RhoA/Rho-Kinase in the Cardiovascular System

Hiroaki Shimokawa, Shinichiro Sunamura, Kimio Satoh

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.

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 Ca^{2+} concentration and Rho-kinase activity[19] 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

Original received September 27, 2015; revision received December 16, 2015; accepted December 21, 2015.
From the Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan.
Correspondence to Hiroaki Shimokawa, MD, PhD, Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan. E-mail shimo@cardio.med.tohoku.ac.jp

Nonstandard Abbreviations and Acronyms

AngII	angiotensin II
ARVC	arrhythmogenic right ventricular cardiomyopathy
CyPA	cyclophilin A
EC	endothelial cells
EDH	endothelium-dependent hyperpolarization
GEFs	guanine nucleotide exchange factors
H_2O_2	hydrogen peroxide
LV	left ventricle
MLC	myosin light chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MMPs	matrix metalloproteinases
MYPT	myosin phosphatase target subunit
NO	nitric oxide
PAC	pulmonary artery constriction
PAH	pulmonary arterial hypertension
PH	pulmonary hypertension
ROS	reactive oxygen species
RV	right ventricle
TRP	transient receptor potential
VSMC	vascular smooth muscle cells

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 Ca^{2+}/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 in its middle portion, and a putative pleckstrin homology domain in the C-terminal domain (Figure 3).[25] To elucidate the functions of the ROCK isoforms in vivo, ROCK1- and ROCK2-deficient mice have been generated.[26,27] Importantly, ROCK1-deficient mice are born with their eyelids opened,[26] whereas ROCK2-deficient mice present placental dysfunction and fetal death.[27,28] Thus, the role of ROCK2, the main isoform in the cardiovascular system, remained to be fully elucidated in vivo. To address this point, we developed tissue-specific knockout mice for ROCK1 and ROCK2. Using VSMC-specific ROCK2 knockout mice, we demonstrated that ROCK2 in VSMC plays a crucial role in the development of hypoxia-induced pulmonary hypertension (PH).[29] In wild-type mice, chronic hypoxia significantly increased ROCK2 expression and ROCK activity in the lung tissues and caused PH and RV hypertrophy, all of which were suppressed in the VSMC-specific ROCK2 knockout mice.[29]

Both ROCK1 and ROCK2 are upregulated by angiotensin II (AngII) via AT_1 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 VSMC.[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. Rnd 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).[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.[37] 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 al[42] demonstrated that phosphoinositide-dependent protein kinase 1 is required for the function of ROCK1. Phosphoinositide-dependent protein kinase 1 binds to and competes with the negative regulator, RhoE, for the same region in ROCK1. 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 Rho-kinase–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 pinocytotic 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

