjects were nonsmokers and free of hypertension, cardiovascular disease, and systemic disorders. Subjects with risk factors (protocols 1 through 3) were older and had similar blood pressures; a greater body mass index (BMI); higher weight, kg; higher systolic blood pressure, mm Hg; higher total cholesterol, mg/dL; higher low-density lipoprotein cholesterol, mg/dL; lower levels of high-density lipoprotein cholesterol (HDL-C), mg/dL; lower body mass index, kg/m²; and lower triglycerides, mg/dL (Table). Subjects in protocol 4 had similar significant differences in cholesterol and its subfractions compared with healthy subjects, but were matched for all other factors. All healthy subjects and those with hypercholesterolemia were not taking any medications; diabetic patients discontinued their insulin or metformin the night before, and 1 subject on sulfonylureas discontinued it before enrollment. Pregnant women were excluded. The study was approved by the Emory Institutional Review Board, and all subjects provided informed consent.

**Measurement of Forearm Blood Flow**

Measurements were performed in subjects after an overnight fast in a quiet temperature-controlled (22°C to 24°C) room. Subjects refrained from exercise, alcohol, and caffeine for at least 24 hours beforehand. After insertion of an intrabrachial arterial cannula for arterial pressure monitoring and delivery of drug infusions, subjects received oral aspirin (975 mg) to inhibit prostacyclin synthesis at least 1 hour before the study.\(^{22}\) Simultaneous forearm blood flow (FFB) measurements were obtained in both arms with a dual-channel venous occlusion strain gauge plethysmograph (model EC6, DE Hokanson, Bellevue, WA) as described previously.\(^{2}\) Flow measurements were recorded for ~7 seconds every 15 seconds up to 8 times and, a mean FBF value in milliliters per minute per 100 mL was computed. Forearm vascular resistance was calculated as the mean arterial pressure divided by FBF and is expressed as millimeters of mercury per 1 mL · min⁻¹ · 100 mL⁻¹.

**Table. Characteristics of Study Subjects in Protocol 1**

<table>
<thead>
<tr>
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<th>Healthy Subjects (n = 103)</th>
<th>Subjects With Risk Factors (n = 71)</th>
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<tr>
<td>Age, y</td>
<td>34 ± 11</td>
<td>46 ± 12*</td>
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<tr>
<td>Male/female</td>
<td>54/49</td>
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<td>Black, n (%)</td>
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<td>Other, n (%)</td>
<td>10 (9)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Hypercholesterolemia, n</td>
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<td>48</td>
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<tr>
<td>Diabetes mellitus, n</td>
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<td>89 ± 18*</td>
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<td>Body mass index, kg/m²</td>
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<td>Glucose, mg/dL</td>
<td>85 ± 10</td>
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<tr>
<td>Triglycerides, mg/dL</td>
<td>87 ± 63</td>
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<td>Total cholesterol, mg/dL</td>
<td>168 ± 28</td>
<td>226 ± 43*</td>
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<tr>
<td>High-density lipoprotein cholesterol, mg/dL</td>
<td>55 ± 11</td>
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<td>Low-density lipoprotein cholesterol, mg/dL</td>
<td>96 ± 22</td>
<td>149 ± 41*</td>
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</table>

Data are mean ± SD.

*Statistically significant (P < 0.05, t test) difference between groups.

