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Diverse Functions of Endothelial NO Synthases System: NO and EDH

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Abstract: Endothelium-dependent relaxations are predominantly regulated by nitric oxide (NO) in large conduit arteries and by endothelium-dependent hyperpolarization (EDH) in small resistance vessels. Although the nature of EDH factors varies depending on species and vascular beds, we have previously demonstrated that endothelial NO synthases (eNOS)-derived hydrogen peroxide (H_2O_2) is an EDH factor in animals and humans. This vessel size-dependent contribution of NO and EDH is, at least in part, attributable to the diverse roles of endothelial NOSs system; in large conduit arteries, eNOS mainly serves as a NO-generating system to elicit soluble guanylate cyclase–cyclic guanosine monophosphate-mediated relaxations, whereas in small resistance vessels, it serves as a superoxide-generating system to cause EDH/H_2O_2-mediated relaxations. Endothelial caveolin-1 may play an important role for the diverse roles of NOSs. Although reactive oxygen species are generally regarded harmful, the physiological roles of H_2O_2 have attracted much attention as accumulating evidence has shown that endothelium-derived H_2O_2 contributes to cardiovascular homeostasis. The diverse functions of endothelial NOSs system with NO and EDH/H_2O_2 could account for a compensatory mechanism in the setting of endothelial dysfunction. In this review, we will briefly summarize the current knowledge on the diverse functions of endothelial NOSs system: NO and EDH/H_2O_2.

Key Words: endothelium-derived relaxing factor, endothelium-dependent hyperpolarization, hydrogen peroxide, nitric oxide, nitric oxide synthase

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INTRODUCTION

The endothelium plays a crucial role in modulating vascular tone by synthesizing and releasing endothelium-derived relaxing factors, including vasodilator prostaglandins (PGs), nitric oxide (NO), and endothelium-dependent hyperpolarization (EDH) factors and also endothelium-derived contracting factors (Fig. 1).[1–4] In 1988, Feletou and Vanhoutte[5] and Chen et al[6] independently demonstrated that a diffusible substance released by the endothelium causes hyperpolarization and relaxation of underlying vascular smooth muscle cells (VSMC), attributing to the existence of putative EDH factors. Since then, a quarter century has passed and now several candidates have been proposed for the nature of EDH factors (Fig. 1).[7] It is widely accepted that the nature of EDH factors varies depending on species and vascular beds examined, including epoxyeicosatrienoic acids (EETs), metabolites of arachidonic P450 epoxygenase pathway,[8,9] electrical communication through gap junctions,[10] K^+ ions,[11] hydrogen sulfide (H_2S),[12] and as we have previously identified, hydrogen peroxide (H_2O_2) (Fig. 1).[13] EETs mainly take part in EDH-mediated relaxations in bovine, porcine, canine and human large coronary arteries, gap junctions in rodents, rabbit, and human mesenteric arteries, K^+ ions in rat hepatic and mesenteric arteries, porcine and canine coronary arteries and human arteries, H_2S in mouse mesenteric arteries, and H_2O_2 in various vascular beds as described below.[2] Intriguingly, the contribution of endothelium-derived relaxing factors (vasodilator PGs, NO, and EDH) to endothelium-dependent vasodilatation markedly varies depending on blood vessel size with the physiological balance between NO and EDH; vasodilator PGs play a small but constant role, and NO predominantly modulates the tone of large conduit vessels, and the contribution of NO decreases as the vessel size decreases, whereas that of EDH increases as the vessel size decreases, which phenomenon is well preserved from rodents to humans.[14–16] Thus, EDH rather than NO plays a dominant role in small resistance vessels where blood pressure and organ perfusion are finely regulated. Indeed, accumulating evidence has demonstrated the critical roles of EDH in modulating blood pressure[17] and vascular metabolic functions[18] in general, and coronary autoregulation[19] and coronary metabolic dilatation[20] in particular. We have previously demonstrated the diverse roles of the NO synthases (NOSs) system in the endothelium depending on blood vessel size; NOS mainly serves as a NO-generating system to elicit soluble guanylate cyclase (sGC)–cyclic guanosine monophosphate (cGMP)-mediated relaxations in large conduit vessels and a superoxide-generating system to cause EDH/H_2O_2-mediated responses in small resistance vessels (Fig. 2).[21] In the clinical settings, it has been reported that chronic nitrate therapy could exert harmful effects in patients with myocardial infarction,[22] and that antioxidant supplements are ineffective

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to prevent cardiovascular events.[23] These lines of evidence suggest the potential importance of the physiological balance between NO and EDH/H_2O_2 through the diverse functions of endothelial NOSs system.

