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Author(s)	Féletou, M; VanHoutte, PM
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EDHF: an update

Michel Féléto and Paul M. Vanhoutte
Department of Angiology, Institut de Recherches Servier, Suresnes, France and Department
of Pharmacology, Faculty of Medicine, University of Hong Kong.

Running title: EDHF

Corresponding Author: Michel Féléto
Department of Angiologie
Institut de Recherches Servier
11 rue des Moulineaux
92150 Suresnes, France
Tel: 01.55.72.22.73
Fax: 01.55.72.80.46
Email: michel.feletou@fr.netgrs.com

Abstract

The endothelium controls vascular tone not only by releasing 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). However, this acronym includes different mechanisms. Arachidonic acid metabolites derived from the cyclooxygenases, lipoxygenases and cytochrome-P450 pathways, H₂O₂, CO, H₂S and various peptides can be released by endothelial cells. These factors activate different families of K⁺ channels and hyperpolarization of the vascular smooth muscle cells contribute to the mechanisms leading to their relaxation. Additionally, another pathway associated with the hyperpolarization of both endothelial and vascular smooth muscle cells contributes also to endothelium-dependent relaxations (EDHF-mediated responses). These responses involve an increase in the intracellular Ca²⁺ concentration of the endothelial cells followed by the opening of Ca²⁺-activated K⁺ channels of small and intermediate conductances (SK_{Ca} and IK_{Ca}). These channels show a distinct subcellular distribution, SK_{Ca} are widely distributed over the plasma membrane while IK_{Ca} are preferentially expressed in the endothelial projections toward the smooth muscle cells. Following SK_{Ca} activation, smooth muscle hyperpolarization is preferentially evoked by electrical coupling through myo-endothelial gap junctions, while following IK_{Ca} activation, potassium efflux can activate smooth muscle Kir2.1 and/or Na⁺/K⁺-ATPase. EDHF-mediated responses are altered by aging and various pathologies. Therapeutic interventions can restore these responses suggesting that the improvement of the EDHF pathway contributes to their beneficial effect. A better characterization of EDHF-mediated responses should allow determining whether or not new drugable targets can be identified for the treatment of cardiovascular diseases.

Key words: endothelium, cell membrane potential, potassium channels, myo-endothelial gap junctions, EDHF.

INTRODUCTION

Endothelial cells synthesize and release factors that regulate angiogenesis, inflammatory responses, hemostasis as well as vascular tone and permeability. The endothelium maintains the balance between inhibition and promotion of the proliferation and migration of smooth muscle cells, between prevention and stimulation of the adhesion and aggregation of the platelets, between thrombogenesis and fibrinolysis as well as between vasodilatation and vasoconstriction. Upsetting this tightly regulated balance leads to endothelial dysfunction [1,2] (figure 1).

Endothelium-dependent relaxations/vasodilatations in response to neuro-humoral mediators and physical forces, such as the shear stress exerted by the flowing blood, are generally attributed to the release of nitric oxide (NO) and/or prostacyclin (PGI₂) [3-6]. However, in numerous blood vessels from different species, including the human, these responses cannot be totally explained by the release of these two mediators. The relaxations observed in the presence of inhibitors of cyclooxygenases and NO-synthases are often associated with the hyperpolarization of the vascular smooth muscle cells and were first attributed to a non-characterized endothelial factor termed EDHF for endothelium-derived hyperpolarizing factor. The acronym "EDHF" turned out to be confusing because it implies that a single diffusible substance mediates this type of endothelium-dependent relaxation. In fact NO itself, but also numerous identified putative endothelium-derived factors including carbon monoxide (CO), hydrogen sulfide (H₂S), reactive oxygen species, peptides and arachidonic acid metabolites derived from the cyclooxygenases (COX), lipoxygenases and cytochrome P450 monooxygenases pathways can hyperpolarize the underlying smooth muscle cells [1].

Hyperpolarization decreases Ca²⁺ influx, either by reducing the open probability of voltage-dependent calcium channels (Ca_v) or the Ca_v-dependent activation of the sarcoplasmic reticulum, which is a powerful mean to produce the relaxation of vascular smooth muscle cells [7-9].

Another pathway, which does not involve the synthesis and the release of a factor *per se*, is associated with the hyperpolarization of both the endothelial and the vascular smooth muscle cells (EDHF-mediated responses) and contributes also to endothelium-dependent relaxations. These responses involve an increase in the intracellular Ca²⁺ concentration of the endothelial cells followed by the opening of Ca²⁺-activated K⁺ channels of small and intermediate conductance (SK_{Ca} and IK_{Ca}, respectively) and the subsequent hyperpolarization of these cells. Then, the endothelium-dependent hyperpolarization of the underlying smooth muscle cells can be evoked by direct electrical coupling through myo-endothelial junctions and/or the accumulation of K⁺ ions in the intercellular space between the two cell types [1]. The present review will briefly summarize the hyperpolarizing effects of the various endothelial-derived factors and focus on this latter mechanism, i.e. EDHF-mediated responses.

ARACHIDONIC ACID METABOLITES CYCLOOXYGENASES

In the endothelial cells, PGI₂ is the principal cyclooxygenase-derived metabolite of arachidonic acid. When activating its preferential receptor, the IP receptor, PGI₂ is a potent antithrombotic and antiplatelet agent and is generally a vasodilator substance [3]. The deletion of PGI₂-synthase generates hypertensive mice with arterial sclerosis while, in response to stress or injury, the IP receptor knockout animals show enhanced platelet activation, thrombosis, intimal hyperplasia, atherosclerosis, restenosis, and are prone to ischemia-reperfusion injury [10].

The vascular relaxation to PGI₂, or its synthetic analogues, is often associated with the concomitant hyperpolarization of the smooth muscle cells, which, depending on the blood vessels and the species, can involve the opening of various populations of potassium channels [11,12]. Therefore, in numerous vascular beds, PGI₂ can act as an endothelium-derived hyperpolarizing substance [13]. Since inhibitors of cyclooxygenases abolish the basal and stimulated generation of PGI₂, and potent and specific antagonist of the IP receptor block its vasodilator responses [14,15], the contribution of PGI₂ in endothelium-dependent responses can reasonably be assessed. This prostaglandin plays a role in flow-mediated vasodilatation [16,17]. However, its contribution to acute endothelium-dependent relaxations in response to neuro-humoral mediators is often minimal [1] or can only be observed when the other endothelial pathways have been inhibited [18,19]. The contribution of PGI₂ to endothelium-dependent responses is increased in eNOS knockout mice [20,21]. Similarly, in human with cardiovascular diseases, COX-2-derived prostaglandins can play a compensatory role for the decreased NO bioavailability [22,23] possibly explaining some of the detrimental cardiovascular effects associated with COX-2 inhibitors [24].

However, in aging and in the course of some cardiovascular diseases PGI₂, along with other prostaglandins, can also act as an endothelium-derived contracting factor by activating smooth muscle thromboxane/endoperoxide receptors (TP-receptors) and thus contribute to endothelial dysfunction [14,15,25] (figure 1).

