EDHF: bringing the concepts together

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Endothelial cells synthesize and release vasoactive mediators in response to various neurohumoral substances (e.g. bradykinin or acetylcholine) and physical stimuli (e.g. cyclic stretch or fluid shear stress). The best-characterized endothelium-derived relaxing factors are nitric oxide and prostacyclin. However, an additional relaxant pathway associated with smooth muscle hyperpolarization also exists. This hyperpolarization was originally attributed to the release of an endothelium-derived hyperpolarizing factor (EDHF) that diffuses to and activates smooth muscle. Originally attributed to the release of an endothelium-derived hyperpolarizing factor (EDHF) that diffuses to and activates smooth muscle. This hyperpolarization was characterized as endothelium-derived relaxing factors are nitric oxide and physical stimuli (e.g. cyclic stretch or fluid shear stress). The best-characterized endothelium-derived relaxing factors are nitric oxide and prostacyclin. However, an additional relaxant pathway associated with smooth muscle hyperpolarization also exists. This hyperpolarization was originally attributed to the release of an endothelium-derived hyperpolarizing factor (EDHF) that diffuses to and activates smooth muscle. Initially, several lines of evidence suggested that the endothelium-dependent hyperpolarization of smooth muscle cells resulted from the opening of K⁺ channels in the smooth muscle plasmalemma. First, the hyperpolarization was abolished by K⁺ concentrations higher than 25 mM [23]. Second, agonists that produced endothelium-dependent hyperpolarizations also stimulated the efflux of ⁴²K⁺ (or ⁸⁶Rb⁺) from pre-loaded arteries [3,4]. Third, a decrease in membrane resistance of the vascular smooth muscle cells was observed during endothelium-dependent hyperpolarizations [23], suggesting that the hyperpolarization was due to the opening of a K⁺ channel rather than the closing of, for example, a Cl⁻ or a nonspecific cationic channel. Collectively, these observations suggested that the endothelium-dependent hyperpolarization of smooth muscle cells involved an increase in K⁺ conductance and that EDHF was an endothelium-derived K⁺ channel opener. However, this interpretation has to be modified in light of more recent experimental observations.

Identification of the K⁺ channels involved in the EDHF pathway

Endothelium-dependent hyperpolarizations (in the presence of inhibitors of NO synthases and cyclooxygenases) are not prevented by glibenclamide [an inhibitor of ATP-dependent K⁺ (K_{ATP}) channels] but are blocked by specific toxins that inhibit Ca²⁺-sensitive (K_{Ca}) K⁺ channels. Indeed, a hallmark of the EDHF-mediated response, first observed by Garland’s group, is its abolition by the combination of apamin [a specific inhibitor of K_{Ca} channels of small conductance (SK_{Ca} channels)] plus charybdotoxin [a nonselective inhibitor of large-conductance (BK_{Ca}) and intermediate-conductance (IK_{Ca}) channels, and of some voltage-dependent K⁺-channels] [24–26], although either toxin can be effective alone in some circumstances [27,28].
The effect of apamin can be mimicked by scyllatoxin, a structurally distinct SK$_{	ext{Ca}}$ channel inhibitor, indicating that SK$_{	ext{Ca}}$ channels are involved in endothelium-dependent hyperpolarizations [26]. However, although iberiotoxin (a specific inhibitor of BK$_{	ext{Ca}}$ channels) can abolish NO- and prostacyclin-independent vasodilation in some vascular beds in vivo [29,30], in arteries in which the combination of charybdotoxin and apamin is essential to inhibit responses, iberiotoxin cannot substitute for charybdotoxin [25,31], which excludes a pivotal role for BK$_{	ext{Ca}}$ channels in many EDHF-mediated responses.

Which cell type expresses the K$_{	ext{Ca}}$ channels involved in the EDHF response?

There are no reports of smooth muscle K$_{	ext{Ca}}$ channels that are sensitive to charybdotoxin but not to iberiotoxin, which suggests that the EDHF-activated IK$_{	ext{Ca}}$ channel is not present on the smooth muscle cells. However, both IK$_{	ext{Ca}}$ and SK$_{	ext{Ca}}$ channels are expressed in endothelial cells [32–34] and endothelial K$^+$ channels are activated by stimuli that produce an increase in [Ca$^{2+}$]i [35]. The hyperpolarization of the endothelial cells elicited by acetylcholine or bradykinin is blocked by the combination of apamin and charybdotoxin [36,37]. In addition, these two toxins prevent EDHF-mediated smooth muscle relaxation when selectively applied to the endothelium [38]. Finally, 1-ethyl-2-benzimidazoline (1-EBIO), an activator of IK$_{	ext{Ca}}$ channels (and possibly SK$_{	ext{Ca}}$, but not BK$_{	ext{Ca}}$, channels) induces endothelial cell hyperpolarization and the endothelium-dependent hyperpolarization of smooth muscle cells, responses that are sensitive to charybdotoxin but insensitive to iberiotoxin [39,40]. Collectively, this evidence suggests that the toxin combination targets two types of K$_{	ext{Ca}}$ channels (IK$_{	ext{Ca}}$ and SK$_{	ext{Ca}}$ channels) expressed on endothelial cells (i.e. prevents endothelial cell hyperpolarization) rather than K$^+$ channels activated by an EDHF and located on smooth muscle cells.

