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INVITED REVIEW

The endothelial saga: the past, the present, the future

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Abstract Endothelium-dependent changes in vasomotor tone, whether evoked by vasoactive agents or physical forces, are recognized as essential for the local hemodynamic control in various normal and pathological circumstances. They are based on a complex signaling network within the vascular wall. In recent years, substantial efforts have been made to analyze how such signals are generated and used in the endothelium-dependent control of vascular smooth muscle. The underlying mechanisms vary with species, age, sex, hormonal status, vascular bed studied, caliber of the blood vessels, triggering stimuli, pre-existing vascular tone, oxidative stress, and pathology. Such aspects and many others will be addressed specifically by the authors contributing to this volume.

Keywords Endothelium · Nitric oxide · EDHF · K_{Ca} · TRP · Oxidative stress

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The endothelial saga: the past

The endothelial saga started with Robert Furchgott [18, 61], who demonstrated that endothelial cells play an essential role in the relaxation evoked by acetylcholine in isolated arteries, which is mediated by activation of endothelial muscarinic receptors. His simple pharmacological experiments have revolutionized not only vascular pharmacology and physiology but science in general, as they lead to the discovery of the role of nitric oxide (NO) in biology [61]. Using "sandwich" bioassay preparations (a layering of arterial strips with and without endothelium whereby the contractile responses are measured only in the strip without endothelium), he demonstrated that the endothelium-dependence of the response to acetylcholine is due to the diffusion of a vasodilator substance from the endothelial cells to the vascular smooth muscle cells [18]. Having ruled out prostacyclin, which is produced by endothelial cells [41], he called the unknown mediator "endothelium-derived relaxing factor" (EDRF). The existence of endothelium-dependent responses was rapidly confirmed in different laboratories around the world [see 37]. More sophisticated superfusion-bioassay systems permitted to apply pharmacological inhibitors to either the endothelial cells or the effector vascular smooth muscle cells [e.g., 50]. The biological half-life of EDRF was found to be disappointingly brief (in the order of seconds), making identification by conventional chemical techniques impossible. Early pharmacological studies indicated that endothelial cells can generate several other signals leading to endothelium-dependent relaxations [8]. The latter multiple signals (Fig. 1) eventually became known as "endothelium-derived hyperpolarizing factor(s)" (EDHF), which play a prominent role in smaller arteries and resistance vessels [7, 15]. In addition, it soon became obvious that, in veins [9], and in