In this review, we will briefly summarize the current knowledge on the diverse functions of endothelial NOSs system: NO and EDH/H_2O_2. As the former is extensively reviewed recently[3] and the space allowed for this article is limited, we will put more focus on the latter with particular reference to clinical implication.

Identification of EDH/H_2O_2

After the original reports on EDH factors in 1988,[5,6] 3 sets of early notions and observations suggesting the similarities between NO and EDH led us to hypothesize that a putative EDH factor could be a non-NO vasodilator substance [possibly reactive oxygen species (ROS)] derived from endothelial NOSs system. First, not only NO-mediated but also EDH-mediated responses are susceptible to vascular injuries caused by various atherosclerotic factors, such as aging, hypertension, diabetes mellitus, and dyslipidemia, with a resultant microvascular dysfunction, and conversely, the treatment of those risk factors restores both NO-mediated and EDH-mediated relaxations.[15,24] Second, calcium/calmodulin is required for the generation of both NO by eNOS and EDH-mediated responses.[25] Third, it is reasonable to consider that endothelial cells use a simple molecule (like NO) rather than complex substances in controlling and adjusting vascular tone in a moment-to-moment manner in response to various physiological demands. In 2000, using eNOS-knockout (eNOS-KO) mice, we identified that endothelium-derived H_2O_2 is an EDH factor in mouse mesenteric arteries[13]; EDH-mediated relaxation and hyperpolarization of underlying VSMC were inhibited by catalase, a specific H_2O_2 inhibitor, in small mesenteric arteries from wild-type mice and were significantly reduced in eNOS-KO mice.[13] This is also the case in human mesenteric[26] and coronary[27] arteries, porcine coronary arteries,[28] canine coronary arteries,[19,20] and piglet pial arterioles,[29] although EDH-independent vasodilating mechanisms by H_2O_2 have also been reported in other vascular beds.[30,31] Notably, the estimated concentration of endothelium-derived H_2O_2 as an EDH factor is in micromolar order,[28,32] which is a much lower concentration than that observed in various pathological conditions.[33,34] Among the possible sources and mechanisms involved in the generation of H_2O_2 in the endothelium,[34,35] Cu–Zn superoxide dismutase (SOD) plays a key role in the synthesis of EDH/H_2O_2; eNOS produces superoxide anions when synthesizing NO from L-arginine and oxygen under physiological conditions, whereas Cu–Zn-SOD dismutates those superoxide anions into H_2O_2, and Cu–Zn-SOD-KO mice show markedly impaired EDH-mediated relaxation and hyperpolarization in mesenteric arteries and coronary circulation without alterations in vasodilator properties of VSMC.[36] Based on the observations that the EDH/H_2O_2-mediated responses are resistant to NOS inhibitors and upregulation of eNOS cofactor tetrahydrobiopterin has no effects on the responses, superoxide anions relevant to EDH/H_2O_2 are not derived from pathologically uncoupled eNOS.[37] This is the case at least in normal mouse mesenteric arteries.[37] Other sources of superoxide anions have been proposed in H_2O_2-mediated vasodilatation; in human coronary arterioles, mitochondrial respiratory chain-derived and nicotinamide adenine dinucleotide phosphate oxidase-derived H_2O_2 is associated with flow-mediated dilation and bradykinin-induced relaxation, respectively.[38,39] To date, several mechanisms have been proposed for H_2O_2-induced vasodilatation.[40] Among them, Burgoyne et al[41] demonstrated a precise mechanism by which EDH/H_2O_2 relaxes underlying VSMC. Briefly, H_2O_2 induces an interprotein disulfide formation between two 1α isoforms of cGMP-dependent protein kinases ($PKG_{1\alpha}$) to enhance the kinase activity through their phosphorylation.

