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Divergent roles of endothelial nitric oxide synthases system in maintaining cardiovascular homeostasis

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ABSTRACT

Accumulating evidence has demonstrated the importance of reactive oxygen species (ROS) as an essential second messenger in health and disease. Endothelial dysfunction is the hallmark of atherosclerotic cardiovascular diseases, in which pathological levels of ROS are substantially involved. The endothelium plays a crucial role in modulating tone of underlying vascular smooth muscle by synthesizing and releasing nitric oxide (NO) and endothelium-dependent hyperpolarization (EDH) factors in a distinct vessel size-dependent manner through the diverse roles of the endothelial NO synthases (NOSs) system. Endothelium-derived hydrogen peroxide (H_2O_2) is a physiological signaling molecule serving as one of the major EDH factors especially in microcirculations and has gained increasing attention in view of its emerging relevance for cardiovascular homeostasis. In the clinical settings, it has been reported that antioxidant supplements are unexpectedly ineffective to prevent cardiovascular events. These lines of evidence indicate the potential importance of the physiological balance between NO and H_2O_2/EDH through the diverse functions of endothelial NOSs system in maintaining cardiovascular homeostasis. A better understanding of cardiovascular redox signaling is certainly needed to develop novel therapeutic strategies in cardiovascular medicine. In this review, we will briefly summarize the current knowledge on the emerging regulatory roles of redox signaling pathways in cardiovascular homeostasis, with particular focus on the two endothelial NOSs-derived mediators, NO and H_2O_2/EDH.

1. Introduction

Reactive oxygen species (ROS) have been considered primarily harmful because of their highly-damaging entity to cells and tissues and pathological implications in various diseased states in humans [1,2]. The detrimental roles of ROS have been well-documented in a wide range of cardiovascular diseases in general, including atherosclerosis, hypertension, heart failure, cardiomyopathy, and coronary artery disease in particular, where endothelial dysfunction is also substantially involved in the pathophysiology [3]. However, accumulating evidence has provided firm foundations for a paradigm shift on the roles of ROS from pathological detriments to crucial physiological signaling molecules [4,5]. Thus, ROS have been re-evaluated as a physiological second messenger in light of recent advances in the better comprehension of their diverse regulatory roles in health and disease [6,7].

Endothelial dysfunction is the hallmark and potential predictor for atherosclerotic cardiovascular diseases and is also noted in patients with metabolic disorders, where prior exposure to various risk factors, such as diabetes mellitus, hypertension, and hypercholesterolemia, causes endothelial dysfunction, leading to the initial step toward atherosclerotic cardiovascular diseases [8]. A typical feature of endothelial dysfunction is reduced production of endothelium-derived relaxing factors, including vasodilator prostaglandins (PGs), nitric oxide (NO), and endothelium-dependent hyperpolarization (EDH) factors (Fig. 1). Although the nature of EDH factors varies depending on species and vascular beds examined, endothelium-derived hydrogen peroxide (H_2O_2) is one of the major EDH factors in various vascular beds in animals and humans and has gained increasing attention in view of its emerging relevance for cardiovascular homeostasis [9]. Importantly, the endothelium synthesizes and releases NO and H_2O_2/EDH to regulate vascular tone in a distinct vessel size-dependent manner through the diverse roles of the NO synthases (NOSs) system; 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-gener-

Abbreviations: AMPK, AMP-activated protein kinase; cGMP, cyclic guanosine monophosphate; EDH, endothelium-dependent hyperpolarization; H_2O_2, hydrogen peroxide; NO, nitric oxide; NOS, nitric oxide synthase; PGs, prostaglandins; PKG, cGMP-dependent protein kinase; ROS, reactive oxygen species; SERCA, sarco/endoplasmic reticulum calcium ATPase; sGC, soluble guanylate cyclase; SOD, superoxide dismutase