**Protocol 1: Contribution of Nitric Oxide and K\(^{+}\)\text{Ca}\ Channel Activation to Resting Vasodilator Tone**

All agents were administered intra-arterially after resting measurements were made during saline infusion (2.5 mL/min). In 37 healthy and 25 subjects with risk factors, FBF was measured during inhibition of NO synthesis during a 5-minute infusion of NG-monomethyl-L-arginine (L-NMMA; Clinalfa, Lauflingen, Switzerland) given at 8 μmol/min, which was previously shown to attenuate resting and agonist-stimulated FBF.\(^{2}\) While L-NMMA was continued, intra-arterial TEA (Clinalfa) at 1 mg/min was infused for 5 minutes to investigate the effect of combined blockade of NO and K\(^{+}_{\text{Ca}}\) channels on resting vessel tone. When given at 0.25 to 1 mg/min, TEA selectively inhibits K\(^{+}_{\text{Ca}}\) channels but reduces FBF with bradykinin only at 1 mg/min (<0.64 μmol/min).\(^{8,12,19,20}\) In separate experiments in 41 healthy subjects and 32 subjects with risk factors, TEA was infused first to investigate the effects of blockade of K\(^{+}_{\text{Ca}}\) channels without prior NO inhibition. This was followed by...
combined infusions of TEA and L-NMMA. Arterial blood pressure and FBF were measured in the last 2 minutes of each intervention.

**Protocol 2: Contribution of Cytochrome P450 Metabolites to Resting Vasodilator Tone**

In 26 healthy subjects and 7 subjects with risk factors, FBF was measured at rest and after fluconazole (Pfizer, New York, NY) given at 0.4 μmol/min for 5 minutes to investigate the effects of epoxide inhibition without prior NO blockade and after combined infusions of fluconazole and L-NMMA 8 μmol/min to investigate the effects of combined blockade of NO and cytochrome P450 epoxide synthesis. In separate experiments in 8 healthy subjects and 7 subjects with risk factors, FBF was measured at rest and after L-NMMA 8 μmol/min for 5 minutes and after combined infusion of L-NMMA and fluconazole.

**Protocol 3: Comparative Contribution of Cytochrome P450 Metabolites and K⁺Ca Channels to Resting Vasodilator Tone**

In 19 healthy subjects, the differential contribution of cytochrome P450–derived epoxides and K⁺Ca channel activation to resting vasodilator tone was investigated. Forearm blood flow was measured at rest and after infusion of fluconazole (0.4 μmol/min) for 5 minutes and finally after combined infusions of fluconazole and TEA at 1 mg/min for 5 minutes.

**Protocol 4: Contribution of K⁺Ca Channel Activation to Bradykinin and Acetylcholine-Mediated Vasodilation in Healthy and Hypercholesterolemic Vasculature**

Fifty-four healthy subjects and 44 subjects with hypercholesterolemia participated in this protocol. In 27 healthy and 24 hypercholesterolemic subjects, FBF was measured at rest and after intra-arterial infusion of the endothelium-dependent vasodilators bradykinin (Clinalfa) at 7.5, 15, and 30 μg/min or acetylcholine (Novartis, East Hanover, NJ) at 100, 200, and 400 ng/min or acetylcholine (Novartis, East Hanover, NJ) at 7.5, 15, and 30 μg/min, followed by intra-arterial infusion of the endothelium-independent vasodilator sodium nitroprusside at 1.6 and 3.2 μg/min for 5 minutes each (Figure 1). This was followed by infusion of L-NMMA (8 μmol/min) for 5 minutes; then, escalating doses of either bradykinin or acetylcholine were repeated. After a 30-minute rest period, TEA 1 mg/min was begun for 5 minutes, and while L-NMMA and TEA infusions were continued, bradykinin or acetylcholine infusions and sodium nitroprusside infusions were repeated. This protocol enabled measurement of the contribution of NO and combined NO and K⁺Ca channel activation to endothelium-dependent and -independent vasodilation.

Finally, to investigate the effect of inhibition of K⁺Ca channels in response to agonist-mediated vasodilation without prior blockade of NO, we studied another 27 healthy and 20 hypercholesterolemic subjects. After measurement of FBF during bradykinin or acetylcholine infusions, TEA was infused followed by repeat infusions of bradykinin or acetylcholine. Finally, combined infusions of TEA and L-NMMA were given, followed by repeat infusions of bradykinin or acetylcholine and sodium nitroprusside (Figure 1). This enabled assessment of the contribution of K⁺Ca channel activation without blockade of NO to agonist-stimulated endothelium-dependent and -independent vasodilation.