Beyond endothelial hyperpolarization

Why have so many vastly different hypotheses been proposed concerning the mechanism of EDHF-mediated responses? Increasing the [Ca$^{2+}$]i in endothelial cells opens not only SK$_{	ext{Ca}}$ and IK$_{	ext{Ca}}$ channels, which results in the efflux and accumulation of K$^+$ in the myo-endothelial space, but also leads to the activation of various enzymes including phospholipases and the subsequent metabolism of arachidonic acid by cytochrome P450 epoxygenases. Currently, experimental evidence favours three explanations for the EDHF phenomenon: (1) the increase in endothelial [Ca$^{2+}$]i triggers the synthesis of a cytochrome P450 metabolite that is essential for the subsequent EDHF-mediated responses; (2) endothelial cell hyperpolarization is transmitted to the vascular smooth muscle via gap junctions; and (3) K$^+$, released from the endothelial cells via K$_{	ext{Ca}}$ channels, induces smooth muscle hyperpolarization by activating K$^+$ channels and/or Na$^+$-K$^+$-ATPase on vascular smooth muscle cells.

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A role for epoxyeicosatrienoic acids?

Epoxyeicosatrienoic acids (EETs) are arachidonic-acid-derived products of cytochrome P450 epoxygenases that appear to play an important role in the regulation of vascular homeostasis [41]. Most of the evidence in favour of EDHF being a short-lived metabolite of arachidonic acid, produced...
via the cytochrome P450 epoxygenase pathway, has been obtained using bovine [42,43], porcine [42,44], canine [45] and human coronary arteries [28,46]. In these blood vessels, EDHF-mediated responses are blocked by inhibitors of cytochrome P450. Although classical cytochrome P450 inhibitors were nonspecific, new selective and structurally different compounds do inhibit EDHF-mediated responses in some arteries [47]. Moreover, EETs relax coronary arteries and hyperpolarize smooth muscle cells by increasing the open-state probability of KCa channels that are sensitive to tetraethylammonium, charybdotoxin or iberiotoxin [43,48–50]. In porcine coronary arteries, endothelial cells express several cytochrome P450 epoxygenases and in this artery as well as in hamster resistance arteries, endothelium-dependent hyperpolarization and relaxation can be inhibited by antisense oligonucleotides against cytochrome P450 2C [50,51]. Furthermore, EDHF-mediated responses are increased by agents that enhance the endothelial expression of cytochrome P450 [44,50]. Taken together, such data strongly suggest that the activation of a cytochrome P450 epoxygenase is a prerequisite for the generation of EDHF-mediated relaxation in the porcine coronary artery.

However, although EETs are generated by endothelial cells both under resting conditions and in response to cell stimulation with agonists, there is little direct evidence demonstrating that these cytochrome P450 metabolites can be released from endothelial cells in amounts sufficient to elicit smooth muscle cell hyperpolarization and relaxation. Whether or not this is due to current technical limitations in measuring the concentration of these metabolites remains to be determined. Supramaximal concentrations of receptor-dependent or -independent agonists, and haemodynamic stimuli such as pulsatile stretch are however able to stimulate the release of EETs from isolated bovine and porcine coronary arteries in addition to from cultured endothelial cells [44,50,52,53]. Under such circumstances, EETs are endothelium-derived hyperpolarizing factors that elicit smooth muscle relaxation by opening BKCa channels. However, the fact that exogenously applied EETs activate BKCa channels, rather than IKCa and SKCa channels (the channels activated in EDHF responses), suggests that a diffusable EET is unlikely to account for the majority of EDHF-mediated responses.

One possibility that might reconcile many of the experimental observations is that hyperpolarization of endothelial cells might be partly regulated by activation of the cytochrome P450. For example, EETs might modulate endothelial Ca2+ influx in response to Ca2+-store depletion [54] and might facilitate the activation of endothelial K+ channels by increasing their sensitivity to Ca2+ [55,56]. Additionally, EETs also exert a biphasic change in gap junctional communication between endothelial cells [57] and their effect on myo-endothelial gap junctions is currently under investigation. Therefore, in coronary arteries, EETs and other products of cytochrome P450 could be reclassified as intracellular second messengers crucial to the initiation and transmission of endothelial cell hyperpolarization, and, as a consequence, to EDHF-mediated hyperpolarization and relaxation of vascular smooth muscle cells.