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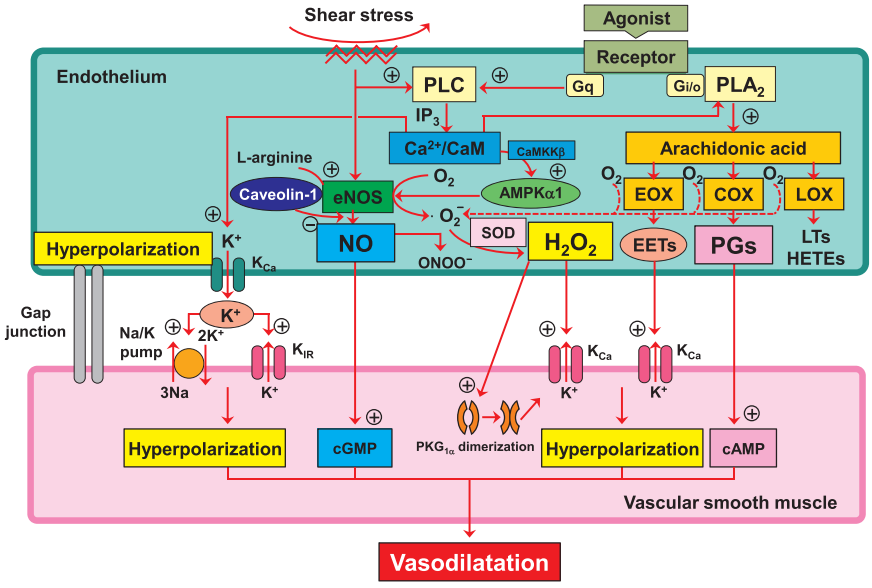

Fig. 1. Mechanisms for synthesis and action of endothelium-derived relaxing factors. In addition to vasodilator prostaglandins (PGs) and nitric oxide (NO), several candidates could act as endothelium-dependent hyperpolarizing (EDH) factor. PGs, NO, and EDH factor cause relaxations of underlying vascular smooth muscle through the mechanisms mediated by cyclic AMP (cAMP), cyclic GMP (cGMP) and hyperpolarization mediated by opening of Ca-activated K (KCa) channels, respectively. Other abbreviations: AMPKα1, 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; PLA_2, phospholipase A_2; PLC, phospholipase C; SOD, superoxide dismutase.

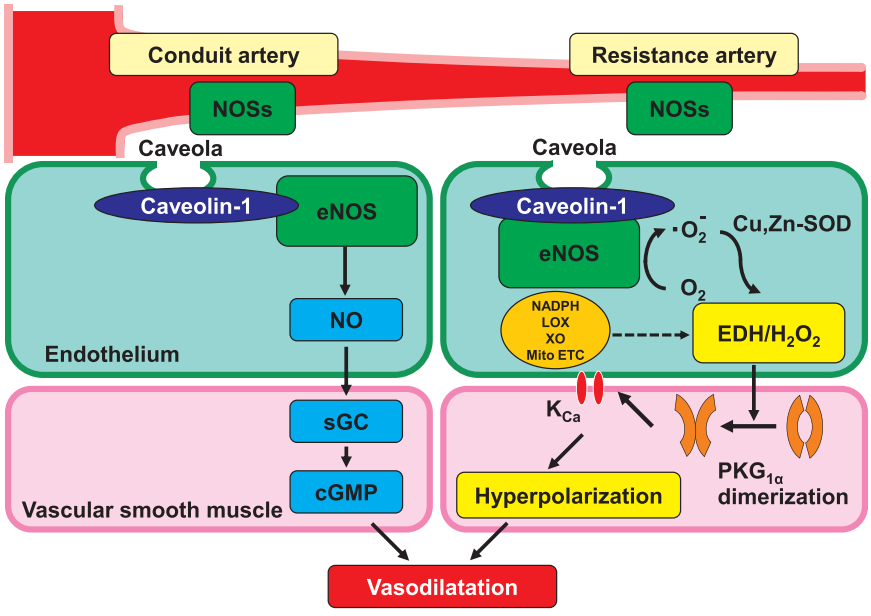

Fig. 2. Diverse roles of endothelial nitric oxide synthases system. In large conduit vessels, NO synthases (NOSs) mainly serve as a NO-generating system to cause vasodilatation through soluble guanylate cyclase (sGC)-cGMP pathway, while in small resistance vessels, they act as a superoxide-generating 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. Other abbreviations: Cu, Zn-SOD, zinc-superoxide dismutase; KCa, 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.

ating system to cause H_2O_2/EDH -mediated responses in small resistance vessels [10] (Fig. 2). In the clinical settings, it has been reported that chronic nitrate therapy could exert harmful effects in patients with ischemia heart disease [11,12] and that antioxidant supplements are unexpectedly ineffective to prevent cardiovascular events [13]. These lines of evidence suggest the potential importance of the physiological balance between NO and H_2O_2/EDH through the diverse functions of endothelial NOSs system in maintaining cardiovascular homeostasis.

