The interaction between endothelial cells (ECs) and vascular smooth muscle cells (VSMC) plays an important role in regulating cardiovascular homeostasis. ECs release vasoactive factors, such as prostacyclin, nitric oxide (NO), and endothelium-derived hyperpolarizing (EDH) factors, which participate in the regulation of vascular tone and resistance.1–3 Twenty years ago, Rho-kinases (Rho-kinase α/ROKα/ROCK2 and Rho-kinase β/ROKβ/ROCK1) were identified as the effectors of the small GTP-binding protein, RhoA, independently by 3 research groups.4–6 Hereafter, both Rho-kinase α/ROKα/ROCK2 and Rho-kinase β/ROKβ/ROCK1 are collectively referred to as Rho-kinase.7,8 Both endothelial NO production and NO-mediated signaling in VSMC are targets and effectors of the RhoA/Rho-kinase pathway. In EC, the RhoA/Rho-kinase pathway negatively regulates NO production. On the contrary, the pathway regulates contraction in VSMC and promotes the development of vascular remodeling.9–12 In addition, we recently demonstrated the Rho-kinase inhibition in the developing heart results in the development of arrhythmogenic right ventricular cardiomyopathy (ARVC).13 Herein, we will review the recent advances about the importance of RhoA/Rho-kinase in the cardiovascular system.

Molecular Roles and Regulation of Rho-Kinase in the Cardiovascular System

During the past 20 years, significant progress has been made in understanding of the molecular mechanisms and therapeutic importance of Rho-kinase in the cardiovascular system. The Rho family of small G proteins comprises 20 members of ubiquitously expressed proteins in mammals, including RhoA, Rac1, and Cdc42.2,14 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 (Figure 1).15 The activity of RhoA is controlled by the guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP.16 In contrast, GTPase-activating proteins stimulate the intrinsic GTPase activity and inactivate RhoA.17 Guanine nucleotide dissociation inhibitors block spontaneous RhoA activation (Figure 1).18

Rho-kinase plays important roles in many intracellular signaling pathways.7,8 Agonists bind to G-protein–coupled receptors and induce contraction by increasing both cytosolic Ca2+ concentration and Rho-kinase activity19 through GEF activation.20 Rho-kinase activity is enhanced by binding to the active GTP-bound RhoA.4 The substrates of Rho-kinase include myosin light chain (MLC), myosin phosphatase target

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Abstract: Twenty years ago, Rho-kinase was identified as an important downstream effector of the small GTP-binding protein, RhoA. Thereafter, a series of studies demonstrated the important roles of Rho-kinase in the cardiovascular system. The RhoA/Rho-kinase pathway is now widely known to play important roles in many cellular functions, including contraction, motility, proliferation, and apoptosis, and its excessive activity induces oxidative stress and promotes the development of cardiovascular diseases. Furthermore, the important role of Rho-kinase has been demonstrated in the pathogenesis of vasospasm, arteriosclerosis, ischemia/reperfusion injury, hypertension, pulmonary hypertension, and heart failure. Cyclophilin A is secreted by vascular smooth muscle cells and inflammatory cells and activated platelets in a Rho-kinase–dependent manner, playing important roles in a wide range of cardiovascular diseases. Thus, the RhoA/Rho-kinase pathway plays crucial roles under both physiological and pathological conditions and is an important therapeutic target in cardiovascular medicine. Recently, functional differences between ROCK1 and ROCK2 have been reported in vitro. ROCK1 is specifically cleaved by caspase-3, whereas granzyme B cleaves ROCK2. However, limited information is available on the functional differences and interactions between ROCK1 and ROCK2 in the cardiovascular system in vivo. Herein, we will review the recent advances about the importance of RhoA/Rho-kinase in the cardiovascular system. (Circ Res. 2016;118:352-366. DOI: 10.1161/CIRCRESAHA.115.306532.)

Key Words: cardiovascular system ▪ GTP-binding protein ▪ inflammation ▪ oxidative stress ▪ rho-associated kinases

The interaction between endothelial cells (ECs) and vascular smooth muscle cells (VSMC) plays an important role in regulating cardiovascular homeostasis. ECs release vasoactive factors, such as prostacyclin, nitric oxide (NO), and endothelium-derived hyperpolarizing (EDH) factors, which participate in the regulation of vascular tone and resistance.1–3 Twenty years ago, Rho-kinases (Rho-kinase α/ROKα/ROCK2 and Rho-kinase β/ROKβ/ROCK1) were identified as the effectors of the small GTP-binding protein, RhoA, independently by 3 research groups.4–6 Hereafter, both Rho-kinase α/ROKα/ROCK2 and Rho-kinase β/ROKβ/ROCK1 are collectively referred to as Rho-kinase.7,8 Both endothelial NO production and NO-mediated signaling in VSMC are targets and effectors of the RhoA/Rho-kinase pathway. In EC, the RhoA/Rho-kinase pathway negatively regulates NO production. On the contrary, the pathway regulates contraction in VSMC and promotes the development of vascular remodeling.9–12 In addition, we recently demonstrated the Rho-kinase inhibition in the developing heart results in the development of arrhythmogenic right ventricular cardiomyopathy (ARVC).13 Herein, we will review the recent advances on the importance and regulation of Rho-kinase in the cardiovascular system.
subunit (MYPT)-1, ezrin/radixin/moesin family, adducin, phosphatase and tensin homolog, endothelial NO synthase (eNOS), Tau and LIM-kinase (Figure 1).21 MLC is crucial for VSMC contraction, which is phosphorylated by Ca2+/calmodulin-activated MLC kinase (MLCK) and is dephosphorylated by MLC phosphatase (MLCP; Figure 2).22

