

Diverse Functions of Endothelial NO Synthases System: NO and EDH

Hiroaki Shimokawa, MD, PhD and Shigeo Godo, MD, PhD

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

(*J Cardiovasc Pharmacol*TM 2016;67:361–366)

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 Vanhoutte⁵ and Chen et al⁶ 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 pressure¹⁷ and vascular metabolic functions¹⁸ in general, and coronary autoregulation¹⁹ and coronary metabolic dilatation²⁰ 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

Received for publication August 24, 2015; accepted November 11, 2015.

From the Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan.

Supported in part by the Grant-in-Aid for Tohoku University Global COE for Conquest of Signal Transduction Diseases with Network Medicine, the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan, and the Grants-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare, Tokyo, Japan.

The authors report no conflicts of interest.

Reprints: Hiroaki Shimokawa, MD, PhD, Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryomachi, Aoba-ku, Sendai 980-8574, Japan (e-mail: shimo@cardio.med.tohoku.ac.jp).

Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved. This is an open-access article distributed under the terms of the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CCBY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially.

to prevent cardiovascular events.²³ These lines of evidence suggest the potential importance of the physiological balance between NO and EDH/H₂O₂ 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/H₂O₂. As the former is extensively reviewed recently³ 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/H₂O₂

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 H₂O₂ is an EDH factor in mouse mesenteric arteries¹³; EDH-mediated relaxation and hyperpolarization of underlying VSMC were inhibited by catalase, a specific H₂O₂ 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 mesenteric²⁶ and coronary²⁷ arteries, porcine coronary arteries,²⁸ canine coronary arteries,^{19,20} and piglet pial arterioles,²⁹ although EDH-independent vasodilating mechanisms by H₂O₂ have also been reported in other vascular beds.^{30,31} Notably, the estimated concentration of endothelium-derived H₂O₂ 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 H₂O₂ in the endothelium,^{34,35} Cu–Zn superoxide dismutase (SOD) plays a key role in the synthesis of EDH/H₂O₂; eNOS produces superoxide anions when synthesizing NO from L-arginine and oxygen under physiological conditions, whereas Cu–Zn-SOD dismutates those superoxide anions into H₂O₂, 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/H₂O₂-mediated responses are resistant to NOS inhibitors and upregulation of eNOS cofactor tetrahydrobiopterin has no effects on the responses, superoxide anions relevant to EDH/H₂O₂ 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 H₂O₂-mediated vasodilatation; in human coronary arterioles, mitochondrial respiratory chain-derived and nicotinamide adenine dinucleotide phosphate oxidase-derived H₂O₂ is associated with flow-mediated dilation and bradykinin-induced relaxation, respectively.^{38,39} To date, several mechanisms have been proposed for H₂O₂-induced vasodilatation.⁴⁰ Among them, Burgoyne et al⁴¹ demonstrated a precise mechanism by which EDH/H₂O₂ relaxes underlying VSMC. Briefly, H₂O₂ induces an interprotein disulfide formation between two 1 α isoforms of cGMP-dependent protein kinases (PKG_{1 α}) 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-K α 1, α 1-subunit of AMP-activated protein kinase; CaM, calmodulin; CaMKK β , Ca²⁺/CaM-dependent protein kinase β ; COX, cyclooxygenase; EETs, epoxyeicosatrienoic acids; eNOS, endothelial NO synthase; EOX, epoxygenase; HETEs, hydroxyeicosatetraenoic acids; H₂O₂, hydrogen peroxide; IP₃, inositol trisphosphate; LOX, lipoxygenase; LTs, leukotrienes; ONOO⁻, peroxynitrite; PKG_{1 α} , 1 α -subunit of protein kinase G; PLA₂, phospholipase A₂; PLC, phospholipase C.