A role for gap junctions?
Gap junctions not only couple endothelial cells to endothelial cells, and smooth muscle cells to smooth muscle cells, but also couple smooth muscle cells to endothelial cells, thus providing a low-resistance electrical pathway between these two cell layers [58]. One study has suggested that the number of these heterocellular myo-endothelial gap junctions increases with the diminution in the size of the artery [59], finding that would parallel the changing importance of the contribution of EDHF to the control of vascular tone [12]. Substances that produce endothelium-dependent hyperpolarization of vascular smooth muscle cells also hyperpolarize endothelial cells with the same time-course [60]. In various microcirculatory beds, the link between the EDHF phenomenon and the spread of an electric current through myo-endothelial gap junction has been established. For example, in submucosal arterioles of the guinea-pig small intestine, acetylcholine induces outward currents in endothelial cells and smooth muscle cells that are blocked by the combination of charybdotoxin plus apamin. After the administration of blockers of gap junctions, acetylcholine elicits an outward current in endothelial cells but no longer in the smooth muscle cells, which suggests that the two cell types are connected electrically and form a functional syncytium [61–63]. In arterioles and feed arteries from the retractor muscle of the hamster, simultaneous measurements of the membrane potential in endothelial cells and smooth muscle cells show that electrical signals travel freely and bidirectionally between the two layers. These electrical signals are associated with vasomotor responses, and hyperpolarization originating in a single endothelial cell can drive the relaxation of smooth muscle cells throughout an entire arterial segment [64,65]. Cell activation by an agonist facilitates myo-endothelial gap junctional communication because a more pronounced myo-endothelial coupling is observed in acetylcholine-stimulated arteries than in arteries in which either smooth muscle or endothelial cells were stimulated by injection of a negative current [64].

In larger blood vessels, the suggestion that EDHF-mediated responses require functional myo-endothelial gap junctional communication is more controversial. The specificity of gap junction inhibitors (carbenoxolone, glycyrrhetinic acid, halothane and heptanol) is often questionable. By contrast, peptides with sequences similar to a portion of the extracellular
loop of the connexon proteins (the so-called gap peptides (e.g. GAP27)) can inhibit gap junctions [66,67]. Although such peptides are assumed to inhibit myo-endothelial gap junctions, this has merely been inferred from their ability to reduce dye coupling in cultured cells that express only connexin 43 and 40 [67]. However, electrical coupling between endothelial cells and smooth muscle cells can be observed in the absence of dye coupling [63]. Furthermore, the assumption that gap junction inhibitors are acting at the level of myo-endothelial junctions could be mistaken. For example, in the porcine coronary artery, endothelium-dependent hyperpolarizations to substance P can be recorded in the smooth muscle cells situated close to the intimal layer or close to the adventitia. GAP27 inhibits the hyperpolarization in the outer layer while the response remains unaffected in the intimal layer, suggesting that smooth muscle rather than myo-endothelial gap junctions were targeted by the peptide [37]. Clearly, there is need for further investigation of the function of gap junctions and for a better understanding of the mechanism of action of these gap junction inhibitors.

Are gap junctions a privileged site for the transfer of electrical charges or do they transfer a messenger molecule (e.g. EDHF)? In some microcirculatory beds, such as the retractor muscle of the hamster, an electrotonic current spread from endothelial cells to smooth muscle cells seems to be the most likely explanation of the EDHF phenomenon [65]. The possibility that gap junctions facilitate the diffusion of an ‘hyperpolarizing factor’, such as cAMP [67], from the endothelium to the smooth muscle raises other questions. First, if cAMP were transferred from endothelial cells to smooth muscle cells the overall response would be similar to that elicited by prostacyclin and should be sensitive to an inhibitor of KIR channels. Second, any ‘factor’ transferred via gap junctions would have to be hydrophilic (to allow its passage through the aqueous pore) and should also be actively regenerated because a significant dilution of the factor would occur from the site of its production during its rapid distribution throughout the smooth muscle layers of the vascular wall. Therefore, should cAMP play a role in the EDHF response, it is more likely that it is involved in the dynamic regulation of gap junctional communication than as acting as an EDHF per se [57].