**Statistical Analysis**

In protocols 1 through 3, to assess the treatment effect of TEA, L-NMMA, fluconazole, and their combination within healthy and risk factor groups, a paired t-test was used to compare single blockade with baseline and combined blockade with single blockade. Then, to compare the magnitude of treatment effects between the healthy and risk factor groups (in protocol 1 and 2), an unpaired t-test was used to compare the changes in single blockade on baseline and the changes of combined blockade on single blockade. Multivariable regression analysis was performed by fitting regression models with each of the factors in the Table (age, sex, body mass index, blood pressure, and glucose and lipid levels) for possible confounding effects. The Pearson correlation coefficient was used to assess the correlation between the percentage changes in FBF with L-NMMA versus TEA.

In protocol 4, the treatment effects in FBF and forearm vascular resistance were assessed within subjects for baseline versus single blockade versus combined blockade by repeated-measures ANOVA with treatment levels as the main effect. Then, the magnitude of the treatment effects was compared between healthy and hypercholesterolemic subjects by repeated-measures ANOVA with health status as the main effect. Possible confounding effects were controlled for age, sex, blood pressure, body mass index, and LDL levels in a stepwise fashion. Reported P values are based on main effects.

Results are presented as mean±SD in the text and as least-square means and SEs in the figures. The Bonferroni method was used for adjustment for multiple comparisons for comparing baseline, TEA, L-NMMA, fluconazole, and combinations. Reported P values are values before adjustment.
Results

Relative Contribution of $K^+_{Ca}$ Channel Activation and Nitric Oxide to Resting Vasodilator Tone

In protocol 1, TEA infusion produced vasoconstriction; resting FBF decreased by $18\pm16\%$ ($P<0.0001$) in healthy subjects and by $24\pm13\%$ ($P<0.0001$) in those with risk factors, indicating a significant contribution of $K^+_{Ca}$ channel activation to resting microvascular vasodilator tone. The magnitude of vasoconstriction with TEA was lower in healthy subjects compared with those with risk factors ($P=0.04$). Addition of L-NMMA to TEA further reduced FBF in both groups (Figure 2A, 2B, 2E, and 2F).

When L-NMMA was infused first, resting FBF decreased by $29\pm17\%$ ($P<0.0001$) in healthy subjects and by $23\pm15\%$ ($P<0.0001$) in those with risk factors. Vasoconstriction with L-NMMA was greater in healthy subjects than in those with risk factors ($P=0.04$). Addition of TEA to L-NMMA further reduced FBF in both groups, indicating a significant contribution of $K^+_{Ca}$ channel activation to resting vasodilator tone, even after NO blockade (Figure 2C, 2D, 2G, and 2H). Thus, there was a greater contribution of $K^+_{Ca}$ channel activation and a lower contribution of NO to resting microvascular vasodilator tone in those with risk factors compared with healthy subjects. Moreover, in the entire population, there was an inverse relationship between the constrictor responses to L-NMMA and TEA, indicating that reduced NO bioavailability is accompanied by higher $K^+_{Ca}$ channel activity and vice versa (Figure 3). The reduction in FBF after dual...
blockade was similar in healthy subjects and those with risk factors (38±17% versus 39±17% reduction in FBF; P=0.78).

Multivariate analysis was performed to determine whether demographic/risk factors, including age, sex, body mass index, total, LDL and high-density lipoprotein cholesterol, glucose, and systolic blood pressure, were determinants of the flow responses to L-NMMA and TEA. The multivariate determinants of the constrictor response with L-NMMA were LDL cholesterol (P=0.0002), total cholesterol (P=0.0003), systolic blood pressure (P=0.0016), and fasting glucose (P=0.003), and multivariate determinants of the vasodilator response to TEA were LDL levels (P=0.05) and the L-NMMA response (P=0.006).