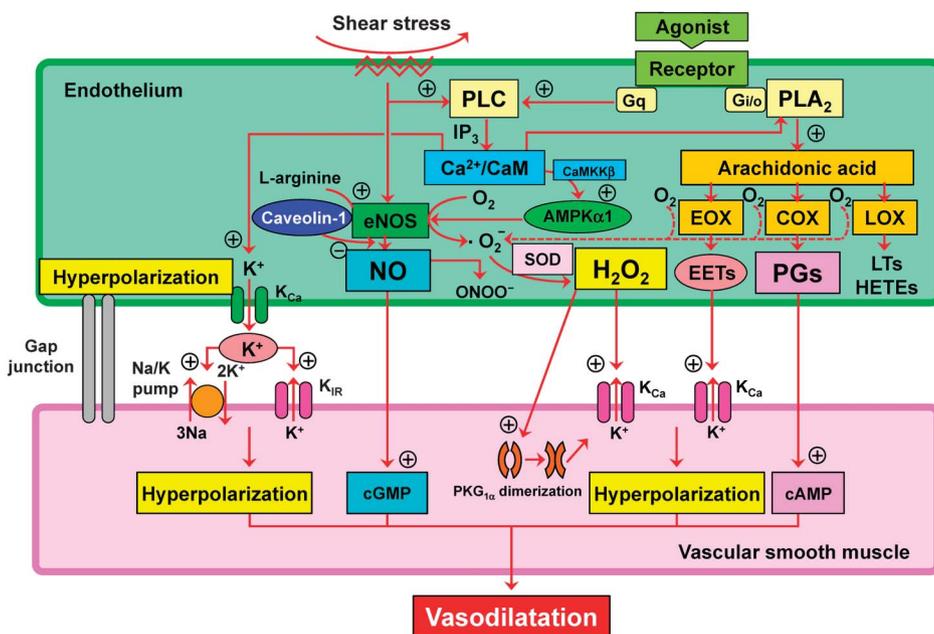
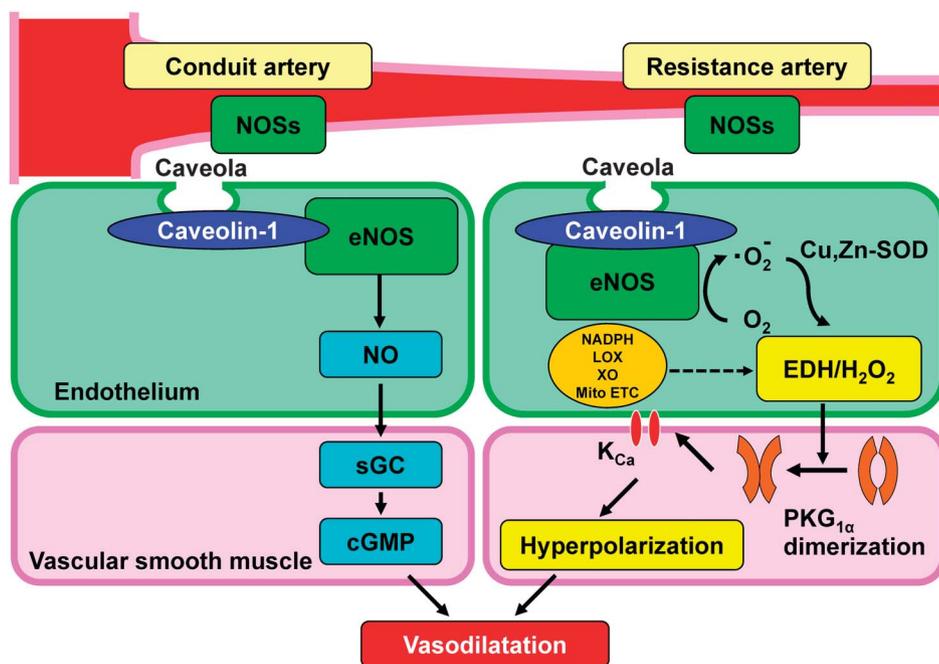


FIGURE 2. Diverse roles of endothelial nitric oxide synthases system in large conduit vessels, NO synthases (NOSs) mainly serve as a NO-generating system to cause vasodilatation through sGC–cGMP pathway, whereas in small resistance vessels, they act as a superoxide-generating system to evoke EDH-mediated responses through H₂O₂-induced PKG_{1α} 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_{1α}, 1α-subunit of protein kinase G; XO, xanthine oxidase.



