Reactive oxygen species in cardiovascular health and disease: special references to nitric oxide, hydrogen peroxide, and Rho-kinase

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The interaction between endothelial cells and vascular smooth muscle cells (VSMC) plays an important role in regulating cardiovascular homeostasis. Endothelial cells synthesize and release endothelium-derived relaxing factors (EDRFs), including vasodilator prostaglandins, nitric oxide (NO), and endothelium-dependent hyperpolarization (EDH) factors. Importantly, the contribution of EDRFs to endothelium-dependent vasodilatation markedly varies in a vessel size-dependent manner; NO mainly mediates vasodilatation of relatively large vessels, while EDH factors in small resistance vessels. We have previously identified that endothelium-derived hydrogen peroxide (H_2O_2) is an EDH factor especially in microcirculation. Several lines of evidence indicate the importance of the physiological balance between NO and H_2O_2/EDH factor. Rho-kinase was identified as the effectors of the small GTP-binding protein, RhoA. Both endothelial NO production and NO-mediated signaling in VSMC are targets and effectors of the RhoA/Rho-kinase pathway. In endothelial cells, the RhoA/Rho-kinase pathway negatively regulates NO production. On the contrary, the pathway enhances VSMC contraction with resultant occurrence of coronary artery spasm and promotes the development of oxidative stress and vascular remodeling. In this review, I will briefly summarize the current knowledge on the regulatory roles of endothelium-derived relaxing factors, with special references to NO and H_2O_2/EDH factor, in relation to Rho-kinase, in cardiovascular health and disease.

Key Words: **endothelium, endothelium-derived relaxing factors, nitric oxide, hydrogen peroxide, Rho-kinase**

The endothelium plays important roles in modulating the tonus of underlying vascular smooth muscle cells (VSMC) by synthesizing and releasing endothelium-derived relaxing factors (EDRFs), including vasodilator prostaglandins (e.g., prostacyclin), nitric oxide (NO), and endothelium-dependent hyperpolarization (EDH) factors (Fig. 1).[1,2] Since the discovery of endothelium-dependent hyperpolarization in 1988,[3,4] several candidates have been proposed as the nature of EDH factors. Importantly, the contribution of EDRFs to endothelium-dependent vasodilatations markedly varies as a function of vessel size; endothelium-derived NO mainly mediates vasodilatation of relatively large, conduit vessels, while EDH factors that of resistance arteries (Fig. 2).[1,5] This vessel-size-dependent contribution of NO and EDH factors is well preserved among species, from rodents to humans, in order to maintain a physiological balance between them.[1,2] Endothelial dysfunction is characterized by impaired production and/or action of EDRFs, reflecting the hallmark and potential predictor for atherosclerotic cardiovascular diseases.[2] Various risk factors (e.g., smoking, diabetes mellitus, hypertension, and dyslipidemia) cause endothelial dysfunction, initiating the step toward atherosclerotic cardiovascular diseases.[1,2]

Rho-kinases (Rho-kinase α/ROKα/ROCK2 and Rho-kinase β/ROKβ/ROCK1) were identified as the effector of the small GTP-binding protein, RhoA, independently by 3 research groups in 1996.[6–8] Hereafter, both Rho-kinase α/ROKα/ROCK2 and Rho-kinase β/ROKβ/ ROCK1 are collectively referred to as Rho-kinase.[1] Accumulating evidence indicates that Rho-kinase plays important roles in the pathogenesis of oxidative stress and cardiovascular diseases.[1,9,10]

In this review, I will briefly summarize the current knowledge on the two endothelium-derived relaxing factors, NO and H_2O_2/EDH factor, and Rho-kinase in cardiovascular health and disease.

Physiological Balance between NO and EDH

EDH factors cause hyperpolarization and subsequent relaxation of underlying VSMC with resultant vasodilatation of small resistance vessels, finely tuning blood pressure and tissue perfusion instantaneously in response to diverse physiological demands.[1,2] As mentioned above, endothelium-derived NO and EDH factors share the important roles in modulating vascular tonus in a distinct vessel size-dependent manner (Fig. 2).[1,5] In this scope, vasodilator prostaglandins play a small but constant role, independent of vessel size in general. In contrast, NO predominantly regulates the tonus of relatively large conduit vessels (e.g., aorta and epicardial coronary arteries), while the importance of EDH factors increases as vessel size decreases (e.g., small mesenteric arteries and coronary microvessels).[1,5] Thus, EDH-mediated vasodilatation is especially important in microcirculation, where blood pressure and tissue perfusion are critically determined. Moreover, such redundant mechanisms in endothelium-dependent vasodilatations are advantageous for maintaining cardiovascular homeostasis with compensatory interactions.[11–14] Indeed, in various pathological conditions with atherosclerotic risk factors, NO-mediated relaxations are easily impaired, where EDH-mediated responses are fairly preserved or even enhanced to serve as a compensatory vasodilator system.[11–14] Multiple mechanisms appear to be involved in the enhanced EDH-mediated responses in small resistance vessels as discussed later.[15]

Endothelium-derived H_2O_2 as an EDH Factor

Identification of endothelium-derived H_2O_2 as an EDH factor. Several EDH factors appear to exist depending on the vascular bed, vessel size, and species studied, including

