ABSTRACT—Investigation of endothelial regulation of vascular reactivity and tone has led to the discovery of chemical mediators such as nitric oxide (NO) and prostacyclin (PGI$_2$). Evidence has emerged indicating another as yet unidentified hyperpolarizing agent (endothelium-derived hyperpolarizing factor or EDHF) that is different from NO and PGI$_2$ and exerts its effects through calcium-activated potassium channels (K$_{Ca}$). Previous studies to identify EDHF have been carried out using inhibitors that block NOS and COX before application of K$_{Ca}$ channel and/or muscarinic receptor antagonists. Such pharmacological manipulation has complicated interpretation of results, clearly pointing to the need for altered approaches to verify previous studies. Evidence has emerged that potential EDHF candidates vary with vessel size, species and tissue beds, indicating that there may be more than one EDHF. To date, the most commonly described and best characterized of them all are a set of arachidonic acid metabolites, epoxyeicosatrienoic acids (EETs). These compounds are synthesized both intra- and extravascularly. Until recently, methodology to detect EETs in the microvasculature has been tedious and expensive, limiting the experimentation that is necessary to confirm EETs as an EDHF. This review describes state-of-the-art methods for assaying EETs in biological samples, after summarizing evidence for EETs as an EDHF and introducing emerging concepts of the role of extravascular EETs in linking neuronal activity to localized blood flow during functional hyperemia.

Keywords: Endothelium, Endothelium-derived hyperpolarizing factor, Fluorescence assay, Epoxyeicosatrienoic acid, Vascular tone

1. Introduction
It has been over 20 years since Furchgott and Zawadzki first described the obligatory role of the endothelium in contributing to vascular smooth muscle function following stimulation of muscarinic receptors (1). Following this discovery, intense research confirmed that the endothelium generated a number of factors that were vaso-relaxing agents, including nitric oxide (NO) and prostaglandins (reviewed in references 2 and 3). Relatively early in these investigations, researchers noticed that the relaxation of blood vessels such as the rabbit aorta were not only dependent on NO but were also sensitive to inhibitors of cyclooxygenase (COX) and nitric oxide synthase (NOS) (4). The factor(s) responsible for P450 sensitivity were labile and synthesized by the endothelium. Similarly, rat intestinal vasodilation could be regulated by application of arachidonic acid (AA) which was partially blocked by the cytochrome P450 inhibitors SKF 525A (10 μg/ml) or metyrapone (100 μg/ml) (5). Interestingly, application of derivatives of AA synthesized by the cytochrome P450 epoxygenase or monooxygenases enzymes, epoxyeicosatrienoic acids (EETs), induced relaxation in the intestinal microcirculation, with potency comparable to adenosine. The EETs are synthesized by endothelium, very labile and treatment with 5,6-EET (32 ± 6 μg/ml), 11,12-EET (25 ± 3 μg/ml) and 8,9-EET (36 ± 2 μg/ml) induce vasodilation of intestinal vessels, fulfilling the criteria defined above (5).

Although the identity of the factor(s) was not confirmed, it was consistent in relaxing vascular smooth muscle in the presence of inhibitors of cyclooxygenase (COX) and nitric oxide synthase (NOS) and so was named endothelium-derived hyperpolarizing factor (EDHF) (6). Some investigators had shown that EDHF functioned by interaction with the Na$^+$/K$^+$ pump (2, 6), while others clearly demonstrated it was mediated by K-channels (see reviews 2, 3).
channels as determined by the patch clamp technique.

EDHF was a cytochrome P450 metabolite that was measured in bovine coronary artery segments once again suggested release until recently. However, studies in porcine and bovine coronary arteries and endothelial cells from the same vessels within a transit time of under 30 ms indicated in this review.

2. Evidence for EETs as EDHF

Investigation of the cerebral circulation has shown that the brain synthesizes EETs and that they influence cerebrovascular tone (7, 8). Studies have shown that at least three EET isomers activate K⁺ channels in vascular smooth muscle, leading to vasodilation, and that blockade of these channels with tetraethylammonium (TEA) attenuates this effect (7), which is consistent with EDHF.