The activated PKG_{1α} subsequently stimulates potassium channels, leading to hyperpolarization and vasodilatation in mouse mesenteric arteries¹⁷ 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_{1α}, whose mutant PKG_{1α} is unable to be activated by H₂O₂-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 H₂O₂ functions as an important endogenous second messenger to elicit EDH-mediated 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 H₂O₂ 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 H₂O₂/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/H₂O₂-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/H₂O₂-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 H₂O₂-induced PKG_{1α} 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 caveolin-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 caveolin-1-dependent mechanism, switching its function from NO synthase to EDH/H₂O₂-generating enzyme (Fig. 2).⁴⁹

Mechanistic Insight into Vessel Size-dependent Contribution of NO and EDH/H₂O₂

As mentioned above, NO and EDH/H₂O₂ share the diverse roles in modulating vascular tone in a distinct vessel size-dependent manner; NO plays a dominant role in conduit

arteries and EDH/H₂O₂ in resistance vessels.¹⁴ Mechanistic insight into vessel size-dependent contribution of NO and EDH/H₂O₂ has been recently demonstrated. As compared with large conduit vessels, eNOS is functionally inhibited by caveolin-1, and relaxation responses of VSMC to H₂O₂ are enhanced through PKG_{1 α} -mediated mechanism in resistance vessels in mice.^{49,50} Indeed, mouse resistance vessels have less NO production and less antioxidant capacity, allowing PKG_{1 α} to be more sensitive to H₂O₂-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 H₂O₂ is likely to be involved because H₂O₂-induced PKG_{1 α} dimerization, a central mechanism of H₂O₂-induced vasodilatation, is inhibited by cGMP-dependent activation of PKG.⁵⁰ Moreover, pharmacological inhibition of sGC sensitizes conduit vessels to H₂O₂-induced vasodilatation in mice.⁵⁰ These observations support the notion that excessive endothelium-derived NO desensitizes blood vessels to EDH/H₂O₂-mediated relaxations. In addition, the actions of other EDH factors may also be inhibited through interaction with NO. For instance, Bauersachs et al⁵² 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 al⁵⁵ 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 H₂S 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 H₂O₂, 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 H₂O₂ have attracted much attention as endothelium-derived H₂O₂ causes

endothelium-dependent vasodilatation and contributes to microvascular homeostasis at its physiological concentrations.^{26,32,33,56} As predicted previously in the commentary article⁵⁸ on our original EDH/H₂O₂ report,¹³ H₂O₂ 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/H₂O₂-mediated relaxations in microcirculations and may provide a clue for better understanding of the harmful effects of antioxidant supplements.^{23,59}

Endothelium-derived H₂O₂ is mainly generated by the dismutation of superoxide anions, which come from various sources in the endothelium, including nicotinamide adenine dinucleotide phosphate 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/H₂O₂-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 Ca²⁺ 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 H₂O₂ 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 vasodilatation 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

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, H₂O₂ also has potent vasodilator properties in coronary resistance vessels, such that impaired EDH/H₂O₂-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/H₂O₂ 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.