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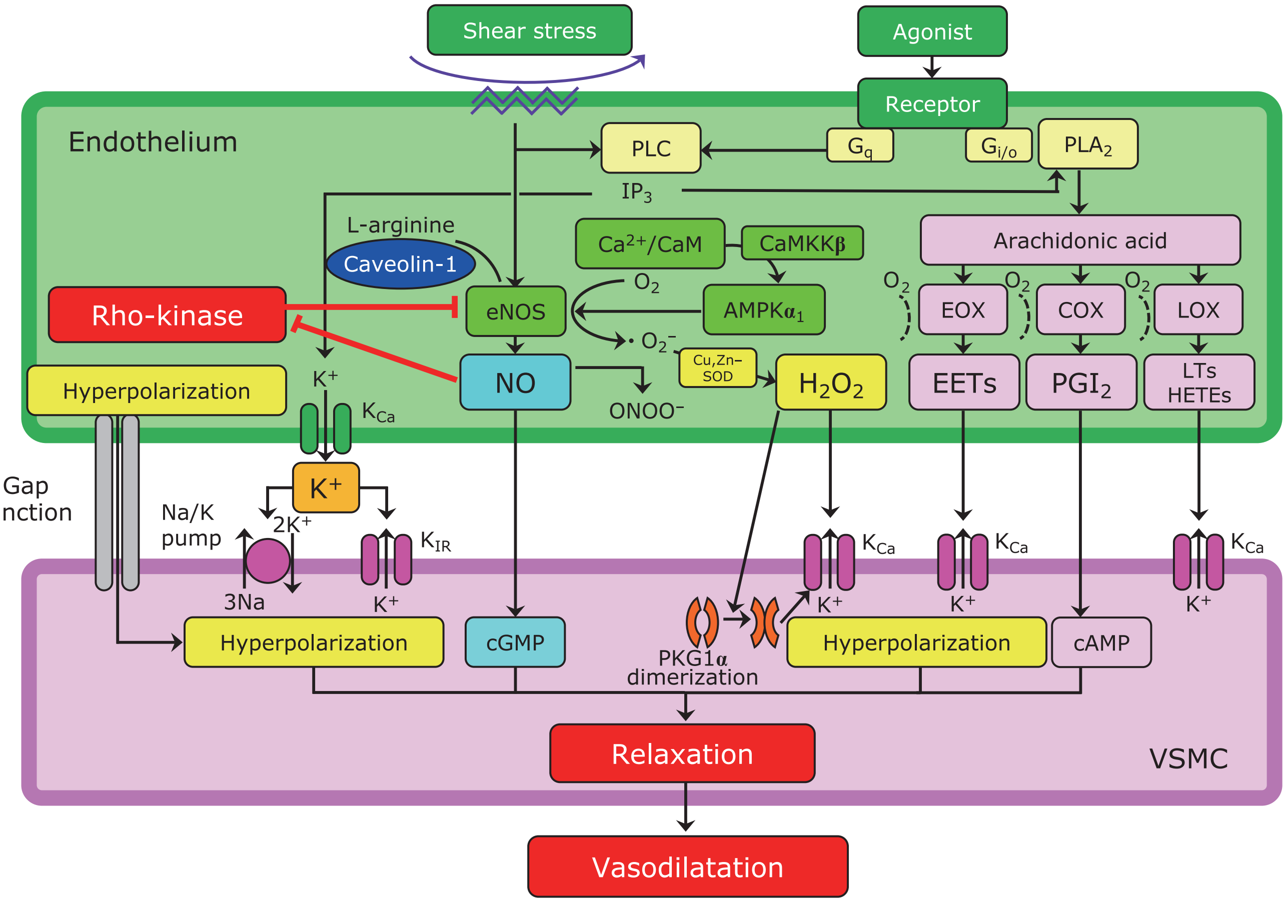

Fig. 1. Endothelium-derived relaxing factors. Endothelial cells synthesize and release nitric oxide and endothelium-dependent hyperpolarization (EDH) factors. Endothelium-derived H_2O_2 is one of the major EDH factors. AMPKα_1, α_1-subunit of AMP-activated protein kinase; CaM, calmodulin; CaMKKβ, Ca^{2+}/CaM-dependent protein kinase β; cAMP, cyclic AMP; cGMP, cyclic GMP; COX, cyclooxygenase; EETs, epoxyeicosatrienoic acids; eNOS, endothelial NO synthase; EOX, epoxygenase; HETEs, hydroxyeicosatetraenoic acids; H_2O_2, hydrogen peroxide; IP_3, inositol trisphosphate; K_{Ca}, calcium-activated potassium channel; K_{IR}, inwardly rectifying potassium channel; LOX, lipoxygenase; LTs, leukotrienes; NO, nitric oxide; $ONOO^-$, peroxynitrite; PGI_2, prostacyclin; PKG1α, 1α-subunit of protein kinase G; PLA_2, phospholipase A_2; PLC, phospholipase C; SOD, superoxide dismutase.

epoxyeicosatrienoic acids, metabolites of arachidonic P450 epoxygenase pathway,[16,17] electrical communication through gap junctions,[18] K^+ ions,[19] and as we demonstrated in 2000, endothelium-derived hydrogen peroxide (H_2O_2) (Fig. 1).[20,21] Indeed, endothelium-derived H_2O_2 at physiologically low concentrations is one of the major EDH factors in mouse and human small mesenteric arteries and human, porcine, and canine coronary arteries.[20–27] Thus, endothelium-derived H_2O_2 attracts increasing attention in view of its emerging relevance for cardiovascular disease.[1,2,20–29] In the clinical settings, it has been repeatedly reported that chronic nitrate therapy has neutral or even harmful effects in patients with cardiovascular diseases and that antioxidant treatments are also ineffective to prevent cardiovascular events.[30–33] These lines of evidence indicate the importance of the physiological balance between NO and EDH factors in maintaining cardiovascular homeostasis and in curing diseases associated with endothelial dysfunction.[1,11]

