Endothelium-Derived Hyperpolarizing Factor
Identification and Mechanisms of Action in Human Subcutaneous Resistance Arteries

Paul Coats, PhD, BSc; Fiona Johnston, BSc; John MacDonald, MD, MRCP, BSc; John J.V. McMurray, MD, FRCP, FESC, BSc; Chris Hillier, PhD, BSc

Background—Both a vascular endothelial cytochrome P450 (CYP450) product of arachidonic acid metabolism and the potassium ion (K⁺) have been identified as endothelium-derived hyperpolarizing factors (EDHFs) in animal vascular tissues. We studied the relative importance of EDHF, nitric oxide (NO), and prostacyclin (PGI₂) as vasodilators in human subcutaneous arteries. We also examined the mechanisms underlying the vasodilator action of EDHF to elucidate its identity.

Methods and Results—Subcutaneous resistance arteries were obtained from 41 healthy volunteers. The contribution of EDHF to the vasodilation induced by acetylcholine was assessed by inhibiting production of NO, PGI₂, and membrane hyperpolarization. The mechanisms underlying the relaxation evoked by K⁺ and EDHF were elucidated. EDHF was found to account for ≈80% of acetylcholine-mediated vasorelaxation. Its action was insensitive to the combination of barium and ouabain, whereas barium and ouabain reversed K⁺-mediated vasorelaxation. EDHF-mediated vasorelaxation, however, was sensitive to the phospholipase A₂ inhibitor oleyloxyethyl phosphorylcholine and the CYP450 inhibitor ketoconazole.

Conclusions—EDHF is the major contributor to endothelium-dependent vasorelaxation in human subcutaneous resistance arteries. A product of phospholipase A₂/CYP450—dependent metabolism of arachidonic acid and not K⁺ is the likely identity of EDHF in human subcutaneous resistance arteries. (Circulation. 2001;103:1702-1708.)

Key Words: endothelium-derived factors □ nitric oxide

Endothelial dysfunction is known to occur in a number of cardiovascular diseases, including atherosclerosis, hypertension, and heart failure.1-6 Therefore, considerable interest is attached to understanding the mechanisms underlying endothelium-dependent vasorelaxation. It is hoped that therapeutic strategies may be developed to counter vascular endothelial dysfunction in these and other diseases.7 The vascular endothelium modulates local blood flow via the dynamic release of numerous vasoactive factors. Receptor-dependent agonists, such as acetylcholine, bradykinin, and substance P, indirectly relax vascular smooth muscle by inducing the release of the known endothelium-derived relaxing factors (EDRFs) nitric oxide (NO), prostacyclin (PGI₁), and endothelium-derived hyperpolarizing factor (EDHF).8-11 At present, there is no clear consensus on the identity of EDHF or the exact mechanisms by which EDHF relaxes vascular smooth muscle. Some evidence, however, demonstrates that the action of EDHF involves calcium-sensitive K⁺ channels (KCa), which are sensitive to the combined effects of the toxins apamin and charybdotoxin.12-15

To date, numerous studies have aimed to identify the nature of EDHF and its mechanisms of action. Experimental evidence suggests that the most likely candidates are the P450 metabolite of arachidonic acid metabolism, 11,12-epoxyeicosatrienoic acid (11,12-EET), and the potassium ion (K⁺).15-19 K⁺ has long been known to possess vasodilator properties.20-22 Recently, Edwards and colleagues15 proposed that K⁺, as an EDHF, was extruded from the endothelium into the myoendothelial gap, leading to activation of vascular smooth muscle inwardly rectifying potassium channels and Na⁺,K⁺-ATPase pumps. This in turn resulted in efflux of K⁺ from the vascular smooth muscle, inducing hyperpolarization and vasorelaxation. Other recent and compelling evidence, however, suggests that the CYP450 enzyme product 11,12-EET, derived from the endothelium-dependent metabolism of arachidonic acid, is an EDHF in porcine coronary arteries and hamster gracilis muscle artery.19,23 The evidence that K⁺ or a P450 enzyme product is an EDHF, however, remains controversial.24-29 Therefore, real uncertainty exists regarding the identification of EDHF.