REFERENCES

- Vanhoutte PM. Endothelial dysfunction: the first step toward coronary arteriosclerosis. *Circ J*. 2009;73:595–601.
- Feletou M, Vanhoutte PM. EDHF: an update. *Clin Sci (Lond)*. 2009;117:139–155.
- Feletou M, Kohler R, Vanhoutte PM. Nitric oxide: orchestrator of endothelium-dependent responses. *Ann Med*. 2012;44:694–716.
- Vanhoutte PM, Shimokawa H, Tang EH, et al. Endothelial dysfunction and vascular disease. *Acta Physiol*. 2009;196:193–222.
- Feletou M, Vanhoutte PM. Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br J Pharmacol*. 1988;93:515–524.
- Chen G, Suzuki H, Weston AH. Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *Br J Pharmacol*. 1988;95:1165–1174.
- Feletou M, Vanhoutte PM. Endothelium-dependent hyperpolarization: no longer an F-word! *J Cardiovasc Pharmacol*. 2013;61:91–92.
- Fisslthaler B, Popp R, Kiss L, et al. Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature*. 1999;401:493–497.
- Campbell WB, Gebremedhin D, Pratt PF, et al. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res*. 1996;78:415–423.
- Griffith TM, Chaytor AT, Edwards DH. The obligatory link: role of gap junctional communication in endothelium-dependent smooth muscle hyperpolarization. *Pharmacol Res*. 2004;49:551–564.
- Edwards G, Dora KA, Gardener MJ, et al. K⁺ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature*. 1998;396:269–272.
- Tang G, Yang G, Jiang B, et al. H₂S is an endothelium-derived hyperpolarizing factor. *Antioxid Redox Signal*. 2013;19:1634–1646.
- Matoba T, Shimokawa H, Nakashima M, et al. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest*. 2000;106:1521–1530.
- Shimokawa H. 2014 Williams Harvey Lecture: importance of coronary vasomotion abnormalities—from bench to bedside. *Eur Heart J*. 2014;35:3180–3193.
- Urakami-Harasawa L, Shimokawa H, Nakashima M, et al. Importance of endothelium-derived hyperpolarizing factor in human arteries. *J Clin Invest*. 1997;100:2793–2799.
- Shimokawa H, Yasutake H, Fujii K, et al. The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *J Cardiovasc Pharmacol*. 1996;28:703–711.
- Prisyazhna O, Rudyk O, Eaton P. Single atom substitution in mouse protein kinase G eliminates oxidant sensing to cause hypertension. *Nat Med*. 2012;18:286–290.
- Nakajima S, Ohashi J, Sawada A, et al. Essential role of bone marrow for microvascular endothelial and metabolic functions in mice. *Circ Res*. 2012;111:87–96.
- Yada T, Shimokawa H, Hiramatsu O, et al. Hydrogen peroxide, an endogenous endothelium-derived hyperpolarizing factor, plays an important role in coronary autoregulation in vivo. *Circulation*. 2003;107:1040–1045.
- Yada T, Shimokawa H, Hiramatsu O, et al. Important role of endogenous hydrogen peroxide in pacing-induced metabolic coronary vasodilation in dogs in vivo. *J Am Coll Cardiol*. 2007;50:1272–1278.
- Takaki A, Morikawa K, Tsutsui M, et al. Crucial role of nitric oxide synthases system in endothelium-dependent hyperpolarization in mice. *J Exp Med*. 2008;205:2053–2063.
- Kojima S, Matsui K, Sakamoto T, et al. Long-term nitrate therapy after acute myocardial infarction does not improve or aggravate prognosis. *Circ J*. 2007;71:301–307.
- Bjelakovic G, Nikolova D, Gluud LL, et al. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. *JAMA*. 2007;297:842–857.
- Shimokawa H, Morikawa K. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in animals and humans. *J Mol Cell Cardiol*. 2005;39:725–732.
- Nagao T, Illiano S, Vanhoutte PM. Calmodulin antagonists inhibit endothelium-dependent hyperpolarization in the canine coronary artery. *Br J Pharmacol*. 1992;107:382–386.
- Matoba T, Shimokawa H, Kubota H, et al. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in human mesenteric arteries. *Biochem Biophys Res Commun*. 2002;290:909–913.
- Miura H, Bosnjak JJ, Ning G, et al. Role for hydrogen peroxide in flow-induced dilation of human coronary arterioles. *Circ Res*. 2003;92:e31–40.
- Matoba T, Shimokawa H, Morikawa K, et al. Electron spin resonance detection of hydrogen peroxide as an endothelium-derived hyperpolarizing factor in porcine coronary microvessels. *Arterioscler Thromb Vasc Biol*. 2003;23:1224–1230.
- Lacza Z, Puskar M, Kis B, et al. Hydrogen peroxide acts as an EDHF in the piglet pial vasculature in response to bradykinin. *Am J Physiol*. 2002;283:H406–H411.
- Hatoum OA, Binion DG, Miura H, et al. Role of hydrogen peroxide in ACh-induced dilation of human submucosal intestinal microvessels. *Am J Physiol*. 2005;288:H48–H54.
- Chaytor AT, Edwards DH, Bakker LM, et al. Distinct hyperpolarizing and relaxant roles for gap junctions and endothelium-derived H₂O₂ in NO-independent relaxations of rabbit arteries. *Proc Natl Acad Sci U S A*. 2003;100:15212–15217.
- Yada T, Shimokawa H, Hiramatsu O, et al. Cardioprotective role of endogenous hydrogen peroxide during ischemia-reperfusion injury in