H_2O_2 is an important physiological signaling molecule serving especially in microcirculation, for blood pressure, coronary microcirculation, and metabolic functions.[25–27,34–36] Reactive oxygen species (ROS) have been considered to be harmful in general because of their highly-damaging effects on cells and tissues and pathological implications in various cardiovascular diseases, including atherosclerosis, hypertension, heart failure, and coronary artery disease.[34–37] However, as exemplified by endothelium-derived H_2O_2/EDH factor, a growing evidence has demonstrated that physiological levels of ROS can serve as crucial signaling molecules in health and disease.[38] The following 4 sets of early observations led us to hypothesize that a putative EDH factor might be a non-NO vasodilator substance (likely ROS) derived from endothelial NO synthases (NOSs) system. First, both NO-mediated and EDH-mediated responses are susceptible to vascular injuries caused by various atherosclerotic factors, and conversely, the treatment of those risk factors can restore both responses.[1,2,39] Second, it was previously demonstrated that endothelium-derived free radicals exert relaxing or contracting effects in an endothelium-dependent manner in canine coronary arteries.[40] Third, both endothelial NOS (eNOS)-derived NO generation and EDH-mediated responses are dependent on calcium/calmodulin.[41] Fourth, a simple molecule (like NO) rather than complex substances may be favorable in instantaneously modulating vascular tone in response to physiological demands in the body. Finally, in 2000, we were able to demonstrate for the first time that eNOS-derived H_2O_2 is an EDH factor in mouse mesenteric arteries, using eNOS-knockout (KO) mice.[20] Subsequently, this was also confirmed in other blood vessels, including human mesenteric and coronary arteries, porcine and canine coronary arteries, and piglet pial arterioles.[21–29,39,42,43]

Endothelial source of H_2O_2/EDH factor. Endothelium-derived H_2O_2 could be generated by the dismutation of superoxide anions, which are derived from various sources in the endothelium, including eNOS, NADPH oxidase, mitochondrial electron transport

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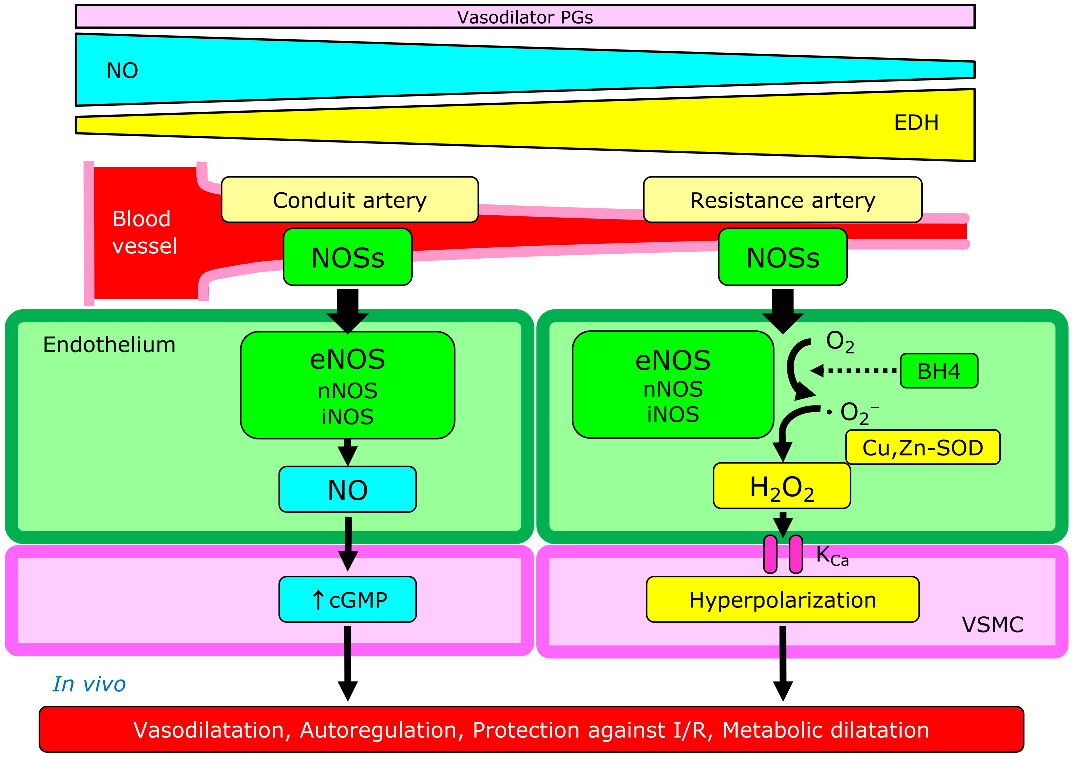

Fig. 2. Vessel size-dependent roles of endothelial nitric oxide synthases system. The contribution of EDRFs to endothelium-dependent vasodilatations markedly varies as a function of vessel size; endothelium-derived NO mainly mediates vasodilatation of relatively large, conduit vessels, while EDH factors that of resistance arteries. BH_4, tetrahydrobiopterin; cGMP, cyclic GMP; Cu,Zn-SOD, copper-zinc superoxide dismutase; eNOS, endothelial nitric oxide synthase; H_2O_2, hydrogen peroxide; iNOS, inducible NOS; I/R, ischemia-reperfusion; K_{Ca}, calcium-activated potassium channel; nNOS, neuronal NOS; NO, nitric oxide; NOSs, nitric oxide synthases; VSMC, vascular smooth muscle cells.

