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CARDIOVASCULAR PHYSIOLOGY

Hydrogen peroxide as an endothelium-derived hyperpolarizing factor

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Abstract The endothelium plays an important role in maintaining cardiovascular homeostasis by synthesizing and releasing several vasodilating substances, including vasodilator prostaglandins, nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF). Since the first report on the existence of EDHF, several substances/mechanisms have been proposed for the nature of EDHF, including epoxyeicosatrienoic acids (metabolites of arachidonic P450 epoxygenase pathway), K ions, and electrical communications through myoendothelial gap junctions. We have demonstrated that endothelium-derived hydrogen peroxide (H_2O_2) is an EDHF in animals and humans. For the synthesis of H_2O_2/EDHF, endothelial NO synthase system that is functionally coupled with Cu,Zn-superoxide dismutase plays a crucial role. Importantly, endothelium-derived H_2O_2 plays important protective roles in the coronary circulation, including coronary autoregulation, protection against myocardial ischemia/reperfusion injury, and metabolic coronary vasodilatation. Indeed, our H_2O_2/EDHF theory demonstrates that endothelium-derived H_2O_2, another reactive oxygen species in addition to NO, plays important roles as a redox-signaling molecule to cause vasodilatation as well as cardioprotection. In this review, we summarize our current knowledge on H_2O_2/EDHF regarding its identification and mechanisms of synthesis and actions.

H_2O_2 as an EDHF.

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Introduction

The endothelium synthesizes and releases several vasodilator substances, including vasodilator prostaglandins, nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF) [20, 50, 60]. Although the nature of EDHF has not been fully elucidated, different EDHFs could exist depending on species, blood vessels, and the size of blood vessels tested [50, 60]. Since the first report on the existence of EDHF [11, 16], several candidates have been proposed for the nature of EDHF, including epoxyeicosatrienoic acids (EETs), metabolites of arachidonic P450 epoxygenase pathway [9, 18], K ions [13], and electrical communication through myoendothelial gap junctions [22] (Fig. 1). We have demonstrated that endothelium-derived hydrogen peroxide (H_2O_2) is an EDHF in mouse [38] and human [39] mesenteric arteries and in porcine [40] and canine [62] coronary microvessels (Fig. 1). Other investigators also have reported that H_2O_2 is an EDHF in the human coronary microvessels [41] and piglet pial arterioles [31]. We also have demonstrated that endothelial Cu,Zn-superoxide dismutase (SOD) plays an important role in the synthesis of H_2O_2 in mouse [42] and human [43] mesenteric arteries and that endothelial NO synthase (eNOS) system plays a crucial role in the synthesis of H_2O_2/EDHF [38, 55]. In this review, I will summarize the current knowledge on our H_2O_2/EDHF theory in terms of the identification and mechanisms of synthesis and actions.