CYTOCHROME P450 MONOOXYGENASES

Epoxyeicosatrienoic acids (EETs), derived from the endothelial cytochrome P450 2C or 2J epoxygenases, are generally, but not necessarily, vasodilator agents [26,27] while 20-hydroxyeicosatetraenoic acid (20-HETE, a metabolite of the cytochrome P450 of the 4A and 4F family preferentially located in vascular smooth muscle cells) is a potent endogenous vasoconstrictor of renal, cerebral, coronary, mesenteric and skeletal muscle arteries [28] (figure 1).

EETs play an important role in endothelium-dependent relaxations either as diffusible factors or as essential endothelial intracellular second messenger(s) [29]. When released, they can act as an autocrine agent eliciting endothelium and NO-dependent relaxations [30] or more generally diffuse toward the underlying vascular smooth muscle cells and produce their relaxation [31,32]. In the latter case, 11,12-EET and 14,15-EET, the two predominant diffusible isoforms, activate large conductance calcium-activated potassium channels (K_{Ca}1.1 or BK_{Ca}) of the vascular smooth muscle cells [33-36]. Experiments performed both *in vitro* and *in vivo* on human blood vessels suggest a contribution of EETs to endothelium-dependent relaxations/vasodilatations in coronary and mammary arteries as well as in peripheral muscular and subcutaneous arterioles [37-40].

Cytochrome P450 metabolites play also an important role in the regulation of the kidney circulation and contribute to the long-term regulation of blood pressure and sodium homeostasis. EETs regulate not only regional blood flow, but activate K_{ATP} channels in cardiomyocytes, limit platelet aggregation, exert anti-inflammatory actions and improve insulin sensitivity and lipid metabolism. Thus, they can protect the kidney vasculature from injury during renal and cardiovascular diseases, exert cardioprotection and prevent/delay metabolic syndrome and atherogenesis [41-43].

EETs are rapidly metabolized by soluble epoxide hydrolase to form the generally less active dihydroxyeicosatrienoic acids (DHETs). In mice, disruption of the soluble epoxide hydrolase gene produces no or minor decreases in basal arterial blood pressure [44] but protects against myocardial ischemia-reperfusion, heart failure and ischemic stroke [45-47]. In rats, soluble epoxide hydrolase plays an essential role in angiotensin II-induced cardiac hypertrophy [48], which in cardiovascular patients is the most common cause of heart failure. Polymorphisms of soluble epoxide hydrolase (*EPHX2*) have been associated with

coronary artery diseases, ischemic stroke and insulin resistance [43,49,50]. Potent and selective inhibitors of this enzyme have been designed and are currently undergoing clinical trials for the treatment of hypertension. The additional anti-inflammatory properties of soluble epoxide hydrolase inhibitors make them also attractive for the treatment of chronic kidney disease in patients with cardiometabolic syndrome [51].

LIPOXYGENASES

In rat and porcine coronary arteries, the 12-lipoxygenase metabolite 12-(S)-hydroxyeicosatetraenoic acid (12-S-HETE) can be released by the endothelial cells and evoke relaxation of the vascular smooth muscle by activating BK_{Ca} [52].

In rabbit arteries, the generation of 11,12,15-trihydroxyeicosatrienoic acid (11,12,15-THETA) by reticulocyte-15-lipoxygenase-I contributes to acetylcholine-induced endothelium-dependent relaxations [53]. In this species, the age-related decrease in hypotension and the endothelium-dependent relaxations induced by acetylcholine are mediated by a decreased synthesis of 11,12,15-THETAs, associated with a down-regulation of 15-lipoxygenase expression and a reduced activity of the enzyme [54,55]. By contrast, short-term hypercholesterolemia, in the absence of atherosclerotic lesions, and chronic hypoxia increase endothelial 15-lipoxygenase expression, 11,12,15-THETAs production as well as acetylcholine-induced endothelium-dependent relaxations and hypotension [56,57] (figure 1).

However, although a strong case for lipoxygenases derivatives acting as endothelium-derived hyperpolarizing substance can be built in specific arteries and especially in those of the rabbit, most of the EDHF-mediated responses, including that in human arteries, do not appear to involve metabolite of arachidonic acid produced by this pathway [1,58].

ENDOCANNABINOIDS

In isolated blood vessels or *in vivo* in anaesthetized animals, endogenous and exogenous cannabinoids usually have vasodilator properties and are likely to play a role in cardiovascular homeostasis [59]. However, the suggestion that anandamide could be an endothelium-derived hyperpolarizing substance has not been substantiated [1,60].

NO-SYNTASES

Three different isoforms of an enzyme are able to synthesize NO from L-arginine and molecular oxygen, NO-synthase I (or neuronal, nNOS, NOS-1), NO-synthase II (or inducible, iNOS, NOS-2) and NO-synthase III (or endothelial, eNOS, NOS-3). Under various circumstances such as the presence of NOS inhibitors, low levels of L-arginine or oxidized tetrahydrobiopterin but also under physiological conditions, NO-synthases can generate superoxide anion ($O_2^{\cdot-}$), which is reduced to hydrogen peroxide (H_2O_2), either spontaneously or enzymatically by superoxide dismutase (SOD). H_2O_2 can act locally close to its site of production or, since it is an uncharged molecule, diffuse through the cell membrane and act on neighboring cells [5,61-63] (figure 1).

NITRIC OXIDE (NO)

NO is a potent vasodilator and a powerful inhibitor of platelet adhesion and aggregation. The relaxations elicited by NO are generally associated with the stimulation of the cytosolic soluble guanylyl cyclase and the subsequent activation of cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) [5]. NO regulates the activity of various potassium channels and, depending on the vascular beds, the hyperpolarizing effects of NO on vascular smooth muscle cells can substantially contribute to their relaxation [1]. The activation of BK_{Ca} channels by NO contributes to the beneficial effects of currently prescribed drugs associated with the L-arginine-NOS-cGMP pathway, for instance NO donors and phosphodiesterase inhibitors such as sildenafil [64].

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 still be produced by the endothelial cells and contributes to the relaxation and/or hyperpolarization of the underlying vascular smooth muscle [65]. Furthermore, NO can also be stored and released independently of the activation of NO-synthase [66-68]. Therefore, it seems likely that the role of NO as an endothelium-derived hyperpolarizing substance may be underestimated by assuming that the presence of an inhibitor of NO-synthases rules out its contribution. A non-NO- non-PGI₂-mediated response should be reported not only when a relaxation and/or hyperpolarization is recorded in the combined presence of inhibitors of cyclooxygenases and NO-synthases but when this response is still observed with the additional presence of a NO scavenger [69]. In *in vivo* studies in human, complete blockade of NO synthase is difficult to achieve (or to demonstrate) and for obvious ethical reasons many of the pharmacological tools used to study EDHF-mediated responses cannot be administered. The limitation of these studies should always be kept in mind when interpreting these data.

In NOS-3 knockout mice, EDHF-mediated responses play a compensatory role for the absence of endothelial NO [70] and this adaptation to NO synthase deletion is gender specific [71]. Similarly, in resistance arteries from female double knockout mice for NOS-3 and cyclooxygenase-1 (COX-1), endothelium-dependent relaxations are preserved by an EDHF-mediated mechanism while, in arteries from the double knockout males, the endothelium-dependent relaxations are impaired severely. In genetically modified female mice, the double deletion of NOS-3 and COX-1 does not affect mean arterial blood pressure while the corresponding males are hypertensive [72].