chain, xanthine oxidase, and lipoxygenase (Fig. 1).[44] There are 3 NOS isoforms; eNOS (NOS3), neuronal NOS (nNOS, NOS1), and inducible NOS (iNOS, NOS2). Using singly-eNOS-KO, doubly-n/eNOS-KO, and triply-n/i/eNOS-KO mice, we have previously demonstrated that EDH-mediated relaxations are progressively reduced as the number of deleted NOS genes increased.[45] Collectively, these results indicate that the 3 NOSs isoforms compensate each other to maintain H_2O_2-mediated EDH-type relaxations (Fig. 2). Thus, in large conduit vessels, NOSs mainly serve as a NO-generating system to cause soluble guanylate cyclase (sGC)-cyclic guanosine monophosphate (cGMP)-mediated vasodilatation, whereas in small resistance vessels, they act as a superoxide-generating system to evoke H_2O_2/EDH factor-mediated responses (Fig. 2).[45–47] In addition, among superoxide dismutase (SOD) isoforms, Cu,Zn-SOD plays a key role in the synthesis of H_2O_2/EDH factor in the endothelium (Fig. 1).[48] Indeed, eNOS produces superoxide anions under physiological conditions when synthesizing NO from L-arginine and oxygen, while Cu,Zn-SOD dismutates those superoxide anions into H_2O_2. Moreover, Cu,Zn-SOD-KO mice show markedly impaired EDH-mediated hyperpolarizations and relaxations in mesenteric arteries and coronary circulation without VSMC dysfunction.[48] Importantly, superoxide anions relevant to H_2O_2/EDH factor are not derived from pathologically uncoupled eNOS because H_2O_2-mediated EDH-type responses are not suppressed by NOS inhibitors and upregulation of eNOS co-factor tetrahydrobiopterin had no effects on the responses.[44] Other sources of superoxide anions in H_2O_2-mediated vasodilatation, such as mitochondrial respiratory chain and NADPH oxidase, have also been identified in human coronary arterioles.[49,50]

A recent study has demonstrated the potential regulatory mechanisms underlying the physiologically relevant H_2O_2 in the endothelium.[51,52] Indeed, 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 for cellular signaling and co-localization of the source and target of H_2O_2 may help to avoid non-specific harmful oxidations.[51,52] One good example of this notion is that only a minor increase in ROS caused by caveolar localization of NADPH oxidase-1 in hypertension is enough to interfere with NO-mediated signaling.[53]

Mode of action of H_2O_2/EDH factor. Oxidative modification of cGMP-dependent protein kinase G (PKG) is a central mechanism by which H_2O_2 induces hyperpolarization and relaxation of underlying VSMC,[43,54] although other modes of action of H_2O_2/EDH factor have also been proposed (Fig. 1).[55] Briefly, H_2O_2 induces dimerization of 1α-isoforms of PKG (PKG1α) through an interprotein disulfide bond formation between them to enhance the kinase activity through phosphorylation. The activated PKG1α subsequently stimulates K^+ channels with resultant hyperpolarization and vasodilatation in mouse mesenteric arteries and human coronary arterioles.[43,54,56] H_2O_2 also promotes the translocation of PKG1α from cytoplasm to membrane in human and porcine coronary arteries.[54,57] Such reversible post-translational modification, like phosphorylation, may be favorable for the fine control of vascular tone in response to demand fluctuation *in vivo*.[58]

Mechanisms for the dominant role of H_2O_2/EDH factor in microcirculation. Accumulating evidence has provided the mechanistic insights into vessel size-dependent contribution of NO and H_2O_2/EDH factor (Fig. 3). Previous studies have shown that pretreatment with NO donors attenuates EDH-mediated vasodilatation in porcine coronary arteries *in vitro* and canine coronary microcirculation *in vivo* and that NO exerts a negative-feedback effect on endothelium-dependent vasodilatation through

