Endothelium-Derived Hyperpolarizing Factor
Where Are We Now?
Michel Féiletou, Paul M. Vanhoutte

Abstract—The endothelium controls vascular tone not only by releasing nitric oxide (NO) and prostacyclin but also by other pathways causing hyperpolarization of the underlying smooth muscle cells. This characteristic was at the origin of the denomination endothelium-derived hyperpolarizing factor (EDHF). We know now that this acronym includes different mechanisms. In general, EDHF-mediated responses involve an increase in the intracellular calcium concentration, the opening of calcium-activated potassium channels of small and intermediate conductance and the hyperpolarization of the endothelial cells. This results in an endothelium-dependent hyperpolarization of the smooth muscle cells, which can be evoked by direct electrical coupling through myo-endothelial junctions and/or the accumulation of potassium ions in the intercellular space. Potassium ions hyperpolarize the smooth muscle cells by activating inward rectifying potassium channels and/or Na⁺/K⁺-ATPase. In some blood vessels, including large and small coronary arteries, the endothelium releases arachidonic acid metabolites derived from cytochrome P450 monoxygenases. The epoxyeicosatrienoic acids (EET) generated are not only intracellular messengers but also can diffuse and hyperpolarize the smooth muscle cells by activating large conductance calcium-activated potassium channels. Additionally, the endothelium can produce other factors such as lipoxygenases derivatives or hydrogen peroxide (H₂O₂). These different mechanisms are not necessarily exclusive and can occur simultaneously. (Arterioscler Thromb Vasc Biol. 2006;26:1215-1225.)

Key Words: CNP ■ cytochrome P450 ■ cyclooxygenase ■ EDHF ■ gap junction ■ lipoxygenase ■ NO ■ potassium channels ■ prostacyclin

Endothelial cells control the tone of the underlying vascular smooth muscle cells by releasing various relaxing and contracting factors.¹ The former include nitric oxide (NO) and prostacyclin.²⁻⁴ However, early experimental evidence suggested that, beside the cyclooxygenase and the NO synthase pathways, an additional endothelial pathway(s) had to be involved to fully explain endothelium-dependent relaxations.⁵ This third endothelium-dependent pathway(s) has been observed in numerous blood vessels from different species, including the human. It is associated with the hyperpolarization of the vascular smooth muscle cells and was then attributed to a noncharacterized endothelial factor termed endothelium-derived hyperpolarizing factor (EDHF).⁶⁻⁷ The coining of this acronym “EDHF” turned out to be
confusing because it implies that a single diffusible substance mediates this type of endothelium-dependent relaxation. In fact, numerous endothelium-derived factors, including NO and prostacyclin themselves, can hyperpolarize the underlying smooth muscle. The purpose of this review is to highlight the different pathways that can lead to endothelium-dependent hyperpolarization of the vascular smooth muscle cells. To clarify the nomenclature of non-NO–non-PGI2–
independent hyperpolarizations of the vascular smooth muscle, we propose that once a endothelial mediator has been identified properly, it should be adequately named (ie, epoxycosatrienoic acid [EET], H2O2) and no longer denominated with the acronym EDHF.5,6

**Prostacyclin**

Prostacyclin is the major metabolite of arachidonic acid produced by cyclooxygenase in endothelial cells.2 It activates IP receptors on vascular smooth muscle and, in most normal arteries, produces relaxation. Depending on the artery and/or the species, a hyperpolarization can occur, which involves the opening of one or more of types of potassium channels. Thus, ATP-sensitive potassium channels (K-ATP) (Figure 1), large conductance calcium-activated potassium channels (BKCa), inwardly rectifying potassium channels (KIR) and/or voltage-activated potassium channels (KV) can be associated with the prostacyclin-induced relaxation.7 Therefore, in numerous vascular beds, the prostanooid can be regarded as an endothelium-derived hyperpolarizing substance. Because most of the available inhibitors of cyclooxygenase abolish the production of prostaglandins in vascular tissues, any endothelium-dependent hyperpolarization observed in the presence of one of these inhibitors is unlikely to involve prostacyclin.7