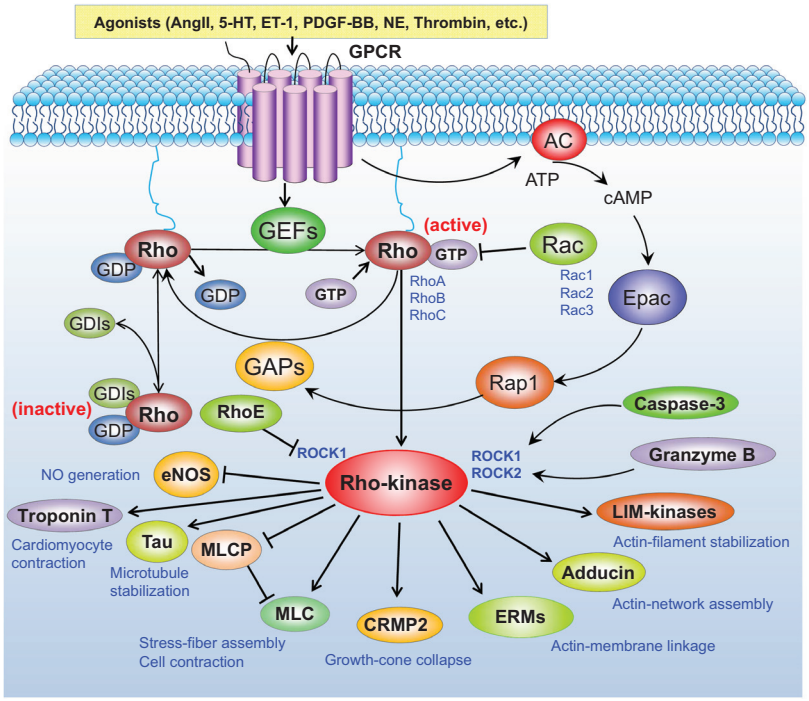

Figure 1. Rho-kinase activation and multiple targets. Rho GTPases, including RhoA, is activated by the guanine nucleotide exchange factors (GEFs) that catalyze exchange of GDP for GTP and inactivated by the GTPase-activating proteins (GAPs). Rho-kinase is an effector of the active form of Rho. Many substrates of Rho-kinase have been identified, including myosin light chain (MLC), MLC phosphatase (MLCP), ezrin/radixin/moesin (ERM) family, adducin, and LIM kinases. 5-HT indicates 5-hydroxytryptamine; AC, adenylyl cyclase; CRMP2, collapsin response mediator protein 2; eNOS, endothelial NO synthase; Epac, exchange protein directly activated by cAMP; ET-1, endothelin; GDI, guanine nucleotide dissociation inhibitor; GPCR, G-protein–coupled receptor; NE, norepinephrine; and PDGF-BB, platelet-derived growth factor-BB.

the activity of RhoA/Rho-kinase signaling.[48] Thus, the RhoA/Rho-kinase signaling pathway is involved in the mechanotransduction mechanism involved in the adherence junction strengthening at EC–EC contacts (Figure 4).[48] 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.[51–54]

Several reports demonstrated that NO and Rho-kinase have opposing effects.[55,56] Rho-kinase–deficient mice revealed preserved EC function in a diabetic model.[56] Moreover, a Rho-kinase inhibitor, fasudil, significantly enhanced the