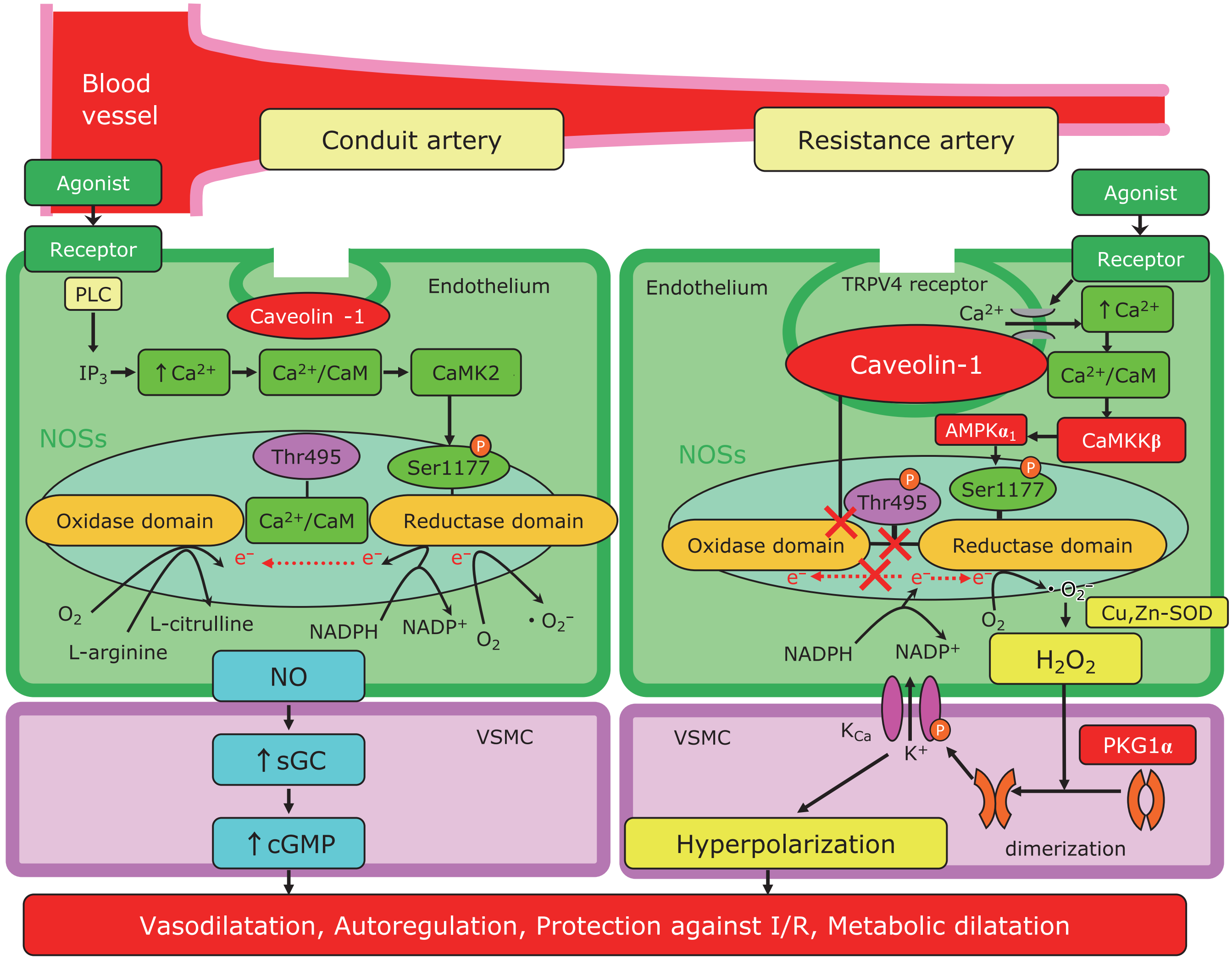

Fig. 3. Molecular mechanisms of enhanced H_2O_2/EDH factor-mediated responses in microvesseles. Multiple mechanisms are involved in the enhanced EDH-mediated responses in microvessels. AMPKα_1, α_1-subunit of AMP-activated protein kinase; CaM, calmodulin; CaMKKβ, Ca^{2+}/CaM-dependent protein kinase β; CaMK2, Ca^{2+}/CaM dependent protein kinase II; cGMP, cyclic GMP; Cu,Zn-SOD, copper-zinc superoxide dismutase; EDH, endothelium-dependent hyperpolarization; H_2O_2, hydrogen peroxide; IP_3, inositol trisphosphate; I/R, ischemia-reperfusion; K_{Ca}, calcium-activated potassium channel; NO, nitric oxide; NOSs, NO synthases; P, phosphorylation; PKG1α, 1α-subunit of protein kinase G; PLC, phospholipase C; sGC, soluble guanylate cyclase; TRPV4, transient receptor potential vanilloid 4, VSMC; vascular smooth muscle cells.

cGMP-mediated desensitization in canine coronary arteries *ex vivo*.[59–61] Multiple mechanisms have been proposed for the dominant role of H_2O_2/EDH factor in microcirculation (Fig. 3). Among them, cGMP-dependent activation of PKG desensitizes VSMC to H_2O_2 by inhibiting H_2O_2-induced PKG1α dimerization, a central mechanism of H_2O_2/EDH factor-mediated vasodilatation, and in turn, pharmacological inhibition of sGC sensitizes conduit vessels, but not resistance vessels, to H_2O_2-induced vasodilatation in mice.[62] Furthermore, mouse resistance vessels have less NO production and less antioxidant capacity, predisposing PKG1α to be more sensitive to H_2O_2-induced activation.[62,63] Other key players for the dominant role of H_2O_2/EDH factor in resistance vessels include endothelial caveolin-1 (a negative regulator of eNOS) and α_1-subunit of endothelial AMP-activated protein kinase (Fig. 3).[63,64] In contrast, phosphorylation at Tyr657 of eNOS in response to H_2O_2 leads to reduction in eNOS activity with resultant reduced NO production.[65] Taken together, these mechanisms are in line with the widely accepted notion that EDH-mediated responses function as a compensatory vasodilator system when NO-mediated relaxations are compromised.[1,2,11] It is important to maintain the vessel size-dependent contribution of NO and EDH factors because excessive endothelial NO production by either caveolin-1 deficiency or eNOS overexpression disrupts the physiological balance between NO and H_2O_2/EDH factors in endothelium-dependent vasodilatation, resulting in impaired cardiovascular homeostasis associated with enhanced nitrative stress in mice *in vivo*.[11,63,66]

Clinical significance of H_2O_2/EDH factor. Endothelium-derived H_2O_2 plays an important role in blood pressure regulation. Pharmacological inhibition of catalase, which decomposes H_2O_2 into O_2 and H_2O_2, decreases arterial blood pressure associated with enhanced PKG1α dimerization *in vivo*.[57] Moreover, the ‘redox-dead’ knock-in mice of Cys42Ser PKG1α, whose mutant PKG1α is unable to be activated by H_2O_2-induced dimerization due to the deletion in its redox-sensitive sulfur, exhibit markedly impaired EDH-mediated hyperpolarization and relaxation in resistance arteries associated with systemic arterial hypertension.[35] Furthermore, H_2O_2 has potent vasodilator properties in coronary resistance vessels and plays important roles in coronary autoregulation,[25] cardioprotection against myocardial ischemia/reperfusion