Obviously, a growing number of recent publications and review articles in this field reflect that our scientific community craves for much better understanding of this complex but promising redox signaling systems and its clinical application for curing diseases associated with oxidative stress [5,14–18]. In this review, we will briefly summarize the current knowledge on the emerging regulatory roles of redox signaling pathways in cardiovascular homeostasis, with particular focus on the two endothelial NOSs-derived mediators, NO and H_2O_2/EDH.

2. Endothelium-derived H_2O_2 as an EDH factor

The endothelium plays a crucial role in modulating vascular tone by synthesizing and releasing endothelium-derived relaxing factors, including vasodilator PGs (e.g. prostacyclin), NO and EDH factors as well as endothelium-derived contracting factors [19]. Among them, EDH factors-mediated responses are the predominant mechanisms of endothelium-dependent vasodilatation of resistance arteries [9,19]. EDH factors cause hyperpolarization and subsequent relaxation of underlying vascular smooth muscle with resultant vasodilatation of small resistance vessels to finely regulate blood pressure and organ perfusion. More precise and comprehensive information regarding EDH-mediated responses is available in the extensive review published recently [8]. We will focus on endothelium-derived H_2O_2 as an EDH factor in detail.

Early observations showing a vasoactive role of endothelium-derived free radicals and H_2O_2 in isolated canine coronary arteries imply that these endothelium-derived ROS can be vasoactive mediators to take part in endothelium-dependent relaxation [20]. After the initial reports on EDH factors in 1988 [21,22], three 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 ROS) derived from endothelial NOSs system. First, not only NO- but also EDH-mediated responses are susceptible to vascular injuries caused by various atherosclerotic factors, such as aging, smoking, hypertension, diabetes mellitus, and dyslipidemia, with a resultant microvascular dysfunction, and conversely, the treatment of those risk factors restores both NO- and EDH-mediated relaxations [23,24]. Second, both eNOS-derived NO and EDH-mediated responses are generated in a calcium/calmodulin-dependent manner [25]. Third, it is a judicious notion that endothelial cells adopt a simple molecule (like NO) rather than complex substances in controlling and adjusting vascular tone in a moment to moment manner in response to diverse physiological demands. In 2000, using eNOS-knockout (eNOS-KO) mice, we were able to demonstrate for the first time that endothelium-derived H_2O_2 is an EDH factor in mouse mesenteric arteries; EDH-mediated relaxation and hyperpolarization of underlying vascular smooth muscle 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 [26]. This is also true for other vascular beds, such as human mesenteric [27] and coronary [28] arteries, porcine coronary arteries [29], canine coronary arteries [30–32] and piglet pial arterioles [33], although EDH-independent vasodilating mechanisms by H_2O_2 have also been reported in other vascular beds [34,35]. Notably, the estimated concentration of endothelium-derived H_2O_2 as an EDH factor is in micro molar order [29,31], which is much lower concentration than that observed in various pathological states [36,37].

Among the possible sources and mechanisms involved in the generation of H_2O_2 in the endothelium [2,36], Cu, Zn-superoxide dismutase (SOD) plays a key role in the synthesis of H_2O_2/EDH; eNOS produces superoxide anions when synthesizing NO from L-arginine and oxygen under physiological conditions, while Cu, Zn-SOD dismutates those superoxide anions into H_2O_2. Indeed, 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 vascular smooth muscle [38]. Cu, Zn-SOD-derived H_2O_2 signaling also plays important roles in metabolic regulation [39]. Based on the observations that the H_2O_2/EDH-mediated responses are resistant to NOS inhibitors and upregulation of eNOS co-factor tetrahydrobiopterin has no effects on the responses, superoxide anions relevant to H_2O_2/EDH are not derived from pathologically uncoupled eNOS [40]. This is the case at least in normal mouse mesenteric arteries [40]. Other sources of superoxide anions have been proposed in H_2O_2-mediated vasodilatation; in human coronary arterioles, mitochondrial respiratory chain- and NADPH-derived H_2O_2 is associated with flow-mediated dilation and bradykinin-induced relaxation, respectively [41,42].