HYDROGEN PEROXIDE

In the mesenteric arteries of mice with disruption of the various NOS isoform genes (single NOS-3 knockout, double NOS-1/NOS-3 knockout and triple NOS-1/NOS-2/NOS-3 knockout) the endothelium-dependent relaxations and hyperpolarizations resistant to inhibitors of NO-synthases and cyclooxygenases are progressively reduced as the number of the disrupted NOS genes increased [73]. The dependency of the responses resistant to inhibitors of NO-synthases and cyclooxygenases towards the NOS systems could not be explained by residual NO release but has been attributed to the production of H₂O₂ [73] (figure 1).

Depending on the tissue, the experimental conditions or the concentrations studied, H₂O₂ possesses dilator or constrictor properties, and can hyperpolarize or depolarize vascular smooth muscle cells [74]. For instance, in the isolated murine mesenteric artery, H₂O₂, at concentrations lower than 50 μM, produces an endothelium-independent relaxation providing that K_{Ca} channels are operational but, at the same concentrations, elicits a potent contractile response if the activity of these channels is compromised [75].

In NOS-disrupted murine arteries, relaxations resistant to inhibitors of NO-synthases and cyclooxygenases are sensitive to catalase, the enzyme that dismutates H₂O₂ into water and oxygen, and have been attributed to the NOS-3/Cu,Zn-SOD-dependent formation of H₂O₂ [76,77] (figure 1). The maintenance of these endothelium-dependent relaxations/hyperpolarizations in NOS-3 knockout mice was explained by the compensatory endothelial expression of other NOS genes, the production of H₂O₂ being preserved up to the total disruption of the three NOS genes [73]. Nevertheless, whether or not the decrease in the EDHF-mediated responses is directly associated with the disruption of the NOS genes or is independently associated with the severe phenotype of these mice, especially with the one observed in the triple knockout mice (hypertension, dyslipidemia, myocardial infarction and nephrogenic diabetes insipidus) remains to be fully assessed (see also the phenotype of mice with deletion of the cystathionine γ-lyase).

The involvement of H_2O_2 in agonist- and flow-induced endothelium-dependent relaxations/vasodilatations has been suggested in other vascular beds [78], including human mesenteric [79] and coronary arteries [80]. In addition, without actually being released by the endothelial cells, H_2O_2 can enhance EDHF-mediated responses by potentiating calcium release from endothelial stores [81]. In various experimental models or in arteries from patients with cardiovascular diseases, H_2O_2 generation can partially compensate the decreased NO production, at least in term of endothelium-dependent relaxations, but in the long term this production of reactive oxygen species may contribute to vascular oxidative injury [82-84].

Depending on the blood vessels, besides NO-synthases several other endothelial enzymes such as cyclooxygenases, lipoxygenases, cytochrome P450 epoxygenases, NADPH oxidases and mitochondrial respiratory enzymes can generate superoxide anions and be at the origin of H_2O_2 production [78]. In murine arteries, endothelial oxidases other than NOS do not appear to be involved in H_2O_2 -mediated responses [85]. However, in human coronary arterioles flow-induced endothelium-dependent dilation is associated with H_2O_2 generated by the mitochondrial respiratory chain, while bradykinin-induced endothelium-dependent relaxation requires NADPH oxidase-derived H_2O_2 [86,87].

Therefore, in presence of nitric oxide synthases and cyclooxygenases inhibitors, residual NO and/or H_2O_2 , both potentially derived from various endothelial NOS isoforms, can act as endothelium-derived hyperpolarizing substances. However, in many arteries EDHF-mediated responses cannot be attributed to the generation of residual NO or to that of H_2O_2 [1,74,88].

OTHER GASEOUS MEDIATORS

Besides NO, CO and H_2S are also water soluble low molecular weight gas that readily cross lipid membranes and therefore diffuse homogeneously and in a non-polarized manner from their production site acting as autocrine and paracrine substances [89].

CARBON MONOXIDE (CO)

The predominant biological source of CO is from the heme degradation by heme-oxygenase (HO), either from the constitutive (HO-2) or the inducible (HO-1) isoform, both being expressed in vascular smooth muscle and endothelial cells [90,91]. In many physiological situations the HO-CO pathway compensates for the decrease NO bioavailability [91]. CO is a potent vasodilator in most, but not all, vascular beds. The mechanisms of CO-induced vasodilatation involve the stimulation of soluble guanylate cyclase, the inhibition of cytochrome P450-dependent production of 20-HETE and/or the activation of various populations of K^+ channels [91]. However, CO is also a tonic inhibitor of NOS, by binding to its prosthetic heme, and can contribute to endothelial dysfunction [92].

HO-1 knockout mice, although normotensive, when subjected to stress or injury show exacerbated responses. In contrast, overexpression of HO-1 plays a protective role in hypoperfusion and ischemia/reperfusion injury and induction of HO-1 expression by transient hemin administration produces a long-lasting normalization of arterial blood pressure in spontaneously hypertensive rats (SHR) [93,94]. HO-2 knockout mice are also normotensive but stroke damage in response to injuries is accentuated in these animals, indicating that HO-2 plays an endogenous neuroprotective role in the brain [95]. Carbon monoxide releasing molecules have vasodilator, anti-ischaemic and anti-inflammatory effects and may present some therapeutic interest in cardiovascular diseases [96].

An endothelial production of CO, contributing to endothelium-dependent relaxations in response to neurohumoral substances, has been demonstrated only in a limited number of arteries and is therefore unlikely to explain most EDHF-mediated responses [1,97,98].

HYDROGEN SULFIDE (H_2S)

Two main enzymes are responsible for the production of hydrogen sulfide (H₂S), cystathionine β-synthase and cystathionine γ-lyase and both use L-cysteine as substrate. The physiological cardiovascular effects of H₂S, which are generally linked to the activation of the latter enzyme, involve anti-inflammatory and anti-oxidant properties, vasodilatation and a decrease in arterial blood pressure [89,99] (figure 1).

Mice with deletion of the cystathionine γ-lyase, an enzyme expressed in multiple tissues, and recently identified in the endothelial cells, are hypertensive and the endothelium-dependent relaxations of their mesenteric artery in response to methacholine is virtually abolished [100]. H₂S is produced and released by endothelial cells, in a calcium-dependent manner, following neuro-humoral stimulation and evokes relaxation and hyperpolarization of vascular smooth muscle cells by activating K_{ATP} channels [100,101]. Thus, these results suggest that H₂S is an endothelium-derived relaxing and hyperpolarizing factor. However, the precise role of this mediator needs to be further substantiated. In most studies, the endothelium-dependent relaxations of the murine mesenteric artery involve NO release and EDHF-mediated responses, which are not necessarily associated with the activation of K_{ATP} channel [1]. The disappearance of both the NO- and EDHF-mediated component of the endothelium-dependent relaxation in cystathionine γ-lyase knockout mice is unexplained at present but could be attributed to the increase in homocysteine levels [102,103]. Again, whether or not the decrease in endothelium-dependent responses (NO-mediated and EDHF-mediated responses) is directly associated with the disruption of the cystathionine γ-lyase gene or is independently associated with the phenotype of these mice remains to be determined (see also multiple NOS-knockout mice).

H₂S donors are currently being synthesized and have therapeutic potential in cardiovascular diseases associated with inflammatory processes such as reperfusion injury, circulatory shock, atherosclerosis, diabetes and possibly hypertension [97,104,105].