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injury,[26] and tachycardia-induced metabolic coronary vasodilatations[27] in dogs *in vivo*. Since coronary vascular resistance is mainly determined by the prearterioles and arterioles,[67] where the effect of EDH-mediated relaxations outweigh that of NO-mediated ones, it is important to maintain the vessel size-dependent contribution of NO and EDH factors for the treatment of coronary artery disease (CAD). Thus, endothelium-derived H_2O_2 functions as an important endogenous second messenger at its physiological low concentrations to elicit EDH-meditated vasodilatations and to maintain vascular homeostasis in the coronary circulation.[1,11,21,39,46,47]

Clinical Implications for Endothelial Functions (H_2O_2/EDH)

Endothelial function tests. Assessment of endothelial functions has been acknowledged as a useful surrogate marker of cardiovascular events in many clinical settings, although it is challenging to accurately assess EDH-mediated responses, especially in humans *in vivo*, because the contribution of EDH factors could be determined only after the blockade of both vasodilator prostaglandins and NO by its definition.[1,2] EDH-mediated vasodilatation can be enhanced to compensate for impaired NO-mediated responses in the early stage of atherosclerotic conditions.[13,21] However, after prolonged exposure to atherosclerotic risk factors, this compensatory role of EDH-mediated responses is finally disrupted to cause metabolic disturbance.[68] Indeed, endothelial dysfunction, as reflected by impaired flow-mediated dilatation (FMD) of the brachial artery or digital reactive hyperemia index (RHI) in peripheral arterial tonometry, is associated with future cardiovascular events in patients with coronary artery disease and one SD decrease in FMD or RHI is associated with doubling of cardiovascular event risk.[69]

H_2O_2/EDH factor and coronary artery disease. Previous studies focused structural and functional abnormalities of "epicardial" coronary arteries in CAD patients because they are easily visible on coronary angiography and amenable to procedural intervention (e.g., percutaneous coronary intervention). However, those of coronary microvasculature, referred to as coronary microvascular dysfunction (CMD), have recently attracted increasing attention due to their unexpectedly high prevalence and significant prognostic impacts in this population.[70] The etiologies of CMD still remain largely unknown and may be heterogeneous, for which several structural (e.g., vascular remodeling, vascular rarefaction, and extramural compression) and functional abnormalities (e.g., endothelial dysfunction, VSMC dysfunction, and microvascular spasm) have been demonstrated.[67,71] Given that H_2O_2 has potent vasodilator properties in coronary resistance vessels where EDH factors play relatively dominant roles than NO, it is highly possible that impaired H_2O_2/EDH factor-mediated vasodilatation is involved in the pathogenesis of CMD. Indeed, in eNOS-KO mice, CMD caused by reduced H_2O_2/EDH factor is substantially involved in the pathogenesis of cardiac diastolic dysfunction.[66] Thus, for the treatment of CAD, it is essential to maintain the physiological balance between NO and H_2O_2/EDH factor, which notion is supported by the fact that significant negative interactions exist between NO and several EDH factors and that nitrates as NO donors are not beneficial for the treatment of CMD.[11,63,66]

Lessons from clinical trials targeting NO: it is a time to change our mind. Although the role of CMD has been implicated in patients with obstructive CAD who underwent successful revascularization,[72] the effects of isosorbide-5-mononitrate were unexpectedly neutral in patients with microvascular ischemia despite successful percutaneous coronary intervention.[33] Besides CAD, recent studies highlighted the high prevalence and pathophysiological relevance of CMD in patients with heart failure with preserved ejection fraction (HFpEF).[73–75] Contrary to the premise that enhancing NO-mediated vasodilatation should exert beneficial effects on patients with HFpEF, the results of systemic and long-term administrations of inorganic nitrite in those patients were disappointing or even harmful in randomized clinical trials.[76] In a recent animal study, genetic ablation of endothelial arginase-1, an inhibitor of NO production, did not improve vasomotor function of resistance arteries in diabetic mice.[77] Similarly, antioxidant therapies for patients with cardiovascular diseases had no benefits.[78] These lines of evidence indicate that we need to change our mind to avoid excessive NO supplementation and to pay more attention to the potential harmful effects of non-specific elimination of ROS by antioxidants.[79,80] Multiple mechanisms may be involved in the failure of antioxidant therapies, including inadequate dose, short treatment duration, and pro-oxidant effects of antioxidants upon supplementation and thus so-called "antioxidant paradox" in clinical trials requires further investigations.[81] An alternative explanation for such "paradox" of NO-targeted therapy may be nitrosative stress induced by an excessive amount of NO,[63,81] again suggesting the importance of physiological balance between NO and EDH factors in endothelium-dependent vasodilatation. Further research is warranted to address how to modulate CMD to improve clinical outcomes of patients with cardiovascular diseases.