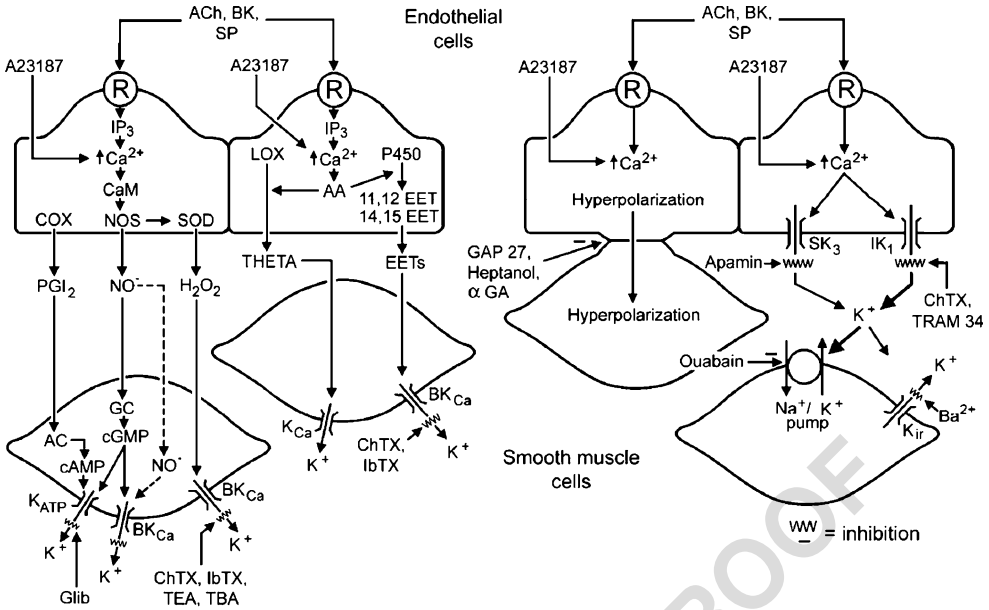

Fig. 1 EDRFs in 2009. Substances such as acetylcholine (*ACh*), bradykinin (*BK*), and substance P (*SP*), through the activation of M_3-muscarinic, B_2-bradykinin, and NK_1-neuroknin receptor subtypes, respectively, and agents that increase intracellular calcium, such as the calcium ionophore A23187, release EDHFs. *CaM* calmodulin, *COX* cyclooxygenase, *EET* epoxyeicosatrienoic acid, *IP_3* inositol trisphosphate, *GC* guanylate cyclase, *NAPE N*-acylphosphaticylethanolamine, *Hyperpol* hyperpolarization, *NOS* NO synthase, *O_2^-* superoxide anions, *PGI_2* prostacyclin, *P450* cytochrome P450 monooxygenase, *R* receptor, *X* putative EDHF synthase. SR141716 is an antagonist of the cannabinoid receptor subtype CB_1. Glibenclamide (*Glib*) is a selective inhibitor of ATP-sensitive potassium channels (*K_{ATP}*). Tetraethylammonium (*TEA*) and tetrabutylammonium (*TBA*) are nonspecific inhibitors of potassium channels when used at high concentrations (>5 mM), while at lower concentrations (1–3 mM), these drugs are selective for calcium-activated potassium channels (*K_{Ca}*). Iberiotoxin (*IBX*) is a specific inhibitor of large conductance K_{Ca} (*BK_{Ca}*). Charybdotoxin (*CTX*) is an inhibitor of BK_{Ca}, intermediate conductance K_{Ca} (*IK_{Ca}*), and voltage-dependent potassium channels. Apamin is a specific inhibitor of small conductance K_{Ca} (*SK_{Ca}*). Barium (*Ba^{2+}*), in the micromolar range, is a specific inhibitor of the inward rectifier potassium channel (*K_{ir}*). GAP 27 is an 11-amino acid peptide possessing conserved sequence homology to a portion of the second extracellular loop of connexin. 18α-glycyrrhetinic acid (*αGA*), and heptanol are gap junction uncouplers (from Vanhoutte et al., 2009. By permission)

arteries as well [36], the endothelium produces "endothelium-derived contracting factors" (EDCF), which add to the difficulty of analyzing endothelium-dependent responses [17, 37, 64]. More physiological stimuli than acetylcholine [including physical forces (increases in shear stress), circulating hormones (catecholamines, vasopressin,), platelet products (serotonin, adenosine diphosphate), autacoids (histamine, bradykinin), prostaglandin E_4, and thrombin] were shown to cause endothelium-dependent relaxations [37, 63]. Of those more physiological stimuli, increases in shear stress [51] explain the endothelium-dependency of flow-mediated vasodilatation, a response that allows the most accurate assessment of endothelial function in humans. Research in the field was fostered by the fact that endothelium-dependent relaxations are reduced under a number of pathological conditions, including myocardial infarction [32] and hypertension [31], which lead to the current conviction that endothelial dysfunction precedes, or at least accompanies, vascular disease and predicts the occurrence of cardiovascular events [39, 63].

It soon appeared that EDRF, whatever its nature, stimulated soluble guanylyl cyclase in vascular smooth muscle [28]. Soluble guanylyl cyclase catalyzes the formation of cyclic guanosine monophosphate (cyclic GMP), which in turn initiates relaxation. The demonstration followed that under superfusion-bioassay conditions superoxide anions scavenge EDRF [21, 52]. Based on the earlier observation that nitric oxide (NO) activates soluble guanylyl cyclase and is scavenged by superoxide anions [42] and on his own work with acidified nitrite, Robert Furchgott proposed in 1986 that his EDRF is NO [19]. Louis Ignarro had reached the same conclusion [27]. One year later, Salvador Moncada and his colleagues demonstrated, using a chemiluminescence technique, that, when cultured endothelial cells are stimulated with bradykinin, they indeed release NO [45]. The biology of NO was born. One crucial finding was that macrophages and endothelial cells transform the semi-essential amino acid L-arginine into NO and citrulline, and then inhibitors of the responsible enzymatic activity were discovered [24, 46, 48]. The access to inhibitors of nitric oxide synthase (NOS) permitted the exploration of the physiological role of NO in isolated tissues and organs, and in the intact organism. The fact that they augment arterial blood pressure in vivo

[49] implied a role for NO in cardiovascular homeostasis. The use of NOS inhibitors in vivo rapidly lead to the conclusion that NO not only is a key player in vasomotor control but it affects almost every bodily function. The next breakthrough came when Salomon Snyder and his group isolated NOS from the brain [e.g., 6]. We now know that there are three isoforms of the enzyme: neuronal NOS (nNOS, NOS 1), inducible NOS (iNOS, NOS 2), and endothelial NOS (eNOS, NOS 3). Paul Huang and colleagues genetically engineered mice with deletion of the eNOS gene [25]. These animals have an increased arterial blood pressure, illustrating the role of NO in the control of cardiovascular homeostasis.