**NO**

NO also can hyperpolarize, or repolarize, vascular smooth muscle cells by activating, in either a cyclic-GMP–dependent or cyclic-GMP–independent manner, potassium channels such as K-ATP (Figure 1), BKCa, KIR, and/or KV. NO interacts with other ionic channels of the smooth muscle, including chloride and cationic channels and also influences the membrane potential of the smooth muscle cells indirectly in an autocrine fashion.7

In contrast to inhibitors of cyclooxygenase, inhibitors of NO synthase do not necessarily fully inhibit the production of NO. Thus, in their presence, residual NO can be produced by
the endothelial cells and contribute to the relaxation and/or hyperpolarization of the underlying vascular smooth muscle.8,9

NO can contribute in another way to responses resistant to inhibitors of cyclooxygenases and NO synthases. NO can also be stored and released independently of the activation of NO synthase. Two such stores have been described. The first one is the so-called photosensitive store that involves the release of NO by ultraviolet light.10 The mechanism of NO release by this store in the absence of ultraviolet light is unknown. Nevertheless, in rabbit and rat mesenteric arteries, the same pool of NO that is released during photoactivation may contribute to those responses.11 The NO-dependent responses are sensitive to guanylyl cyclase inhibitors or NO scavengers but not to NO synthase inhibitors. The second store is located in the adventitia. It is generated by the formation of protein-bound dinitrosyl non-heme iron complexes and S-nitrosated proteins, when elevated concentrations of NO are produced, particularly when NOS-2 has been induced by lipopolysaccharides.12 Low-molecular-weight thiols displace NO from these stores and transfer it to various target membrane proteins including potassium channels and may therefore function as hyperpolarizing factors.13

Therefore, it is obvious that the role of NO as an endothelium-derived hyperpolarizing substance has certainly been underestimated assuming that the presence of an inhibitor of NO synthase (NOS) rules out its contribution. Residual NO is even more of an issue in human studies performed in vivo where typically only relatively low doses of one of the weakest inhibitors of NOS (L-NMMA) can be administered. Despite these caveats, the presence of a third endothelial pathway, besides prostacyclin and NO, contributing to the endothelium-dependent relaxation of the smooth muscle cells has been demonstrated beyond reasonable doubt in various blood vessels.7,14

Non-Prostanoid–Dependent, Non-NO–Dependent Endothelium-Dependent Hyperpolarizations

In NOS-3 knockout and NOS-3/COX-1 double knockout mice, EDHF-mediated responses compensate for the absence of endothelial NO.15–18 The importance of this compensation depends on the gender. In resistance arteries of NOS-3/COX-1 double knockout female mice, endothelium-dependent relaxations are preserved whereas in arteries from corresponding males they are impaired. In female mice, the deletion of NOS-3 and COX-1 does not affect mean arterial blood pressure, whereas the males with these deletions are hypertensive.18 These results suggest that EDHF-mediated responses contribute to the overall regulation of arterial blood pressure.
Characterization of EDHF-Mediated Responses

Agonists stimulating G protein–coupled receptors as well as the calcium ionophore A 23187, thapsigargin, and cyclopiazonic acid increase the endothelial intracellular calcium concentration ([Ca^{2+}]_i), hyperpolarize the endothelial cells, and in some blood vessels evoke endothelium-dependent hyperpolarization of the underlying vascular smooth muscle cells.\(^{19–23}\) In most arteries, such EDHF-mediated responses involve the activation of SKCa (blocked by apamin, scyllatoxin, tubocurarine, or UCL 1684) and/or IKCa (blocked by charybdotoxin, TRAM-34 or TRAM-39).\(^{23–27}\) but not that of BKCa (blocked by charybdotoxin and iberiotoxin).\(^{28,29}\)