A role for K+ ions?
One consequence of the opening of the endothelial cell K$_\text{IR}$ channel is the accumulation of K$^+$ ions in the intercellular space between endothelial cells and smooth muscle cells. A moderate increase in the myo-endothelial K$^+$ concentration, such as that which occurs on stimulation of endothelial cells in rat hepatic artery [36], can induce the hyperpolarization of vascular smooth muscle cells by activating the inwardly rectifying K$^+$ (KIR) channels and the Na$^+$--K$^+$-ATPase [68,69]. In the rat hepatic and mesenteric arteries, the endothelium-dependent smooth muscle hyperpolarization is partially inhibited by ouabain (an inhibitor of Na$^+$--K$^+$-ATPase) and barium (at a concentration that selectively inhibits KIR channels), and is abolished by the application of both inhibitors together [36]. The maintenance of the endothelial cell hyperpolarization despite the abolition of smooth muscle hyperpolarization in the presence of barium plus ouabain indicates that these inhibitors act on the smooth muscle [36]. The ouabain sensitivity of the EDHF response suggests that the type of Na$^+$--K$^+$-ATPase involved is likely to contain a type 2 or 3 α-subunit [39]. At this point, it is important to note that submicromolar concentrations of ouabain selectively target the Na$^+$--K$^+$-ATPase and inhibit EDHF-mediated responses without affecting the resting membrane potential or gap junctional communication [70,71].

Nevertheless, the proposal that EDHF might simply be endothelium-derived K$^+$ has been refuted [72–75] and indeed K$^+$-induced relaxations and hyperpolarizations are not observed in some blood vessels that exhibit EDHF-mediated responses [76]. However, in many vessels, these apparent discrepancies can be explained. The hypothesis of K$^+$ as an EDHF has been established on the basis of electrophysiological experiments, whereas the criticisms were based on experiments measuring vascular tone. In comparison to electrophysiological studies, tension recordings are more difficult to interpret because of the obligatory presence of a contractile agonist to induce tone. The elevation of smooth muscle[Ca$^{2+}$], might result in the diffusion of Ca$^{2+}$- through myo-endothelial gap junctions and thus influence endothelial cell Ca$^{2+}$-dependent processes [77]. Experiments in which tension and membrane potential were simultaneously measured clearly demonstrated that the ability of K$^+$ to cause hyperpolarization, and thus act as an EDHF, decreases as the level of agonist-dependent stimulation increases [78]. The depolarization produced by the agonist increases the [Ca$^{2+}$], that stimulates K$^+$ efflux from smooth muscle. This efflux might form a ‘K$^+$ cloud’ that surrounds the myocytes and prevents any effect of additionally added K$^+$ or any component of the EDHF response that could have resulted from a direct effect of K$^+$ on the smooth muscle [79]. Under these circumstances, the contribution of gap junctions to the EDHF phenomenon dominates. Almost certainly, the obligatory use of contractile agonists in the studies involving the measurement of tension in rat arteries could account for observations that gap junctions have essentially no role (electrophysiological measurements) [76] as opposed to a major role (tension study) [67] in EDHF-mediated responses and that K$^+$ is (electrophysiological investigations) [36,76] or is not (tension experiments) [72–75] an EDHF.

In vivo most arteries are contracted to some extent depending on the level of sympathetic activation or...
of circulating hormones. However, in healthy men infusion of barium, ouabain or their combination reduce brachial artery blood flow and K\textsuperscript{+} infusion produces vasodilatation sensitive to these inhibitors [80], suggesting that K\textsuperscript{+} as an EDHF could play a physiological role.

**Conclusion:** bringing the concepts together

There is now good evidence that EDHF-mediated responses are initiated by an increase in the endothelial [Ca\textsuperscript{2+}] and the consequent activation of endothelial SK\textsubscript{Ca} and IK\textsubscript{Ca} channels, which elicits the hyperpolarization of the endothelial cells (Fig. 2). In some tissues, the hyperpolarization of the endothelial cells might be regulated by the activation of cytochrome P450 and the resulting generation of EETs. The endothelial hyperpolarization could then spread to the adjacent smooth muscle cells through myo-endothelial gap junctions (which might also be modulated by EETs) or the efflux of K\textsuperscript{+} through the endothelial SK\textsubscript{Ca} and IK\textsubscript{Ca} channels could elicit the hyperpolarization of the surrounding myocytes by activating K\textsubscript{IR} channels and/or the Na\textsuperscript{+}–K\textsuperscript{+}-ATPase. These mechanisms are not necessarily mutually exclusive and, in a given blood vessel, they could occur simultaneously or sequentially and might act synergistically. The relative proportion of each mechanism almost certainly depends on numerous parameters including the state of activation of the vascular smooth muscle, the density of myo-endothelial gap junctions and the level of the expression of cytochrome P450 and the appropriate isoforms of Na\textsuperscript{+}–K\textsuperscript{+}-ATPase and/or K\textsubscript{IR} channels.
A transient increase in the level of cAMP might play a permissive role in the EDHF-mediated responses by enhancing the electrotonic spread of endothelial hyperpolarization through the vessel wall [82]. This study further suggests that cAMP might act as a modulating factor at the level of myo-endothelial gap junctions and gap junctions coupling smooth muscle cells.

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