C-TYPE NATRIURETIC PEPTIDE (CNP),

Endothelial cells can theoretically synthesize numerous vasoactive peptides. Among them CNP, a member of the natriuretic peptide family, evokes relaxations and hyperpolarizations of vascular smooth muscle cells, including those of human forearm resistance vessels. The vasodilator effects of CNP are generally attributed to the activation of natriuretic peptide receptors B subtype (NPR-B) on the smooth muscle, followed by the stimulation of particulate guanylate cyclase, leading to accumulation of cGMP and the subsequent opening of BK_{Ca} and K_{ATP} channels [106-108].

Additionally, it has been suggested that CNP could contribute to EDHF-mediated responses. CNP would activate the NPR-C receptor subtype and evoke hyperpolarization of the smooth muscle cell via the cGMP-independent activation of G-protein regulated inward-rectifier K⁺ channels (GIRK) [109,110]. However, this hypothesis had not been confirmed [111-113] and in mice deficient for the NPR-C gene, EDHF-mediated responses are not altered [114].

Therefore, CNP is unlikely to act as an endothelium-derived relaxing/hyperpolarizing substance and contribute to moment-to-moment endothelium-dependent regulation of vascular tone. Nevertheless, this peptide plays a key role in preventing smooth muscle proliferation, leukocyte recruitment and platelet reactivity. As such, CNP is likely to exert an anti-atherogenic influence on the blood vessel walls [115,116].

EDHF-MEDIATED RESPONSES

EDHF-mediated responses are endothelium-dependent relaxations resistant to inhibitors of NO-synthases and cyclooxygenases, which do not involve one of the identified mediators

mentioned above (arachidonic acid metabolites, residual or stored NO, H₂O₂, CO, H₂S, CNP), and which require the activation of endothelial calcium-activated potassium channels.

CALCIUM-ACTIVATED POTASSIUM CHANNELS

The three subtypes of calcium-activated potassium channels of large (BK_{Ca}), intermediate (K_{Ca}3.1 or IK_{Ca}) and small conductance (K_{Ca}2.3 isoform or SK_{Ca}) are present in the vascular wall but with very specific cellular and subcellular localization (figure 2).

BK_{Ca} channels are expressed in virtually all vascular smooth muscle cells [1] while in most endothelial cells, when freshly isolated, BK_{Ca} channels are at best poorly expressed and iberiotoxin-sensitive currents are observed only at very positive potentials [117-119]. In smooth muscle cells, BK_{Ca} channels, often clustered in groups of 20-100 units, are activated by a general increase in intracellular calcium or by calcium sparks, localized elemental calcium release events from internal calcium stores, which then generate spontaneous transient outward currents (STOC) [120,121]. BK_{Ca} channels are often colocalized in discrete smooth muscle area with endoplasmic reticulum and form signal complex, physically associated with cationic channels such the canonical transient receptor potential channel 1 (TRPC1) [122] or indirectly associated with the vanilloid transient receptor potential channel 4 (TRPV4). The Ca²⁺ influx through TRPV4 preferentially stimulates ryanodine receptors located on the endoplasmic reticulum increasing the frequency of Ca²⁺ sparks. In some arteries, EETs-induced hyperpolarization involves the latter Ca²⁺-signaling complex [36] (figure 2). Conversely, there is little evidence for a functional role of SK_{Ca} channels in vascular smooth muscle cells, although a non-identified apamin-sensitive conductance has been reported in some arteries [123-125]. Similarly, in healthy and freshly isolated vascular smooth muscle cells IK_{Ca} channels are not or very poorly expressed. However, in proliferating cells, as seen in culture or after vascular injury, the expression of this channel increases dramatically [126,127].

By contrast, the IK_{Ca} and SK_{Ca} channels (especially the SK3 α subunit) are constitutively expressed in endothelial cells [117,118,128,129], but show a very different spatial distribution. SK_{Ca} are diffusely distributed over the plasma membrane with preferential locations at sites of homocellular endothelial gap junctions and caveolin-rich domains and are associated with various connexins (Cx). IK_{Ca} are localized preferentially within the endothelial projections through the internal elastic lamina at the sites of myo-endothelial gap junctions [113,130-133] (figure 2).

Agonists that stimulate G protein-coupled receptors and compounds, such as the calcium ionophore A23187, thapsigargin, and cyclopiazonic acid, evoke EDHF-mediated responses. These substances share the property to increase endothelial intracellular Ca²⁺ concentration and to activate endothelial SK_{Ca} channels (blocked by apamin, scyllatoxin, or UCL 1684) and/or IK_{Ca} channels (blocked by charybdotoxin or TRAM-34) [1]. 1-Ethyl-2-benzimidazolinone (1-EBIO), a non-specific activator of K_{Ca} [134], which activates endothelial IK_{Ca} and SK_{Ca} [118], but not BK_{Ca} channels of vascular smooth muscle, hyperpolarizes endothelial cells and produces endothelium-dependent hyperpolarization [135,136]. Similar results were obtained with more potent analogues of 1-EBIO, such as DC-EBIO or NS-309 [137,138] and with derivatives of the neuroprotective agent riluzole such as SK-20 or SK-31 [139], indicating that activation of endothelial K_{Ca} channels and/or endothelial cell hyperpolarization elicit(s) EDHF-mediated responses.

The hyperpolarization of the endothelial cells in turn favours the entry of calcium by increasing the driving force for this ion [140,141]. Therefore, endothelial K_{Ca} channels are not only key players in EDHF-mediated responses but also contribute to the activation of calcium-sensitive enzymes such as eNOS and thus to the generation of NO [142,143] (figure 2).

HYPERPOLARIZATION OF VASCULAR SMOOTH MUSCLE

The involvement of two populations of endothelial K_{Ca} channels in EDHF-mediated responses has been puzzling for a long time but the discrete role of each channel is now better appreciated.

In the blood vessel wall, gap-junctions link smooth muscle with other smooth muscle cells, endothelial with other endothelial cells and in many blood vessels smooth muscle with endothelial cells. The connexins (Cx) 37, 40 and 43 are the predominant isoforms of gap-junction proteins expressed in the vascular wall and, in rodents the Cx37 and Cx40 isoforms are involved preferentially in myoendothelial gap junction communication [144-146] (figure 2). The number of myo-endothelial gap junctions increases with a reduction in the size of the artery [147], a phenomenon that parallels the contribution of the EDHF-mediated responses to endothelium-dependent relaxations [148,149]. Endothelium and smooth muscle cells can communicate via these myo-endothelial gap junctions physically, as Ca^{2+} and IP_3 can diffuse from one cell type to another [150-152], and electrically, since depolarization and hyperpolarization are conducted bi-directionally from one cell type to the other [151-156]. However, endothelium-dependent dilatations do not simply propagate electronically but involve a regenerative mechanism [155,157]. Blockers of gap junctions abolish or partially inhibit EDHF-like responses in many arteries and in the rat mesenteric artery, antibodies directed against Cx40, when loaded selectively in the endothelial cells, block EDHF-mediated responses [144,158-160]. Furthermore, in mice, Cx40 is essential for the acetylcholine-activated regenerative endothelium-dependent vasodilatation [157,162]. Activation of either SK_{Ca} or IK_{Ca} leads to endothelium-dependent hyperpolarizations and relaxations of vascular smooth muscle cells, but in quiescent arteries (in the absence of vasoconstrictor stimulation) EDHF-mediated responses are associated with the preferential activation of SK_{Ca} and the contribution of myoendothelial gap junctions [113,131,133,163].