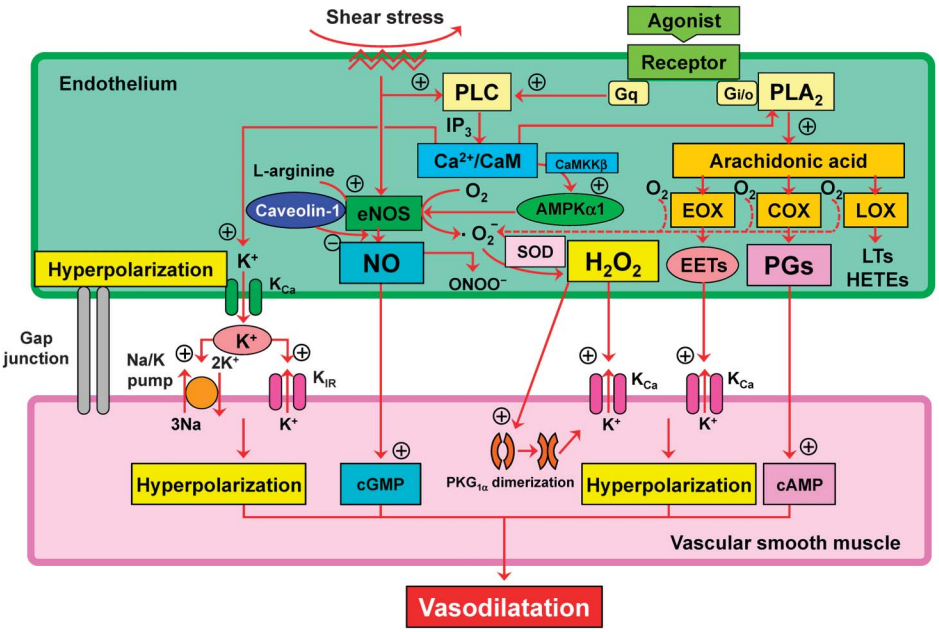

FIGURE 1. Mechanisms for synthesis and action of endothelium-derived relaxing factors in addition to vasodilator PGs and NO; several candidates could act as endothelium-dependent hyperpolarization (EDH) factor. PGs, NO, and EDH factor cause relaxations of underlying vascular smooth muscle through the mechanisms mediated by cyclic AMP (cAMP), cyclic GMP (cGMP), and hyperpolarization mediated by opening of Ca-activated K (K_{Ca}) channels. AMP-Kα1, α1-subunit of AMP-activated protein kinase; CaM, calmodulin; CaMKKβ, Ca^{2+}/CaM-dependent protein kinase β; COX, cyclooxygenase; EETs, epoxyeicosatrienoic acids; eNOS, endothelial NO synthase; EOX, epoxygenase; HETEs, hydroxyeicosatetraenoic acids; H_2O_2, hydrogen peroxide; IP_3, inositol trisphosphate; LOX, lipoxygenase; LTs, leukotrienes; $ONOO^-$, peroxynitrite; $PKG_{1\alpha}$, 1α-subunit of protein kinase G; PLA2, phospholipase A2; PLC, phospholipase C.

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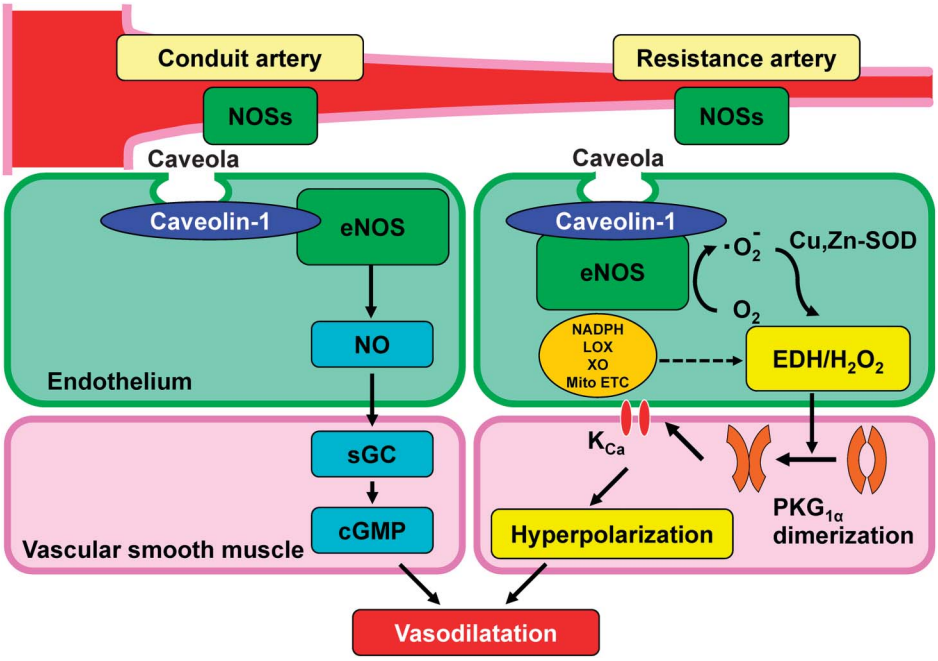