- canine coronary microcirculation in vivo. *Am J Physiol*. 2006;291:H1138–H1146.
33. Burgoyne JR, Oka S, Ale-Agha N, et al. Hydrogen peroxide sensing and signaling by protein kinases in the cardiovascular system. *Antioxid Redox Signal*. 2013;18:1042–1052.
 34. Schroder E, Eaton P. Hydrogen peroxide as an endogenous mediator and exogenous tool in cardiovascular research: issues and considerations. *Curr Opin Pharmacol*. 2008;8:153–159.
 35. Lassegue B, San Martin A, Griendling KK. Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *Circ Res*. 2012;110:1364–1390.
 36. Morikawa K, Shimokawa H, Matoba T, et al. Pivotal role of Cu,Zn-superoxide dismutase in endothelium-dependent hyperpolarization. *J Clin Invest*. 2003;112:1871–1879.
 37. Takaki A, Morikawa K, Murayama Y, et al. Roles of endothelial oxidases in endothelium-derived hyperpolarizing factor responses in mice. *J Cardiovasc Pharmacol*. 2008;52:510–517.
 38. Larsen BT, Bubolz AH, Mendoza SA, et al. Bradykinin-induced dilation of human coronary arterioles requires NADPH oxidase-derived reactive oxygen species. *Arterioscler Thromb Vasc Biol*. 2009;29:739–745.
 39. Liu Y, Zhao H, Li H, et al. Mitochondrial sources of H₂O₂ generation play a key role in flow-mediated dilation in human coronary resistance arteries. *Circ Res*. 2003;93:573–580.
 40. Shimokawa H. Hydrogen peroxide as an endothelium-derived hyperpolarizing factor. *Pflugers Arch*. 2010;459:915–922.
 41. Burgoyne JR, Madhani M, Cuello F, et al. Cysteine redox sensor in PKG1 α enables oxidant-induced activation. *Science*. 2007;317:1393–1397.
 42. Zhang DX, Borbouse L, Gebremedhin D, et al. H₂O₂-induced dilation in human coronary arterioles: role of protein kinase G dimerization and large-conductance Ca²⁺-activated K⁺ channel activation. *Circ Res*. 2012;110:471–480.
 43. Liu Y, Bubolz AH, Mendoza S, et al. H₂O₂ is the transferrable factor mediating flow-induced dilation in human coronary arterioles. *Circ Res*. 2011;108:566–573.
 44. Forstermann U, Sessa WC. Nitric oxide synthases: regulation and function. *Eur Heart J*. 2012;33:829–837.
 45. Forstermann U, Li H. Therapeutic effect of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling. *Br J Pharmacol*. 2011;164:213–223.
 46. Stuehr D, Pou S, Rosen GM. Oxygen reduction by nitric-oxide synthases. *J Biol Chem*. 2001;276:14533–14536.
 47. Huang PL, Huang Z, Mashimo H, et al. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature*. 1995;377:239–242.
 48. Morishita T, Tsutsui M, Shimokawa H, et al. Nephrogenic diabetes insipidus in mice lacking all nitric oxide synthase isoforms. *Proc Natl Acad Sci U S A*. 2005;102:10616–10621.
 49. Ohashi J, Sawada A, Nakajima S, et al. Mechanisms for enhanced endothelium-derived hyperpolarizing factor-mediated responses in microvessels in mice. *Circ J*. 2012;76:1768–1779.
 50. Burgoyne JR, Prysazhna O, Rudyk O, et al. cGMP-dependent activation of protein kinase G precludes disulfide activation: implications for blood pressure control. *Hypertension*. 2012;60:1301–1308.
 51. Enkhjargal B, Godo S, Sawada A, et al. Endothelial AMP-activated protein kinase regulates blood pressure and coronary flow responses through hyperpolarization mechanism in mice. *Arterioscler Thromb Vasc Biol*. 2014;34:1505–1513.