The endothelial saga: the present

The advent of genetically modified animals and of inhibitors of NOS permits the systematic exploration of the role of NO in vascular health and disease, considerably increasing our knowledge (Fig. 2). In a given blood vessel, the level of activity of eNOS and the amounts of endothelium-derived NO released are not constant. They can be upregulated by chronic increases in shear stress (exercise), hormones (estrogens), and diet (ω_3-unsaturated fatty acids or polyphenols of red wine, green tea, and dark chocolate). The endothelial production of NO is reduced by high glucose (diabetes) and increased oxidative stress (hypertension) [see 3, 63]. NO not only affects the tone of vascular smooth muscle, but also inhibits platelet aggregation, in synergy with endothelium-derived prostacyclin [47], and the growth of the media [55]. It reduces the endothelial production of endothelin-1 [62] and of cyclooxygenase-derived EDCF [14]. NO inhibits the expression of endothelial adhesion molecules and, thus, the adhesion of platelets and white blood cells [47, 63]. It modulates angiogenesis [3, 68]. The signaling cascade, in particular, the role of Akt, in the phosphorylation that leads to activation of eNOS is unraveled [3, 16, 29, 33]. The original concept that the eNOS is a strictly Ca^{2+}-dependent enzyme, and, thus, that endothelium-dependent relaxations rely entirely on an increase in intracellular Ca^{2+}-concentration, has been challenged [3, 16]. Moreover, in vivo responses to acetylcholine in arterioles consist of two phases: (a) a rapidly conducted vasodilatation initiated by a local rise in endothelial Ca^{2+} but independent of endothelial Ca^{2+}-signaling at remote sites and (b) a slower complementary dilatation associated with a Ca^{2+}-wave that propagates along the endothelium [57]. In the mouse aorta, calcium-imaging shows that only some clusters of endothelial cells respond to acetylcholine, which represent only one third of the total number of cells, but this is enough for endothelium-dependent relaxation [4]. The importance of the caveolae for the activity of eNOS is now established [20, 40]. The formation of NO-metabolites constitutes a non-enzymatic source of activators of soluble guanylyl cyclase [38]. Beyond NO itself, derivatives such as nitroxyl (HNO) and nitrosothiols have also emerged as EDRFs and HNO may be as important as NO in rodent small arteries [2]. The binding of NO to superoxide anions, with the formation of peroxynitrite, is a major player in genesis of endothelial dysfunction [23, 30, 58, 67]. The progressive inability of endothelial cells, prematurely aged by the exposure to risk factors, to generate sufficient NO may well be the initial step permitting the inflammatory response that leads to atherosclerosis [see 63]. The most widely used therapeutic agents for the treatment of cardiovascular disease enhance the ability of the endothelial cells to produce NO [26, 59, 64].