SKCa and IKCa channels are present in freshly isolated endothelial cells from various arteries (Figures 2, 3).\(^{26,30–34}\) In porcine coronary endothelial cells, messenger RNAs encoding IK1 as well as the SK2 and SK3 subunits of SKCa can be detected by RT-PCR. Western blot analysis indicates that the SK3 protein is abundant, whereas immunofluorescent labeling confirms that IK1 and SK3 (Figure 3) are expressed at the plasmalemma of endothelial cells but not in that of vascular smooth muscle. Substance P and bradykinin, both of which initiate EDHF-mediated responses in the porcine coronary artery, activate an outward current sensitive to the combination of blockers of IKCa and SKCa. By contrast, in freshly isolated vascular smooth muscle cells from various species, IKCa and SKCa are not or very poorly expressed, whereas functional BKCa are constitutively present.\(^{35,36}\) These observations demonstrate that the 2 Ca^{2+}-activated potassium channels involved in EDHF-mediated responses are located on the endothelial plasma membrane.\(^{26,32–34}\) In the mouse, the level of expression of SK3 channels on the endothelial cells correlates with the cell membrane potential of both endothelial and vascular smooth muscle cells, with the tone of isolated mesenteric arteries and the diameter of these arteries in situ, as well as with the arterial blood pressure of the animals.\(^{37}\)

Depolarization with KCl and inhibition of SKCa and/or IKCa by 2 different maneuvers that fully prevent the hyperpolarization of both the endothelial and the smooth muscle cells, produce no or only minimal inhibition in the sustained phase of the increase in endothelial [Ca^{2+}]_i,\(^{38–40}\) Conversely, 1-ethyl-2-benzimidazolinone (1-EBIO, an activator of SKCa and/or IKCa, but not BKCa)\(^{41}\) evokes EDHF-mediated responses without increasing endothelial [Ca^{2+}]_i.\(^{23}\) Thus, the increase in [Ca^{2+}]_i after endothelial receptors activation, stimulates KCa channels to hyperpolarize the endothelial cells. This endothelial hyperpolarization is a prerequisite to obtain the endothelium-dependent hyperpolarization of the underlying vascular smooth muscle.

Beyond Endothelial Hyperpolarization

Two mechanisms have been proposed to explain how the activation of endothelial SKCa and/or IKCa leads to the hyperpolarization of the smooth muscle cells: (1) hyperpolarization of the endothelial cell transmitted directly to the vascular smooth muscle by means of gap junctions; and (2) accumulation in the intercellular cleft of K^+ ions released from the endothelial cells through opening of KCa, leading to hyperpolarization of the smooth muscle by activating K^+ conductances and/or Na^+/K^+-ATPase in the latter.

Gap Junctions

Gap junction channels are formed by the docking of 2 connexons present in adjacent cells leading to the creation of an aqueous central pore that permits the transfer of ions and polar molecules. This provides an electrical continuity allowing a uniform membrane potential among coupled cells. Endothelium and smooth muscle cells communicate via myo-endothelial gap junctions.\(^{42–48}\)

In arteries exhibiting EDHF-mediated responses, the endothelium-dependent hyperpolarization of vascular smooth muscle cells and the hyperpolarization of the endothelial cells follow the same time course.\(^{49,50}\) A close correlation exists between the expression of myo-endothelial gap junctions and
the occurrence of EDHF-mediated responses.51–53 Furthermore, the number of myo-endothelial gap junctions increases as the size of the artery decreases,54,55 a phenomenon that parallels the contribution of the EDHF-mediated responses in endothelium-dependent relaxations.56,57 In many blood vessels, blockers of gap junctions (glycyrrhetinic acid and some of its derivative such as carbenoxolone, HEPES and other taurine-based buffers and Gap peptides), partially inhibit or abolish EDHF-mediated responses.45,58,59 However, many questions remain unanswered. To date, what flows through myo-endothelial gap junction is basically unknown. The fact that a single layer of endothelial cell can drive the hyperpolarization of a multiple layers of smooth muscle cells suggests the involvement of an undefined active membrane process in the conducted hyperpolarization.7,60 Some EDHF-mediated responses are associated with a small but significant early and transient endothelium-dependent increase in the cyclic-AMP content of the smooth muscle cells. The stimulation of a calcium-sensitive adenyl cyclase isofrom results in an increase in the production of cyclic-AMP with subsequent enhancement of gap junctional communication.61,62