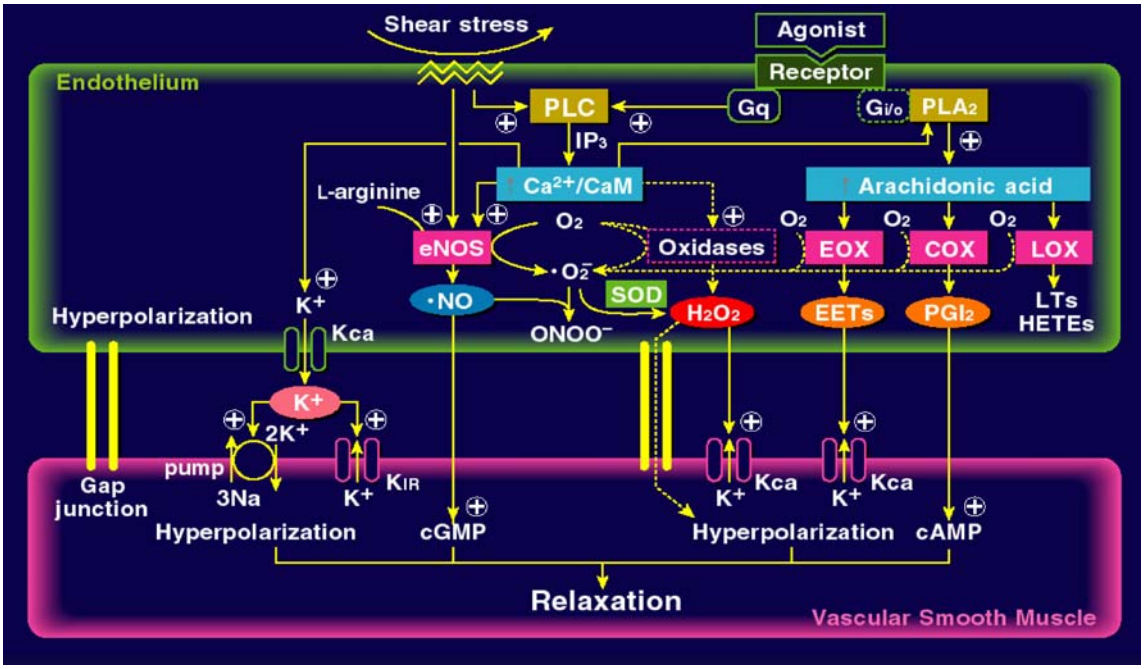

Fig. 1 H_2O_2/EDHF hypothesis on the nature of EDHF. EDHF hyperpolarizes vascular smooth muscle by opening K channels and then elicits vasodilatation. Major candidates for the nature of EDHF include (1) epoxyeicosatrienoic acids (*EETs*), metabolites of arachidonic P450 epoxygenase pathway, (2) K ions released from the endothelium through endothelial K_{Ca} channels that activates Na,K ATPase of vascular smooth muscle, and (3) electrical communication through myoendothelial gap junctions. We also have identified that (4) endothelium-derived H_2O_2 is an EDHF, for which eNOS that is functionally coupled with Cu,Zn SOD is an important source. In contrast, our findings suggest that other endothelial oxidases may not play a major role for the EDHF-mediated responses

History of EDHF research

It was known that acetylcholine induces hyperpolarization of vascular smooth muscle of isolated blood vessels in an endothelium-dependent manner [6, 30]. In 1988, Feletou and Vanhoutte [16] and Chen et al. [11] independently demonstrated that a diffusible substance released by the endothelium causes hyperpolarization of underlying vascular smooth muscle, proposing the existence of endothelium-derived hyperpolarizing factor, EDHF [11, 16].

Nature and mechanisms of action of EDHF

Endothelium-derived NO mediates vascular relaxation of relatively large, conduit arteries (i.e., aorta and epicardial coronary arteries), while EDHF plays an important role in modulating vascular tone in small resistance arteries in vitro [8, 50, 51, 60] and in human forearm microcirculation in vivo [28]. EDHF causes vascular relaxation by opening K channels and then hyperpolarizing membrane of vascular smooth muscle [8, 50, 60]. EDHF is synthesized not only upon stimulation by agonists but also by shear stress [56], and its synthesis and release are stimulated by increase in intracellular calcium in the endothelium [50, 52, 60], although calcium-independent endothelial cell hyperpolarization has also been reported [57]. NO and vasodilator prostaglandins elicit hyperpolarization of underlying vascular smooth muscle and NO may activate large conductance K_{Ca} channels in some blood vessels [5]; however, those responses to NO and vasodilator prostaglandins are largely inhibited by the inhibition of ATP-sensitive potassium (K_{ATP}) channels [8, 60]. Importantly, substantial endothelium-dependent hyperpolarization exists even after the blockade of the synthesis of NO and vasodilator prostaglandins [8, 50, 60]. Thus, EDHF appears to be different from vasodilator prostaglandins or NO, and EDHF-mediated responses are classically defined as the endothelium-dependent responses (relaxations and hyperpolarizations) after the blockade of the synthesis of vasodilator prostaglandins and NO [50, 52, 60].