Although characterization of the structure of EDHF was attempted, there has been little success in measuring its release until recently. However, studies in porcine and bovine coronary artery segments once again suggested that EDHF was a cytochrome P450 metabolite that was sensitive to a phospholipase A₂ (PLA₂) inhibitor, quinacrine (30 μM), or P450 inhibitors SKF525A (30 – 100 μM) and clotrimazole (100 μM) (9, 10). In addition, methacholine was shown to induce relaxation and hyperpolarization in bovine coronary arteries that could be blocked by TEA and high [K⁺]o (10). This data correlated with biochemical data showing that methacholine stimulated the release of a number of AA metabolites including the EETs, thus again supporting the concept that EETs or a P450 metabolite could be EDHF. Furthermore, it was shown that bovine coronary arteries and endothelial cells cultured from these vessels were able to metabolize AA via COX, lipoxygenase and cytochrome P450 pathways (10, 11), with 14,15-, 11,12-, 8,9- and 5,6-EETs being the major metabolites.

The exact nature of EDHF was still difficult to confirm in the absence of a convenient and sensitive bioassay for these products. Several studies were performed using endothelium-intact donor vessels artificially placed in line with endothelium-denuded vessels. These experiments indicated that the hyperpolarizing factor was diffusible (12). This was elegantly demonstrated in cannulated bovine coronary arteries, with intact endothelium, which were treated with inhibitors of NO and prostaglandin synthesis (13). Perfusion from this source was applied to isolated smooth muscle cells from the same vessels within a transit time of under 10 s. Addition of bradykinin to the donor vessel induced a 1.2 – 5-fold increase in the open state probability of 240 picoSiemen (pS) single (Ca²⁺)-activated potassium (K⁺) channels as determined by the patch clamp technique.

Removal of the endothelium or inhibition of cytochrome P450 in the donor arterial segment abolished this increase in channel activity. These findings (13) strongly support the role of a P450 product as an EDHF in bovine coronary arteries because it directly measured the effect of the diffusible agent on its target, the KCa channel. Further studies demonstrating a diffusible cytochrome P450 intermediate as the EDHF have recently been reviewed (2, 3).

Most evidence supporting EETs as EDHF have been obtained using pharmacological inhibitors. Unfortunately, none of these inhibitors have a high degree of specificity, and the possibility remains that they may have effects on ion channels or other downstream pathways involved in the EDHF response. Thus, non-pharmacological approaches to the study of EDHF have been very helpful to support the existing evidence (14). Porcine coronary endothelial cells were examined for the presence of messenger RNA of known epoxygenase enzymes. Even though there are a number of epoxygenase genes reported in endothelial cells (e.g., isoform 2J2 (15), which demonstrated anti-inflammatory activity), candidate enzymes were screened for the ability to be up-regulated by β-naphthoflavone, a compound that increased EDHF-mediated relaxation and hyperpolarization of porcine coronary arteries. The porcine P450 gene 2C34 (homologous to human 2C8) was found to be induced by β-naphthoflavone in these arteries and could not be detected by the reverse transcriptase polymerase chain reaction (RT-PCR) in endothelium-denuded vessels (14), indicating that the gene is expressed solely in endothelial cells.

EDHF activity of these pig vessels was inhibited by sulphaphenazole, a selective inhibitor of human CYP 2C8/9. Endothelial cells are capable of making 11,12- and 8,9-EETs, the production of which is induced by β-naphthoflavone. Charybdoxin and apamin (inhibitors for large and small conductance K⁺ channels, respectively) blocked the EDHF response in porcine coronary arteries, but ouabain and barium (inhibitors of the Na⁺/K⁺-ATPase pump) had no effect (14). Furthermore, an antisense oligonucleotide directed against the 2C8 isoform (homologous to porcine 2C34) inhibited the bradykinin-induced EDHF-mediated relaxation and hyperpolarization. Interestingly, this antisense oligonucleotide enhanced NO-mediated relaxation to bradykinin, suggesting that a by-product of EET synthesis such as superoxide may decrease the production of NO (14). In summary, synthesis of endothelial EETs and hyperpolarization-induced relaxation of porcine coronary arteries were selectively modulated by altering expression of the 2C34 (human 2C8) gene product using a molecular approach, substantiated by pharmacological induction of the same gene (14).