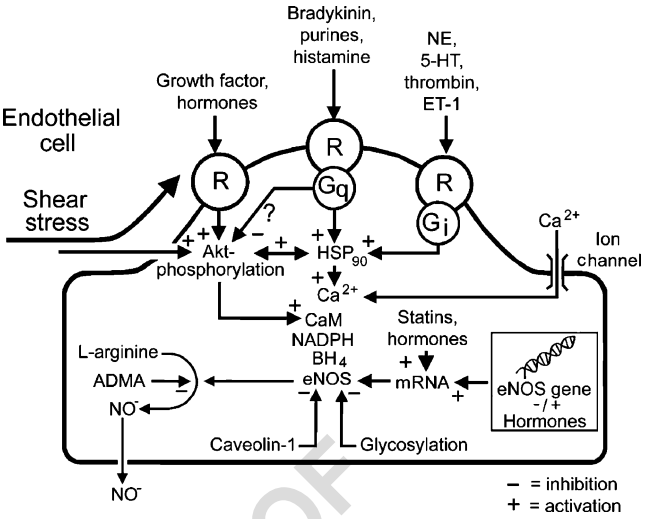

Fig. 2 Possible mechanisms by which production of nitric oxide is regulated in endothelial cells. Nitric oxide is produced through enzymatic conversion of L-arginine by NOS (endothelial or type III, *eNOS*). The transcription of this enzyme is regulated genomically by hormones and growth factors. Stability of eNOS mRNA is modulated by statins and hormones. eNOS enzyme activity requires calcium, calmodulin, nicotinamide adenine dinucleotide phosphate (*NADPH*), and 5, 6, 7, 8-tetra-hydrobiopterine (BH_4). Enzyme activity is regulated by complexing to these proteins in microdomains of the endothelial cell. Association with this complex of heat shock protein 90 (HSP_{90}) increases enzyme activity. Stimulation of specific receptors on the endothelial surface (*R*) complexed with guanine nucleotide regulatory proteins, which are sensitive to pertussis toxin (G_i) or insensitive to pertussis toxin (G_q), activate intracellular pathways that modulate eNOS activity posttranslationally through heat shock protein 90 or Akt-phosphorylation. Association of eNOS with caveolin-1 or glycosylation of the enzyme reduces activity. A metabolite of L-arginine, asymmetric dimethyl arginine (*ADMA*) decreases production of the nitric oxide through competitive binding to eNOS. Thus, this endogenous amine may be a risk factor for the development of cardiovascular disease. *Plus signs* indicate stimulation, *minus signs* indicate inhibition, *question marks* indicate those pathways in which the regulation is unknown (from Vanhoutte et al., 2009. By permission)

We now appreciate better the importance and the complexity of endothelium-dependent hyperpolarization in the local control of vascular tone [7]. Although EDHF has been considered to be of particular importance in smaller arteries, we have to recognize that its contribution to vasodilatation may be merely transient [22]. Nevertheless, coordinated increases in small artery diameter occur by means of flow-mediated vasodilatation (shear-stress-induced and NO-dependent) combined with the conducted vasodilatation resulting from electrotonic propagation of hyperpolarization in the endothelium [56]. At the level of endothelial protrusions, functional cooperation ensures the EDHF-component of endothelium-dependent vasodilatation, which is mediated by K^+ released from endothelium and involves endothelial $K_{Ca}2.3$ and $K_{Ca}3.1$, local interstitial Ca^{2+}, Ca^{2+}-sensing receptors co-localized with $K_{Ca}3.1$ in caveolin-poor regions of endothelial cells, myo-endothelial gap junctions, and the Na/K pump and $K_{ir}2.1$ of the vascular smooth muscle [11]. Experiments in dysgenic mice suggest that $K_{Ca}2.3$ and $K_{Ca}3.1$ have important but different contributions to endothelium-dependent vasodilatation and, thus, represent novel therapeutic targets for the treatment of hypertension [5, 66]. Inositol 1,4,5-endothelial trisphosphate receptors in the endothelial protrusions subserve local Ca^{2+}-release events ("pulsars"), which activate the functionally co-localized $K_{Ca}3.1$ [34]. Activators of the small and intermediate conductance K channels constitute useful pharmacological tools and potential new drugs for the treatment of hypertension [54].

The Ca^{2+}-dependent component of local vasodilation obviously depends on Ca^{2+} influx into endothelial cells. One of the most attractive candidate influx pathways has been the store-operated Ca^{2+} entry (SOC), which could be mediated by transient receptor potential (TRP) channels [see 43 for a critical review]. SOC was indeed identified in endothelium [1, 13], but a direct relation to NO production and release is still under evaluation [4]. Non-store-operated channels seem to play a more important role in regulation of NO release [65]. The involvement of TRPV4-channels in flow-induced endothelium-dependent vasodilatation is now generally accepted [35, see also 44 for a review]; the mechanism requires an active CYP epoxygenase and channel translocation to the cell membrane, where it is associated with caveolin-1. Moreover, the expression of caveolin-1 is required for EDHF-related relaxation, by modulating the membrane location and activity of TRPV4 channels and connexins, which are both implicated at different steps in the EDHF-signaling pathway [53]. The TRPV4 channels of both endothelial and vascular smooth muscle cells are critically involved in endothelium-dependent vasodilatation of mesenteric arteries and in TRPV4-knockout mice the hypertension induced by NOS inhibition is greater than in wild-type animals [12].

The endothelial saga: the future

Much remains to be learned about the precise regulation of NO release by endothelial cells and also about the consequences of its perturbation within the complex chain of events leading to the vascular dysfunction characteristic of hypertension, diabetes, and atherosclerosis [64]. We still do not completely understand the exact role of EDHF-mediated responses in physiology and pathology, as we are still unable to selectively interfere with them in vivo [7]. We still do not fully comprehend the importance of EDCFs in endothelial dysfunction [60]. Finally, we have to unravel the complex interactions between the different endothelium-derived signals. For example, in diabetic mice, hyperglycemia-induced changes in endothelial function are linked to COX2 and oxidative stress (enhanced NADPH oxidase and decreased SOD expression), uncoupling of eNOS, and changes in its expression and regulation, while EDHF-mediated vasodilatation can be maintained, but with a modified profile [10]. Whatever the future of endothelial research will yield, we should not forget that this extraordinary scientific saga started with the very simple pharmacological experiments of Robert Furchgott [18, 61], whose memory we honor in this special issue.

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