 52. Bauersachs J, Popp R, Hecker M, et al. Nitric oxide attenuates the release of endothelium-derived hyperpolarizing factor. *Circulation*. 1996;94:3341–3347.
 53. Nishikawa Y, Stepp DW, Chilian WM. Nitric oxide exerts feedback inhibition on EDHF-induced coronary arteriolar dilation in vivo. *Am J Physiol*. 2000;279:H459–H465.
 54. Olmos L, Mombouli JV, Illiano S, et al. cGMP mediates the desensitization to bradykinin in isolated canine coronary arteries. *Am J Physiol*. 1995;268:H865–H870.
 55. Mustafa AK, Sikka G, Gazi SK, et al. Hydrogen sulfide as endothelium-derived hyperpolarizing factor sulfhydrates potassium channels. *Circ Res*. 2011;109:1259–1268.
 56. Satoh K, Godo S, Saito H, et al. Dual roles of vascular-derived reactive oxygen species—with a special reference to hydrogen peroxide and cyclophilin A. *J Mol Cell Cardiol*. 2014;73:50–56.
 57. Wong WT, Tian XY, Huang Y. Endothelial dysfunction in diabetes and hypertension: cross talk in ras, BMP4, and ROS-dependent COX-2-derived prostanoids. *J Cardiovasc Pharmacol*. 2013;61:204–214.
 58. Vanhoutte PM. Endothelium-derived free radicals: for worse and for better. *J Clin Invest*. 2001;107:23–25.
 59. Bjelakovic G, Nikolova D, Gluud C. Antioxidant supplements to prevent mortality. *JAMA*. 2013;310:1178–1179.
 60. Shao D, Oka S, Brady CD, et al. Redox modification of cell signaling in the cardiovascular system. *J Mol Cell Cardiol*. 2012;52:550–558.
 61. van Rooij E, Olson EN. MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles. *Nat Rev Drug Discov*. 2012;11:860–872.
 62. Trajkovski M, Hausser J, Soutschek J, et al. MicroRNAs 103 and 107 regulate insulin sensitivity. *Nature*. 2011;474:649–653.
 63. Sun HX, Zeng DY, Li RT, et al. Essential role of microRNA-155 in regulating endothelium-dependent vasorelaxation by targeting endothelial nitric oxide synthase. *Hypertension*. 2012;60:1407–1414.
 64. Kozai D, Ogawa N, Mori Y. Redox regulation of transient receptor potential channels. *Antioxid Redox Signal*. 2014;21:971–986.
 65. Nishida M, Hara Y, Yoshida T, et al. TRP channels: molecular diversity and physiological function. *Microcirculation*. 2006;13:535–550.
 66. Ozkor MA, Murrow JR, Rahman AM, et al. Endothelium-derived hyperpolarizing factor determines resting and stimulated forearm vasodilator tone in health and in disease. *Circulation*. 2011;123:2244–2253.
 67. Matsuzawa Y, Sugiyama S, Sugamura K, et al. Digital assessment of endothelial function and ischemic heart disease in women. *J Am Coll Cardiol*. 2010;55:1688–1696.
 68. Kitta Y, Obata JE, Nakamura T, et al. Persistent impairment of endothelial vasomotor function has a negative impact on outcome in patients with coronary artery disease. *J Am Coll Cardiol*. 2009;53:323–330.
 69. Crea F, Lanza G, Camici P. Mechanisms of coronary microvascular dysfunction. In: *Coronary Microvascular Dysfunction*. Milan, Italy: Springer; 2014:31–47.
 70. Ambrosio G, Del Pinto M, Tritto I, et al. Chronic nitrate therapy is associated with different presentation and evolution of acute coronary syndromes: insights from 52,693 patients in the global registry of acute coronary events. *Eur Heart J*. 2010;31:430–438.