Taken into conjunction, the results of these *in vitro* experiments provide compelling evidence for a major contributing role of myoendothelial gap junction in EDHF-mediated responses [164]. However, experiments performed *in vivo* generally failed to demonstrate such a significant role for myoendothelial gap junctions [165,166]. The origin of this discrepancy is unknown but may involve the type and size of arteries studied *in vivo*, the presence of shear stress, sympathetic innervation and circulating hormones as well as confounding factors such as the use of anesthetics which inhibit gap junctions [131,164].

Additionally, the efflux of K^+ ions associated with the activation of endothelial K_{Ca} can contribute to EDHF-mediated responses [166]. The resultant moderate increase in the extracellular K^+ concentration (1 to 15 mM) can provoke the relaxation of vascular smooth muscle cells [167] by activating K_{IR} [168] and the Na^+/K^+ pump [169]. The activation of K_{IR} and the Na^+/K^+ pump overcomes the small depolarizing effects linked to the increase in potassium ions *per se* and the net resultant is hyperpolarization and thus relaxation of the smooth muscle cells. This hypothesis was first demonstrated successfully in the hepatic and mesenteric arteries of the rat [166] and observed in many other blood vessels including human arteries [170-177].

However, this phenomenon is likely to occur only in specialized microdomains situated in the endothelial projections associated with myo-endothelial gap junctions. Section of endoplasmic reticulum densely expressing 1,4,5-trisphosphate (IP_3) receptors, Cx40, IK_{Ca} channels and calcium-sensing receptors are collocated in these endothelial projections [113,130-133,178]. Repetitive localized calcium events (pulsars), driven by IP_3 and/or Ca^{2+} ions (calcium-induced calcium release), originate from these endothelial calcium stores. IP_3 can be generated by the endothelial cells (for instance following acetylcholine stimulation) or by the smooth muscle cells (for instance following phenylephrine stimulation). In the latter case, IP_3 would diffuse toward the endothelial cells (possibly with Ca^{2+} ions) through the myoendothelial gap junctions [113,133,179,180]. The closely situated IK_{Ca} channels are

activated by these calcium pulsars and the resultant endothelial hyperpolarization can be transmitted to the smooth muscle cells either via the myo-endothelial gap junctions, as described previously, or be elicited by the K^+ ions accumulating in the restricted extracellular space surrounding these endothelial projections (figure 3). In the rat mesenteric artery, K^+ ion accumulation preferentially activates the Na^+/K^+ pump [113,181].

The precise role of the calcium-sensing receptor at the site of these endothelial projections is not completely understood. Stimulation of the calcium-sensing receptor results in selective IK_{Ca} -dependent endothelial hyperpolarization and endothelium-dependent vascular smooth muscle hyperpolarization [178,182,183]. In quiescent arteries (in the absence of vasoconstrictor stimulation), the calcium-sensing receptor would be fully stimulated by the concentration of calcium bathing the endothelial cells and IK_{Ca} inactivated [113]. Stimulation of smooth muscle (for instance by phenylephrine) opens CaV and, in the small extracellular space surrounding myoendothelial projections, could create a localized calcium sink. The endothelial calcium-sensing receptor would detect the changes in extracellular calcium and allow the recruitment of endothelial IK_{Ca} . The subsequent endothelium-dependent hyperpolarization would restrain excessive activation of the smooth muscle [113] (figure 3). Indeed, reducing the extracellular calcium concentration enables IK_{Ca} activation by acetylcholine [113,163]. Therefore, endothelial projections and myo-endothelial gap junctions are key structures intrinsically associated with extracellular and intracellular calcium homeostasis in both endothelial and vascular smooth muscle cells [113,131,133].

However, in some blood vessels, K^+ does not evoke, or inconsistently produces relaxations and hyperpolarizations [136,159,160,184,185], indicating that in these blood vessels the contribution of K^+ ions in EDHF-mediated responses must be, if anything, minimal. The involvement of gap junctions and K^+ ions are not necessarily mutually exclusive. The relative proportion of each mechanism almost certainly depends on numerous parameters including the extracellular concentrations in potassium and calcium ions associated with the state of activation of the underlying vascular smooth muscle cells, the density of myo-endothelial gap junctions and the level of the expression of the appropriate isoforms of Na^+/K^+ -ATPase and/or K_{IR} [1,131].

GENETICALLY MODIFIED ANIMALS

In transgenic mice with SK3 gene expression under the control of dietary doxycycline, the suppression of SK3 expression in the endothelial cells depolarizes both the endothelial and the vascular smooth muscle cells, reduces the diameter of resistance vessels *in situ* and increases arterial blood pressure, a reversible phenotype upon restoration of endothelial SK3 expression [186]. Disruption of the IK1 gene reduces the hyperpolarization of endothelial and smooth muscle cells in response to acetylcholine and decreases the associated vasodilatation, because of a substantial reduction in EDHF-mediated responses. Moreover, the IK1 deletion also led to a significant increase in arterial blood pressure and to mild left ventricular hypertrophy [187]. In double knockout mice, lacking both SK3 and IK1, an addition of the detrimental effects provoked by the deletion of either gene is observed [188,189]. These results confirm that in mice endothelial SK_{Ca} and IK_{Ca} channels are fundamental determinants of endothelial hyperpolarization and EDHF signaling and indicate that they actively control vascular tone and contribute to the overall regulation of the circulation.

Connexins 37 and 40 are the predominant gap junction proteins in murine endothelial cells [190]. Connexin 40 proteins are involved in endothelial homocellular gap junctions and also in heterocellular gap junctions linking endothelial cells not only to smooth muscle cells but also to renin-producing juxtaglomerular cells. The presence of the latter gap junction communication is required in order to maintain the calcium-dependent inhibitory effects of angiotensin II and that of intrarenal pressure on renin secretion and synthesis, suggesting that the endothelium is strongly involved in the regulation of the renin system. Mice deficient for

connexin 40 are hypertensive. However, alteration in the control of renin release only partially explained the hypertension observed in connexin 40 knockout mice [191]. The arterioles of these animals also exhibit a reduced spread of dilatation in response to endothelium-dependent vasodilators and irregular arteriolar vasomotion [192-194].

These results show that deletion of each key molecular component of EDHF-mediated responses is associated with hemodynamic alterations suggesting that this endothelial pathway contributes to the overall regulation of arterial blood pressure.

ENDOTHELIAL DYSFUNCTION AND THERAPEUTIC INTERVENTIONS

Endothelial dysfunction, observed in various cardiovascular diseases, is associated with a decrease of NO synthesis and/or a loss of its biological activities. However, alteration of the EDHF pathway can also contribute to these endothelial dysfunctions or conversely compensate for the loss in NO bioavailability. Alterations of EDHF-mediated responses have been reported with aging and under various pathological conditions (hypertension, atherosclerosis, hypercholesterolemia, heart failure, ischemia-reperfusion, angioplasty, eclampsia, diabetes, sepsis) [1,2,195].