**Contribution of Cytochrome P450 Metabolites to Resting Vasodilator Tone**

In protocol 2, infusion of fluconazole decreased FBF by 13±16% (P=0.001) in healthy subjects and by 17±13% (P=0.04) in those with risk factors, indicating a significant contribution of cytochrome P450 metabolites to resting vasodilator tone. Infusion of L-NMMA after fluconazole reduced FBF by an additional 25±13% (P=0.02) in healthy subjects, changes that were similar to those observed in subjects with risk factors. When L-NMMA was infused before fluconazole, resting FBF decreased by 37±12% (P=0.002) in healthy subjects and by 31±14% (P=0.009) in subjects with risk factors. In the presence of L-NMMA, infusion of fluconazole further reduced FBF by 26±22% (P=0.03) in both groups. Reduction in FBF with fluconazole tended to be greater after inhibition of NO than when given in the presence of NO (26±22% versus 13±16% reduction in FBF; P=0.07).

**Comparative Contribution of Cytochrome P450 Metabolites and K⁺Ca Channel Activation**

To assess the relative contribution of the 2 potential hyperpolarization mechanisms (cytochrome P450 metabolites and K⁺Ca channel activation) to resting vasodilator tone, individual and combined blockade with fluconazole and TEA was studied in protocol 3. In healthy subjects, fluconazole infusion reduced FBF by 12±13% (P=0.002), and the addition of TEA further reduced FBF by 22±23% (P=0.01), indicating that hyperpolarizing factor(s) beyond cytochrome P450 metabolites are activating K⁺Ca channels at rest (Figure 4A and 4B).

**Contribution of K⁺Ca Channel Activation and Nitric Oxide to Bradykinin Responses**

Studies were performed in healthy and hypercholesterolemic subjects in protocol 4. Bradykinin infusion resulted in dose-related forearm vasodilation that was similar in magnitude in healthy (n=29) and hypercholesterolemic (n=26) subjects (both P<0.0001; Figure 5). We found that L-NMMA lowered FBF by 17% (P=0.0003) in healthy subjects (Figure 5A and 5C) and by 33% (P<0.0001) in hypercholesterolemic subjects (Figure 5E and 5G). Bradykinin-mediated vasodilation was also attenuated by TEA in the absence of L-NMMA in both groups. Thus, in both groups, FBF was 25%, P<0.0001 lower (Figure 5B, 5D, 5F, and 5H) after TEA. Bradykinin-induced vasodilation was further attenuated after the addition of L-NMMA to TEA in both groups.

**Contribution of K⁺Ca Channel Activation and Nitric Oxide to Acetylcholine Responses**

Acetylcholine produced dose-related forearm vasodilation that was significantly lower in hypercholesterolemic compared with healthy subjects (P=0.05), as previously described. We found that L-NMMA attenuated the acetylcholine-stimulated FBF increase by 28% (P=0.0006; Figure 6A and 6C) in healthy subjects and by 26% (P=0.0002) in those with hypercholesterolemia (Figure 6E and 6G). Addition of TEA to L-NMMA produced no further inhibition of acetylcholine-induced vasodilation (P=0.19) in healthy subjects (Figure 6A and 6C), but in

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**Figure 3.** Relationship between contribution of nitric and K⁺Ca channel activation to resting vasodilator tone. Correlation between the percent change in forearm blood flow (FBF) with N⁴-monomethyl-L-arginine (L-NMMA) and tetraethylammonium chloride (TEA; n=131).

**Figure 4.** Contribution of cytochrome P450 metabolites and K⁺Ca channel activation to resting forearm blood flow (FBF) and vascular resistance. Change in (A) resting FBF and (B) vascular resistance with fluconazole alone and combined infusions of fluconazole and tetraethylammonium chloride (TEA). Values are mean±SEM.
those with hypercholesterolemia, there was a 10% reduction in FBF ($P=0.09$) and a 21% increase in forearm vascular resistance ($P=0.04$; Figure 6E and 6G).