To date, several mechanisms have been proposed for H_2O_2-induced vasodilatation [15,43]. Most notably, Burgoyne et al. demonstrated a precise mechanism by which H_2O_2/EDH relaxes underlying vascular smooth muscle [44]. Briefly, H_2O_2 induces an interprotein disulfide bond formation between two 1α-isoforms of cGMP-dependent protein kinases ($PKG_{1\alpha}$) to enhance the kinase activity through their phosphorylation. The activated $PKG_{1\alpha}$ subsequently stimulates potassium channels, leading to hyperpolarization and vasodilatation in mouse mesenteric arteries [45] as well as in human coronary arterioles [46,47] (Fig. 2). H_2O_2 also promotes the translocation of $PKG_{1\alpha}$ from cytoplasm to membrane in porcine coronary arteries [48]. Such reversible post translational modulation [49] like phosphorylation gains much advantage in the fine control of vascular tone in vivo [50]. The 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 due to the absence of its redox-sensitive sulfur, show markedly impaired EDH-mediated relaxation in resistance arteries ex vivo and systemic hypertension in vivo [45]. In addition, H_2O_2/EDH also plays important roles with potent vasodilator properties in coronary resistance vessels. Since coronary vascular resistance is predominantly determined by the prearterioles (from approx. 500–100 μm in diameter) and arterioles (less than 100 μm in diameter) where the effect of EDH-mediated responses on vascular tone takes over that of NO-mediated relaxations [51], maintaining the vessel size-dependent contribution of NO and EDH is essential for the treatment of coronary artery disease. Furthermore, the emerging roles of H_2O_2/EDH in coronary circulation include coronary autoregulation [30], cardioprotection during coronary ischemia reperfusion injury [31], and tachycardia-induced metabolic coronary vasodilator responses [32] in dogs in vivo. 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.

3. Molecular mechanisms for the diverse functions of endothelial NO synthases system

Next, we will discuss the diverse roles of endothelial NOSs system. There are three NOS isoforms, including neural NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial NOS (eNOS, NOS3) [52,53]. Although three NOS isoforms are expressed in cardiovascular system, eNOS is the dominant NOS isoform in blood vessels [54]. NOSs have been known to generate superoxide anions from reductase domain under physiological conditions [55], where superoxide anions are converted to H_2O_2 to cause EDH-mediated responses (Fig. 1). Because heme reduction rate in eNOS is much slower than that in other NOS isoforms, reductase domain-mediated superoxide generation is a significant alternative in eNOS [55]. 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 described above, genetic deletion of eNOS gene in mice causes impaired EDH-mediated relaxations associated with systemic hypertension [56]. 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 arteries, the remaining relaxations are still sensitive to catalase [26]. We speculated that the three NOSs compensate each other to maintain endothelium-dependent relaxations. Using doubly-n/eNOS-KO and triply-n/i/eNOS-KO mice [57], we have previously demonstrated that the EDH-mediated relaxations are progressively reduced in accordance with the number of NOS genes ablated; as compared with wild-type mice, the H_2O_2/EDH-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 vascular smooth muscle [58]. In contrast, NO-mediated relaxations are totally absent in all three

genotypes of NOS-KO mice [58]. 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 potassium channels, leading to hyperpolarization and vasodilatation (Fig. 2).

Mechanistic insight into vessel size-dependent contribution of NO and H_2O_2/EDH has been recently emerging. First, 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 H_2O_2/EDH- generating enzyme [59] (Fig. 2). Second, relaxation responses of vascular smooth muscle to H_2O_2 are enhanced through a $PKG_{1\alpha}$-mediated mechanism in resistance vessels in mice [59,60]. 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 vascular smooth muscle [60]. Third, 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 [61]. Fourth, it has been previously reported that NO donors attenuate EDH-mediated responses in porcine coronary arteries in vitro [62] and canine coronary microcirculation in vivo [63]. Furthermore, NO exerts a negative-feedback effect on endothelium-dependent relaxations through cGMP-mediated desensitization in canine coronary arteries ex vivo [64]. Multiple mechanisms may be involved in the negative interactions between NO and H_2O_2/EDH. For instance, desensitization of vascular smooth muscle 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 [60], and in turn, pharmacological inhibition of sGC sensitizes conduit vessels to H_2O_2-induced vasodilatation in mice [60]. These observations support the notion that excessive endothelium-derived NO desensitizes blood vessels to H_2O_2/EDH-mediated relaxations. In addition, the actions of other EDH factors may also be inhibited through interaction with NO. Mustafa et al. have reported that NO exerts a direct inhibitory effect on cystathionine γ-lyase activity in vitro [65]. Considering that cystathionine γ-lyase is a biosynthetic enzyme of hydrogen sulfide, which is another oxidant species serving as one of EDH factors in mouse mesenteric arteries [65,66], it is conceivable that this mechanism is also involved in the negative feedback of NO on EDH-mediated relaxations. Collectively, these results are consistent with the widely accepted view 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 compromised [19].