Received July 13, 2000; revision received October 4, 2000; accepted October 16, 2000.
From the Department of Biological and Biomedical Sciences, Glasgow Caledonian University (P.C., F.J., C.H.), and the Department of Medicine and Therapeutics, Western Infirmary (J.M., J.J.V.M), Glasgow, Scotland.
Correspondence to Dr Paul Coats, School of Biological and Biomedical Sciences, Glasgow Caledonian University, Cowcaddens Road, Glasgow, Scotland, UK. E-mail p.coats@gcal.ac.uk
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Circulation is available at http://www.circulationaha.org

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All subsequent acetylcholine experiments were carried out with arteries preincubated with 100 μmol/L L-NOARG + 30 μmol/L indomethacin. CCRCs were then constructed after intraluminal incubation with either (1) glibenclamide (1 μmol/L), (2) norepinephrine + acetylcholine (10 μmol/L), (3) norepinephrine + acetylcholine + L-NOARG (100 μmol/L), or (4) norepinephrine + acetylcholine + ODQ (10 μmol/L). All experiments were performed in the presence of isobutylmethylxanthine (10 μmol/L).

### Pharmacological Protocols

All studies were carried out in arteries preconstricted with norepinephrine to 80% of maximum response. In experiments in which K⁺-modified PSS was used, the concentration of norepinephrine was reduced to maintain a preconstriction diameter similar to previous measures, comparison of curves: *P<0.05, response vs ACh, n=12.

### Identification of K⁺ Channels

All subcutaneous artery segments (>3 mm) were isolated from 3 volunteers and incubated in PSS in the presence of either (1) norepinephrine (1 μmol/L), (2) norepinephrine + acetylcholine (10 μmol/L), (3) norepinephrine + acetylcholine + L-NOARG (100 μmol/L), or (4) norepinephrine + acetylcholine + ODQ (10 μmol/L). All experiments were performed with Student’s paired t test followed by multiple comparisons by Bonferroni’s test where appropriate. Not calculable (NC) appears where the pEC₅₀ could not be determined. Comparison of

| TABLE 1. Basic Clinical Details of Volunteers Who Provided Biopsies |
|-----------------------------|-----------------|
| Age, y                      | 65±1            |
| Creatinine, μmol/L          | 93±2.5          |
| Urea, mmol/L                | 5.6±0.2         |
| Cholesterol, mmol/L         | 5.18±0.2        |
| Glucose, mmol/L             | 5.31±0.2        |
| Systolic BP, mm Hg          | 151±24          |
| Diastolic BP, mm Hg         | 81±7            |

BP indicates blood pressure.
CCRCs was by one-way ANOVA for repeated measures. Statistical significance was assumed at a value of $P<0.05$.

**Results**

The effects of L-NOARG, indomethacin, and 25 mmol/L $K_\text{1}$PSS on acetylcholine-mediated vasorelaxation are shown in Figure 1. Incubation with L-NOARG significantly reduced both the maximum relaxation and $pEC_{50}$ (137 ± 24 versus 72 ± 14 nmol/L, $P<0.05$) to acetylcholine. Subsequent incubation with indomethacin failed to modify this response further. Exchange of PSS with 25 mmol/L $K_\text{1}$ PSS, however, abolished the relaxation response to acetylcholine (Figure 1).

Figure 2 summarizes the results of the cGMP radioimmunoassay. As expected, acetylcholine substantially increased cGMP generation. Incubation with L-NOARG or ODQ abolished the acetylcholine-dependent liberation of cGMP. Both L-NOARG (100 μmol/L) and ODQ (10 μmol/L) were equipotent at inhibiting the generation of cGMP.

Incubation with 10 μmol/L glibenclamide had no effect on the acetylcholine-dependent response (Figure 3). Apamin or charybdotoxin alone also had little effect on the response to acetylcholine (Figure 4). Incubation with the combination of apamin and charybdotoxin, however, abolished the relaxation to acetylcholine. Substitution of charybdotoxin with iberiotoxin had no effect on the relaxation response (Figure 5). Cumulative addition of KCl on preconstricted arteries initially produced a concentration-dependent relaxation (4.6 to 14 mmol/L). This was reversed at higher concentrations (16 to 20 mmol/L) to a vasoconstriction response (Figure 6). Ba$^{2+}$ (30 μmol/L) or ouabain (1 mmol/L) alone was unable to significantly modify the $K_\text{1}$-mediated responses (data not presented). When combined, however, Ba$^{2+}$ and ouabain reversed $K_\text{1}$-induced vasorelaxation, resulting in vasoconstriction at all concentrations of KCl (Figure 6). In contrast, Ba$^{2+}$ and ouabain either alone (data not shown) or combined, at concentrations that reversed $K_\text{1}$-mediated vasorelaxation, had no effect on acetylcholine-mediated vasorelaxation (Figure 7).