Connexins 37 and 40 are the predominant gap junction proteins in the endothelial cells of the mouse. Mice deficient for connexin 40 are hypertensive and exhibit a reduced spread of dilatation in response to endothelium-dependent vasodilators and electrical stimulation, as well as irregular arteriolar vasomotion.63,64 Antibodies directed against connexin 40, when loaded selectively in the endothelial cells of the murine mesenteric artery, block EDHF-mediated responses.65 By contrast, mice subjected to specific deletion of endothelial connexin 43 do not present any major alteration in blood pressure, possibly because this connexin is not the major one expressed in their endothelial cells.

Taken in conjunction, the available data show that transmission of the hyperpolarization via gap junctions contributes to many EDHF-mediated responses whereby, at least in the mouse, connexin 40 appears to play a predominant role. However, in a number of EDHF-mediated responses, the involvement of gap junctions appears minimal implying the occurrence of other mechanisms.7,67

**Potassium Ions**

Another mechanism to achieve hyperpolarization and relaxation of the underlying vascular smooth muscle cells is linked directly to the hyperpolarization of the endothelial cells. Indeed, the activation of endothelial IKCa and/or SKCa causes an efflux of potassium ions from the intracellular compartment toward the extracellular space. Potassium is an endogenous metabolic vasodilator involved in exercise hyperemia and in active hyperemia of the brain due to increased neuronal activity. A moderate increase in the extracellular potassium concentration (1 to 15 mmol/L) causes relaxation of vascular smooth muscle.68 This observation is counterintuitive because, as a result of such an increase in the extracellular potassium ion concentration, the Nernst equation predicts depolarization and a subsequent contraction. However, small increases in the extracellular concentration of potassium ions can also activate Kir and the Na+/K+ pump.69,70 The activation of these 2 systems overcomes the minor depolarizing effects linked to the increase in potassium ions per se. The net result is hyperpolarization and thus relaxation of the smooth muscle cells. The endothelium is a thin monolayer and any efflux of potassium from this small cell mass into the lumen of the blood vessel is washed away by the flowing blood. However, an efflux of potassium in the abluminal direction could lead to accumulation of those ions in the small intercellular space between endothelial and smooth muscle cells and to reach levels sufficient to activate Kir and the Na+/K+ pump on the latter. Therefore, potassium ions could contribute to EDHF-mediated responses.

This hypothesis was demonstrated successfully in the hepatic and mesenteric arteries of the rat.71 This study provided evidence that the K+ channels involved were located on the endothelial cells, that potassium ions indeed accumulate in the intercellular space between endothelial and smooth muscle cells and that the potassium efflux associated with the activation of endothelial IKCa and/or SKCa channels produces hyperpolarization by activating both Kir and the Na+/K+ pump on the vascular smooth muscle cells.71 The contribution of K+ in EDHF-mediated responses was demonstrated in many blood vessels including human arteries.72 Reverse-transcription polymerase chain reaction and immunohistochemistry studies indicate the Kir channel in vascular smooth muscle involved in potassium ion-induced hyperpolarization is composed of the Kir2.1 α-subunits.73,74 In cerebral arteries of mice (an artery in which potassium-induced relaxation does not involve the activation of the Na+/K+–ATPase) potassium-induced dilation are no longer observed in mice subjected to the deletion of Kir2.1 whereas these responses are preserved Kir2.2 knockout in mice,75 supporting a predominant role of Kir2.1 α-subunits.

However, in many arteries potassium does not consistently produces a relaxation and/or a hyperpolarization, suggesting that in these blood vessels EDHF-mediated responses do not rely on the accumulation of interstitial potassium.6 The involvement of gap junctions and K+ ions are not necessarily mutually exclusive. They can occur simultaneously or sequentially and also may act synergistically (Figure 4).