Measuring EETs is further complicated by the fact that EETs in the vascular endothelium can be bound to phos-
pholipids (16). Indeed, inhibitors of PLA₂ and PLC attenuate the EDHF response, indicating that at least part of EET production is actually release from phospholipid storage pools. Thus, compounds such as adrenergic receptor agonists could stimulate the phospholipases to release AA from the phospholipid pool to be available foro conversion to EETs or to liberate esterified EETs that have been incorporated into vascular membrane pools (16). This effect could explain the action of thimerosal-induced EDHF-like activity, since this agent blocks acetyltransferases that could esterify EETs back into phospholipids (see review 3). Another level of complexity in this paradigm is that some of the EDHF-like properties have been assigned to metabolites of EETs, the DiHETEs (vic-dihydroxyeicosatrienoic acids) that are also synthesized by endothelial cells (11, 16).

In summary, EETs satisfy the profile for EDHF in that: i) they are diffusible (13); ii) EETs are synthesized by the endothelium (11, 14); iii) the production of EETs is increased after stimulation by agonists that elicit an EDHF response (10, 13, 14); iv) exogenous EETs mimic the actions of EDHF (7, 8); and v) convincing molecular evidence revealed an epoxygenase gene product that upon induction increased the EDHF response (14). Still, it remains controversial whether EETs are the EDHF in the coronary and other vascular beds.

3. Other candidates as EDHFs

There is evidence against EETs as EDHF and a number of other compounds have been proposed to be candidates for EDHF (2, 3). The greatest criticism against EETs as EDHF are the non-specific actions of the cytochrome P450 inhibitors used in the studies and their failure to inhibit the EDHF response in some instances. In addition, there have been disparities between responses by agonists known to induce EDHF versus other hyperpolarizing factors. Furthermore, studies have shown that EETs lack potency as vasodilators in specific vascular beds.

Other putative candidates suggested to be EDHFs are also being investigated. By definition, NO and COX products are eliminated because the EDHF response is measured in the presence of inhibitors of the enzymes that synthesize these compounds. A report of lipoxygenase-dependent EDHF activity in rabbit aorta describes a different eicosatrienoic acid (THETA or 11(S),12(S),15(S)-trihydroxyeicosatrienoic acid) as an EDHF (17).

One candidate with considerable evidence supporting its role as EDHF is K⁺. These ions are released from the endothelium of rabbit hepatic and mesenteric arteries and activate vascular smooth muscle Na⁺/K⁺-ATPase and Kᵢᵡ (inward rectifying potassium channels) to induce hyperpolarization (18). However, inhibitors like ouabain and Ba²⁺, which block both pathways of hyperpolarization brought about by K⁺, do not inhibit the EDHF response in porcine coronary arteries (14).

Cannabinoids, including anandamide (a compound involving AA for its synthesis) have also been suggested to be EDHF. These compounds have been shown to regulate vasodilation in rat cerebral arterioles (19) and have been implicated in other EDHF-like responses in rat vessels. In mice, however, anandamide agonists inhibited the EDHF response (20). Therefore, a systematic characterization of the agonist-induced release and electrophysiological effects of cannabinoids must be carried out.

A number of investigators have studied the role of vascular gap junctions, as mediators of an electro-coupled EDHF (2, 3, 20). These are intercellular channels belonging to the connexin family of proteins, which allow ionic movement between cells to propagate action potentials. Although gap junctions have been located in vascular tissue and their inhibitors have impaired EDHF responses in certain instances, there is not enough data yet to convincingly show their participation in the EDHF response. These proteins show considerable promise as playing a role in the EDHF response, but the chemical component that is communicated through the channels from endothelial cells to the underlying smooth muscle remains to be identified.

Recently, there is added evidence supporting H₂O₂ as an EDHF (21). This has been shown in eNOS knock-out mice, where the mesenteric arteries had a compromised ability to produce EDHF. H₂O₂ production by the endothelium was documented by confocal microscopy. In addition, exogenous H₂O₂ relaxed and hyperpolarized vessels that were denuded of endothelium (21). In fact, reactive oxygen species have been shown to be products of epoxygenase activity in porcine coronary arteries (22).