H_2O_2/EDHF hypothesis

NO and EDHF share many similarities in terms of the influence by atherosclerotic risk factors and the synthesis pathway. Indeed, various atherosclerotic risk factors attenuate both NO- and EDHF-mediated responses [50, 59, 60], and the treatment of those risk factors improve both NO- and EDHF-mediated responses [17, 50, 60]. In various pathological situations, the production of reactive oxygen

species (ROS) is increased, while NO-mediated relaxations are attenuated. EDHF-mediated relaxations are temporarily enhanced to compensate the reduced NO-mediated relaxations; however, the EDHF-mediated responses also are subsequently reduced during the pathological process [50, 52]. The calcium/calmodulin pathway is involved in endothelial synthesis of both NO and EDHF [46]. These lines of evidence led us to hypothesize that EDHF is a non-NO vasodilator substance (possibly ROS other than NO) derived from eNOS [50, 52].

The endothelium produces several kinds of ROS, including superoxide, hydrogen peroxide (H_2O_2), NO, peroxynitrite, and hydroxyl radicals [14, 33, 34, 49]. ROS modulate vascular tone by several mechanisms, including alteration in K channel conductance [24]. Superoxide itself attenuates endothelium-dependent relaxations by scavenging NO [48], while it also causes relaxations of cat cerebral arteries [61]. Peroxynitrite, a potent ROS produced by the reaction of NO with superoxide, acts as a relaxing factor at its lower concentrations by activating sarco/endoplasmic reticulum Ca-ATPase in rabbit carotid arteries [1] and by activating cGMP pathway in canine coronary arteries [34]. Peroxynitrite also inhibits K_{Ca} channel activity of vascular smooth muscle [35]. H_2O_2 exerts a direct hyperpolarizing effect on vascular smooth muscle [4]. H_2O_2 elicits hyperpolarization of porcine coronary microvessels by opening large conductance K_{Ca} channels [2] and relaxes bovine pulmonary arteries by activating guanylate cyclase [7]. Thus, we examined the possible involvement of each ROS in the EDHF-mediated responses and finally were able to identify that endothelium-derived H_2O_2 is an EDHF [38, 52].

Vasodilating effect of H_2O_2

H_2O_2 causes vasodilatation through several mechanisms, including cGMP in bovine pulmonary arteries [7], cyclooxygenase and cyclic AMP in canine cerebral arteries [27], and phospholipase A_2 in porcine coronary microvessels [3]. Exogenous H_2O_2 also causes vasodilatation by opening several K channels, including K_{ATP} channels in cat cerebral [61] and rabbit mesenteric arteries [26] and K_{Ca} channels in rat cerebral arteries [53]. It has been reported that H_2O_2 causes both vasodilatation and vasoconstriction (when hyperpolarization is compromised) of perfused mouse mesenteric arteries [37] and may be distinct from EDHF in rabbit femoral arteries [10] and that H_2O_2 stimulates the release of a chemically distinct EDHF in human submucosal intestinal microvessels [25]. H_2O_2 has also been reported to induce endothelium-dependent vasodilatation through COX-1-mediated release of PGE_2 and to directly relax smooth muscle by hyperpolarization through K_{Ca} channel activation [58].

