Endothelium-Derived Hyperpolarizing Factor in Human Internal Mammary Artery Is 11,12-Epoxyeicosatrienoic Acid and Causes Relaxation by Activating Smooth Muscle BKCa Channels

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Background—Left internal mammary arteries (LIMAs) synthesize endothelium-derived hyperpolarizing factor (EDHF), a short-lived K+ channel activator that persists after inhibition of nitric oxide (NO) and prostaglandin synthesis. EDHF hyperpolarizes and relaxes smooth muscle cells (SMCs). The identity of EDHF in humans is unknown. We hypothesized that EDHF (1) is 11,12-epoxyeicosatrienoic acid (11,12-EET); (2) is generated by cytochrome P450-2C, CYP450-2C; and (3) causes relaxation by opening SMC large-conductance Ca2+-activated K+ channels (BKCa).

Methods and Results—The identity of EDHF and its mechanism of action were assessed in 120 distal human LIMAs and 20 saphenous veins (SVs) obtained during CABG. The predominant EET synthesized by LIMAs is 11,12-EET. Relaxations to exogenous 11,12-EET and endogenous EDHF are of similar magnitudes. Inhibition of EET synthesis by chemically distinct CYP450 inhibitors (17-octadecynoic acid, N-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide), or a selective EET antagonist (4,15-epoxyeicosa-5(Z)-enoic acid) impairs EDHF relaxation. 11,12-EET activates a BKCa current and hyperpolarizes LIMA SMCs. Inhibitors of BKCa but not inward-rectifier or small-conductance K+ channels abolish relaxation to endogenous EDHF and exogenous 11,12-EET. BKCa and CYP450-2C mRNA and proteins are more abundant in LIMAs than in SVs, perhaps explaining the lack of EDHF activity of the SV. Laser capture microdissection and quantitative RT-PCR demonstrate that BKCa channels are primarily in vascular SMCs, whereas the CYP450-2C enzyme is present in both the endothelium and SMCs.

Conclusions—In human LIMAs, EDHF is 11,12-EET produced by an EDHF synthase CYP450-2C and accounting for ~40% of net endothelial relaxation. 11,12-EET causes relaxation by activating SMC BKCa channels. (Circulation. 2003;107:769-776.)

Key Words: lasers ■ ion channels ■ bypass ■ cytochromes

Human left internal mammary arteries (LIMAs) are relatively resistant to atherosclerosis and make superb bypass conduits with better long-term patency rates than saphenous vein (SV) grafts.1 The advantage of the LIMA may be the result of its superior endothelial function. LIMAs produce more of the antithrombotic vasodilators prostacyclin and nitric oxide (NO) and more endothelium-derived hyperpolarizing factor (EDHF) than SVs.2 Although bioassays suggest that EDHF is present in LIMAs, its identity and mechanism of relaxation remain unknown. EDHF is a short-lived, endogenous, endothelium-derived vasodilator that acts independently of the NO-cGMP pathway and persists after effective inhibition of NO synthase (NOS) and prostaglandin H synthase (PGHS).3 EDHF activity is elicited by acetylcholine (ACH) and bradykinin (BK). EDHF activates K+ channels and causes hyperpolarization of arterial smooth muscle cells (SMCs), thereby inhibiting voltage-gated Ca2+ channels, lowering cytosolic Ca2+, and promoting relaxation (Figure 1; Reference 3).

In animals, EDHF has been variously identified as an epoxyeicosatrienoic acid (EET) synthesized by one or more isoforms of cytochrome P450 (CYP450),4,5 an endogenous cannabinoid (anandamide),6 or even the K+ ion itself.7 The subcellular mechanism for the effects of EDHF is also controversial. Proposed effector pathways include activation of SMC K+ channels (either Ca2+-sensitive K+ channels, KCa,
or an inward rectifier K+ channel, KCa, and/or gap junctions. The bioassay properties of EDHF vary between species, among vascular beds, and with the agonist used; consequently, defining the human EDHF pathway is best done in the relevant human vessels. We examined 3 hypotheses: (1) EDHF is 11,12-epoxyeicosatrienoic acid (11,12-EET); (2) EDHF is generated by cytochrome P450-2C, CYP450-2C; and (3) EDHF causes relaxation by opening large-conductance KCa channels (BKCa) in SMCs.