**Other Identified Endothelium-Derived Hyperpolarizing Substances**

Besides the production of NO, prostacyclin and potassium ions, the endothelial cells can release other relaxing factors. The contribution of these depends on the species and/or the vascular bed studied. The hyperpolarization of the smooth muscle cells evoked by these other endothelium-derived hyperpolarizing factors can be the major mechanism underlying the relaxation or only contribute partially to it.

**Metabolites of Arachidonic Acid**

**Products of Cytochrome P450 Monoxygenase**

EETs, derived from cytochrome P450 2C or 2J epoxygenases, and possibly their epoxide hydrolase metabolites dihydroxyeicosatrienonic acids, contribute to endothelium-dependent relaxations of various blood vessels, including large and small coronary arteries.76–79
EETs hyperpolarize coronary arterial smooth muscle cells and increase the open-state probability of BKCa. Although EETs activate BKCa through a G protein–signaling cascade, the existence of a specific cell membrane receptor(s) for EETs in vascular smooth muscle has not been established. The observation that some EET analogues, 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE) and 14,15-epoxyeicosa-5(Z)-enoic-methylsulfonylimide (14,15-EEZE-mSi), act as inhibitors of the action of EETs support the existence of such receptors. Additionally, EETs activate smooth muscle vanilloid transient receptor potential channel (TRPV4), which increase the frequency of calcium sparks and subsequently that of spontaneous transient outward currents. This EET-dependent activation of a calcium-signaling complex (TRPV4-ryanodine receptors-BKCa) hyperpolarizes and relaxes the smooth muscle cells.

In many arteries, inhibitors of cytochrome P450 2C9 derived metabolites are not involved in the regulation of basal blood flow and in these non-NO–non-PGI2–mediated responses. In people, a cytochrome P450 metabolite, in association with NO, plays a role in the regulation of the radial artery diameter in vivo and in exercise-induced increases in lower limb skeletal muscle blood flow. However, in the forearm of healthy volunteers and patients with various pathologies, the cytochrome P450 inhibitor sulfaphenazole does not influence basal tone or does not reduce the bradykinin-induced vasodilation alone or in presence of inhibitors of cyclooxygenase and NO synthase. These observations suggest that cytochrome P450 2C9 derived metabolites are not involved in the regulation of basal blood flow and in these non-NO–non-PGI2–mediated responses. Furthermore, in patients with coronary artery disease, inhibition of cytochrome P450 2C9 improves the endothelium-dependent, NO-mediated vasodilation, most likely because this enzyme generates superoxide anion. Additionally, in numerous vascular preparations from various species, EETs evoke no or minor relaxations and/or hyperpolarization, indicating that in these arteries non-NO–non-PGI2–mediated responses are unlikely to rely on this metabolite of arachidonic acid.

The lack of selectivity of the available tools has made it difficult to determine whether the activation of the endothelial potassium channels IKCa and/or SKCa or the release of EETs underlies non-NO–non-PGI2–mediated responses. For instance, clotrimazole, a reference inhibitor of cytochrome P450 epoxygenases, also blocks IKCa. Charybdotoxin, a reference blocker of IKCa, also blocks the BKCa in smooth muscle that are opened by EETs. The use of more selective inhibitors that are resistant to the presence of inhibitors of NO synthases and cyclooxygenases. Likewise, endothelium-dependent hyperpolarizations and relaxations can be inhibited by antiseNSE oligonucleotides directed against cytochrome P450–2C8–9. Conversely, these responses are increased by agents that enhance the endothelial expression of cytochrome P450.

In porcine and bovine coronary arteries, the non-NO–non-PGI2–mediated hyperpolarizations are inhibited by 14,15-EEZE-mSi, a preferential antagonist of 5,6-EET and 14,15-EET. 14,15-EET is released from the endothelium, which then diffuse to and hyperpolarize the vascular smooth muscle cells by activating BKCa. In porcine and bovine coronary arteries, the non-NO–non-PGI2–mediated hyperpolarizations are inhibited by 14,15-EEZE-mSi, a preferential antagonist of 5,6-EET and 14,15-EET. 14,15-EET is released from the endothelium in response to bradykinin stimulation, strongly suggesting that, at least in this artery, this EET contributes to endothelium-dependent hyperpolarizations.