No drug is available which has been designed to target EDHF-mediated responses. Nevertheless, therapeutic interventions, with beneficial effects on the cardiovascular system such as angiotensin converting enzyme inhibitors, antagonists of angiotensin receptors and phosphodiesterase-3 inhibitors [1,195] can restore these responses, suggesting that the improvement of the EDHF pathway contributes to the observed beneficial effect. Similarly, various so-called non-pharmacological therapeutic strategies including exercise and supplementation with estrogens, omega-3 polyunsaturated fatty acids, polyphenol derivatives, potassium and/or calcium help to reverse endothelial dysfunction including blunted EDHF-mediated responses [1,195].

The improvement or restoration of EDHF responses has not been, yet, the direct purpose of any pharmaceutical effort. SKA-31, a preferential activator of murine IK_{Ca} , potentiates EDHF-mediated responses in vitro and lowers mean arterial blood pressure in normotensive and in angiotensin II-hypertensive mice [139]. However, IK_{Ca} channels are required for the differentiation of vascular smooth muscle cells, as well for their proliferation and migration [126,127,197,198]. Selective blockade of IK_{Ca} with TRAM-34 [199] prevents phenotypic changes of smooth muscle and coronary artery neointimal formation in two different models of post-angioplasty restenosis and the development of atherosclerosis in ApoE(-/-) mice [126,198,200]. IK_{Ca} are also involved in the proliferation of endothelial [201] and various cancerous cells [202,203]. Therefore, activators of IK_{Ca} may have some unwanted detrimental effects.

Additionally, activation of endothelial TRP and SK_{Ca} channels, calcium sensing receptors, smooth muscle K_{IR} and/or specific isoform(s) of Na^+/K^+ -ATPase as well as facilitating myo-endothelial communication and increasing the expression of appropriate connexins, channels and receptors may represent new potential targets. However, the precise role of these various molecular elements is far from being completely understood. For instance, TRPV4 channel could appear as a promising target in cardiovascular diseases since this cationic channel is involved in calcium entry following endothelial stimulation. [204]. Indeed, the arterial responses to shear stress critically depend on the activation of this endothelial channel and both the NO and the EDHF-mediated components of acetylcholine-induced vasodilatation are attenuated in TRPV4-deficient mice [205,206]. However, GSK1016790A, a specific and potent agonist, which as expected increases endothelial intracellular calcium concentration and produces endothelium-dependent relaxations, also causes endothelial failure, circulatory collapse and death [207]. Rotigaptide (ZP123), an antiarrhythmic peptide that prevents uncoupling of Cx43-mediated gap junction communication [208], has no effect on basal vascular tone and does not enhance endothelium-

dependent or independent vasodilatation in the forearm arterial circulation of healthy subjects [209]. Whether or not augmenting Cx43 communication would improve endothelial function in patients with vascular disease and whether or not Cx40, in human, would be a more appropriate target than Cx43 remain to be determined.

CONCLUSION AND PERSPECTIVES

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 (e.g; prostacyclin, EETs, lipoxygenase 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 numerous animal species and in the human. They can act as a back up system when NO is inhibited or reduced but this is not necessarily the case.

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. The mechanisms underlying endothelium-responses must be carefully dissected in order to be properly identified. The synthesis of more selective compounds such as non-peptidic inhibitors and activators of SK_{Ca} and IK_{Ca} may in the future allow the selective blockade/activation of EDHF-mediated responses and hence the proper determination of their physiological role in the human circulation. The limited information available suggests that if better (i.e. more potent, more specific and if possible orally active) pharmacological tools were developed to modulate the role of the various molecular constituents underlying EDHF-mediated responses, it may be possible to determine whether or not putative cardiovascular targets identified within this pathway are drugable.

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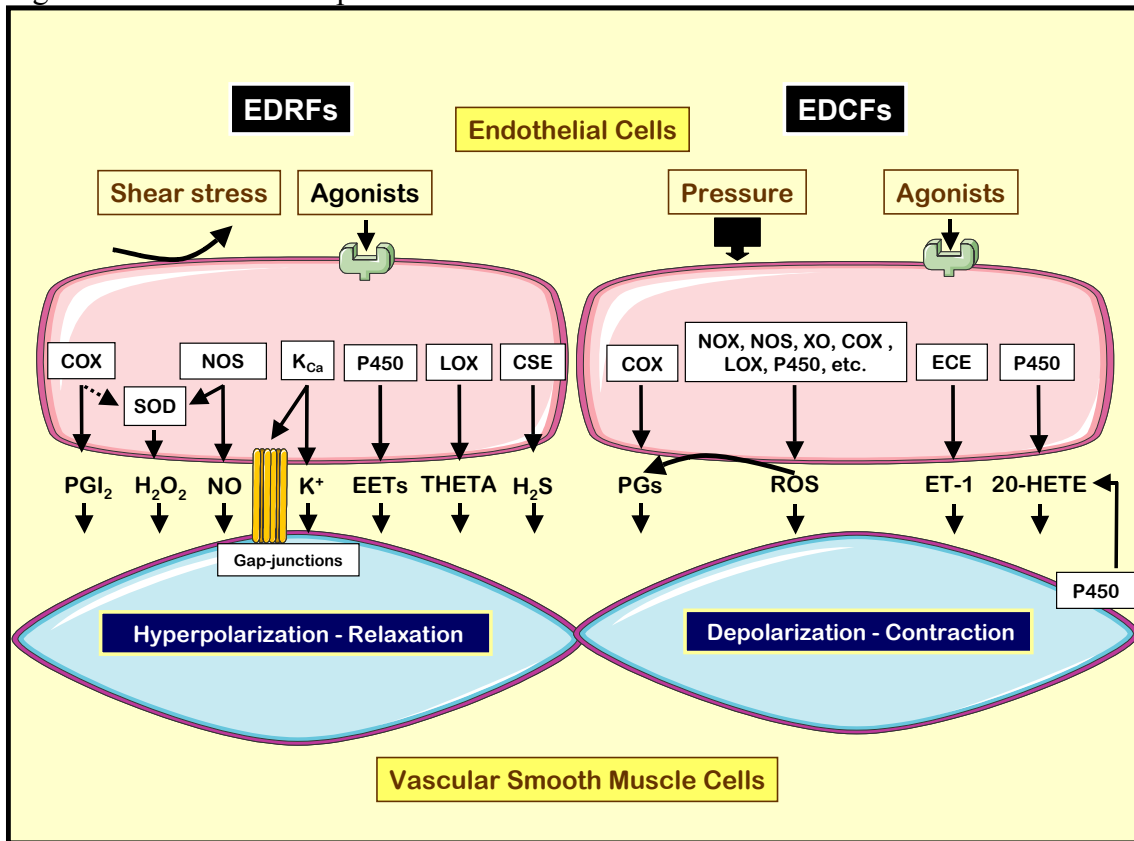
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Figure legends