FIGURE 2. Diverse roles of endothelial nitric oxide synthases system in large conduit vessels, NO synthases (NOSs) mainly serve as a NO-generating system to cause vasodilatation through sGC–cGMP pathway, whereas in small resistance vessels, they act as a superoxide-generating system to evoke EDH-mediated responses through H_2O_2-induced $PKG_{1\alpha}$ dimerization and subsequent activation of potassium channels, leading to hyperpolarization and vasodilatation. K_{Ca}, calcium-activated potassium channel; LOX, lipoxygenase; Mito ETC, mitochondrial electron transport chain; NADPH, reduced nicotinamide adenine dinucleotide phosphate oxidase; $ONOO^-$, peroxynitrite; $PKG_{1\alpha}$, 1α-subunit of protein kinase G; XO, xanthine oxidase.

The activated $PKG_{1\alpha}$ subsequently stimulates potassium channels, leading to hyperpolarization and vasodilatation in mouse mesenteric arteries[17] and also in human coronary arterioles (Fig. 2).[42,43] This oxidant-mediated signaling is essential for blood pressure control because the "redox-dead" knock-in mice of Cys42Ser $PKG_{1\alpha}$, whose mutant $PKG_{1\alpha}$ is unable to be activated by H_2O_2-induced dimerization because of the absence of its redox-sensitive sulfur, show markedly impaired EDH-mediated relaxation in resistance arteries ex vivo and systemic hypertension in vivo.[17] Taken together, endothelium-derived H_2O_2 functions as an important endogenous second messenger to elicit EDH-meditated relaxations, modulate vascular tone, and maintain vascular homeostasis.

Diverse Roles of Endothelial NOS System

There are 3 NOS isoforms, including neural NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3).[3,44] Although 3 NOS isoforms are expressed in cardiovascular system, eNOS is the dominant NOS isoform in blood vessels.[45] NOSs have been known to generate superoxide anions from reductase domain under physiological conditions,[46] where superoxide anions are converted into H_2O_2 to cause EDH-mediated responses. Because heme reduction rate in eNOS is much slower than that in the other NOS isoforms, reductase domain-mediated superoxide generation is a significant alternative in eNOS.[46] Based on these observations, it is conceivable that eNOS is the most important isoform in generating H_2O_2/EDH in the endothelium. Indeed, as mentioned above, genetic deletion of eNOS gene in mice causes impaired EDH-mediated relaxations associated with systemic hypertension.[47] Although singly eNOS-KO mice exhibit abolished NO-mediated relaxations in the aorta and markedly reduced (but not abolished) EDH-mediated relaxations in the mesenteric artery, the remaining relaxations are still sensitive to catalase.[13] We speculated that the 3 NOSs compensate each other to maintain endothelium-dependent responses. To clarify the roles of endothelial NOSs in EDH/H_2O_2-mediated responses, we generated doubly n/eNOS-KO and triply n/i/eNOS-KO mice.[48] Interestingly, the EDH-mediated relaxations are progressively reduced in accordance with the number of NOS genes ablated; as compared with wild-type mice, the EDH/H_2O_2-mediated relaxations of small mesenteric arteries are reduced almost by half in singly eNOS-KO mice, further diminished in doubly n/eNOS-KO mice, and are finally completely abolished in the triply n/i/eNOS-KO mice without functional alterations of underlying VSMC.[21] In contrast, NO-mediated relaxations are totally absent in all 3 genotypes of NOS-KO mice.[21] These findings provide a novel concept on the diverse roles of endothelial NOSs system; in large conduit vessels, they mainly serve as a NO-generating system to cause vasodilatation through sGC–cGMP pathway, whereas in resistance vessels, they act as a superoxide-generating system to evoke EDH-mediated responses through H_2O_2-induced $PKG_{1\alpha}$ dimerization and subsequent activation of K_{Ca} channels, leading to hyperpolarization and vasodilatation (Fig. 2). Furthermore, at least in mice under physiological condition, the extent of eNOS bound to cavelion-1 (a negative regulator of eNOS) is greater in mesenteric arteries than in the aorta, and thus, eNOS is functionally suppressed in resistance vessels through a cavelin-1–dependent mechanism, switching its function from NO synthase to EDH/H_2O_2-generating enzyme (Fig. 2).[49]