Methods
The University of Alberta Human Studies Committee approved the use of discarded vascular segments, and patients provided consent for the use of their discarded tissues for research.

Tissue Isolation
The distal 1 cm of LIMAs (n=120) or SVs (n=20) was obtained at the time of CABG, immediately placed in iced physiological saline, and transported to the laboratory for study. Patients were excluded from the study if the LIMA was not used as a conduit, if it was not received in iced saline within 1 hour of harvest, or if it relaxed <10% to ACh and BK.

Vascular Ring Studies
Vessels were divided into 3-mm rings and equilibrated in 10-mL tissue baths for 45 minutes in Krebs’ solution (pH 7.35 to 7.45, PO2 120 mm Hg, PCO2 40 mm Hg, 37°C) before the protocol was initiated. Optimal tension, defined as the tension at which maximum constriction to phenylephrine (PE) occurred, was determined experimentally (LIMA, 2000 mg; SV, 1500 mg).

Figure 2. Greater endothelium-dependent and EDHF relaxation in LIMA versus SV. A, Representative traces showing relaxation to 11,12-EET, BK, and ACh. In some cases, contraction induced by PE is tonic (top), but in most cases, it is phasic (lower 2 panels). Phasic pattern occurs independent of maximal tone achieved or inhibitors used. It occurs in rings treated with vehicle alone or with L-NAME (L) plus Meclo (M). B, Representative trace of experiments (n=3) showing that LIMAs synthesize NO, as detected by an NO microelectrode, in response to PE. Note reduced but persistent production of NO in response to PE after L-NAME. Residual NO is scavenged by reduced Hb. C and D, ACh relaxation in absence (n=8) and presence (n=14) of NOS and PGHS inhibitors. *P<0.05, LIMA relaxes more than SV.
Protocols

Comparative Importance of NOS-PGHS Versus EDHF

ACh and BK were used to stimulate endothelial relaxation under 2 basic conditions: control (no inhibitors) and EDHF mode (meclofenamate, Meclo, 10⁻⁵ mol/L, plus N⁵-nitro-L-arginine methyl ester, L-NAME, 10⁻⁴ mol/L). In additional experiments, reduced hemoglobin (Hb, 20 μmol/L) was added to L-NAME and Meclo to scavenge any residual NO that persisted. A 30-μm NO electrode (World Precision Instruments) was placed in the lumen of the LIMA to allow measurement of NO. Rings were constricted with PE (10⁻⁵ mol/L) for 10 minutes and then exposed to ACh (10⁻⁵ to 10⁻⁷ mol/L) or BK (10⁻⁷ to 10⁻⁵ mol/L). Vehicle controls did not alter tone.

Target of EDHF

To identify the family of K⁺ channels involved in EDHF relaxation, the effects of the following K⁺ channel blockers were assessed: tetraethylammonium (TEA, 5 mmol/L, a nonspecific K⁺ inhibitor), apamin (10⁻⁶ mol/L, a specific small-conductance K⁺ inhibitor, SK₉₆), charybdotoxin (10⁻⁷ mol/L, an inhibitor of BK₉₆, intermediate-conductance [IK₉₆], and some voltage-gated K⁺ channels, Kᵥ),iberiotoxin (IBTx, 10⁻⁹ mol/L, a specific BK₉₆ inhibitor), or BaCl₂ (100 μmol/L), an inward rectifier K⁺ channel (Kᵢ) inhibitor.

Identity of EDHF

Relaxation to exogenous 11,12-EET (10⁻⁸ to 10⁻⁶ mol/L) was assessed in endothelium-intact and -denuded LIMAs and in the presence of IBTx. Arteries were denuded by gently passing a knotted suture through the lumen. The effects of inhibiting EET synthesis were assessed by use of the CYP450 inhibitor 17-octadecynoic acid (ODYA, 10⁻⁵ mol/L). Because ODYA can inhibit production of 20-hydroxyeicosatrienoic acid (20-HETE), a vasoconstrictor and K⁺ channel inhibitor produced by CYP450-4A, (Figure 1), a selective EET-synthesis inhibitor, N-methylsulfonyl-6-(2-propargyloxyphenyl)-hexanamide (MSPPOH; 20 μmol/L) was tested. The effects on relaxation to ACh, BK, and exogenous 11,12-EET of 14,15-epoxyeicosa-5(Z)-enoic acid, a specific EET antagonist that does not alter EET synthesis, were tested.