To summarize the current hypotheses of the nature of EDHF, most reports suggest that there may be more than one EDHF, and this would explain the divergent data and variable responses recorded between species, vessels of different size and vascular beds.

4. EETs and functional hyperemia in the brain

An exciting extension of the role of EETs as an EDHF is the hypothesis that EETs mediate cerebral functional hyperemia (reviewed in ref. 23), which is the change in blood flow in response to local changes in neuronal metabolic activity. Functional hyperemia induced by application of a second exogenous neurotransmitter, glutamate, to the intact, anesthetized rat brain was inhibited by the cytochrome P450 inhibitor miconazole, as well as by antisense oligonucleotides to the monoxygenase, 2C11 (24). This enzyme has been detected in cultured rat astrocytes (25), which have also been shown to synthesize EETs from radioactive AA precursors (25). In addition, mouse brain has been reported to synthesize 14,15- and 5,6-EET (8). However, in the case of cultured rat astrocytes, EET
synthesis was induced threefold by the neurotransmitter glutamate (26). Vasodilation of rat pial vessels induced by addition of exogenous NMDA (N-methyl d-aspartate) in vivo has been shown to be sensitive to the specific EET inhibitors MS-PPOH (N-methylsulfonyl-6-(2-propargyl-oxophenyl) hexanamide) and miconazole (27). These experiments provide molecular as well as pharmacological evidence that neurotransmitters liberated during cognitive neuronal stimulation may induce release of astrocytic EETs. Since astrocytes extend foot processes onto cerebral blood vessels, the EETs derived from astrocytes have the same potential as their endothelial counterparts to regulate vascular smooth muscle activity, and relax cerebral microvessels, thus increasing cerebral blood flow on demand. The synthesis of EETs may be regulated differentially in variable cell types. Tissue-specific regulation of EETs expression and activity may explain the divergent results observed in many studies. Therefore, cerebral vascular flow may be coupled to endothelial factors as well as extraneous EETs produced by astrocytic release triggered by spillovers during neurotransmission.

5. Variations in EDHF response
The controversies over the nature of EDHF result largely because of the diversity in responses recorded, depending on the species, size and tissue source of vessels. There are reports that EDHF activity is greater in smaller versus larger arteries (28). In one study, three vessels of varying sizes, rat aorta, proximal and distal mesenteric arteries, were used to record EDHF-induced hyperpolarization. The roles of EDHF and other relaxing factors in inducing this response were determined in each vessel (28) using inhibitors. The contribution by PGI2 was negligible in all three blood vessels tested. The EDHF response was most prominent in the distal mesenteric arteries, while NO activity and expression was maximal in the aorta and lowest in the distal mesenteric. These results indicate that EDHF increases as vessel size decreases, while the NO system dominates in larger blood vessels (28). This tends to confirm other reports in which smaller peripheral vessels have shown higher EET production and sensitivity (see reviews 2 and 3 for other examples). Further complicating the issue, different vascular beds exhibit varied responsiveness to the different EET isoforms. For example the labile 5,6-EET is the most potent EET isomer to dilate rat coronary vessels (2), while bovine coronary arteries relax to the same extent in the presence of all four regioisomers (7).

6. Measurement of EETs in biological samples
To verify the nature of EDHF, one of the most important steps is to measure its release after stimulation of a vessel with defined agonists. This is difficult, especially in small vessels where EETs have been identified pharmacologically as the prominent EDHF. Laboratory measurements to determine cytochrome P450 enzymes have been classically carried out after incubation of radiolabeled AA with tissue homogenates, or more sensitive microsomal preparations from these extracts, in the presence of P450 cofactors. The products have been resolved by reverse-phase high-performance liquid chromatography (rpHPLC) systems that were equipped with flow through radioisotope detectors to monitor the EETs. These were further separated to reveal the individual isomers by normal-phase HPLC and actually measured the enzymatic conversion of AA to EETs and HETEs in a given tissue under in vitro conditions. Small blood vessels are difficult to use in these assays because it is hard to obtain tissue free of surrounding contaminating cells and to be able to make enough microsomal samples for the assay. Also, homogenization could easily alter the balance of regulatory factors to yield artificial levels of enzyme activity. In order to assess the role of EETs as EDHF, it became necessary to quantitate endogenous levels of these compounds in biological samples. The EETs have very weak capability to absorb light either in the UV or visible wavelengths and are not fluorescent. For a long time, the only way to measure endogenous levels was by gas chromatography-mass spectrometry (GC-MS; for an example, see reference 11), which is discussed below, along with other methods that reflect new approaches for these assays. Insight into the pros and cons of each assay are included.