Mechanistic Insight into Vessel Size-dependent Contribution of NO and EDH/H_2O_2

As mentioned above, NO and EDH/H_2O_2 share the diverse roles in modulating vascular tone in a distinct vessel size-dependent manner; NO plays a dominant role in conduit

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arteries and EDH/H_2O_2 in resistance vessels.[14] Mechanistic insight into vessel size-dependent contribution of NO and EDH/H_2O_2 has been recently demonstrated. As compared with large conduit vessels, eNOS is functionally inhibited by caveolin-1, and relaxation responses of VSMC to H_2O_2 are enhanced through $PKG_{1\alpha}$-mediated mechanism in resistance vessels in mice.[49,50] Indeed, mouse resistance vessels have less NO production and less antioxidant capacity, allowing $PKG_{1\alpha}$ to be more sensitive to H_2O_2-induced activation and subsequent hyperpolarization and relaxation of VSMC.[50] Furthermore, endothelial AMP-activated protein kinase (AMPK) modulates EDH-mediated responses in resistance arteries, but not in conduit arteries, to regulate blood pressure and coronary flow responses in mice in vivo.[51]

Interactions Between NO and EDH

It has been previously reported that NO donors attenuate EDH-mediated responses in porcine coronary arteries in vitro[52] and canine coronary microcirculation in vivo.[53] Furthermore, NO exerts a negative-feedback effect on endothelium-dependent relaxations through cGMP-mediated desensitization in canine coronary arteries ex vivo.[54] Multiple mechanisms may be involved in the negative interactions between NO and EDH. Among them, desensitization of VSMC to H_2O_2 is likely to be involved because H_2O_2-induced $PKG_{1\alpha}$ dimerization, a central mechanism of H_2O_2-induced vasodilatation, is inhibited by cGMP-dependent activation of PKG.[50] Moreover, pharmacological inhibition of sGC sensitizes conduit vessels to H_2O_2-induced vasodilatation in mice.[50] These observations support the notion that excessive endothelium-derived NO desensitizes blood vessels to EDH/H_2O_2-mediated relaxations. In addition, the actions of other EDH factors may also be inhibited through interaction with NO. For instance, Bauersachs et al[52] showed that exogenous NO donors attenuate EDH-mediated relaxations in vitro. A possible mechanism involved in this phenomenon is the inhibitory effect of NO on EET production through inhibition of cytochrome P450 epoxygenase activity.[52] More recently, Mustafa et al[55] have reported that NO exerts a direct inhibitory effect on cystathionine γ-lyase activity in vitro. Considering that cystathionine γ-lyase is a biosynthetic enzyme of H_2S that has been suggested to be one of EDH factors in mouse mesenteric arteries,[12,55] and it is conceivable that this mechanism is also involved in the negative feedback of NO on EDH-mediated relaxations. These results are consistent with the widely accepted notion that EDH functions as a compensatory vasodilator system when NO-mediated relaxations are hampered. Thus, EDH dominance in microcirculation is a vital mechanism to maintain sufficient tissue perfusion in the setting of pathological conditions where NO-mediated responses are jeopardized.[14]

Dual Roles of ROS

Endothelium-derived ROSs, including superoxide anions, NO, peroxynitrite, hydroxyl radicals, and H_2O_2, modulate vascular tone through multiple mechanisms in health and disease.[14,56,57] Although these ROSs have been regarded to be primarily harmful, the vasoprotective roles of H_2O_2 have attracted much attention as endothelium-derived H_2O_2 causes endothelium-dependent vasodilatation and contributes to microvascular homeostasis at its physiological concentrations.[26,32,33,56] As predicted previously in the commentary article[58] on our original EDH/H_2O_2 report,[13] H_2O_2 is a physiological signaling molecule serving as an EDH factor especially in microcirculations to modulate blood pressure,[17] metabolic coronary vasodilatation,[20] and metabolic functions.[18] These findings shed new light on the significance of maintaining EDH/H_2O_2-mediated relaxations in microcirculations and may provide a clue for better understanding of the harmful effects of antioxidant supplements.[23,59]