Infusion of TEA alone did not change acetylcholine-mediated vasodilation in healthy subjects ($P=0.97$; Figure 6B and 6D). In contrast, in hypercholesterolemic subjects, acetylcholine-stimulated FBF was lowered by 18% ($P=0.04$; Figure 6E and 6H). The addition of L-NMMA to TEA significantly attenuated acetylcholine-stimulated vasodilation in both groups. Thus, unlike bradykinin, there was no contribution of $K^+_{Ca}$ channel activation to acetylcholine-mediated vasodilation in healthy subjects; however, there was a significant $K^+_{Ca}$ channel activation with acetylcholine in hypercholesterolemia. Moreover, the magnitude of constriction with NO blockade in the presence of $K^+_{Ca}$ channel blockade was lower in the hypercholesterolemic group compared with healthy subjects (9% versus 37%; $P=0.023$).

**Figure 5.** Contribution of nitric oxide and $K^+_{Ca}$ channel activation to bradykinin-stimulated vasodilation in healthy and hypercholesterolemic forearm vasculature. FBF (A) and forearm vascular resistance (C) in response to increasing doses of bradykinin (100, 200 and 400 ng/min) alone, after initial infusion of L-NMMA, and combined blockade with $N^\omega$-monomethyl-L-arginine (L-NMMA) and tetraethylammonium chloride (TEA) in healthy (n=17) subjects. FBF (B) and vascular resistance (D) during infusion of bradykinin alone and after initial infusion of TEA, followed by combined TEA and L-NMMA infusions in healthy (n=12) subjects. FBF (E) and vascular resistance (G) in response to increasing doses of bradykinin alone, after initial infusion of L-NMMA, and combined blockade with L-NMMA and TEA in hypercholesterolemic (n=16) subjects. FBF (F) and vascular resistance (H) during infusion of bradykinin alone and after initial infusion of TEA, followed by combined TEA and L-NMMA infusions in hypercholesterolemic (n=10) subjects. Data presented as mean±SEM.

**Contribution of $K^+_{Ca}$ Channel Activation and Nitric Oxide to Sodium Nitroprusside Responses**

Vasodilation with sodium nitroprusside was similar in healthy and hypercholesterolemic subjects (FBF at the peak dose, 10.4±4 versus 10.9±5 mL·min⁻¹·100 mL⁻¹, respectively; $P=0.63$). Importantly, infusions of L-NMMA and TEA did not alter the vasodilator responses to sodium nitroprusside in either group (Figure 7).

Throughout each experiment, there was no change in mean arterial blood pressure or FBF in the control, noninfused arm.
Discussion

Here, we report first that EDHFs contribute to resting vasodilator tone in vivo via activation of $K_{Ca}^+$ channels. The contribution of NO to resting tone in the human forearm microcirculation is greater compared with activation of $K_{Ca}^+$ channels in healthy subjects, but in those with multiple risk factors, $K_{Ca}^+$ channel activation contributes equally as much as NO. Thus, there is an inverse relationship between tonic basal NO and EDHF activities. Second, a candidate EDHF, cytochrome P450–derived EET, also contributes to resting vasodilator tone in the healthy microcirculation, and in the presence of NO deficiency, EET-mediated vasodilation is upregulated. Third, sources other than EETs activate $K_{Ca}^+$ channels because there was additional constriction with TEA after fluconazole. Fourth, bradykinin-stimulated vasodilation is mediated by both NO and activation of $K_{Ca}^+$ channels in healthy and hypercholesterolemic subjects. Finally, in contrast to bradykinin, $K_{Ca}^+$ channel activation does not contribute to acetylcholine-stimulated vasodilation in healthy, but in hypercholesterolemia, there is reduced NO release and a significant contribution of $K_{Ca}^+$ channel activation to the acetylcholine response. Thus, enhanced EDHF activity in conditions of NO deficiency contributes to maintenance of resting and agonist-stimulated vasodilation.