**Functional Differences Between ROCK1 and ROCK2**

Rho-kinase is a serine/threonine kinase with a molecular weight of ≈160 kDa.7,8 Two isoforms of Rho-kinase encoded by 2 different genes have been identified.4,23,24 In humans, ROCK1 and ROCK2 genes are located separately on chromosome 18 and chromosome 2, respectively. ROCKs consist of 3 major domains, including a kinase domain in the N-terminal domain, a coiled-coil domain that includes a Rho-binding domain, and a pleckstrin homology domain, a coiled-coil domain that includes a Rho-binding domain.37,38 The small G-protein RhoE specifically binds to the N-terminal region of ROCK1 at the kinase domain, whereas the MYPT-1 binds to ROCK2.39,40 RhoE binding to ROCK1 inhibits its activity and prevents RhoA binding to the Rho-binding domain.32 For active cell movement, ROCK1 must be catalytically active and localized to the plasma membrane. RhoA is critical for the recruitment of ROCK1 to the plasma membrane.41 In addition, Pinner et al42 demonstrated that phosphoinositide-dependent protein kinase 1 is required for the function of ROCK1. Phosphoinositide-dependent protein kinase 1 binds to and blocks the function of ROCK1 but not that of ROCK2 (Figure 1).7,8 The small G-protein RhoE specifically binds to the N-terminal region of ROCK1 and inhibits its activity. ROCK1 and ROCK2 are upregulated by angiotensin II (AngII) via AT1 receptor stimulation and by interleukin-1β.30 Functional differences between ROCK1 and ROCK2 have been reported. ROCK1 is specifically cleaved by caspase-3, whereas granzyme B cleaves ROCK2 (Figure 3).31,32 During the development of erythroblasts, ROCK1 is activated by caspase-3–mediated cleavage, allowing terminal maturation through phosphorylation of the light chain of myosin II.33 Granzyme B is a serine protease expressed in the granules of cytotoxic lymphocytes, basophils, mast cells, and VSMCs.34 Granzyme B induces inflammation by cytokine release and contributes to the extracellular matrix remodeling. Thus, granzyme B–mediated activation of ROCK2 may be involved in cardiovascular homeostasis and diseases. Rho proteins negatively regulate the RhoA/Rho-kinase signaling to the cytoskeleton.35,36 Specifically, RhoE (Rnd3) can bind to and block the function of ROCK1 but not that of ROCK2 (Figure 1).7,8 The small G-protein RhoE specifically binds to the N-terminal region of ROCK1 and inhibits its activity. Thus, when RhoE is present and phosphoinositide-dependent protein kinase 1 is absent, RhoA-GTP does not induce prolonged activation of ROCK1 at the plasma membrane.42 Many Rho-kinase substrates have been identified,43 and ROCK2–mediated substrate phosphorylation causes actin filament formation, organization, and cytoskeleton rearrangement (Figure 1).44 The N-terminal regions, upstream of the kinase domains of ROCKs, may play a role in determining substrate specificity of the 2 Rho-kinase isoforms (Figure 3).44

**Opposing Effects of NO and Rho-Kinase in EC Function**

In EC, the RhoA/Rho-kinase pathway negatively regulates NO production, whereas in VSMC, the pathway enhances MLC phosphorylation through inhibition of MYPT-1 of MLCP and promotes VSMC contraction (Figures 2 and 4). The RhoA/Rho-kinase pathway is critically involved in actin dynamics.45 Cyclic strain stimulates RhoA activation and enhances cell contractility. Mechanical activation of the RhoA/Rho-kinase system renders cells more sensitive to external stimuli.46 Thus, RhoA/Rho-kinase–mediated actin contractility may contribute to vascular function as a mechanosensor. Rho-kinase has opposing activities in the regulation of the endothelial barrier function at the cell margins and contractile F-actin stress fibers.47 On the contrary, disruption of the endothelial barrier could lead to increased endothelial permeability,48 promoting organ damage in various diseases.49,50 The quantity of pinocytic vesicles and permeability in EC are regulated by the expression and phosphorylation of caveolin-1 and caveolin-2 in EC, as well as the levels of p-Src and...
the activity of RhoA/Rho-kinase signaling. Thus, the RhoA/Rho-kinase signaling pathway is involved in the mechano-transduction mechanism involved in the adherence junction strengthening at EC–EC contacts (Figure 4). This endothelial mechanosensing is required for EC alignment along the flow direction, which contributes to vascular homeostasis.