Measurement of EETs by GC-MS: This method (used in ref. 11) involves the extraction of lipids from biological samples followed by separation using thin layer chromatography, rpHPLC or normal-phase HPLC. The purified products have been derivatized to methyl or pentabenzylfluoro esters, followed by conversion to trimethylsilyl derivatives. The protocols include synthesis and/or addition of a deuterated internal standard to correct for variable extraction and derivatization efficiencies. The multi step process results in poor recoveries, which reduces the detection limits to the nanogram range. The instrumentation used for this technique is very expensive and the entire analysis is time consuming.

Fluorescence polarization immunoassay: In the last three years, other investigators have come up with detection methods that are specific for one of the four regioisomers of EETs. A fluorescence polarization immunoassay for the determination of 14,15-EET and 14,15-DiHETE was developed using a specific antibody that was raised in rabbits using antigenic 14,15-DiHETE conjugated to thyroglobulin (29). The tissue samples are first hydrolyzed to convert the 14,15-EET to the corresponding DiHETE. These products are used to competitively displace fluorescein labeled 14,15-DiHETEs in a fluoroimmunoassay. In the presence of the unlabeled ligand, there is a change in
fluorescence polarization that is proportional to the amount of unlabelled ligand competitively bound to the antibody. This is advantageous to the assay because it eliminates the need for additional steps to separate bound antibody from the free form. The assay is very sensitive with detection in the picogram range. A second advantage is that it is specific for the 14,15 isomer, though it does not detect any other form of EET. Also, it cannot differentiate endogenous DiHETE from EET, which is a problem with many assays since EETs are readily converted into DiHETEs.

Determination of 5,6-EET using the lactone as an intermediate in the formation of the diol: This method (30) takes advantage of the capacity of 5,6-EET and its DiHETE derivative to form 5,6-β-lactone. This intermediate is resolved by HPLC and converted to 5,6-DiHETE that could be quantified by GC-MS. Even though this method is reliable and sensitive, it is once again only specific for a single regioisomer, cannot differentiate between endogenous 5,6-EET and DiHETE and is also expensive and laborious.

Analysis of P450 metabolites by liquid chromatography-mass spectrometry (LC-MS) with Ion Trap MS: The advantage of this method (31) over GC-MS is that the samples do not have to be derivatized, and it is therefore more suitable for labile compounds as well as fatty acid esters. The technique is made possible due to the development of powerful electrospray ionization and atmospheric pressure chemical ionization detectors. Like GC-MS, this method utilizes the characteristic ion fragmentation patterns for the identification of the metabolites of AA. Even though this method is reliable and less labor intensive, the equipment that is used is costly.

Determination of EETs using microbore liquid chromatography with fluorescence detection: The AA metabolites are derivatized with 2-(2,3-naphthalimino) ethyl trifluoroacetic acid (N,N-diisopropylmethylammonium carbonate). The products are separated by rpHPLC and have a detection limit in the nanogram range. This method has similar advantages as the previous one, but it requires standard HPLC instrumentation. However, users need to be cautious of contaminants in the assays since this is sensitive to all carboxy terminal lipids. We have applied this method successfully to measure EETs, as described in the next section.