Contribution of Nitric Oxide and $K_{Ca}^+$ Channel Activation to Resting Microcirculatory Tone

We confirm previous findings that basal NO contributes substantially to vasodilator tone of the microcirculation.\textsuperscript{7,21} For the first time, we demonstrate that the presence of multiple risk factors, including raised LDL cholesterol, hy-
perglycemia, and higher systolic blood pressure, even within the normal range, are all associated with reduced basal NO activity in the microcirculation, findings previously described in conductance vessels and with agonists such as acetylcholine.13,23,24 In our study, the most important determinants of TEA responses were serum LDL and the amount of basal NO activity.

Previous controversies regarding the contribution of EDHF in the human vasculature resulted largely from the use of nonspecific antagonists for the inhibition of endogenous EDHFs.6,12 We found that activation of K[Ca] channels plays an important role in the maintenance of resting vasodilator tone, contributes less than NO in healthy subjects, but contributes as much as NO in those with multiple cardiovascular risk factors. Thus, in the presence of endothelial dysfunction, typified by decreased NO activity, preservation of residual endothelium-dependent vasodilation is via K[Ca] channel signaling in humans (Figure 3), a finding previously demonstrated in experimental animals.13,25

**Contribution of Endothelium-Derived Cytochrome P450 Metabolites to Basal Tone**

Endothelium-derived cytochrome P450 metabolites of arachidonic acid hyperpolarize membranes primarily by activating the K[Ca] channels, although the identity of the specific EETs remains controversial.26–28 In addition, EETs may act in an autocrine fashion, promoting amplification of endothelial hyperpolarization, the initial step in the EDHF phenomenon. Using fluconazole, we have demonstrated an important contribution of cytochrome P450–dependent EET synthesis to microvascular dilator tone in vivo in both the presence and absence of NO and in both individuals with healthy vasculature and those with risk factors. We found no difference in the magnitude of inhibition with fluconazole between healthy subjects and those with risk factors, whereas greater K[Ca] channel activation was present in the latter. This suggests that EDHFs other than EETs that activate K[Ca] channels are upregulated in subjects with risk factors. Previous studies investigating the contribution of EETs to resting tone have been contradictory, partly because of differences in inhibitors used or the small numbers of subjects studied. For example, sulfaphenazole and miconazole had no effect on resting blood flow in small groups of subjects. Moreover, both decreases in and no change in conductance vessel diameter and flow have been previously reported with cytochrome P450 inhibition.11,21,29,30 Other studies have demonstrated a significant contribution of cytochrome P450–derived metabolites to resting vasodilator tone in coronary and renal arteries from several species and, in the human, mammary artery, forearm vasculature, and skeletal muscle circulations.31,32

Because inhibition of EETs had a lesser effect than inhibition of K[Ca] channels on resting tone and because further inhibition of vasodilator tone was observed after TEA, it is clear that sources of K[Ca] channel activation other than EETs are contributing to the EDHF phenomenon, which in experimental studies has been attributed to hydrogen peroxide, gap junctions, or elevations in K+ release from the endothelial cells.13,17,18 Another possibility is that stores of EETs in the endothelial layer may be released once the cell is activated, independently of cytochrome P450 epoxygenases.33

**Contribution of Nitric Oxide and Endothelium-Derived Hyperpolarizing Factor to Bradykinin- and Acetylcholine-Mediated Vasodilation**

Having confirmed our previous findings that the response to bradykinin is similar between healthy and hypercholesterolemic subjects, we now demonstrate that both K[Ca] channel activation and NO contribute to the bradykinin response.2 In contrast, the response to acetylcholine is diminished in hypercholesterolemia, which in experimental studies is believed to be associated with defects in NO bioavailability, no change in prostaglandins, and increased contribution of EDHF.2,25,34–36 We now demonstrate not only that there is no contribution of K[Ca] channel activation to acetylcholine-mediated vasodilation in healthy subjects but also that there is increased signaling via K[Ca] channel activation in hypercholesterolemia. Similar findings of increased contribution of cytochrome P450 2C9 products with acetylcholine have been reported in hypertension.37