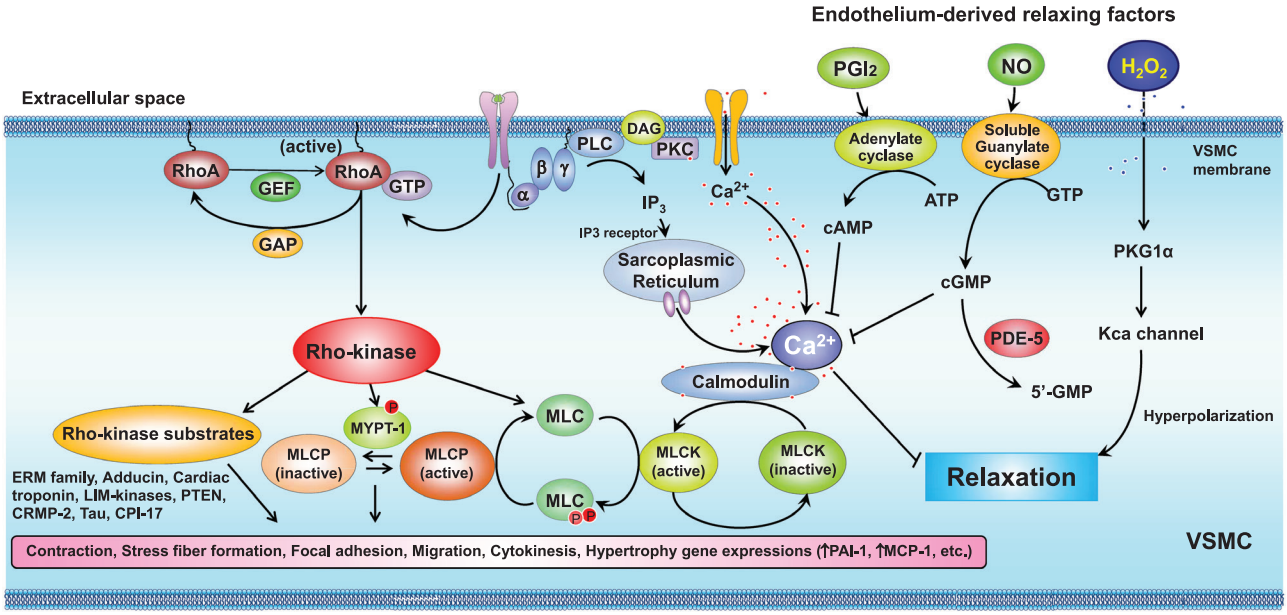

Figure 2. Input from endothelial cells (ECs) to vascular smooth muscle cells (VSMCs) through endothelium-derived relaxing factors. Rho-kinase is a downstream effector of the active form of RhoA. Phosphorylation of myosin light chain (MLC) is a key event in the regulation of VSMC contraction. MLC is phosphorylated by Ca^{2+}-calmodulin-activated MLC kinase (MLCK) and dephosphorylated by MLC phosphatase (MLCP). Rho-kinase mediates agonist-induced VSMC contraction. H_2O_2 rapidly reaches VSMC, stimulates the 1-α isoform of cGMP-dependent protein kinase ($PKG_{1\alpha}$) to form the disulfide form, and opens Ca-activated K channels (K_{Ca}) with subsequent VSMC hyperpolarization and relaxation. CRMP2 indicates collapsin response mediator protein 2; DAG, diacylglycerol; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; IP_3, 1,4,5-triphosphate; MCP, monocyte chemoattractant protein; PAI-1, plasminogen activator inhibitor type 1; PDE, phosphodiesterase; PGI_2, prostacyclin; PKC, protein kinase C; PLC, phospholipase C; and PTEN, phosphatase and tensin homolog.

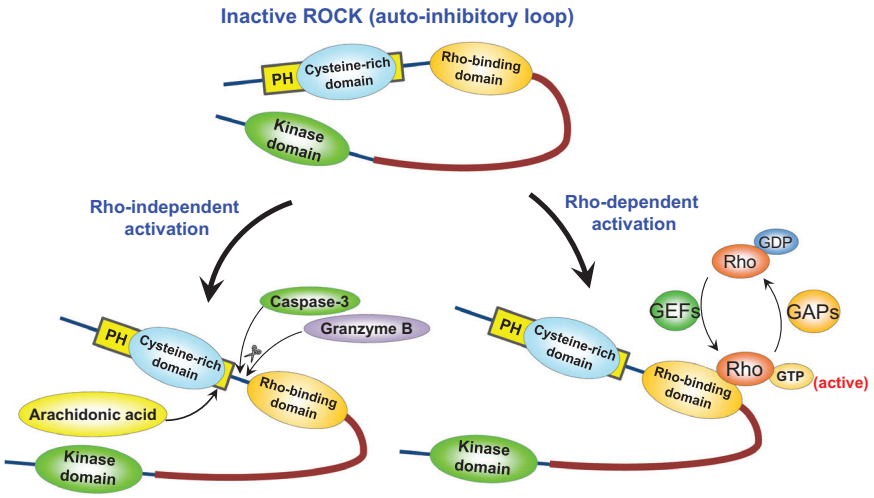

Figure 3. Molecular structure of Rho-kinase isoforms. There are 2 isoforms of Rho-kinase, ROCK1 and ROCK2, which consist of 3 major domains, including a kinase domain in its N-terminal domain, a coiled-coil domain with Rho-binding domain in its middle portion, and a putative pleckstrin homology (PH) domain in its C-terminal domain. ROCK1 and ROCK2 are highly homologous with an overall amino acid sequence identity of 65%. There are 2 types of activation; Rho-dependent and Rho-independent activation. ROCK1 is specifically cleaved by caspase-3, whereas granzyme B cleaves ROCK2. GAP indicates GTPase-activating protein; and GEF, guanine nucleotide exchange factor.