The phospholipase A$_2$ inhibitor OOPC reduced both sensitivity and maximum relaxation responses to the L-NOARG/
indomethacin-insensitive component of acetylcholine-mediated vasorelaxation in a concentration-dependent manner (Figure 8; EC₅₀: ACh, 0.2 ± 0.1 μmol/L; 30 μmol/L OOPC, 1.0 ± 0.6 μmol/L; 130 μmol/L OOPC, NC; P < 0.05, 30 μmol/L OOPC versus ACh). OOPC had no effect on the sensitivity to norepinephrine, because the concentration used for preconstriction remained relatively constant. Furthermore, OOPC failed to inhibit the response to cumulative addition of sodium nitroprusside (Table 2). Incubation with the highest OOPC concentration (100 μmol/L) failed to modify the relaxation response to the ATP-sensitive K⁺ channel opener pinacidil and the Ca²⁺-sensitive K⁺ channel opener EBIO (Table 2).

The cytochrome P450 inhibitor ketoconazole also resulted in a concentration-dependent inhibition of the L-NOARG/indomethacin-insensitive relaxation to acetylcholine (Figure 9; EC₅₀: ACh 0.3 ± 0.1 μmol/L; 1 μmol/L ketoconazole, 1.0 ± 0.6 μmol/L; 10 μmol/L ketoconazole, 3.5 ± 1.7 μmol/L; 30 μmol/L ketoconazole, NC; 100 μmol/L ketoconazole, NC; P < 0.05, 1 μmol/L ketoconazole versus ACh and 10 μmol/L ketoconazole versus 1 μmol/L ketoconazole). Again, ketoconazole did not modify the sensitivity of the tissue to the preconstricting agonist norepinephrine. Also, sodium nitroprusside-, EBIO-, and pinacidil-dependent responses were unaffected by the highest concentration of ketoconazole (100 μmol/L, Table 3).

**Discussion**

This study has 2 important findings. First, it demonstrates that EDHF in human subcutaneous resistance arteries is the major component of endothelium-dependent vasorelaxation. Second, a product of arachidonic acid metabolism, probably a cytochrome P450 product rather than the potassium ion, is likely to be an EDHF in these arteries.

A number of studies have suggested that a substance independent of NO and PGI₂ is the major EDRF(s) in resistance arteries. Our results demonstrate that the L-NOARG/indomethacin-insensitive component of acetylcholine-mediated relaxation is the major component of endothelium-dependent relaxation in human subcutaneous resistance arteries. The NO component, ie, the L-NOARG-sensitive component, accounted for only ~20% of the maximum relaxation response to acetylcholine. Furthermore, indomethacin had no effect on the endothelium-dependent relaxation, indicating that PGI₂ plays no role in the relaxation response in these ex vivo conditions. In contrast, the major component of the endothelium-dependent relaxation was sensitive to 25 mmol/L K⁺ PSS. This sensitivity to high

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**Table 2. Sensitivity (pEC₅₀) and Maximum Relaxation Response to Pinacidil (n=3), Sodium Nitroprusside (SNP, n=3), and EBIO (n=3) Before and After Incubation With 100 μmol/L OOPC**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pEC₅₀, mmol/L</th>
<th>Maximum Relaxation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinacidil</td>
<td>160±120</td>
<td>78±9</td>
</tr>
<tr>
<td>Pinacidil + 100 μmol/L OOPC</td>
<td>190±140</td>
<td>78±8</td>
</tr>
<tr>
<td>SNP</td>
<td>40±30</td>
<td>93±2</td>
</tr>
<tr>
<td>SNP + 100 μmol/L OOPC</td>
<td>51±48</td>
<td>96±3</td>
</tr>
<tr>
<td>EBIO</td>
<td>NC</td>
<td>86±4</td>
</tr>
<tr>
<td>EBIO + 100 μmol/L OOPC</td>
<td>NC</td>
<td>84±4</td>
</tr>
</tbody>
</table>

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**Figure 6.** Effect of increasing [K⁺] from 4.6 to 20 mmol/L in small steps before and after incubation with combination of Ba²⁺ (30 μmol/L) and ouabain (1 mmol/L, n=6).

**Figure 7.** Vasorelaxation to acetylcholine (ACh) in presence of 100 μmol/L L-NOARG + 30 μmol/L indomethacin before and after incubation with Ba²⁺ and ouabain (n=6).