Roles of Rho-kinase in the Cardiovascular System

Molecular regulation of Rho-kinase. Rho-kinase (ROCKs) is an important downstream effector of the small GTP-binding protein RhoA (Fig. 4). During the past 20 years, significant progress has been made regarding the molecular mechanisms and therapeutic importance of Rho-kinase in cardiovascular medicine.[1,82–87] The Rho family of small G proteins includes 20 members of ubiquitously expressed proteins, including RhoA, Rac1, and Cdc42.[1,82–87] Among them, RhoA acts as a molecular switch that cycles between an inactive GDP-bound and an active GTP-bound conformation interacting with downstream targets (Fig. 4). RhoA is activated by the guanine nucleotide exchange factors (GEFs) that catalyze exchange of GDP for GTP and is inactivated by the GTPase activating proteins (GAPs).[88] There are 2 isoforms of Rho-kinase, Rho-kinase α/ROCK α/ROCK2 and Rho-kinase β/ROCK β/ROCK1, which were identified as the effector of Rho and have been shown to play important roles in the pathogenesis of cardiovascular diseases.[1,9,10] Phosphorylation of myosin light chain (MLC) is crucial for VSMC contraction. MLC is phosphorylated by Ca^{2+}/calmodulin-activated MLC kinase (MLCK) and is dephosphorylated by MLC phosphatase (MLCP) (Fig. 4).[89] Agonists bind to G-protein–coupled receptors and induce contraction by increasing both cytosolic Ca^{2+} concentration and ROCK activity through mediating GEF.[88–92] The substrates of ROCK include MLC, myosin phosphatase target subunit (MYPT)-1, ezrin/radixin/moesin family, adducin, phosphatase and tensin homolog, eNOS, Tau, and LIM-kinases (Fig. 4).[88–92] Recently, functional differences between ROCK1 and ROCK2 have been reported *in vitro*. ROCK1 is specifically cleaved by caspase-3, whereas granzyme B cleaves ROCK2.[93,94]

Negative interactions between NO and Rho-kinase. The RhoA/Rho-kinase pathway negatively regulates NO production in endothelial cells, while it enhances contraction of VSMC by MLC phosphorylation through inhibition of MYPT-1 of MLCP and promotes VSMC contraction (Fig. 4).[82–87] Rho-kinase has opposing activities in the regulation of the endothelial barrier function at the cell margins and contractile F-actin stress fibers.[95] Thus, disruption of the endothelial barrier results in increased endothelial permeability, promoting organ damage in various diseases.[1,86,87] The RhoA/Rho-kinase signaling pathway is involved in the mechano-transduction mechanism for the adherence junction strengthening at endothelial contacts.[96] This endothelial mechanosensing is important for endothelial alignment along the flow direction, which contributes to vascular homeostasis. Indeed, a disturbed flow promotes endothelial dysfunction and the development of atherosclerosis.[97,98] Several studies demonstrated that