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

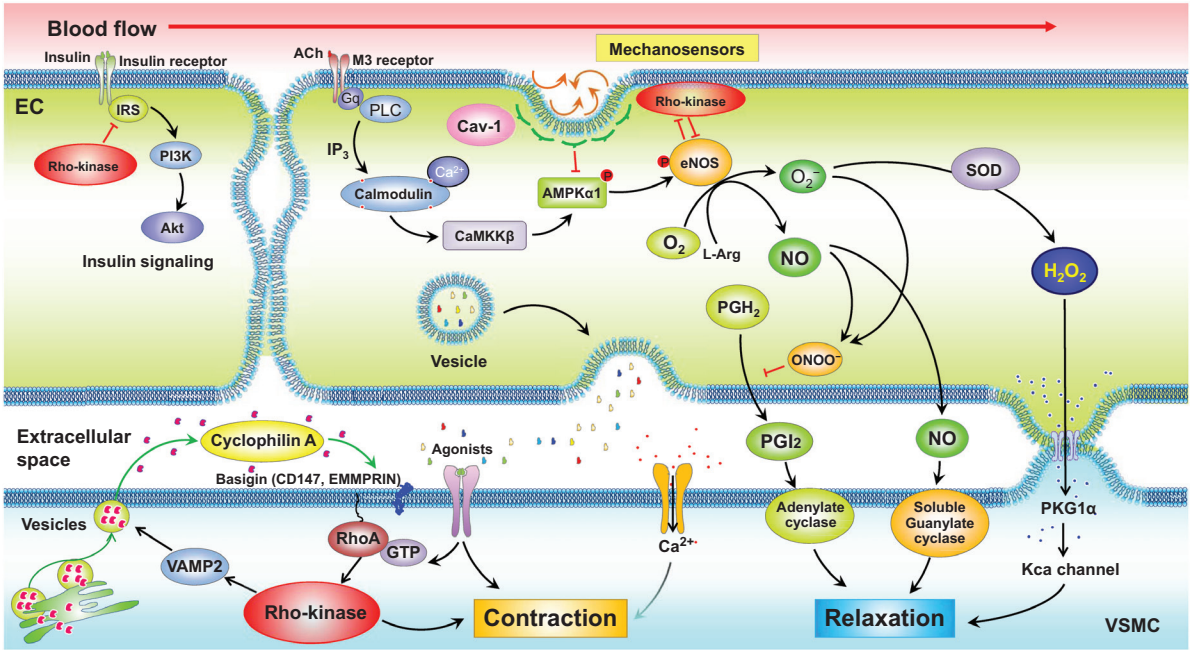

Figure 4. Interactions between endothelial cells (ECs) and vascular smooth muscle cells (VSMCs). Intracellular signaling pathways for Rho-kinase activation, ROS production, and cyclophilin A (CyPA) secretion are closely linked through VAMP2 vesicle formation. H_2O_2 has been reported to cause vasodilatation through several mechanisms. H_2O_2 rapidly reaches VSMC with subsequent VSMC hyperpolarization and relaxation. Oxidative stress promotes CyPA secretion from VSMC. Secreted CyPA promotes ROS production, contributing to the augmentation of oxidative stress. AMPK indicates AMP-activated protein kinase; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase; EMMPRIN, extracellular matrix metalloproteinases inducer protein; IP_3, 1,4,5-triphosphate; IRS, insulin receptor substrate; PGH2, prostaglandin H2; PGI_2, prostacyclin; PI3K, phosphoinositide-3-kinase; PKG1α, protein kinase G, subunit 1α; PLC, phospholipase C; SOD, superoxide dismutase; and VAMP, vesicle-associated membrane protein.

previously demonstrated that endothelium-derived hydrogen peroxide (H_2O_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_2O_2. Importantly, the production of endothelial H_2O_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_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.[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 kinase[72] and small GTPases.[68,73]

The dual roles of ROS, particularly H_2O_2, as both protective and pathological agents, are important in vascular homeostasis.[74] At low concentrations, H_2O_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_2O_2 to EDH-dependent vasodilation of resistance vessels[62–64] 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_2O_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 EDHF-mediated responses in murine microvessels (Figure 4).[82] Bone marrow and adiponectin derived from adipose tissues also contribute to the modulation of microvascular EDH responses.[83] The role of H_2O_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 phosphatase[22] and the small GTPase Rho and its target, Rho-kinase (Figure 2).[7,19]