The activity of eNOS is modulated through an array of post-translational modifications (e.g. phosphorylation, thiopalmitoylation, S-nitrosylation, acetylation, glycosylation, and S-glutathionylation) [50]. Importantly, some of them are susceptible to and dysregulated by increased levels of ROS in ageing, hypertension, diabetes mellitus, and heart failure [50]. For example, oxidative stress not only causes oxidative conversion of an essential eNOS cofactor tetrahydrobiopterin to dysfunctional dihydrobiopterin but also induces S-glutathionylation of eNOS in a reversible manner, converting its function from NO synthase to superoxide-generating enzyme [67,68]. These oxidative modulations lead to eNOS uncoupling, where endothelium-derived NO-mediated responses are impaired with reseuultant development of cardiovascular diseases. These lines of evidence suggest a possible importance of physiological redox balance in vivo and a potential therapeutic application of thiol-reducing agents like hydrogen sulfide for the treatment of cardiovascular diseases associated with oxidative stress. Further comprehensive discussions on the regulatory mechanisms of eNOS functions are available in recently published extensive reviews [50,52,53,67].

4. Dual roles of reactive oxygen species

A disequilibrium between prooxidants and antioxidants in favor of oxidants is referred to as oxidative stress and has been recognized as a distinct clinical entity from a physiological condition. This recognition is due to the damaging properties of ROS in numerous experimental models and its pathological implications in a variety of cardiovascular diseases, cancers and aging [1,2]. Nevertheless, accumulating evidence has unveiled the diverse regulatory roles of ROS in vivo [6]. As predicted previously [69] following our original H_2O_2/EDH report [26], H_2O_2 is a physiological signaling molecule serving as an EDH factor especially in microcirculation to modulate blood pressure [45], coronary circulation [30–32] and metabolic functions [70]. We will focus on the physiological roles of H_2O_2, one of the most plentiful and steady form of ROS in our body, in the regulation of vascular homeostasis.

Endothelium-derived ROS, including superoxide anions, NO, peroxynitrite, hydroxyl radicals and H_2O_2, modulate vascular tone through multiple mechanisms in health and disease [19,71,72]. These ROSs have been regarded to be primarily harmful in vascular biology. For example, H_2O_2 at high concentrations causes endothelium-dependent vasoconstriction through multiple mechanisms including release of cyclooxygenase-derived thromboxane [73,74], and overproduction of superoxide anions reacts with and scavenges NO to form peroxynitrite, a potent oxidant with toxic entity to cells and tissues [75]. In striking contrast, 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 low concentrations [27,31,37,72]. The estimated concentration of endothelium-derived H_2O_2 as an EDH factor is in micro molar order [29,31], which is much lower concentration than that observed in various pathological states [36,37]. When applied exogenously in organ chamber experiments, 10–100 μmol/L of H_2O_2 causes vasodilatation of human coronary arterioles [46,47] and in mouse small mesenteric arteries [26,59], while higher concentrations of H_2O_2 elicit vasoconstriction. Note that only 10–15% of H_2O_2 applied exogenously reaches the intracellular targets due to endogenous antioxidants and membrane impedance [76]. Similarly, peroxynitrite shows a dual role working as an endogenous mediator in a distinct concentration-dependent manner [77]. Adachi et al. have demonstrated that peroxynitrite at low concentrations (10–50 μmol/L) increases the activity of sarco/endoplasmic reticulum calcium ATPase (SERCA) through S-glutathiolation of the reactive thiol on Cys674 and contributes to NO-mediated vasodilatation, while peroxynitrite at higher concentrations (> 100 μmol/L) inhibits the SERCA activity [77]. Furthermore, eNOS-transgenic/apoE-knockout mice fed with high-cholesterol diet exhibited more advanced atherosclerotic lesion formation compared with apoE-knockout mice [78], suggesting a potential downside of excessive NO. More recently, we also have demonstrated that excessive endothelial NO production by either caveolin-1-deficiency or eNOS overexpression disrupts the physiological balance between NO and EDH in microcirculation, resulting in impaired cardiovascular homeostasis in mice [79]. Taken together, these apparent double-edge sword effects of ROS may provide a clue to the development of novel therapeutic strategies for cardiovascular diseases associated with oxidative stress.