Taken in conjunction, these observations support the concept that, in some blood vessels, and particularly in coronary arteries, EETs are endothelium-derived hyperpolarizing factors eliciting the relaxation of smooth muscle by opening BKCa (Figure 5). However, this does not imply that the release of this EET explains all the non-NO–non-PGI2–mediated responses in bovine and porcine coronary arteries. For instance, in the latter, the endothelium-dependent hyperpolarizations invoked by substance P involve exclusively the activation of endothelial SKCa and IKCa potassium channels. The response to bradykinin involves 2 mechanisms, the opening of these 2 endothelial potassium channels and, additionally and independently, the release of 14,15-EET with the subsequent activation of BKCa on the smooth muscle cell (Figure 5).

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The endothelial formation of epoxyeicosatrienoic acids (EETs) can have multiple effects in the vascular wall. EETs are endothelium-derived hyperpolarizing substances activating smooth muscle BKCa by different means directly via a G protein-signaling cascade in bovine and porcine coronary arteries or indirectly by the calcium signaling complex, TRPV4 - ryanodine receptors - BKCa, in rat cerebral artery. Additionally, BKCa or cytochrome P450 (TRAM 34 and TRAM 39) activate the metabolism of arachidonic acid via cytochrome P450 monooxygenase. The endothelial formation of epoxyeicosatrienoic acids; SK3, small conductance calcium-activated potassium channel formed by SK3; IK1, intermediate conductance calcium-activated potassium channel formed by IK1 \( \alpha \)-subunits; BKCa, large conductance calcium-activated potassium channels; RyR, ryanodine receptors.

**Figure 5.** Bradykinin and substance P induced endothelium-dependent hyperpolarization in the porcine coronary artery. Both peptides stimulate endothelial SKCa and IKCa and evoke the subsequent hyperpolarization of the smooth muscle cells (see Figure 6). Additionally, bradykinin, but not substance P, activates the metabolism of arachidonic acid via cytochrome P450 monoxygenase. The endothelial formation of epoxyeicosatrienoic acids (EETs) can have multiple effects in the vascular wall. EETs are endothelium-derived hyperpolarizing substances activating smooth muscle BKCa by different means directly via a G protein-signaling cascade in bovine and porcine coronary arteries or indirectly by the calcium signaling complex, TRPV4 - ryanodine receptors - BKCa, in rat cerebral artery. Additionally, EETs can modulate EDHF-mediated responses by regulating the intracellular calcium concentration, potassium channel activity and gap junctional communications. NK1 indicates neurokinin 1 receptor subtype; B2, bradykinin B2 receptor subtype; PLC, phospholipase C; DAG, diacylglycerol; AA, arachidonic acid; SR, sarcoplasmic reticulum; TRPV4, transient receptor channel vanilloid-4; SOC, store-operated channel; AC, adenylyl cyclase; cAMP, cyclic-AMP; P450, cytochrome P450 monooxygenase; EETs, epoxyeicosatrienoic acids; SK3, small conductance calcium-activated potassium channel formed by SK3 \( \alpha \)-subunits; IK1, intermediate conductance calcium-activated potassium channel formed by IK1 \( \alpha \)-subunits; BKCa, large conductance calcium-activated potassium channels; RyR, ryanodine receptors.

**Products of Lipoxygenases**

Under various physiological and/or pathophysiological conditions, endothelial cells express different lipoxygenases, which can metabolize arachidonic acid into relaxing and contracting substances. Thus, 12-(S)-HETE is released from the endothelium by different stimuli and activates BKCa on the smooth muscle cells. This lipoxygenase derivative activates BKCa on the smooth muscle, an apamin-sensitive but charybdotoxin-insensitive BKCa in the aorta and BKCa in the mesenteric artery.