Phosphorylation of the regulatory MLC activates myosin 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).[22] Phosphorylation of the second site of MLC is known to further increase the actin-activated Mg^{2+}-ATPase activity of myosin in vitro.[86,87] These results indicate that enhanced MLC phosphorylation plays a central role in the augmentation 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 cardiovascular 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.[19,86,94] 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.[95,96] 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 factor[97] and induces VSMC contraction[98] 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.[98] 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]

and urotensin[108] (Figure 1). It has previously been shown that statins enhance eNOS mRNA by cholesterol-independent mechanisms, involving the inhibition of Rho geranyl-geranylation.[59] We also demonstrated that statins and Rho-kinase inhibitors completely block the secretion of cyclophilin A (CyPA) from VSMC.[109,110] Rho-kinase plays an important role in mediating various cellular functions, not only VSMC contraction[111,112] but also actin cytoskeleton organization,[113] adhesion, and cytokinesis.[14] Thus, Rho-kinase plays a crucial role in the development of cardiovascular disease through ROS production, inflammation, EC damage, and VSMC contraction and proliferation (Figure 1). Rho-kinase inhibitors have excellent vasodilator activity and can induce vasodilation, especially when the vasoconstrictor tone is increased by a variety of mechanisms, including enhanced Ca^{2+} entry through activation of G-protein–coupled receptors, ventilatory hypoxia, and NOS inhibition.[114]

Physiological and Pathological Roles of Rho-Kinase in the Cardiovascular System

Cardiovascular diseases often result from imbalances in the levels of intracellular ROS.[74,115] The O_2^--producing oxidases in the vascular system, including eNOS, cyclooxygenase, lipoxygenase, P-450 monooxygenase, and nicotinamide adenine dinucleotide phosphate oxidases,[116] 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.[117] In this process, transient receptor potential (TRP) channels also substantially contribute to the ROS augmentation in response to external stimuli.[118] A class of TRP channels works as sensors of ROS and gaseous messenger molecules, including oxygen (O_2), hydrogen sulfide (H_2S), and carbon dioxide (CO_2).[119] H_2O_2 triggers the production of ADP-ribose, which activates TRPM2. TRPC5, TRPV1, and TRPA1 are also activated by H_2O_2. NO regulates TRP channels via cGMP/PKG-dependent phosphorylation.[119] Excessive ROS target multiple biomolecules, causing numerous cellular complications, including lipid peroxidation, protein oxidation/inactivation, and DNA damage/mutations.[117] Furthermore, increased O_2^- levels attenuate endothelium-dependent relaxation and enhance VSMC contraction through the formation of hydroxyl radicals.[120,121] Although H_2O_2 is important for vascular homeostasis at physiological low concentrations,[62,64] excessive ROS are hazardous to the cells, leading to endothelial dysfunction and VSMC proliferation.[74,115,122]

Recent evidence suggests that many other stimuli that modulate VSMC functions, including ROS, promote VSMC growth by inducing autocrine/paracrine growth mechanisms.[12,122] Among the autocrine/paracrine factors, CyPA has been identified as an ROS responsive protein that is secreted by VSMC on activation of the RhoA/Rho-kinase system (Figure 4).[109,123] The extracellular CyPA decreases eNOS expression,[124] suggesting the indirect role of the RhoA/Rho-kinase pathway for the negative regulation of endothelial NO production. Accumulating evidence indicates that Rho-kinase plays important roles in the pathogenesis of a wide range of cardiovascular diseases.[14,125,126] Indeed, the RhoA/Rho-kinase pathway not only mediates VSMC hypercontraction through inhibition of MLCP but also promotes cardiovascular diseases by enhancing ROS production.[2,3,14,125,126] The beneficial effects of long-term inhibition of Rho-kinase for the treatment of cardiovascular disease have been demonstrated in various animal models, such as coronary artery spasm, arteriosclerosis, restenosis, ischemia/reperfusion injury, hypertension, PH, stroke, and cardiac hypertrophy/heart failure.[2,14,112,125] Gene transfer of dominant-negative Rho-kinase reduced neointimal formation of the coronary artery in pigs.[127] Long-term treatment with a Rho-kinase inhibitor suppressed neointimal formation after vascular injury in vivo,[128,129] monocyte chemoattractant protein-1–induced vascular lesion formation,[130] constrictive remodeling,[131,132] in-stent restenosis,[133] and development of cardiac allograft vasculopathy.[134]