7. Fluorescent measurements of increased EETs in cultured human coronary endothelial cells after stimulation with bradykinin

Diffusible EDHF is synthesized in endothelial cells after stimulation with agonists. Using early passage (P4) human coronary artery endothelial cells purchased from Clonetics (Walkersville, MD, USA) we examined levels of EETs before and after treatment of these cells with bradykinin (100 μM) for 30 min. The EETs were assayed using modifications of the fluorescent HPLC techniques (32, 33). The cells were grown in endothelial growth media (Clonetics) and treated overnight with serum-free media (SFM) containing 20% bovine serum albumin (BSA) and 5 μM AA. After 18 h, the cells were washed and incubated with SFM-BSA (0.5%) and then stimulated with bradykinin. The samples were processed (33) after adding a known amount of internal standard, tridecanoic acid (TA, 150 pmol). The acidic lipids were extracted and reacted with NT and catalyst, purified by a Sep-Pak Vac (Waters Corporation, Milford, NJ, USA) (33) and then resuspended in rpHPLC mobile phase (methanol: water: acetic acid, 82:18:0.1, vol/vol/vol). An aliquot was separated under isocratic conditions on a 4.6 × 250-mm Symmetry C18 rpHPLC column using methanol: water: acetic acid 82:18:0.1 vol/vol/vol as the mobile phase. The eluate was monitored for fluorescence (excitation wavelength = 259 nm and emission = 394 nm). The fluorescent intensity values corresponding to peaks that migrated at retention times similar to known EETs as well as the internal standard TA, were used to compare levels of EETs produced after stimulation. Figure 1 shows the chromatogram from cells treated with bradykinin, the sharp peaks at retention times 68.213, 78.267 and 94.633 min represent 14,15-, 11,12- and 5,6-EETs, respectively. There was no detectable 8,9-EET, although it is possible that this isomer could have co-migrated with the 11,12- or 5,6-EETs, respectively. There was no detectable 8,9-EET, although it is possible that this isomer could have co-migrated with the 11,12- or 5,6-EETs, respectively. The TA (internal standard) eluted at 106.647 min. Table 1 shows levels of EETs (in relative fluorescent units) before and after stimulation with bradykinin. The values were computationally normalized each sample with the TA standard (150 pmol). There was a 31% increase of 14,15-EET after stimulation with bradykinin. However, there was a 7.5-fold and 9-fold increase in 11,12- and 5,6-EETs, respectively, in the human coronary artery endothelial cells after 30 min treatment with 100 μM bradykinin. The data clearly demonstrate a significant increase in EET formation in these endothelial cells after stimulation with bradykinin.
Fig. 1. Chromatogram depicting elution profile of fluorescent-EETs as well as internal standard, tridecanoic acid (TA) in human coronary artery endothelial cells after 30-min incubation with bradykinin. The X-axis shows retention time in min and the Y-axis depicts relative fluorescent intensity monitored by a fluorescent detector in millivolts. The EETs eluted from 65 to 95 min with prominent peaks at 68.2, 78.2 and 94.6 min, corresponding to 14,15-, 11,12- and 5,6-EETs, respectively. There was a very low level of endogenous 8,9-EET, while the 5,6-isomer was the most abundant. The internal standard TA (150 pmol) eluted at 106.6 min.

Table 1. Effects of bradykinin (100 μM) on the levels of epoxyeicosatrienoic acids (EETs) in human coronary artery endothelial cells

| Metabolite | Levels of EETs in human coronary artery endothelial cells (relative fluorescence units) |
|------------|-----------------------------------------------------------------------------------|
| 14,15-EET  | Control 6.5 | Bradykinin-treated 8.5 |
| 11,12-EET  | 20.9 | 158.1 |
| 5,6-EET    | 17.2 | 157.9 |
| TA*        | 150 | 150 |

*Tridecanoic acid (internal standard).

8. Conclusions

The search for EDHF has been complicated by the diversity of the vascular systems studied. The need to finely tune vessel reactivity invokes regulation at multiple levels that vary with size of vessel, source of vessel, agonist/stimulant, species, etc. This has proved to be challenging for researchers who have defined a number of candidates for EDHF. In order to establish the current hypotheses for EETs being the EDHF, there is a need for newer simple, sensitive and reproducible assays coupled with molecular approaches to confirm the wealth of existing pharmacological evidence.

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