Indeed, a disturbed flow promotes EC dysfunction and the development of atherosclerosis.

Several reports demonstrated that NO and Rho-kinase have opposing effects. Rho-kinase–deficient mice revealed preserved EC function in a diabetic model. Moreover, a Rho-kinase inhibitor, fasudil, significantly enhanced the...
phosphorylation of AMP-activated protein kinase and changed lipid metabolism.57,58 Statins upregulate eNOS by cholesterol-independent mechanisms, involving the inhibition of Rho geranyl-geranylation.59 In addition, small GTP-binding protein dissociation stimulator plays a central role in the pleiotropic effects of statins, independently of the Rho-kinase pathway.60 On the basis of these recent findings, we need to consider the complex interactions between Rho-kinase and NO signaling for endothelial homeostasis in vivo (Figure 4).

Role of Rho-Kinase on Vascular Reactive Oxygen Species

The balance between oxidants and antioxidants maintains redox status equilibrium in the cardiovascular system.61 We...
previously demonstrated that endothelium-derived hydrogen peroxide (H$_2$O$_2$) is an EDH factor in animals and humans (Figures 2 and 4).62–64 In contrast, excessive reactive oxygen species (ROS; oxidative stress) damage mitochondrial proteins and further increase intracellular ROS, thus forming a vicious cycle of ROS augmentation. In addition to ROS generation in mitochondria, several enzymes generate intracellular ROS, including nicotinamide adenine dinucleotide phosphate oxidases (Nox) that produce O$_2^−$ and H$_2$O$_2$. Importantly, the production of endothelial H$_2$O$_2$ for EDH responses largely depends on eNOS functions.64,65 Enhanced Rho-kinase activity downregulates eNOS, resulting in impaired endothelial responses to NO and EDH (Figure 4).2,3,14 eNOS produces NO with the resultant production of cyclic GMP (cGMP). NO can react with O$_2^−$ to produce peroxynitrite (ONOO$^-$).66 Among ROS, H$_2$O$_2$ can easily penetrate the cell membrane and act as a second messenger. Peroxiredoxin is regenerated by the antioxi- dant protein thioredoxin 1 and reduces H$_2$O$_2$ levels, thus balancing the intracellular redox state.67 Thioredoxin 1 also functions as a signaling intermediate that can sense redox state imbalances.61 Here, fluid shear stress plays a crucial role in the regulation of EC stress fiber formation with decreased stress fibers in areas of disturbed flow when compared with steady flow areas.68 Importantly, stress fibers are critical for several EC functions, including cell shape, mechanosignal transduction, EC–EC junction integrity,69 and inflammation.70,71 A key mediator of steady flow–induced stress fiber formation is Src, which regulates downstream signaling mediators such as focal adhesion kinase72 and small GTPases.69,73

The dual roles of ROS, particularly H$_2$O$_2$, as both protective and pathological agents, are important in vascular homeostasis.74 At low concentrations, H$_2$O$_2$ plays an important role in endothelial functions and vascular relaxation. Endothelium-dependent relaxation is mediated primarily by prostacyclin, NO, and EDH factor (Figures 2 and 4).2,50,75–77 The contribution of H$_2$O$_2$ to EDH-dependent vasodilation of resistance vessels64–66 can be attributed to the oxidation of protein kinase G, subunit 1α in VSMCs (Figures 2 and 4).78 EDH responses are more prevalent in resistance than in conduit blood vessels.2,50,76,79 Burgoyne et al.80 demonstrated that PKG activation depends on the oxidation mechanism, where the homodimer complex forms an interprotein disulfide bond. In EC, PKG activity is also regulated by intracellular cGMP levels, which can be modified by NO produced by shear stress and agonists such as bradykinin, acetylcholine, and adenosine.81 The mechanism of H$_2$O$_2$-induced hyperpolarization is complex and varies depending on the type of blood vessels. For example, Ca$^{2+}$/calmodulin-dependent protein kinase kinase β and caveolin-1 in EC and protein kinase G, subunit 1α in VSMC play substantial roles for the enhanced EDH-mediated responses in murine microvessels (Figure 4).82 Bone marrow and adiponecin derived from adipose tissues also contribute to the modulation of microvascular EDH responses.83 The role of H$_2$O$_2$ as an EDH factor has led to extensive research on the importance and complexity of endothelium-derived relaxing factors.