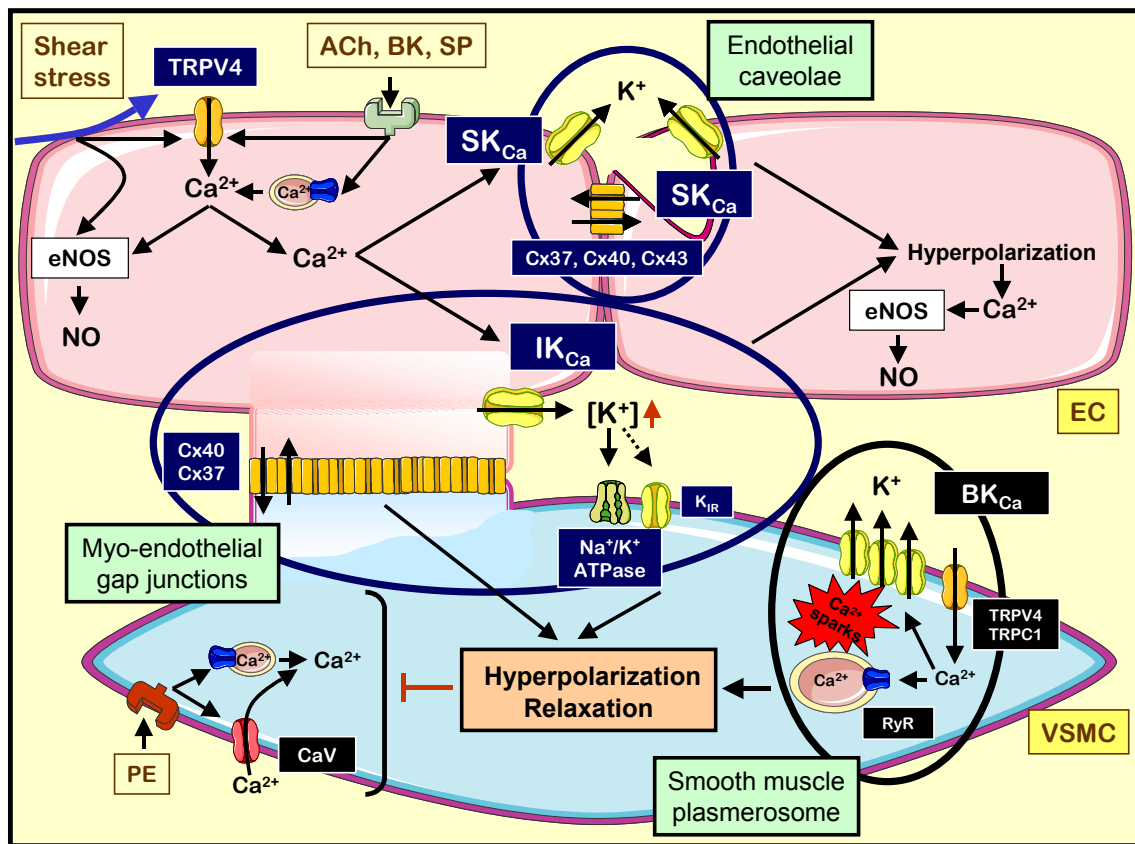
Figure 1: Endothelium-dependent relaxations and contractions



Endothelial cells synthesize and release various vasoactive factors and therefore maintain the balance between vasodilatation and vasoconstriction. Upsetting this tightly regulated balance contributes to endothelial dysfunction [2].

EDRFs: endothelium-derived relaxing factors; EDCFs: endothelium-derived contracting factors; COX: cyclooxygenases; SOD: superoxide dismutases; NOS: nitric oxide synthases; K_{Ca}: calcium-activated potassium channels; P450: cytochrome P450; LOX: lipoxygenases; CSE: cystathionine γ -lyase; XO: xanthine oxidase; ECE: endothelin converting enzyme; PGI₂: prostacyclin; H₂O₂: hydrogen peroxide; K⁺: potassium ions; EETs: epoxyeicosatrienoic acids; THETA: trihydroxyeicosatrienoic acid; H₂S: hydrogen sulfide; PGs: prostaglandins; ROS: reactive oxygen species; ET-1: endothelin-1; 20-HETE: 20-hydroxyeicosatetraenoic acid.

Figure 2: Calcium-activated potassium channels in the vascular wall and EDHF-mediated responses

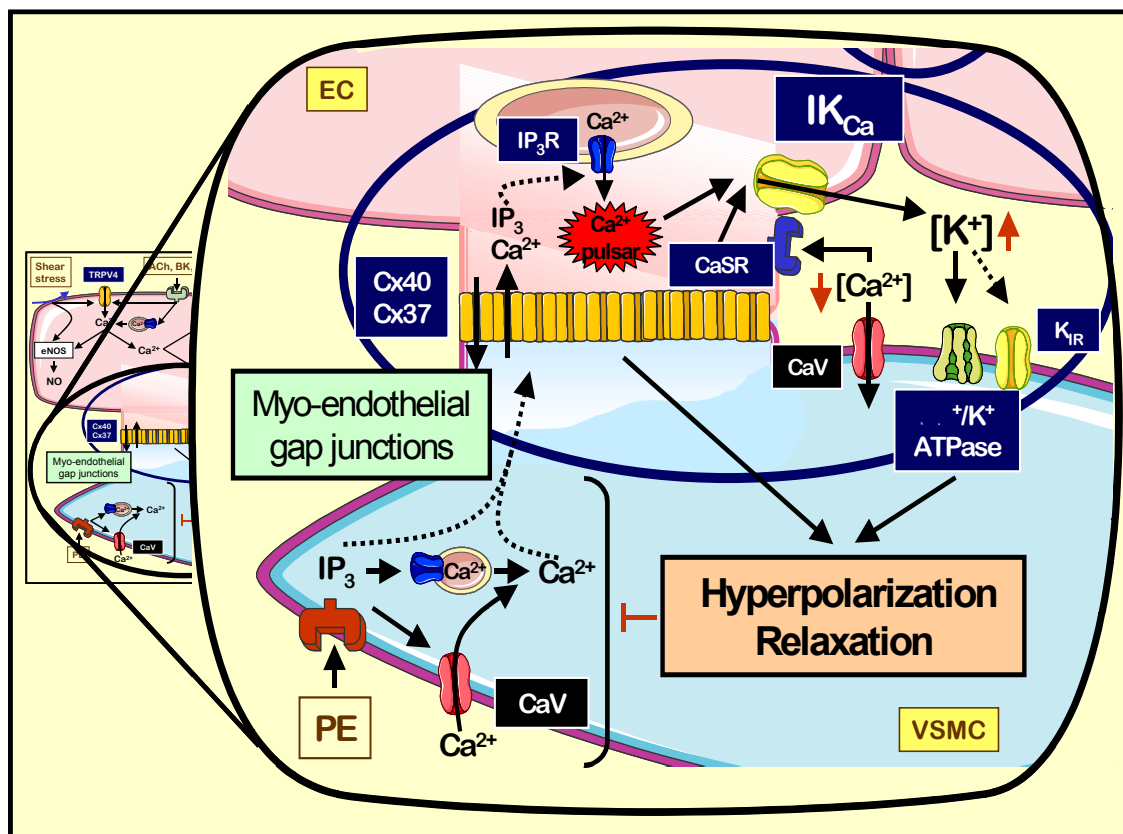


The three subtypes of calcium-activated potassium channels of large (BK_{Ca}), intermediate (IK_{Ca}) and small conductance (SK_{Ca}) are present in the vascular wall but with very specific cellular and subcellular localization. BK_{Ca} channels are expressed preferentially in discrete vascular smooth muscle area, smooth muscle plasmersome, associated with endoplasmic reticulum. They form signal complexes with canonical or vanilloid transient receptor potential channels 1 (TRPC1 or TRPV4). The IK_{Ca} and SK_{Ca} channels (especially the SK3 α subunit) are constitutively expressed in endothelial cells. SK_{Ca} are diffusely distributed over the plasma membrane with preferential locations at sites of homocellular endothelial gap junctions and caveolin-rich domains and are associated with various connexins (Cx) (Endothelial caveolae). IK_{Ca} are preferentially localized within the endothelial projections through the internal elastic lamina (myo-endothelial gap junctions).