Identification of endothelium-derived H_2O_2 as an EDHF

We have demonstrated that EDHF-mediated relaxations and hyperpolarizations are inhibited by catalase, a specific inhibitor of H_2O_2, in mesenteric arteries of normal mice and are significantly reduced in $eNOS^{-/-}$ mice [38]. The specific inhibitory effect of catalase on H_2O_2 was confirmed as it lost its inhibitory effect when inactivated by aminotriazole [38]. We also have demonstrated that exogenous H_2O_2 elicits relaxations and hyperpolarizations of vascular smooth muscle of mouse mesenteric arteries by opening K_{Ca} channels and that acetylcholine causes endothelial H_2O_2 production in mouse mesenteric arteries [38]. Thus, we concluded that H_2O_2 fulfills the criteria for an EDHF in mouse mesenteric arteries [38] (Fig. 1). We subsequently confirmed that H_2O_2 is an EDHF in human mesenteric arteries [39] and porcine [40] and canine [62] coronary microvessels. Furthermore, we were able to directly demonstrate endothelial H_2O_2 production in porcine coronary microvessels by using electron spin resonance imaging [40]. The estimated concentration of endothelium-derived H_2O_2 is in micromolar range, which is consistent to its concentrations to cause EDHF-mediated responses [38, 40]. Subsequently, other investigators reported that endothelium-derived H_2O_2 also is an EDHF in human coronary microvessels [41] and piglet pial arterioles [31], although other mechanisms for endothelium-dependent and endothelium-independent relaxation in response to H_2O_2 have been reported [10, 25, 58]. We also have recently demonstrated that endothelium-derived H_2O_2 plays important cardioprotective roles in the canine coronary microcirculation in vivo, including coronary autoregulation [62], myocardial protection against ischemia/reperfusion injury [63], and metabolic coronary vasodilatation [64]. Thus, we have confirmed that endothelium-derived H_2O_2 acts as an EDHF, especially in microvessels [50, 52, 56], although the mechanisms for the endothelial production of H_2O_2/EDHF remains to be elucidated.

Mechanisms for endothelial synthesis of H_2O_2/EDHF

Several previous studies have addressed the mechanisms for the synthesis of EDHF (Fig. 1). In porcine epicardial coronary arteries, cytochrome P450 2C has been reported to act as an EDHF synthase to synthesize EETs as an EDHF [8, 18]. cAMP is reported to enhance gap junctional electrical communication for EDHF-mediated responses [21]. It also is reported that agonist stimulation opens endothelial K_{Ca} channels with a resultant release in K ion into myoendothelial space as an EDHF [13].

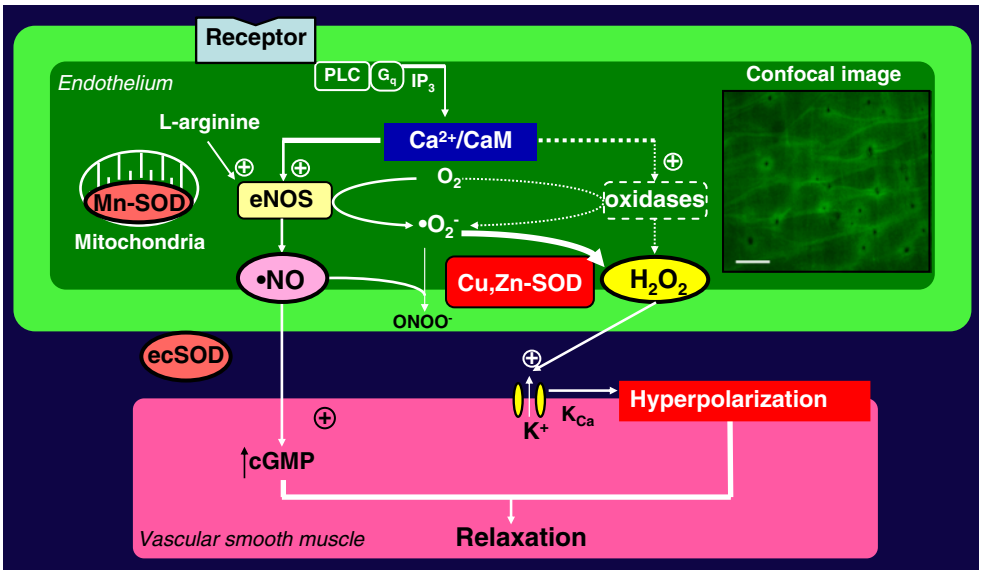

Fig. 2 Endothelial SOD isoforms and H_2O_2/EDHF responses. In the endothelium, three SOD isoforms are present, including Cu,Zn-SOD (cell membrane), Mn-SOD (mitochondria), and ecSOD (extracellular). Our findings indicate that Cu,Zn-SOD plays a major role to synthesize H_2O_2/EDHF from NOSs-derived superoxide anions. This notion also is supported by the confocal images with peroxidase-sensitive dye indicating the H_2O_2 signals originating from the endothelial cell membrane where both NOSs and Cu,Zn-SOD are co-located