**Roles of Rho-Kinase in VSMC Function**

When agonists bind to their receptors, phospholipase C is activated, leading to the formation of inositol 1,4,5-triphosphate and diacylglycerol by the hydrolysis of phosphatidyl-inositol 4,5-bis-phosphate (Figure 2).84 1,4,5-triphosphate then binds to an 1,4,5-triphosphate receptor on the membrane of the sarcoplasmic reticulum to mobilize the stored calcium ions (Ca$^{2+}$) from the sarcoplasmic reticulum into the cytosol. Diacylglycerol activates protein kinase C, which causes vasoconstriction and augments the Ca$^{2+}$ sensitivity of contractile proteins.85 Several mechanisms are involved in the Ca$^{2+}$ sensitivity of myosin filaments, including myosin phospha- tase$^2$ and the small GTPase Rho and its target, Rho-kinase (Figure 2).7,19

Phosphorylation of the regulatory MLC activates myo- sin Mg$^{2+}$-ATPase and permits cross-bridge cycling, which leads to force generation and contraction.22 The level of MLC phosphorylation is determined by a balance between MLC phosphorylation by MLCK and dephosphorylation by MLCP (Figure 2).82 Phosphorylation of the second site of MLC is known to further increase the actin-activated Mg$^{2+}$-ATPase activity of myosin in vitro.65,67 These results indicate that enhanced MLC phosphorylation plays a central role in the aug- mentation of vascular tone. The phosphorylated site of MLC is MLCK-dependent Ser19 for MLC monophosphorylation and MLCK-dependent Ser19/Thr18 for MLC diphosphorylation.88

Phenotype modulation of VSMC (from contractile type to synthetic type) has been demonstrated in the neointimal regions of the atherosclerotic artery.89–91 In cultured VSMC, MLC diphosphorylation is higher in actively growing cells than in growth-arrested cells.87 Thus, phenotype changes of arterial VSMC may be an important mechanism of cardio-vascular diseases. The generation of diphosphorylated MLC is caused, in part, by MLCP inhibition in VSMC.92 In vitro studies demonstrated that a GTP-binding protein regulates the receptor-mediated sensitization of MLC phosphorylation,93 and that small GTPase Rho is involved in GTP-enhanced Ca$^{2+}$ sensitivity of VSMC contraction.94,95,96 Recent studies further demonstrated that Rho regulates MLC phosphorylation through its target, Rho-kinase, and the MYPT-1 of MLCP.7,8 Smooth muscle MLCP consists of a 38-kDa catalytic subunit, 130-kDa MYPT-1, and a 20-kDa subunit.97,98 Activated Rho-kinase subsequently phosphorylates MYPT-1, thereby inactivating MLCP (Figure 2).7 Rho-kinase itself might also phosphorylate MLC at the site phosphorylated by MLCK and activate myosin ATPase in vitro.8 The activated form of Rho-kinase enhances the transcriptional regulation of serum response factor97 and induces VSMC contractility98 and stress fiber formation.99 Some studies suggest that both inhibition of MLCP and direct phosphorylation of MLC contribute to the increase in MLC phosphorylation.99 Rho-kinase has been implicated in the pathogenesis of cardiovascular diseases, in part, by promoting VSMC proliferation.100–102 Changes in the vascular redox state are a common pathway involved in the pathogenesis of atherosclerosis, aortic aneurysm, and vascular stenosis. Vascular ROS formation can be stimulated by mechanical stretch, pressure, shear stress, environmental factors (eg, hypoxia), and growth factors (eg, AngII).103 Importantly, Rho-kinase is substantially involved in the vascular effects of various vasoactive factors, including AngII,104 thrombin,105 platelet-derived growth factor,106 extracellular nucleotides,107...
Physiological and Pathological Roles of Rho-Kinase in the Cardiovascular System

Cardiovascular diseases often result from imbalances in the levels of intracellular ROS. The O$_2^-$-producing oxidases in the vascular system, including eNOS, cyclooxygenase, lipoxygenase, P-450 monooxygenase, and nicotinamide adenine dinucleotide phosphate oxidases, can be stimulated to produce excessive ROS (oxidative stress) by external stimuli, such as mechanical stretch, pressure, shear stress, and hypoxia, and by humoral factors, such as AngII. In this process, transient receptor potential (TRP) channels also substantially contribute to the ROS augmentation in response to external stimuli. A class of TRP channels works as sensors of ROS and gaseous messenger molecules, including oxygen (O$_2$), hydrogen sulfide (H$_2$S), and carbon dioxide (CO$_2$). H$_2$O$_2$ triggers the production of ADP-ribose, which activates TRPM2. TRPC5, TRPV1, and TRPA1 are also activated by H$_2$O$_2$. NO regulates TRP channels via cGMP/PKG-dependent phosphorylation. Excessive ROS target multiple biomolecules, causing numerous cellular complications, including lipid peroxidation, protein oxidation/inactivation, and DNA damage/mutations. Furthermore, increased O$_2^-$ levels attenuate endothelium-dependent relaxation and enhance VSMC contraction through the formation of hydroxyl radicals. Although H$_2$O$_2$ is important for vascular homeostasis at physiological low concentrations, excessive ROS are hazardous to the cells, leading to endothelial dysfunction and VSMC proliferation.