**Figure 8.** Vasorelaxation to acetylcholine (ACh) in presence of 100 μmol/L L-NOARG + 30 μmol/L indomethacin after luminal incubation with phospholipase A₂ inhibitor OOPC. ANOVA for repeated measures, comparison of curves: *P<0.05, ACh response vs previous ACh response (n=6).
concentrations of K⁺ PSS, which has been shown to be a characteristic of EDHF, accounted for ≈75% of the maximum response to acetylcholine. [13,36,38,41,42] This study confirms the relative importance of EDHF and that NO and PGI₂ play relatively minor roles in endothelium-dependent relaxation of human resistance arteries. [11,34,43,44]

In vessels in which NO synthase and cyclooxygenase-1 are inhibited, glibenclamide, a Kₐₐₚ channel inhibitor previously shown to inhibit EDHF-mediated vasorelaxation in rabbit cerebral arteries, had no measurable effect on EDHF-mediated relaxation to acetylcholine in human subcutaneous resistance arteries. [45] This indicates that Kₐₐₚ channels have no role in this response.

One of the few points of consensus to emerge in EDHF research has been that preincubation of arteries with either apamin, an inhibitor of large-conductance calcium-sensitive K⁺ channels (BK ca), or charybdotoxin, an inhibitor of intermediate-conductance calcium-sensitive K⁺ channels (IK ca), alone has little effect on the EDHF-mediated relaxation to acetylcholine; when combined, however, apamin and charybdotoxin together abolish the EDHF-mediated relaxation. [13,15,46] Our results confirm that the EDHF-mediated relaxation in human subcutaneous resistance arteries is similarly resistant to apamin or charybdotoxin alone but is abolished by the combination of the 2 toxins. Interestingly, substitution of charybdotoxin with iberiotoxin, a selective inhibitor of BK ca, in the presence of apamin had no effect on the relaxation response. [47] Interpretation of these findings is difficult. Inhibition of IK ca or BK ca alone clearly does not inhibit the actions of EDHF, suggesting that there is a BK ca/IK ca channel codependency involved in this mechanism of vasorelaxation. Alternatively, there may be an as yet unidentified BK ca/IK ca-like heteromultimeric K⁺ channel associated with an EDHF-mediated response. [48] Charybdotoxin has inhibitory action at BK ca and a number of voltage-sensitive K⁺ channels. [49] The fact that charybdotoxin, as opposed to iberiotoxin, is required in combination with apamin to block the relaxation response insensitive to L-NOARG and indomethacin suggests that Ca²⁺-sensitive K⁺ channels and voltage-sensitive K⁺ channels are involved in this response.

Elevation of the extracellular potassium concentration, [K⁺] o, has been shown to dilate isolated arteries from rat cerebral, coronary, hepatic, and mesenteric vascular beds. [15,21] The mechanism of this response involves K⁺ efflux from vascular smooth muscle via the inwardly rectifying potassium channel (Kir) and the Na⁺·K⁺-ATPase pump, leading to hyperpolarization and vasorelaxation. Using the protocols established by Edwards and colleagues, we mimicked the effects of EDHF by increasing [K⁺] o. In our study, elevation of [K⁺] o resulted in a small vasorelaxation of preconstricted arteries. The maximum relaxation response occurred at ≈14 to 16 mmol/L [K⁺] o; increasing [K⁺] o further resulted in vasoconstriction. The [K⁺] o-dependent vasodilation observed was sensitive to the combination of Ba²⁺, an inhibitor of Kir, plus ouabain, an inhibitor of the Na⁺·K⁺·ATPase pump, but not Ba²⁺ or ouabain alone. In fact, elevation of [K⁺] o in the presence of Ba²⁺ plus ouabain resulted in a concentration-dependent vasoconstriction at all increments of [K⁺] o and in all arteries studied. This observation confirms Kir as having vasodilator properties and confirms the role of the Kir and the Na⁺·K⁺-ATPase pump in K⁺-dependent vasodilation in these vessels. Ba²⁺ plus ouabain, however, had no effect on the EDHF-mediated relaxation to acetylcholine. This observation shows that EDHF is insensitive to inhibition of the Kir and the Na⁺·K⁺-ATPase pump.

These results demonstrate that the EDHF component of the acetylcholine-mediated endothelium-dependent relaxation is mediated via mechanisms different from those through which K⁺ mediates vasorelaxation. If K⁺ were an EDHF in human subcutaneous resistance arteries, this response should have been sensitive to the combination of Ba²⁺ plus ouabain.