In the endothelium, H_2O_2 is synthesized by either spontaneous dismutation from superoxide or dismutation of superoxide by SOD [15]. In blood vessels, there are three SOD isoforms that dismutate superoxide into H_2O_2 [48] (Fig. 2). Cu,Zn-SOD is located mainly in the cytosol, nucleus, and, to a lesser extent, in mitochondria [15], dismutating superoxide and prolonging the half-life of NO [45] (Fig. 2). The Cu,Zn-SOD activity approximately accounts for 50–80% of all SOD activities in vascular wall [12]. Mn-SOD is located in mitochondria and dismutates superoxide derived from respiratory chains [15] (Fig. 2). Extracellular SOD (ecSOD) is located extracellularly and dismutates extracellular superoxide to protect the diffusion of NO [19] (Fig. 2). As mentioned above, we have demonstrated that eNOS is one of the major contributors for the synthesis of H_2O_2 as an EDHF [38]. eNOS produces superoxide when it synthesizes NO from L-arginine, while Cu,Zn-SOD dismutates those superoxide anions into H_2O_2 [54]. Since heparin, which inhibits ecSOD activities, had no effect on EDHF-mediated response, we excluded ecSOD as a source of EDHF [42]. Mn-SOD is located in mitochondria and does not seem to be involved in EDHF synthesis because in mouse mesenteric arteries, endothelium-derived H_2O_2 is mainly derived from membrane, where Cu,Zn-

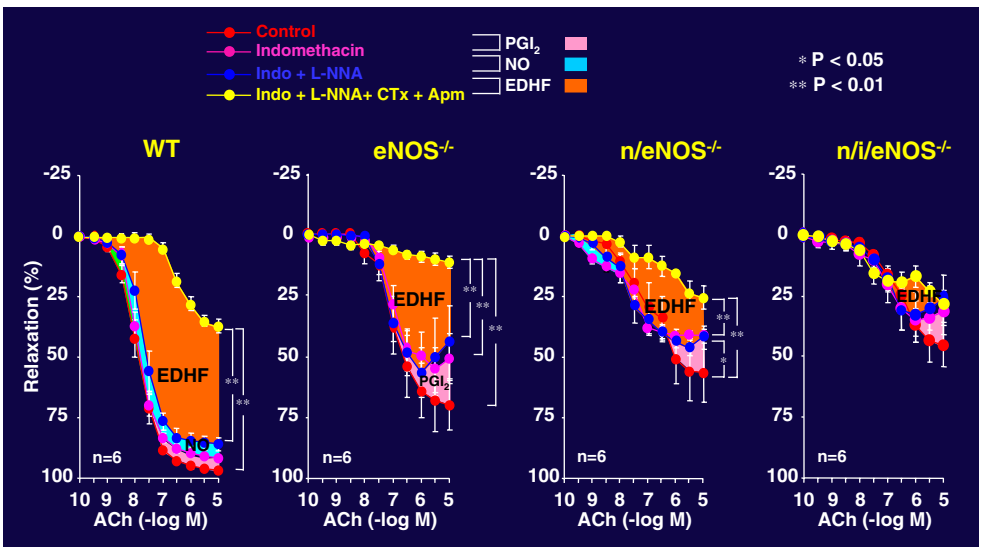

Fig. 3 Endothelium-dependent relaxations of mesenteric arteries from normal and NOS-deficient mice. EDHF-mediated relaxations can functionally be evaluated by the inhibitory effects of the K_{Ca} blockade with charybdotoxin (*CTx*, an intermediate K_{Ca} blocker) and apamin (*Apm*, a small K_{Ca} channel blocker) in the presence of indomethacin and L-arginine analog (*L-NNA*). The EDHF-mediated relaxations were progressively reduced as the number of NOS genes deleted was increased and finally in the triply n/i/eNOSs$^{-/-}$ mice, EDHF-mediated relaxations were abolished, whereas vasodilator functions of vascular smooth muscle were fairly preserved (modified from [55] with permission)