Recent evidence suggests that many other stimuli that modulate VSMC functions, including ROS, promote VSMC proliferation by inducing autocrine/paracrine growth mechanisms. Rho-kinase augments inflammation by inducing proinflammatory molecules, including interleukin-6, monocyte chemoattractant protein-1, and sphingosine-1-phosphate. In EC, Rho-kinase downregulates eNOS and substantially activates proinflammatory pathways, including enhanced expression of adhesion molecules. The expression of Rho-kinase is accelerated by inflammatory stimuli, such as AngII and interleukin-1β, and by remnant lipoproteins in human coronary VSMC. Rho-kinase also upregulates NAD(P)H oxidases (Nox1, Nox4, gp91phox, and p22phox) and augments AngII-induced ROS production.

Several growth factors are secreted by VSMC in response to oxidative stress. Among them, CyPA has been identified as a protein that is secreted by VSMC, inflammatory cells, and activated platelets in a Rho-kinase–dependent manner (Figure 4). ROS activate a pathway containing vesicles, resulting in CyPA secretion. Secreted extracellular CyPA stimulates extracellular signal-regulated kinase 1/2, Akt, and JAK in VSMC, contributing to ROS production and creating a vicious cycle of ROS augmentation. CyPA is secreted by VSMC via a highly regulated pathway that involves vesicle transport and plasma membrane binding (Figure 4). Rho GTPases, including RhoA, are key regulators in signaling pathways linked to actin cytoskeletal rearrangement. RhoA plays a central role in vesicular trafficking pathways by controlling the organization of the actin cytoskeleton. The active participation of Rho GTPases is required for secretion. Myosin II is involved in secretory mechanisms as a motor for vesicle transport. Rho-kinase mediates myosin II activation via phosphorylation and inactivation of myosin II light chain phosphoryase. These results suggest that myosin II–mediated vesicle transport is required for CyPA secretion from VSMC in a Rho-kinase–dependent manner. CyPA is transported to the plasma membrane and colocalizes with VAMP2 (vesicle-associated membrane protein) in response to ROS stimulation (Figure 4).
In addition to the effects on vascular cells, CyPA has been shown to be a direct chemoattractant for inflammatory cells, promoting matrix metalloproteinases (MMPs) activation. All of these roles of CyPA can also be explained by the activation of Rho-kinase in the cardiovascular system (Figure 4). CyPA plays an important role as a Ca²⁺ regulator in platelets. Moreover, extracellular CyPA activates platelets via basigin (CD147)–mediated phosphoinositide-3-kinase/Akt signaling, leading to enhanced adhesion and thrombus formation. Moreover, thrombin suppresses eNOS in EC via Rho-kinase pathway. Thus, CyPA and Rho-kinase function in concert, leading to the development of vascular diseases. Indeed, CyPA may be a key mediator of Rho-kinase that generates a vicious cycle of ROS augmentation, affecting EC, VSMC, and inflammatory cells (Figure 4).

Importantly, CyPA plays a crucial role in the translocation of Nox enzymes, such as p47phox, contributing to VSMC proliferation and vascular diseases. Because ROS production by Nox enzymes activates other oxidase systems, CyPA and Nox enzymes amplify ROS formation in a synergistic manner, leading to augmentation of oxidative stress. In addition, CyPA secretion from VSMC requires ROS production, RhoA/Rho-kinase activation, and vesicle formation. Thus, both intracellular and extracellular CyPA contribute to ROS production in a 3-legged race with Rho-kinase activation. Furthermore, basigin has been identified as an extracellular receptor for CyPA in inflammatory cells and VSMC. Further knowledge of the extracellular CyPA receptors on vascular cells will contribute to the development of novel therapies for cardiovascular diseases.

Furthermore, the identification of CyPA as a mediator of oxidative stress-induced tissue damage provided some additional insight into the mechanisms of several therapies. For example, Rho-kinase inhibitor and simvastatin significantly reduce CyPA secretion from VSMC. Indeed, Rho-kinase is an important therapeutic target in cardiovascular diseases. On the basis of role of extracellular CyPA, we think that it is logical to consider that agents that prevent CyPA receptor binding and reduce circulating CyPA may have therapeutic potentials. Blocking the vicious cycle that increases ROS production through autocrine/paracrine CyPA signaling pathway mediated by Rho-kinase could be a novel therapeutic tool for controlling cardiovascular diseases (Figure 4).

Rho-Kinase in Systemic and PH
Rho-kinase–mediated Ca²⁺ sensitization is involved in the pathophysiology of hypertension. Short-term administration of Y-27632, another Rho-kinase inhibitor, preferentially reduces systemic blood pressure in a dose-dependent manner in a rat model of systemic hypertension, suggesting an involvement of Rho-kinase in the pathogenesis of increased systemic vascular resistance in hypertension. The expression of Rho-kinase is significantly increased in resistance vessels of spontaneously hypertensive rats. Rho-kinase is also involved in the central mechanisms of sympathetic nerve activity.