Phospholipase A₂-dependent metabolism of membrane-bound phospholipids is a primary source of arachidonic acid and contributes to EDHF-mediated vasorelaxation in rabbit mesenteric arteries. [50] In turn, the metabolism of arachidonic acid by P450 enzymes into vasoactive substances is a commonly identified phenomenon in vascular physiology. [51–55] In the present study, luminal incubation with OOPC, a specific inhibitor of phospholipase A₂, had a profound effect on endothelium-dependent relaxation to EDHF. Likewise, luminal incubation with the P450 enzyme inhibitor ketoconazole resulted in a concentration-dependent inhibition. The OOPC and ketoconazole results provide strong evidence that the endothelium-dependent relaxation to EDHF is a product

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**TABLE 3. Sensitivity (pEC₉₀) and Maximum Relaxation Response to Pinacidil (n=3), Sodium Nitroprusside (SNP, n=3), and EBIO (n=3) Before and After Incubation with 100 µmol/L Ketoconazole (KETO)**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pEC₉₀</th>
<th>Maximum Relaxation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pinacidil</td>
<td>147±115</td>
<td>78±9</td>
</tr>
<tr>
<td>Pinacidil + 100 µmol/L KETO</td>
<td>160±130</td>
<td>78±8</td>
</tr>
<tr>
<td>SNP</td>
<td>52±4</td>
<td>92±4</td>
</tr>
<tr>
<td>SNP + 100 µmol/L KETO</td>
<td>38±50</td>
<td>93±3</td>
</tr>
<tr>
<td>EBIO</td>
<td>NC</td>
<td>87±4</td>
</tr>
<tr>
<td>EBIO + 100 µmol/L KETO</td>
<td>NC</td>
<td>89±3</td>
</tr>
</tbody>
</table>
of phospholipase A2/arachidonic acid/P450 enzyme metabolism in human subcutaneous resistance arteries.

We are confident that these results reflect specifically endothelium-dependent mechanisms, because the vasorelaxation response to the endothelium-independent vasodilator sodium nitroprusside was unaffected by the highest concentrations of OOPC and ketoconazole. Moreover, the endothelium-independent mechanisms of hyperpolarization were unaffected by this treatment, because the K\textsubscript{ATP} channel opener pinacidil and the Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channel opener EBIO produced potent vasorelaxation unaffected by either OOPC or ketoconazole.

In this study, we were unable to measure smooth muscle membrane potential and therefore have no direct evidence that the acetylcholine-mediated relaxation insensitive to L-NOARG and indomethacin is definitely the result of hyperpolarization. Raising [K\textsuperscript{+}]\textsubscript{o} to 25 mmol/L, however, completely blocked the L-NOARG/indomethacin-insensitive response to acetylcholine. Previous studies have shown that raising [K\textsuperscript{+}]\textsubscript{o} has little effect on NO and PGI\textsubscript{1}-mediated vasorelaxation, but rather it specifically antagonizes the actions of EDHF by counterbalancing smooth muscle cell membrane potential.

Experimental evidence suggests that the commonly used concentrations of NO synthase inhibitors may not be completely effective in blocking all NO production.\textsuperscript{56,57} This is important, because NO has been shown to have a hyperpolarizing effect via cGMP in vascular smooth muscle and the response insensitive to L-NOARG may be as a consequence of residual NO production. L-NOARG (100 \mu \text{mol/L}) in this tissue, however, abolished the acetylcholine-dependent liberation of cGMP. Therefore, we are confident that there was no residual NO and that the relaxation responses observed were independent of NO. Moreover, NO has been shown to hyperpolarize vascular tissue via cGMP- and cAMP-dependent mechanisms sensitive to ibetrixotin and glibenclamide, respectively. Both ibetrixotin and glibenclamide had no effect on the relaxation response insensitive to L-NOARG and indomethacin. Therefore, we are confident that the acetylcholine-mediated relaxation insensitive to L-NOARG and indomethacin is truly an NO/prostanoid-independent phenomenon and is likely to be mediated by an EDHF.

This study shows that endothelium-dependent relaxation to EDHF is mediated via mechanisms different from those mediating K\textsuperscript{+} vasorelaxation; therefore, it is unlikely that K\textsuperscript{+} is an EDHF in human subcutaneous resistance arteries. Moreover, this mechanism is sensitive to phospholipase A2 and P450 enzyme inhibition, thus indicating that EDHF in human subcutaneous resistance arteries is likely to be a P450 enzyme–dependent product of arachidonic acid metabolism. Also, vasodilation depends largely on K\textsuperscript{+} channels, suggesting that selective use of appropriate potassium channel drugs may provide a useful therapeutic approach for the treatment of vascular pathologies.

Acknowledgments
This study was supported by the Barnwood House Trust. Dr Coats was supported by a Glasgow Caledonian University (UK) Research Studentship. The authors thank Dr David Bunton for his assistance with cGMP radioimmunoassay.

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_Circulation_. 2001;103:1702-1708
doi: 10.1161/01.CIR.103.12.1702

_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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