**Hydrogen Peroxide (H\(_2\)O\(_2\))**

Endothelial, but also smooth muscle, cells generate significant amounts of reactive oxygen species. Superoxide either spontaneously or enzymatically through dismutation by superoxide dismutase is reduced to H\(_2\)O\(_2\), which, depending on the tissue and the experimental conditions or the concentrations studied, possesses dilator or constrictor properties and hyperpolarizes or depolarizes smooth muscle.

The role of H\(_2\)O\(_2\) as an endothelium-derived hyperpolarizing factor comes from the observation that, in certain blood vessels, the agonist- and flow-induced responses attributed to non-NO–non-PGI\(_2\)–mediated phenomenon are partially or totally prevented by catalase. In those arteries the agonist-induced non-NO–non-PGI\(_2\)–mediated responses are accompanied by the endothelial production of H\(_2\)O\(_2\). Endothelial Cu Zn superoxide dismutase probably plays a major role in producing H\(_2\)O\(_2\) to elicit endothelium-dependent hyperpolarizations.

A critical appraisal of the available evidence linking H\(_2\)O\(_2\) to EDHF-mediated responses requires a word of caution. H\(_2\)O\(_2\) is a rather weak relaxing and hyperpolarizing substance. One of the major achievements that has improved the understanding of non-NO–non-PGI\(_2\)–mediated responses has been the identification and localization of the potassium channels involved in these responses. In the various arteries where the hyperpolarizations in response to H\(_2\)O\(_2\) and non-NO–non-PGI\(_2\)–mediated responses were compared, none of the specific potassium channels blockers has been studied, and it is not yet known which population(s) of potassium
channels are activated by H$_2$O$_2$ or whether they are situated on the endothelial or the smooth muscle cells. The absence of this information undermines any conclusion as to the potential role of H$_2$O$_2$ in non-NO–non-PGI$_2$–mediated responses. In particular, the questions remain unanswered whether H$_2$O$_2$ is a diffusible factor activating potassium channels on the smooth muscle or an intracellular messenger involved in the activation of endothelial potassium channels. Likewise, although H$_2$O$_2$ is produced in response to an increase in endothelial [Ca$^{2+}$], it is uncertain whether this is truly a pathway linked to activation of endothelial IK$_{Ca}$ and SK$_{Ca}$ or whether this is an independent (epi)phenomenon. Finally, catalase does not inhibit non-NO–non-PGI$_2$–mediated responses in all arteries, and H$_2$O$_2$ does not relax or hyperpolarize all vascular smooth muscle cells.

**C-Type Natriuretic Peptide**

C-type natriuretic peptide (CNP) causes relaxation and hyperpolarization of arterial and venous smooth muscle cells and opens BK$_{Ca}$. This endothelium-derived natriuretic peptide has therefore been proposed as a putative hyperpolarizing factor. However, at least in the porcine coronary artery, the characteristics and the amplitude of the hyperpolarizations evoked by exogenous CNP are by no means comparable to those observed during EDHF-mediated responses. Experiments in the rat mesenteric artery suggest that acetylcholine releases CNP from the endothelial cells, which in turn activates NPR-C receptors on vascular smooth muscle. Hyperpolarization of the smooth muscle cell is obtained by the cyclic-GMP–independent activation of a G-protein regulated inward-rectifier potassium channel (GIRK).

The hypothesis proposing that CNP is an endothelium-derived hyperpolarizing substance requires the validation of various concepts. Whether the CNP-dependent activation of NPR-C in vascular smooth muscle cells produces a cyclic GMP-independent, pertussis toxin-sensitive signaling is unknown. The expression and activity of GIRK has been well-characterized in neurons and cardiac myocytes, but the protein expression and the complete characterization of this channel in vascular smooth muscle cells await full demonstration. Finally, there is no evidence to date, in any cell type, that CNP can activate GIRK.