Rho-kinase may also be involved in the pathogenesis of PH as it is associated with hypoxic exposure, endothelial dysfunction, VSMC proliferation, enhanced ROS production, and inflammatory cell migration. Chronic exposure to hypoxia induces vascular remodeling in mice. We demonstrated that pulmonary vascular dysfunction plays a crucial role in the development of hypoxia-induced PH, for which Rho-kinase plays a crucial role. Rho-kinase promotes CyPA secretion from VSMC, and extracellular CyPA stimulates VSMC proliferation in vitro and in vivo (Figure 4). Extracellular CyPA induces EC adhesion molecule expression and apoptosis and is a chemoattractant for inflammatory cells. Thus, extracellular CyPA may contribute to hypoxia-induced PH. Long-term treatment with fasudil suppresses the development of monocrotaline-induced PH in rats and hypoxia-induced PH in mice. On the contrary, statins and Rho-kinase inhibitor reduce the secretion of CyPA from VSMCs and pravastatin ameliorates hypoxia-induced PH in mice. Thus, the inhibition of CyPA secretion by statins or Rho-kinase inhibitors may be involved in the therapeutic effects of these medications on PH. Furthermore, we recently demonstrated the crucial role of ROCK2 in the development of hypoxia-induced PH using VSMC-specific ROCK2 knockout mice. Consistently, we observed Rho-kinase activation in patients with pulmonary arterial hypertension (PAH). Furthermore, fasudil significantly reduced pulmonary vascular resistance in patients with PAH.

Chronic hypoxia significantly increased ROCK2 expression and ROCK activity in the lung tissues from wild-type mice. The development of PH and RV hypertrophy caused by chronic hypoxia in vivo was evident in wild-type mice, but was suppressed in VSMC-specific ROCK2 knockout mice. Because CyPA secretion is regulated by Rho-kinase, we further determined whether CyPA contributes to the development of PH in mice and humans. Importantly, we demonstrated that extracellular CyPA and its receptor, basigin (Bsg, CD147), are crucial for hypoxia-induced PH. In addition, PH severity was exacerbated in Bsg⁺/⁻ versus Bsg⁺/⁺ mice. Mechanistic studies demonstrated that Bsg⁻/⁻ VSMCs secreted less cytokines/chemokines and growth factors (eg, platelet-derived growth factor-BB). On the basis of these findings, we proposed a novel mechanism for hypoxia-induced PH in which hypoxia induces growth-promoting genes in VSMCs through a CyPA/Bsg-dependent pathway (Figure 4).

These results suggest that extracellular CyPA and vascular Bsg are crucial for PH development and could be potential therapeutic targets. Intravenous injection of many different Rho-kinase inhibitors reduces systemic and pulmonary arterial pressure even under resting conditions. Furthermore, we demonstrated that the combination therapy using fasudil and sildenafil showed synergistic effects through inhibition of Rho-kinase activity for the treatment of PH in rats. Indeed, we obtained direct evidence of Rho-kinase activation in patients with PAH. Finally, both intravenous infusion and oral administration of fasudil significantly reduced pulmonary vascular resistance in patients with PAH, indicating an involvement of Rho-kinase and its downstream signaling in the pathogenesis of PAH in humans.

Rho-Kinase in Vascular Diseases
Rho-kinase plays a crucial role in ROS augmentation and vascular inflammation. ROS are involved in the pathogenesis
of neointima formation, in part, by promoting VSMC growth and stimulating proinflammatory events. Accumulating evidence indicates that Rho-kinase–mediated pathway is substantially involved in EC dysfunction. VSMC hypercontraction, VSMC proliferation and migration in the media, and accumulation of inflammatory cells in the adventitia. These Rho-kinase–mediated cellular responses lead to the development of vascular disease. In fact, mRNA expression of ROCKs is enhanced in the inflammatory and arteriosclerotic arterial lesions in animals and humans. In the context of atherosclerosis, Rho-kinase should be regarded as a proinflammatory and proatherogenic molecule. Indeed, recent studies demonstrated that ROCK inhibition by statins could lead to improved endothelial function and decreased atherosclerosis.

Rho-kinase plays a crucial role in the pathogenesis of coronary artery spasm. Coronary spasm plays an important role in variant angina, myocardial infarction, and sudden death. Long-term treatment with cortisol, one of the important stress hormones, causes coronary hyper-reactivity through the activation of Rho-kinase in pigs in vivo. The activity and the expression of Rho-kinase are enhanced at the inflammatory/arteriosclerotic coronary lesions. Intracoronary administration of fasudil and hydroxyfasudil inhibits coronary spasm in a porcine model. To further elucidate the molecular mechanism of coronary spasm in our porcine model, experiments were performed to examine whether Rho-kinase is upregulated at the spastic site and how it induces VSMC hypercontraction if it is upregulated. Reverse transcriptase polymerase chain reaction analysis demonstrated that the expression of Rho-kinase mRNA and, to a lesser extent, that of RhoA mRNA was upregulated in the spastic site than the control coronary site. Western blot analysis showed that, during the serotonin-induced contractions, the extent of MYPT-1 phosphorylation was significantly greater in the spastic site than in the control site. Furthermore, another Rho-kinase inhibitor, Y-27632, also inhibited not only serotonin-induced contractions in vivo and in vitro but also the increase in MYPT-1 phosphorylation. Importantly, there was a highly significant positive correlation between the extent of MYPT-1 phosphorylation and that of contractions in the spastic site, but not in the control site. These results indicate that Rho-kinase is upregulated at the spastic site and plays a key role in inducing VSMC hypercontraction by inhibiting MLCP through MYPT-1 phosphorylation (Figure 1). Hydroxyfasudil causes dose-dependent inhibition of serotonin-induced coronary spasm both in vitro and in vivo in the porcine model through suppression of serotonin-induced increases in MLC mono- and diphosphorylation. Thus, the hydroxyfasudil-sensitive Rho-kinase–mediated pathway plays an important role in the enhanced MLC phosphorylation in the spastic coronary artery (Figures 1 and 2).