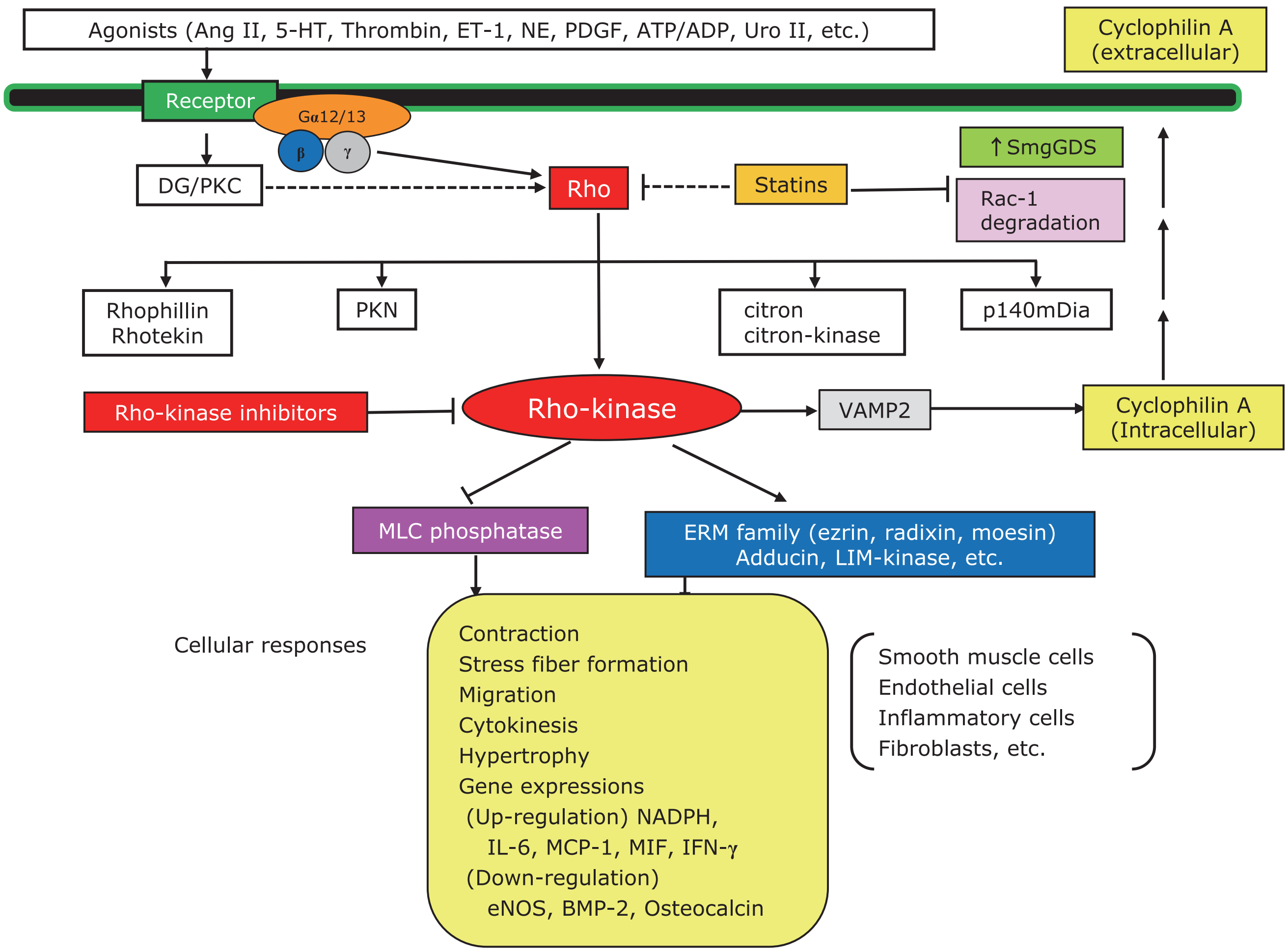

Fig. 4. Role of Rho/Rho-kinase pathway in the pathogenesis of cardiovascular diseases. Rho/Rho-kinase–mediated pathway plays an important role in the signal transduction initiated by many agonists, including angiotensin II (Ang II), serotonin (5-HT), thrombin, endothelin-1 (ET-1), norepinephrine (NE), platelet-derived growth factor (PDGF), adenosine triphosphate (ATP)/adenosine diphosphate (ADP), and urotensin II (Uro II). Through the modulation of its target effectors, Rho-kinase is substantially involved in vascular smooth muscle contraction (via inhibition of myosin phosphatase) and in the pathogenesis of arteriosclerosis (via activation of ERM, adducin, and other effectors). Whereas statins inhibit Rho at their relatively higher concentrations, they simultaneously inhibit pathways mediated by other G proteins, such as Ras and Rac. By contrast, Rho-kinase inhibitors selectively inhibit Rho-kinase pathway. Solid line indicates proven pathway and dashed line proposed pathway. DG, diacylglycerol; MLC, myosin light chain; PKC, protein kinase C; SmgGDS, small GTP-binding protein dissociation stimulator.

NO and Rho-kinase have opposing effects.[99,100] Rho-kinase-KO mice showed preserved endothelial functions in a diabetic model.[100] Moreover, NO and Rho-kinase exert opposing effects on the phosphorylation of AMP-activated protein kinase in lipid metabolism and the insulin receptor substrate-1 in insulin signaling.[101–103] Statins upregulate eNOS by cholesterol-independent mechanisms, involving the inhibition of Rho geranyl-geranylation and hydroxyfasudil reversed hypoxia-induced upregulation of Rho-kinase and eNOS downregulation in human endothelial cells.[104,105] In addition, small GTP-binding protein dissociation stimulator (SmgGDS) plays a central role in the pleiotropic effects of statins, independently of the Rho-kinase pathway, through Rac1 degradation (Fig. 4).[106] Thus, we need to consider the complex interactions between Rho-kinase and NO signaling for vascular homeostasis *in vivo*.

Role of Rho-kinase on vascular reactive oxygen species. The balance between oxidants and antioxidants maintains redox status equilibrium in the cardiovascular system.[107] The RhoA/Rho-kinase pathway is one of the major intracellular pathways that enhance the expressions of molecules for oxidative stress (NADPH, IL-6, MCP-1, MIF, and IFN-γ), thrombosis (PAI-1 and tissue factor), and tissue fibrosis (TGF-β1 and Bcl-2), while the pathway also markedly downregulates eNOS and osteogenesis-related molecules (BMP-2 and osteocalcin) (Fig. 1 and 4).[85–87,105,108–112] Oxidative stress by excessive ROS damages mitochondrial proteins and further increase intracellular ROS, thus forming a vicious cycle of ROS augmentation. In addition to ROS generation in mitochondria, several enzymes also generate intracellular ROS, including NADPH that produce O_2^- and H_2O_2. Importantly, enhanced Rho-kinase activity downregulates eNOS, resulting in impaired endothelial responses to NO and EDH (Fig. 1 and 4).[1,86,87] eNOS produces NO with resultant production of cGMP, and NO can react with O_2^- to produce peroxynitrite ($ONOO^-$).[113] Among ROS, H_2O_2 can easily penetrate the cell membrane and act as a second messenger. Peroxiredoxin is regenerated by the antioxidant protein thioredoxin 1 and reduces H_2O_2 levels, thus balancing the intracellular redox state.[114] The details of the interactions between Rho-kinase and NO/H_2O_2 as an EDH factor remain to be fully elucidated in future studies.

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Conclusions

This review highlights the potential importance of the physiological balance between NO and H_2O_2/EDH factor in a distinct vessel size-dependent manner through the diverse functions of endothelial NOSs system in maintaining cardiovascular homeostasis. It remains an open question how to improve endothelial functions without affecting the delicate balance between NO and EDH factors. Further characterization and better understanding of endothelium-dependent vasodilatations are important to this end, which helps us develop novel therapeutic strategies in cardiovascular medicine. The identification of Rho-kinase as an important mediator of oxidative stress in cardiovascular health and disease provides insight into the development of new therapies. Indeed, accumulating evidence indicates that Rho-kinase is substantially involved in the pathogenesis of a wide variety of cardiovascular diseases, suggesting that it is an important therapeutic target in cardiovascular medicine.

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Abbreviations

BMP-2	bone morphogenetic protein-2
cGMP	cyclic guanosine monophosphate
CMD	coronary microvascular dysfunction
EDH	endothelium-dependent hyperpolarization
EDRF(s)	endothelium-derived relaxing factor(s)
eNOS	endothelial nitric oxide synthase
GAPs	GTPase activating proteins
GEFs	guanine nucleotide exchange factors
H_2O_2	hydrogen peroxide
MCP-1	macrophage chemoattractant protein-1
MIF	macrophage inhibitory factor
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MYPT-1	myosin phosphatase target subunit-1
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
PAI-1	plasminogen activator inhibitor-1
PKG	cGMP-dependent protein kinase
ROS	reactive oxygen species
sGC	soluble guanylate cyclase
SmgGDS	small GTP-binding protein dissociation stimulator
SOD	superoxide dismutase
TGF-β1	transforming growth factor-β1
VSMC	vascular smooth muscle cell

Conflict of Interest

No potential conflicts of interest were disclosed.

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