**Conclusion**

Endothelial cells control the tone of the underlying vascular smooth muscle by releasing numerous vasoactive substances, including NO, reactive oxygen species, potassium ions, and metabolites of arachidonic acid (eg, prostacyclin, EETs, lipoxxygenase derivatives). Furthermore, the endothelial monolayer behaves as a conductive tissue propagating an electrical signal along the axis of the blood vessel by means of homocellular gap junctions and throughout the vascular wall itself by means of myo-endothelial gap junctions.

Endothelium-dependent relaxations, independent of the production of NO and prostacyclin, probably play an important role in cardiovascular physiology in the animal and in the human. They can act as a backup system when NO is inhibited or reduced but this is not necessarily the case. On the one hand, the production of superoxide anion and subsequently H$_2$O$_2$ is increased when the eNOS is dysfunctional (decrease substrate and/or cofactor concentration) and the generation of EETs can be enhanced if the tonic repression of the cytochrome P450 activity is alleviated by a reduced NO availability. On the other hand, EDHF-mediated responses, dependent on endothelial IK$_{Ca}$ and SK$_{Ca}$ activation, appear independent of the activity of eNOS. However, it is often difficult to reach a conclusion as to the true importance of endothelium-dependent hyperpolarizations because of the use of unspecific pharmacological tools and the lack of electrophysiological measurements. Clarity could be improved by applying simple rules.

**Demonstrate the Existence of a Non-NO–Non-PGI$_2$–Mediated Responses Unequivocally**

A non-NO–non-PGI$_2$–mediated responses response should be reported only when the evidence for a third pathway, besides the L-arginine-NO-synthase and the arachidonic acid-cyclooxygenase pathways, is demonstrated beyond any doubt. Demonstration of resistance to the combined presence of inhibitors of NO synthases and cyclooxygenases does not necessarily prove the existence of an EDHF-mediated response. Ideally, the proof that NO generation has been abolished should be brought. However, if a relaxation and/or hyperpolarization is still observed in the combined presence of inhibitors of cyclooxygenase and NO synthase, as well as an NO scavenger (eg, oxyhemoglobin, carboxy-PTIO), it is reasonable to consider that the blood vessel studied exhibits non-NO–non-PGI$_2$–mediated responses. These experiments are needed to rule out the possible involvement of residual NO production, either from NO synthase itself or from NO synthase–independent sources (NO stores).

**Determine the Mechanism Underlying the Suspected Non-NO–Non-PGI$_2$–Mediated Responses**

These responses often encompass different endothelial mediators or pathways that occur independently or in some cases coexist. The generation of H$_2$O$_2$ and the production of EETs by cytochrome P450 or that of CNP do not necessarily require the hyperpolarization of endothelial cells, although in a similar manner to the release of NO itself, the membrane potential of the endothelial cells can be a regulatory factor. H$_2$O$_2$, EETs, CNP, or adenosine, again like NO, are released by the endothelial cells and activate potassium channels on the vascular smooth muscle cells. Appropriate tools are now available to properly identify these mediators. Once their involvement is confirmed in a given vascular bed, they must be referred to by their proper name, ie, endothelium-derived NO, prostacyclin, H$_2$O$_2$, EETs, and CNP, and no longer should be termed “EDHF.” Only the responses requiring the activation of endothelial SK$_{Ca}$ and/or IK$_{Ca}$ and hyperpolarization of the endothelial cells should be referred to as “EDHF-mediated” responses because there is, at present, no better way to name them. Obviously, such EDHF-mediated responses must be characterized and the involvement of gap junction and/or potassium ions must be determined whenever possible. To implement this simple nomenclature will simplify the interpretation of incoming studies.
Use the Most Specific Pharmacological Tools Available

The use of nonselective drugs, particularly inhibitors of cytochrome P450, which also inhibit calcium-activated potassium channels, and charybdotoxin, which inhibits both IKCa and BKCa, has clouded the EDHF field. The use of more specific inhibitors of cytochrome P450 and of antagonists of the EETs such as the EEZE compounds, iberiotoxin to specifically inhibit BKCa and TRAM 34 or TRAM 39 to will allow proper identification of the non-NO–non-PGI2–cytochrome P450, which also inhibit calcium-activated potassium channels.

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