Endothelium-derived H_2O_2 is mainly generated by the dismutation of superoxide anions, which come from various sources in the endothelium, including nicotinamide adenine dinucleotide phosphate oxidase oxidase, mitochondrial electron transport chain, xanthine oxidase, lipoxygenase, and NOS.[33,60] Although the precise mechanisms underlying the physiological regulation of ROS production in the endothelium have not yet fully understood, recent studies have provided novel potential mechanisms relevant to modulation of endothelium-dependent responses. For instance, microRNAs, which are small noncoding RNAs regulating gene expressions through degradation or translational repression of mRNA, have emerged as important regulators in cardiovascular system.[61] Among the key players in EDH/H_2O_2-mediated responses, miR-103/107 have been shown to target caveolin-1 to downregulate its expression,[62] and miR155 is substantially involved in the negative regulation of NO production and endothelium-dependent vasodilatation by directly targeting eNOS.[63] Moreover, a class of transient receptor potential channels plays important role in regulating intracellular Ca^{2+} concentration and membrane potentials to control vascular tone and thereby blood flow through EDH-mediated mechanisms.[2] Notably, several transient receptor potential subfamilies serve as redox sensor to be controlled by endogenous ROS including H_2O_2 and NO.[64,65] Further studies are needed to understand how endothelium-derived ROS are finely regulated to participate in endothelium-dependent responses under physiological conditions.

Clinical Implications

It is difficult to accurately assess the in vivo importance of EDH in humans because the contribution of EDH is determined only after the blockade of both vasodilator PGs and NO.[1–3] However, evaluation of endothelial function in humans has attracted much attention in the clinical settings. Endothelial dysfunction is often noted in patients with atherosclerotic risk factors and cardiovascular diseases; antecedent exposure to various risk factors disables endothelial cells to produce sufficient NO, leading to the initial step toward inflammatory responses and atherosclerosis.[1] Although NO-mediated relaxations are easily impaired in the early stage of atherosclerotic conditions, EDH-mediated responses are fairly preserved or even temporarily enhanced to maintain vascular homeostasis.[2,40] This is well exemplified in the studies showing that enhanced EDH-mediated vasodilation compensates for reduced NO-mediated responses in hypercholesterolemic subjects.[15,66] In other clinical studies, endothelial dysfunction, as evaluated by impaired digital reactive hyperemia in peripheral arterial

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tonometry or flow-mediated dilation of the brachial artery, are associated with future cardiovascular events in patients with coronary artery diseases.[67,68] These observations suggest that endothelial functions in peripheral vascular beds could predict future cardiovascular events. In addition, H_2O_2 also has potent vasodilator properties in coronary resistance vessels, such that impaired EDH/H_2O_2-mediated vasodilatation could lead to coronary microvascular dysfunction.[69] Coronary vascular resistance is mostly determined by the prearterioles (approximately 100–500 μm in diameter) and arterioles (less than 100 μm in diameter) where the contribution of EDH-mediated responses is greater than NO-mediated ones.[69] Thus, it is important to maintain the vessel size-dependent contribution of NO and EDH for the treatment of cardiovascular disease. Indeed, a number of clinical studies have failed to show that chronic nitrate therapy, as a NO donor, has beneficial prognostic effects for patients with ischemic heart disease.[22,70] Similarly, long-term antioxidant therapy for patients with hypertension failed to lower systemic blood pressure or improve mortality rate.[23,59] These results in the clinical studies indicate the importance of the physiological balance between NO and EDH/H_2O_2 in humans. Notably, standard therapeutic agents used for the treatment of cardiovascular diseases in the current era share the pleiotropic effects on endothelial function by enhancing NO-mediated vasodilatations, including angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, and statins.[44] Further studies are warranted for improving EDH-mediated responses in microcirculations to develop a novel therapeutic strategy beyond NO.

CONCLUSIONS

Experimental and clinical studies in our and other laboratories have demonstrated that endothelial NOSs have diverse functions to maintain cardiovascular homeostasis, where the physiological balance between NO and EDH is important. The new research avenue of EDH that was opened a quarter century ago by Vanhoutte et al has greatly contributed to our better understanding of endothelial functions and to develop novel diagnostic and therapeutic strategies in cardiovascular medicine.

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