Aortic aneurysm is formed by chronic inflammation of the aortic wall, associated with medial VSMC loss and progressive destruction of structural components, particularly the elastic lamina. Key mechanisms include VSMC senescence, oxidative stress, increased local production of proinflammatory cytokines, and increased MMPs activities that degrade the extracellular matrix. Chronic AngII infusion into apolipoprotein E knockout mice promotes aortic aneurysm formation. In animal models of aortic aneurysm, genetic and pharmacological inhibition of ROS production and MMPs suppressed the development of aneurysm. Chronic inhibition of Rho-kinase by fasudil reduces AngII-induced aortic aneurysm formation in mice. Rho-kinase activation promotes CyPA secretion from VSMC, and extracellular CyPA stimulates VSMC migration and proliferation and MMP activation. Extracellular CyPA is also a chemoattractant for inflammatory cells and further activates vascular Rho-kinase (Figure 4). We demonstrated that Rho-kinase–mediated CyPA augments AngII-induced ROS production, MMP activation, and inflammatory cell recruitment into the aortic VSMC, contributing to the aortic aneurysm formation in these animal models. Our findings suggest that the Rho-kinase/CyPA signaling pathway is a novel therapeutic target for aortic aneurysm. AngII induces Rho-kinase activation and promotes CyPA secretion. Secreted extracellular CyPA augments Rho-kinase activity in a synergistic manner. Thus, secreted CyPA, acting as a proinflammatory cytokine, synergistically augments AngII-mediated ROS production, contributing to the onset of vascular inflammatory cell migration and aortic aneurysm formation.

Rho-Kinase in Cardiac Hypertrophy and Failure

AngII plays a key role in many physiological and pathological processes in cardiac cells, including cardiac hypertrophy. Understanding the molecular mechanisms of AngII-induced myocardial disorders is important to develop new therapies for cardiac dysfunction and failure. ROS production is one important mechanism now recognized to be involved in AngII-induced cardiac hypertrophy. ROS production and AngII-induced aortic aneurysm formation. Chronic inhibition of Rho-kinase by fasudil reduces AngII-induced aortic aneurysm formation in mice. Rho-kinase phosphorylates troponin and inhibits tension generation in cardiac myocytes. Indeed, Rho-kinase inhibition suppresses the development of cardiac hypertrophy and diastolic heart failure in Dahl salt-sensitive rats. Because ROS stimulates myocardial hypertrophy, matrix remodeling, and cellular dysfunction, Rho-kinase and CyPA may function together to promote ROS production and AngII-induced cardiac hypertrophy (Figure 4). In fact, CyPA is required for AngII-mediated cardiac hypertrophy as it directly potentiates ROS production, stimulates proliferation and migration of cardiac fibroblasts, and promotes cardiac myocyte hypertrophy in mice. ROS production and Rho-kinase activation play crucial roles in myocardial damage after ischemia/reperfusion. We demonstrated that pretreatment with fasudil before reperfusion prevents endothelial dysfunction and reduces the extent of myocardial infarction in dogs in vivo. The beneficial effect of fasudil has also been demonstrated in a rabbit model of myocardial ischemia induced by intravenous administration of endothelin-1, a canine model of pacing-induced myocardial ischemia, and a rat model of vasopressin-induced chronic myocardial ischemia.
Different Roles and Regulation of ROCKs in Cardiac Hypertrophy