Electrophysiology

Freshly dispersed LIMA SMCs were isolated and studied by the whole-cell, amphotericin perforated-patch technique, as previously described. Cells were voltage-clamped at a holding potential of -60 mV, and currents were evoked by stepped, 200-ms test pulses (-100 to +50 mV). Membrane potential was measured by the current-clamp mode. The effects of 11,12-EET were assessed after administration of vehicle, the Kᵥ blocker 4-aminopyridine, and IBTx.

Immunoblotting and Immunohistochemistry

These techniques were performed on freshly isolated LIMAs and veins (n=5 each) as previously described.
RT-PCR Representative of 5 Samples
Total RNA (2 μg) was isolated from LIMAs with the RNeasy mini Kit (Qiagen) and reverse-transcribed with Qiagen Omniscript reverse transcriptase as previously described and validated. 13

Quantitative Real-Time PCR
Quantitative real-time (qRT)-PCR was used to quantify human CYP450-2C9 (GenBank NM-000771) and BKCa (GenBank U13913) mRNA. Total RNA was extracted from LIMA and SV tissue with the RNeasy Mini Kit (Qiagen). Sense, antisense, and TaqMan probes for CYP450-2C9 targeted nucleotides 909 to 931, 978 to 995, and 947 to 973, respectively. Sense, antisense, and TaqMan probes for BKCa targeted nucleotides 1475 to 1495, 1527 to 1551, and 1557 to 1574, respectively. A BLAST search confirmed the specificity of both probes for their targeted genes. qRT-PCR was performed with an ABI PRISM 7700 (Applied Biosystems), and mRNA expression was presented as $2^{-\Delta\Delta C_{t}}$ (ie, abundance relative to a calibrator normalized to a housekeeping gene, glyceraldehyde phosphate dehydrogenase). 13

Laser Capture Microdissection
The PixCell II laser capture microdissector (LCM) (Arcturus Engineering) uses a microscope platform combined with a low-energy, infrared laser to melt a plastic capture film onto selected structures on 5-μm-thick LIMA sections, allowing targeted removal of vessel wall components (endothelium versus media). 13 The LCM sample is captured in a cap (Figure 8) and analyzed by qRT-PCR. 13 To confirm the cellular origins of the sample, we measured cell-specific markers (endothelium, von Willebrand’s factor, vWF; SMCs, α-smooth muscle actin) simultaneously with the genes of interest (BKCa and CYP450-2C) by use of qRT-PCR.

Measurement of 11,12-EET
LIMAs were incubated in Krebs’ solution and, after 10 minutes of incubation with PE or PE + ACh, a mL aliquot was frozen at −80°C for high-performance liquid chromatography (HPLC) analysis. Eicosanoids were separated by reverse-phase HPLC using a water and acetonitrile mobile phase. 15 The presumptive HPLC peak was identified by an atmospheric pressure ionization–mass spectroscopy method using electrospray ionization and detection in the negative ion mode. 15 Deuterated EETs served as internal standards.

Drugs
Drugs were obtained from Sigma-Aldrich and were dissolved in saline, except 11,12-EET and MSPPOH, which were dissolved in ethanol. All antibodies were from Alomone except the CYP450-2C antibody, which was a gift from Dr Ingrid Fleming (Institut für Kardiovaskulare Physiologie, Frankfurt, Germany).
Statistics

Values are expressed as mean±SEM. Intergroup differences are assessed by repeated-measures or factorial ANOVA, as appropriate, with post hoc analysis using Fisher’s protected least significant differences test. A value of \( P<0.05 \) was considered statistically significant.

Results

Comparative Importance of the NOS-PGHS and EDHF Pathways

PE caused LIMAs to contract to a plateau tension in either a stable or an oscillatory pattern (Figure 2A). The rhythmic pattern was never seen with SVs. ACh reduced PE tone by 80% in control rings versus 40% in the presence of L-NAME/Meclo (Figure 2, B and C). L-NAME eliminated virtually all basal and more than 60% of PE-induced NO production (Figure 2D). Reduced Hb eliminated the NO signal without altering the EDHF relaxation to ACh or BK (Figures 2D and 3, C and D). This indicates that the endothelial relaxation persisting after L-NAME/Meclo is caused by EDHF, not residual NO.