The activation of endothelial receptors and the shear stress exerted by the flowing blood increase endothelial $[Ca^{2+}]_i$ and activates endothelial NO-synthase (eNOS) as well as SK_{Ca} and IK_{Ca} channels. The subsequent endothelial hyperpolarization favors the entry of calcium as a positive feedback loop. The hyperpolarization can be conducted through myo-endothelial gap junctions composed of Cx40 and possibly Cx37 to the underlying vascular smooth muscle. Additionally, accumulation of potassium ions in the intercellular space can hyperpolarize the smooth muscle cells by activating Na^+/K^+ -ATPase and inwardly rectifying potassium channels (K_{IR}).

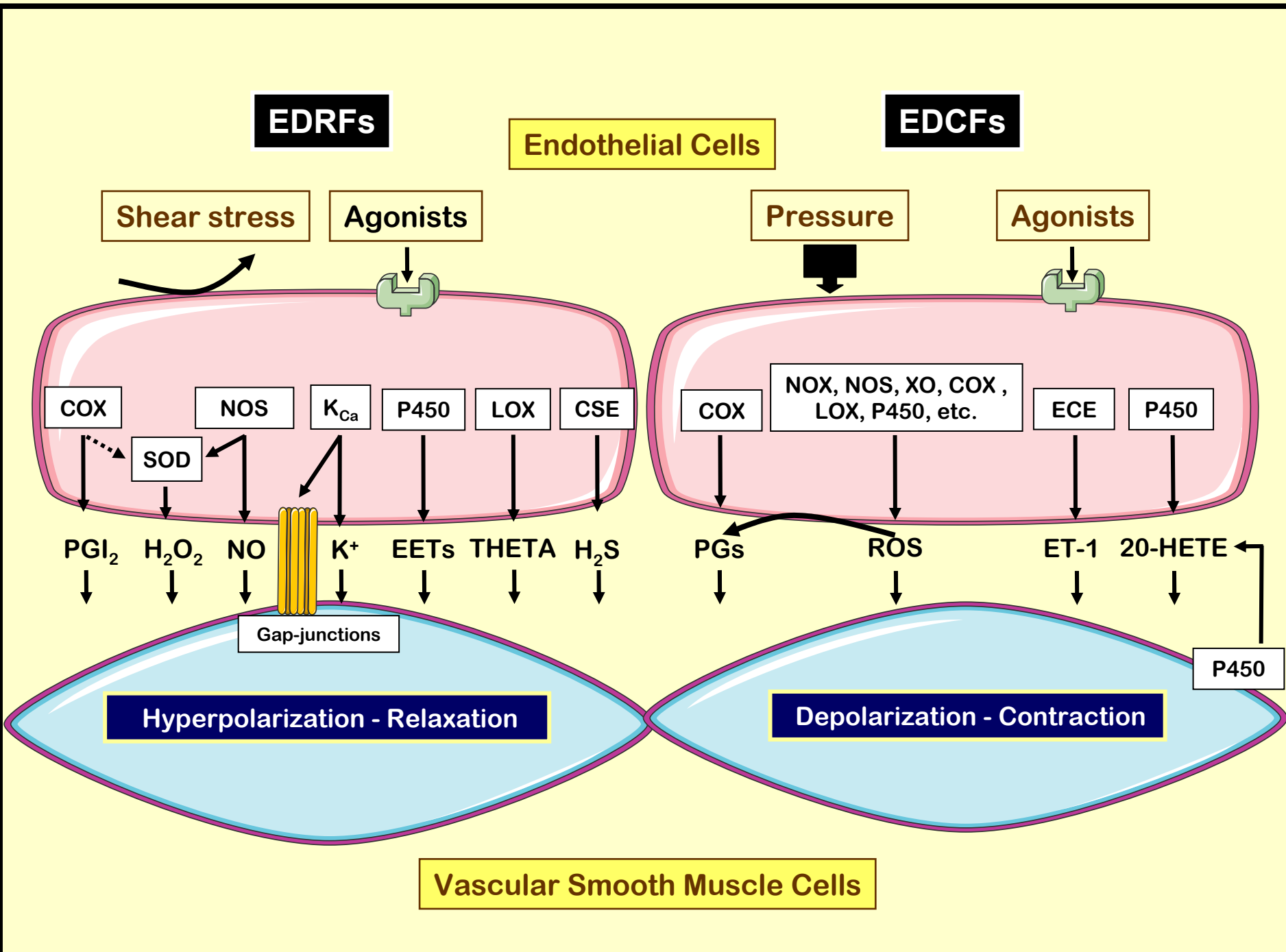
EC: endothelial cells; VSMC: vascular smooth muscle cells; ACh: acetylcholine, BK: bradykinin; SP: substance P; PE: phenylephrine; RyR: ryanodine receptor; CaV: voltage-activated calcium channels.

Figure 3: Myoendothelial projections



Stimulation of vascular smooth muscle cells by contractile agonists, for instance phenylephrine (PE) increases the intracellular Ca^{2+} concentration via the release of calcium from internal stores, through the production of inositol trisphosphate (IP_3), and via the entry of calcium, through voltage-activated calcium channels (CaV). In order to prevent excessive contractions, various negative feedback mechanisms leading to smooth muscle hyperpolarization can operate, i.e. the increase in Ca^{2+} can activate smooth muscle cells BK_{Ca} , Ca^{2+} and IP_3 can diffuse to the endothelial cells through myo-endothelial gap-junctions and activate IK_{Ca} (and possibly SK_{Ca}) either directly or via the generation of calcium pulsars, depletion of Ca^{2+} in the intercellular space at sites of myo-endothelial projections, via the activation of local CaV, can be sensed by the calcium sensing receptor (CaSR) and thus unable the activation of IK_1 . Therefore, intracellular Ca^{2+} concentrations (and IP_3) as well as intercellular concentrations of Ca^{2+} and K^+ , as surrogates of cellular activation, could finely regulate the membrane potential of endothelial and vascular smooth muscle cells and therefore vascular tone (schematic based on references 113 and 133).

EC: endothelial cells; VSMC: vascular smooth muscle cells; Cx: connexins; IK_{Ca} : calcium-activated potassium channels of intermediate conductance; K_{IR} : inwardly rectifying potassium channels; IP_3R : IP_3 receptor;



EDRFs

Endothelial Cells

EDCFs

Shear stress

Agonists

Pressure

Agonists

COX

NOS

K_{Ca}

P450

LOX

CSE

COX

NOX, NOS, XO, COX, LOX, P450, etc.

ECE

P450

SOD

PGI_2

H_2O_2

NO

K^+

EETs

THETA

H_2S

PGs

ROS

ET-1

20-HETE

Gap-junctions

P450

Hyperpolarization - Relaxation

Depolarization - Contraction

Vascular Smooth Muscle Cells