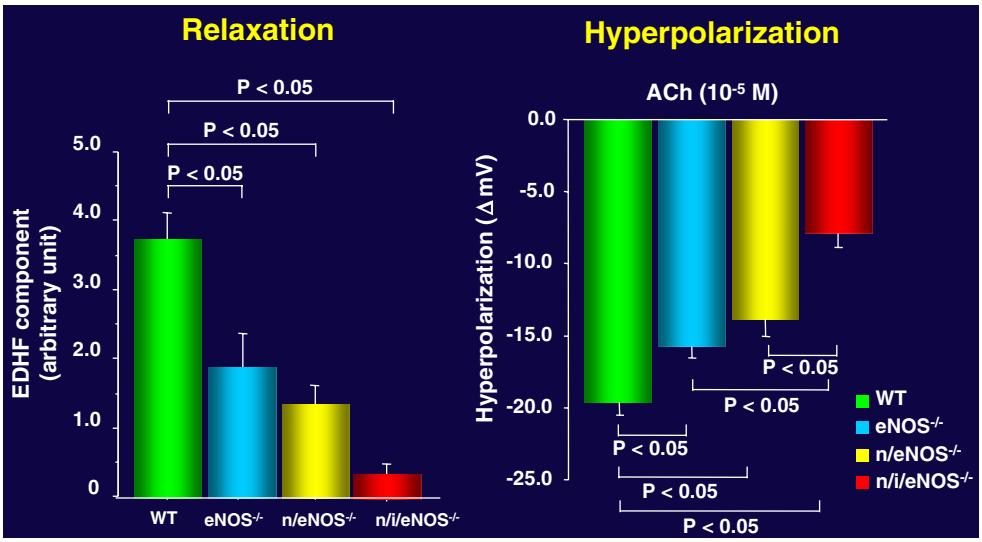

Fig. 4 Endothelium-dependent responses of mesenteric arteries from normal and NOS-deficient mice. EDHF-mediated responses (relaxations and hyperpolarizations) in the presence of indometacin and L-NNA were progressively reduced as the number of NOS genes deleted was increased and finally in the triply n/i/eNOSs$^{-/-}$ mice, those responses were abolished, whereas vasodilator and hyperpolarizing functions of vascular smooth muscle were fairly preserved (modified from [55] with permission)

SOD is located [42] (Fig. 2). Finally, in Cu,Zn-SOD$^{-/-}$ mice, EDHF-mediated relaxations and hyperpolarizations are markedly attenuated in mesenteric arteries and coronary microvessels compared with control mice without alteration in vasodilator properties of vascular smooth muscle [42]. Thus, we concluded that endothelial Cu,Zn-SOD plays an important role for the synthesis of H_2O_2 as an EDHF synthase [42] (Fig. 2).

Furthermore, supplement of SOD mimetics, Tempol [29], restores EDHF-mediated responses [42]. These results may reflect the restoration of contribution of H_2O_2 as an EDHF and/or improved myoendothelial communication as a result of a reduction in ROS generation [23]. Similarly, in human mesenteric arteries, supplement of another SOD mimetics, Tiron [32], enhances EDHF-mediated relaxations and hyperpolarizations [43]. H_2O_2 also is an EDHF in porcine coronary microvessels, where the EDHF-mediated relaxations are enhanced by the pretreatment with Tiron [40]. In human-isolated coronary arterioles, it was suggested that H_2O_2 derived from mitochondria is involved in flow-mediated dilatation [36]. Thus, our H_2O_2/EDHF theory has uncovered the new and important role of endothelial Cu,Zn-SOD as an EDHF synthase, in addition to its classical role to scavenge superoxide to prolong the half-life of NO [42, 43].