Target of EDHF

Relaxation to ACh was eliminated by TEA but not BaCl2 or apamin (Figure 3). Relaxations EDHF and exogenous 11,12-EET were inhibited by IBTx (Figure 6B). These data argue against a role for SKCa or Kir channels in the EDHF pathway of the LIMA and indicate an obligatory role for BK Ca channels.

Identity of EDHF

LIMAs relax in response to exogenous 11,12-EET in a concentration-dependent manner, with a maximum relaxation similar to that achieved during agonist-induced EDHF release (≈40% of PE tone; Figure 4). Inhibition of CYP450 synthesis by 2 chemically distinct agents, ODYA and MSPPOH, or a specific EET antagonist, 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE), markedly reduces ACh- or BK-induced EDHF activity (Figures 1A, 3, 4, and 7). 14,15-EEZE also reduces relaxation to exogenous 11,12-EET but not to the NO donor diethylamine-NO (Figure 7). Together, these data implicate an EET as the mediator of EDHF. Further supporting this, LIMAs (both at baseline and after ACh) synthesized a compound that comigrated with and had a mass spectrum identical to authentic 11,12-EET (Figure 4, D and E). LIMAs synthesized primarily 11,12-EET, although the regioisomers 8,9-EET and 14,15-EET were detected; Figure 4D). ACh tended to increase 11,12-EET levels (Figure 4E, inset, \( P=0.39 \)), and although ACh (10\(^{-7}\) to 10\(^{-5}\) mol/L) did not cause a dose-dependent increase in 11,12-EET synthesis (not shown), the LIMAs that made the most 11,12-EET also relaxed the most (Figure 4G).

Expression of Components of the EDHF Pathway

Neither removal of the endothelium (Figure 4C) nor treatment with L-NAME–Meclo (not shown) reduces the vasodilator effect of exogenous 11,12-EET, suggesting that the K\(_{Ca}\) channels targeted by EDHF are in the SMCs. Conventional and qRT-PCR and immunoblotting indicate that BK Ca channel and CYP450-2C expression is greatest in the LIMA (Figure 5). In addition, LCM shows that the BK Ca channels are expressed predominantly in the LIMA SMCs (α-SM actin–positive, vWF-negative sample), whereas CYP450-2C is found in both SMCs and endothelial cells (Figure 8).

Electrophysiology

LIMA SMCs display both K\(_{V}\) and BK Ca current (Figure 6). However, the same concentration of 11,12-EET that relaxes LIMA rings increases whole-cell K\(_{+}\) current in LIMA SMCs, even in the presence of 4-aminopyridine (a K\(_{+}\) channel blocker; Figure 6). 11,12-EET also causes membrane hyperpolarization by activating SMC BK Ca channels (ie, hyperpolarization is blocked by IBTx but not by 4-aminopyridine; Figure 7).
Discussion

The primary finding of this study is that human LIMAs synthesize an EDHF that accounts for ~40% of net endothelium-dependent relaxation. This EDHF is 11,12-EET, and it causes relaxation by activating SMC BKCa current, leading to SMC hyperpolarization. This study also confirms that the endothelial relaxation of the LIMA is superior to that of SVs, consistent with the superior NO and EDHF production of the LIMA. NO is the predominant endothelium-derived dilator of the LIMA (Figure 2, B and C) and is synthesized after stimulation with the vasoconstrictor PE, constituting an apparently homeostatic mechanism that serves as a brake on vasoconstriction (Figure 2D). Because NO and PGI2 are endothelium-derived vasodilators that act, in part, by activation of KCa channels, a strength of this study is the characterization of the EDHF pathway after documented suppression of NO synthesis (Figure 2D).