**Limitations**

Our findings are limited to the forearm microcirculation; thus, other vascular beds, including conductance arteries, warrant further investigation. Nevertheless, it is known that the contribution of EDHF is smaller in conductance vessels than in the microvessels.13,14,38 Second, L-NMMA, TEA, and fluconazole are competitive inhibitors and may not com-

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**Figure 7. Contribution of nitric oxide and K[Ca] channel activation to nitroprusside-stimulated vasodilation in the healthy and hypercholesterolemic forearm vasculature.** FBF (A) in response to increasing doses of sodium nitroprusside alone and after combined blockade with N3-monomethyl-L-arginine (L-NMMA) and tetraethylammonium chloride (TEA) in healthy (n=42) subjects. FBF (B) during infusion of sodium nitroprusside alone and after combined blockade with L-NMMA and TEA in hypercholesterolemic subjects (n=38). Data presented as mean±SEM.
pletely inhibit NO, K\textsuperscript{+}, Ca channels, and cytochrome P450 pathways and thus may underestimate the importance of these mediators in vivo. Third, other endothelin-derived vasoactive mediators may play a compensatory role after blockade of NO, K\textsuperscript{+}, Ca channels, and cytochrome P450 epoxygenase and need to be investigated. Finally, our investigations were conducted on a background of cyclooxygenase inhibition. Although we have previously demonstrated that vasodilator prostanooids contribute to resting vasodilator tone, we found that cyclooxygenase products did not contribute to the endothelial dysfunction of hypertensive or hypercholesterolemic patients.\textsuperscript{36} 

**Implications**

Because of the known protective role of NO on the vessel wall that not only promotes blood flow, but also impedes thrombosis and atherosclerosis, research has focused predominantly on developing strategies that enhance NO bioavailability. Understanding the pathophysiology of endothelial dysfunction beyond NO, in particular with respect to EDHF in these disease states, is crucial in both understanding the pathophysiology of atherosclerosis and developing novel therapies.

**Sources of Funding**

This work was supported by National Institutes of Health research grant ROI HL79115, and in part by PHS grant UL1 RR025008 from the Clinical and Translational Science Award Program, PHS grant RO1 HL79115, and in part by PHS grant UL1 RR025008 from the National Institutes of Health, National Center for Research Resources, British Cardiovascular Society Research Fellowship, and the National Blood Foundation.

**Disclosures**

None.

**References**


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**CLINICAL PERSPECTIVE**

The endothelium maintains vasodilator tone by releasing paracrine factors, including nitric oxide, prostaglandins, and endothelium-derived hyperpolarizing factors (EDHFs). Although the first 2 factors have been widely studied, the role of EDHFs in the human circulation remains unknown. In the human forearm circulation, we have investigated the role of EDHFs by using antagonists of K^+^ channels and epoxyeicosatrienoic acid synthesis, both purported EDHF pathways. Our results demonstrate first that EDHFs, by activating K^+^ channels, together with nitric oxide, contribute to resting vasodilator tone. The contribution of EDHFs compared with nitric oxide is greater in those with atherosclerosis risk factors compared with healthy subjects. Second, activation of K^+^ channels is only partly through epoxyeicosatrienoic acid release, indicating the presence of other EDHFs. Third, endothelium-dependent vasodilation with bradykinin is by stimulation of both nitric oxide and EDHF, whereas with acetylcholine, vasodilation is not EDHF mediated in healthy subjects, but there is a clear contribution of EDHF in those with hypercholesterolemia. Thus, enhanced EDHF activity in conditions of nitric oxide deficiency contributes to maintenance of resting and agonist-stimulated vasodilation.
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_Circulation_. 2011;123:2244-2253; originally published online May 9, 2011;
doi: 10.1161/CIRCULATIONAHA.110.990317
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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