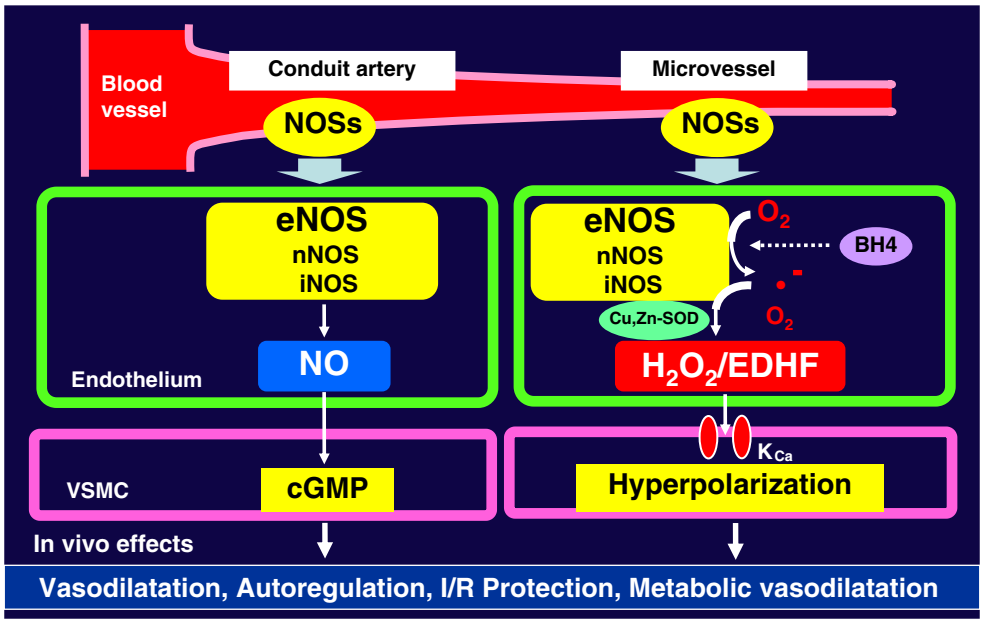

Fig. 5 Novel concept on the roles of endothelial NO synthases system. Accumulating evidence from our laboratory indicates that endothelial NO synthases (NOSs) system plays divergent roles, functioning as the NO-generating system in large conduit arteries, whereas acting as the H_2O_2/EDHF-generating system in microvessels, contributing to the maintenance of cardiovascular homeostasis

There are three NO synthase isoforms, including eNOS, neuronal NOS, and inducible NOS, and they apparently compensate each other [50, 55, 60]. Indeed, in the singly eNOS$^{-/-}$ mice, in addition to abolishment of NO-mediated relaxations in the aorta, EDHF-mediated relaxations of the mesenteric artery were markedly reduced but not abolished, and the remaining relaxations also were sensitive to catalase [38], suggesting some compensatory involvement of other NOS isoform-derived H_2O_2/EDHF. In order to fully understand the role of endothelial NOSs in the H_2O_2/EDHF-mediated responses, we generated mice that are deficient of all three NOS isoforms [44]. Interestingly, the EDHF-mediated responses were progressively impaired in accordance with the NOS genes deleted and finally in the triply n/i/eNOSs$^{-/-}$ mice, the responses were abolished while vasodilator and hyperpolarizing functions of vascular smooth muscle were fairly well preserved (Figs. 3 and 4) [55]. These results have provided us with the novel concept that endothelial NOSs system plays an important role as the EDHF-generating system in microvessels, while the system acts as NO-generating system in large conduit arteries in its original meaning (Fig. 5) [55]. Recently, we have demonstrated that the triply n/i/eNOSs$^{-/-}$ mice exhibit typical characteristics of metabolic syndrome in humans, including visceral obesity, hypertension, diabetes mellitus, and dyslipidemia with a resultant reduced survival due to spontaneously myocardial infarction [47]. Again, these findings indicate the important roles of the NOSs system to maintain cardiovascular and metabolic homeostasis [47].

Conclusions

We have identified that endothelium-derived H_2O_2 is an EDHF in animals and humans and plays an important role as a redox-signaling molecule to cause vasodilatation as well as cardioprotection (Fig. 5).

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