The sources and regulatory mechanisms of physiologically relevant H_2O_2 is in debate [6]. It is conceivable that local subcellular concentrations at microdomains rather than net cellular concentrations may be critical to determine whether the effects of ROS can be detrimental or beneficial to cellular processes and that co-localization of the source and target of H_2O_2 may help prevent non-specific injurious oxidations

[80,81]. For instance, caveolar localization of NOX1 in hypertension causes just a minor increase in ROS but is enough to interrupt NO-mediated responses [82]. Based on the idea that specific cystein residues can function as redox-dependent switches, an interesting mechanism by which ROS-mediated signalings can be regulated have been proposed from the Eaton laboratory [36,37,83]. Cells and tissues are equipped with several free radical scavengers, including SOD, catalase, glutathione peroxidase and peroxiredoxins. Peroxiredoxins are one of the most abundant proteins in some cells and, like other redox-reactive proteins, are characterized by their cysteine residue enclosing a thiol in basal ionized state, which is prone to oxidative post-translational modifications. They contribute to decompose physiological concentrations of H_2O_2, however, when exposed to as high as 100–200 μmol/L of H_2O_2, hyperoxidation of their catalytic cysteine to sulfinic acid occurs to preclude the decomposition of H_2O_2, leading to an initial step toward cardiovascular dysfunctions [36,37,83]. Regarding the sources, endothelium-derived H_2O_2 is mainly generated by the dismutation of superoxide anions, which come from various sources in the endothelium, including NADPH oxidase, mitochondrial electron transport chain, xanthine oxidase, lipoxygenase and NOS [37,84]. 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 non-coding RNAs regulating gene expressions through degradation or translational repression of mRNA, have emerged as important regulators in cardiovascular system [85]. Among the key players in H_2O_2/EDH-mediated responses, miR-103/107 have been shown to target caveolin-1 to downregulate its expression [86] and miR155 is substantially involved in the negative regulation of NO production and endothelium-dependent vasodilatation by directly targeting eNOS [87]. Moreover, a class of transient receptor potential (TRP) 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 [9]. Notably, several TRP subfamilies serve as redox sensor to be controlled by endogenous ROS including H_2O_2 and NO [88,89]. Further studies are certainly needed to understand how endothelium-derived ROS are finely regulated to participate in endothelium-dependent responses under physiological conditions.

5. Clinical implications

It is difficult to accurately assess the in vivo importance of H_2O_2/EDH in humans because the contribution of EDH is determined only after the blockade of both vasodilator PGs and NO [9]. 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 amount of NO, leading to the first step toward inflammatory responses and atherosclerosis [8]. 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 [9,43]. This is well exemplified in the studies showing that enhanced EDH-mediated vasodilation compensates for reduced NO-mediated responses in hypercholesteremic subjects [23,90]. After a prolonged exposure to atherosclerotic risk factors, this compensatory roles of EDH-mediated responses are eventually disrupted to cause metabolic disturbance [91]. In other clinical studies, endothelial dysfunction, as evaluated by impaired digital reactive hyperemia in peripheral arterial tonometry or flow-mediated dilation of the brachial artery, are associated with future cardiovascular events in patients with coronary artery diseases [92,93]. These observations suggest that endothelial functions in peripheral vascular beds could predict future cardiovascular events.

On the basis of the premise that reducing oxidative stress could exert beneficial effects on various diseased states associated with elevated levels of ROS in general and cardiovascular diseases in particular, numerous clinical trials of antioxidants have been conducted [13,94,95]. Contrary to thousands of in vitro and animal studies and acute beneficial effects on endothelial functions in humans [16], the results of systemic and long-term administrations of antioxidants have been disappointing in many clinical trials. Indeed, long-term antioxidant therapy for patients with hypertension failed to lower systemic blood pressure or to improve mortality rate [13,96]. A supplementation of tetrahydrobiopterin, an essential co-factor for NOSs to produce NO, was neutral [97,98], and an iNOS inhibitor increased the mortality rate in patients with septic shock [99]. Although the reason for such 'antioxidant paradox' in cardiovascular diseases remains largely unknown, these results in the clinical studies indicate potential harm of non-specific elimination of ROS as well as the importance of the physiological balance of endogenous antioxidant systems in humans. Available evidence suggests that vitamin C seems at least better than other antioxidants; favorable effects on endothelial function in both acute and chronic phase [16], beneficial effects in subjects with low concentrations of vitamin C at baseline [100], and relatively poor reactivity with H_2O_2 [14]. In addition, antioxidant paradox can be explained, at least in part, by reductive stress [101].

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 with modest antioxidant capacities as well, including angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, and statins [53].

6. Conclusions

In 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 H_2O_2/EDH is important. Further characterization and better understanding of cardiovascular redox signaling is required to develop novel therapeutic strategies in cardiovascular medicine.

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