K+ Channels and EDHF

EDHF-induced relaxation is blocked by IBTx but not apamin (Figure 3B), consistent with EDHF-induced activation of BKCa channels. The central role for the BKCa channel is confirmed by electrophysiology experiments showing that 11,12-EET hyperpolarizes SMCs by an IBTx-sensitive, 4-aminopyridine–resistant mechanism (Figures 6 and 7). The failure of BaCl2 to attenuate EDHF relaxation is strong evidence against a role for KCa channels in the EDHF pathway of the LIMA (Figure 3A). In human coronary, cerebral, renal, omental, and subcutaneous arteries, EDHF relaxation is blocked either by TEA or by the combination of charybotoxin plus apamin. Furthermore, muscarinic receptor activation causes endothelium-dependent relaxation, EET synthesis, and SMC hyperpolarization in bovine coronary arteries. ACh and BK cause endothelium-dependent hyperpolarization of coronary arterial SMCs by increasing KCa channel activity, and this can be abolished by TEA, charybotoxin, or IBTx. There are other important K+ channels in the LIMA, including KCa channels (inhibition of which depolarizes SMCs; Figure 6). Furthermore, LIMAs often display an oscillatory pattern of contraction (Figure 1A) and express the human ether-a-go-go channel (HERG; Figure 5A). HERG is involved with pacemaker activity in the heart but has not previously been reported in human blood vessels.
association merits further investigation, particularly in light of the clinical problem of LIMA spasm at the time of CABG.

**Identification of EDHF**

To identify EDHF as 11,12-EET, it is necessary to satisfy 4 criteria: (1) EDHF bioactivity is inhibited when EET synthesis is inhibited, (2) EDHF bioactivity is mimicked by exogenous 11,12-EET, (3) no other vasodilator accounts for the relaxation, and (4) LIMAs synthesize 11,12-EET. This study satisfied each of these criteria, although further study of criterion 4 is necessary. The first criterion was satisfied by the finding that 2 structurally distinct CYP450 inhibitors and a highly specific EET antagonist, 14,15-EEZE, inhibit EDHF relaxation (Figures 3, 4, and 7). Despite the ability of 14,15-EEZE to suppress EDHF, relaxation to exogenous NO is preserved (Figure 7). Consistent with the second criterion, EDHF relaxation is mimicked by exogenous 11,12-EET. Indeed, exogenous 11,12-EET causes relaxation of the same magnitude as achieved by the EDHF agonists ACh and BK (Figures 3 and 4). These concentrations of 11,12-EET also activate a BK Ca current in LIMA SMCs, resulting in hyperpolarization (Figure 7), a sine qua non for EDHF that has not previously been documented in LIMAs.\footnote{Relevant to the third criterion, particular care was taken to exclude a confounding effect of residual NO synthesis. Hb scavenged the NO that persists after a large dose of L-NAME. Although Hb did not greatly affect basal NO levels, already lowered by L-NAME, it blunted the transient burst of NO synthesis elicited by PE (Figure 2B). The fact that addition of Hb (Figure 3, C and D) did not decrease EDHF relaxation (Figure 3B) supports the adequacy of L-NAME+Meclo at these doses for the study of EDHF.}

The present study partially satisfies criterion 4. LIMAs synthesize 11,12-EET (Figure 4, D and E). The fact that the reduction in 11,12-EET levels by MSPPOH was not statistically significant may relate to either subendothelial EET release or the observed variability in 11,12-EET production among LIMAs. Individual variability in EET synthesis in a CABG population is not surprising in light of their high prevalence of endothelial dysfunction.

In the porcine coronary artery, there is strong evidence that CYP450-2C is an EDHF synthase.\footnote{This CYP450 isoform is found in human endothelial cells,\footnote{We report, for the first time, that LIMAs are enriched in both this putative EDHF synthase and the target BK Ca channels relative to SV (Figure 5C). This is also the first demonstration that the BK Ca channels relevant to EDHF are present primarily in SMCs (Figures 1 and 8), whereas CYP450-2C is present in both endothelial cells and SMCs. The relative importance of endothelial versus SMC CYP450-2C is interesting in light of the unusually complete internal elastic lamina in the LIMA, which separates the vessel wall into compartments, and merits further study.}

**Limitations**

This study does not define a universal EDHF pathway. In rat mesenteric artery and guinea pig carotid artery, several groups have failed to find an EET-mediated or ODYA-sensitive EDHF.\footnote{Although EETs can increase opening of K Ca channels in endothelial cells (Figure 1), this is not obligatory to EDHF activity in human LIMAs, because denuding the LIMA did not significantly impair relaxation to exogenous 11,12-EET (Figure 4C).}

Additional work is required to assess how 11,12-EET activates BK Ca channels. In bovine coronary artery SMCs, this occurs through a guanine nucleotide–binding protein, $G_{i/o}$, independent of cGMP, suggesting that SMCs may have EET receptors.\footnote{This is consistent with our finding that 14,15-EET, an antagonist that does not alter EET synthesis, inhibits EDHF vasodilation.}
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**References**


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