The fundamental functional difference between RV and left ventricular (LV) failure remains unclear. Thus, our knowledge and strategies for the treatment of RV failure are still limited. We recently addressed this fundamental issue by comparing the responses of both ventricles to chronic pressure overload in mice. Interestingly, there were significant differences in the induction pattern and localization of oxidative stress after pressure overload. Pulmonary artery constriction rapidly induced oxidative stress in the RV without significant changes in the LV, whereas transverse aortic constriction slowly induced oxidative stress in the LV without significant changes in the RV. Furthermore, ROCK2 was promptly upregulated in the RV after PAC and was colocalized with ROS induction. Thus, it is conceivable that the increased ROCK2 expression in the RV after PAC contributes, at least in part, to the vulnerability of the RV to pressure overload and constitutes the characteristic difference between the 2 ventricles. Currently, the roles of ROCK1 and ROCK2 in the pathogenesis of RV and LV failure remain unclear. Mechanical stretch stimulates integrins, which activate the RhoA/Rho-kinase pathway through Rho-GEFs. Mechanotransduction through integrins leads to the activation of the RhoA/Rho-kinase pathway, which induces hypertrophic gene activation. In contrast, mechanosensing by actin filaments causes actin cytoskeleton remodeling through small GTPases of the Rho/Rac/Cdc42 family. However, the detailed mechanisms about mechanoresponses and the link between integrins, Rho-GEFs, and the downstream targets of the RhoA/Rho-kinase pathway are not fully elucidated. In mechanotransduction through integrin-β induced by pressure overload, adhesion of α-actinin, talin, and vinculin to actin filaments may potentially contribute to the activation of FGD2 (Rho-GEF) preferentially in the RV after PAC. Our microarray analysis suggested that there is a special signaling cascade in the RV that connects the FGD2 and RhoA/ROCK2 signaling downstream of integrin-β, which may be the difference between the RV and the LV in response to mechanical stretch.

AngII plays a key role in many physiological and pathological processes in cardiac cells, including cardiac hypertrophy. Understanding the molecular mechanisms involved in AngII-induced myocardial disorders is important to develop new therapies for cardiac dysfunction. ROS production is involved in AngII-induced cardiac hypertrophy. However, the precise mechanism by which ROS cause myocardial hypertrophy and dysfunction still remains to be fully elucidated. In addition, our recent study demonstrated a synergy between CyPA and Rho-kinase to increase ROS generation. Because ROS stimulate myocardial hypertrophy, matrix remodeling, and cellular dysfunction, Rho-kinase and CyPA may promote ROS production and AngII-induced cardiac hypertrophy in a synergistic manner.

Role of Rho-Kinase in ARVC

ARVC is a genetically determined myocardial disease characterized by fibrofatty replacement, predominantly affecting the RV, resulting in ventricular arrhythmias and an increased risk of sudden death, particularly in young people and athletes. Thus, ARVC has been recognized as a disease of the desmosome. We recently demonstrated that Rho-kinase inhibition during cardiac development causes ARVC in mice. Rho-kinase regulates a wide range of cellular functions, including actin cytoskeleton assembly, cell contractility, proliferation, and differentiation, as well as gene expression. In addition, the RhoA/Rho-kinase pathway plays an important role in the regulation of adipogenesis. Indeed, the RhoA/Rho-kinase pathway negatively regulates adipogenesis through interacting with Wnt signaling. Activation of the canonical Wnt/β-catenin signaling pathway is known to inhibit adipogenesis. The less well-characterized noncanonical β-catenin-independent pathway, which involves the activation of small G proteins and their downstream effectors, including the RhoA/Rho-kinase system, may play a more complex role. Interestingly, Wnt signaling downregulation has been recently implicated in the development of ARVC in mice. Finally, we demonstrated that these Rho-kinase-deficient mice spontaneously developed unique phenotypes fulfilling the criteria of ARVC in humans, including cardiac dilatation and dysfunction, myocardial fibrofatty changes, ventricular arrhythmias, and sudden death.

Rho-Kinase as a Therapeutic Target

Fasudil and Y-27632, both Rho-kinase inhibitors, have been shown to inhibit Rho-kinase activity by competing with ATP at the Rho-binding site. Hydroxyfasudil, a major active metabolite of fasudil, exerts a more specific inhibitory effect on Rho-kinase. The role of the Rho-kinase pathway has been emerging, and the indications of Rho-kinase inhibitors have been expanding in cardiovascular medicine. Indeed, the secretion of a variety of cytokines/chemokines and growth factors was significantly reduced by fasudil treatment. The identification of CyPA as a novel mediator of Rho-kinase associated with inflammation provides insight into the mechanisms of several therapies. Currently, many pharmaceutical companies and manufacturers have strong interests in the RhoA/Rho-kinase signaling and the development of its inhibitors. Among them, Akama et al performed a kinome-wide screen to investigate the members of the benzoxaborole family and identified Rho-kinase as a target. They observed a competitive behavior, with respect to ATP, and determined the ROCK2-drug cocrystal structure. On the basis of the role of Rho-kinase in disease processes, we found that the target and therapeutic applications for Rho-kinase inhibitors are mainly in the field of cardiovascular diseases. However, our recent study demonstrated a crucial role for Rho-kinase in cardiac development, which may warn against the use of Rho-kinase inhibitors during pregnancy as in the case of inhibitors of the renin–angiotensin system. To date, we demonstrated that several medications, including statins, calcium channel blockers, and eicosapentaenoic acid, have an indirect inhibitory effect on Rho-kinase. Thus, higher doses of these drugs during pregnancy might potentially cause the development of congenital heart diseases.
Conclusions
Rho-kinase is substantially involved in the pathogenesis of a wide range of cardiovascular diseases, and Rho-kinase inhibitors may be useful for the treatment of these cardiovascular diseases.

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Disclosures
None.

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Rho-Kinase in Cardiovascular System


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