Rho-Kinase–Mediated Development of Cardiovascular Diseases

Growth factors secreted from VSMC play an important role in mediating various cellular responses in the development of cardiovascular diseases.[9–11] Recent evidence suggests that many other stimuli that modulate VSMC functions, including ROS, promote VSMC proliferation by inducing autocrine/paracrine growth mechanisms.[12] Rho-kinase augments inflammation by inducing proinflammatory molecules, including interleukin-6,[135] monocyte chemoattractant protein-1,[136] macrophage migration inhibitory factor,[134,137] and sphingosine-1-phosphate.[138] In EC, Rho-kinase downregulates eNOS[139] 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,[30] and by remnant lipoproteins in human coronary VSMC.[140] Rho-kinase also upregulates NAD(P)H oxidases (Nox1, Nox4, gp91phox, and p22phox) and augments AngII-induced ROS production.[74,104,115]

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).[141–143] ROS activate a pathway containing vesicles, resulting in CyPA secretion.[109,141] 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.[144,145] CyPA is secreted by VSMC via a highly regulated pathway that involves vesicle transport and plasma membrane binding (Figure 4).[109] Rho GTPases, including RhoA, are key regulators in signaling pathways linked to actin cytoskeletal rearrangement.[146] 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.[147] Rho-kinase mediates myosin II activation via phosphorylation and inactivation of myosin II light chain phosphatase.[7] 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,[148] promoting matrix metalloproteinases (MMPs) activation.[149] 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 Ca^{2+} regulator in platelets.[150] Moreover, extracellular CyPA activates platelets via basigin (CD147)–mediated phosphoinositide-3-kinase/Akt signaling, leading to enhanced adhesion and thrombus formation.[151,152] Moreover, thrombin suppresses eNOS in EC via Rho-kinase pathway.[153] 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).[143]

Importantly, CyPA plays a crucial role in the translocation of Nox enzymes, such as p47phox,[154] contributing to VSMC proliferation and vascular diseases.[117] 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.[126] 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[155] and VSMC.[156] 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.[109,123] Indeed, Rho-kinase is an important therapeutic target in cardiovascular diseases.[2,3,14] 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).[157]

Rho-Kinase in Systemic and PH

Rho-kinase–mediated Ca^{2+} sensitization is involved in the pathophysiology of hypertension.[158] 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.[158,159] The expression of Rho-kinase is significantly increased in resistance vessels of spontaneously hypertensive rats.[160] Rho-kinase is also involved in the central mechanisms of sympathetic nerve activity.[161,162]

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.[163–169] Chronic exposure to hypoxia induces vascular remodeling in mice.[170] We demonstrated that pulmonary vascular dysfunction plays a crucial role in the development of hypoxia-induced PH,[123,171] for which Rho-kinase plays a crucial role.[29,172,173] Rho-kinase promotes CyPA secretion from VSMC, and extracellular CyPA stimulates VSMC proliferation in vitro[141,142] and in vivo[110] (Figure 4). Extracellular CyPA induces EC adhesion molecule expression[174] and apoptosis[124] and is a chemoattractant for inflammatory cells.[110,175] Thus, extracellular CyPA may contribute to hypoxia-induced PH. Long-term treatment with fasudil suppresses the development of monocrotaline-induced PH in rats[176] and hypoxia-induced PH in mice.[177] On the contrary, statins and Rho-kinase inhibitor reduce the secretion of CyPA from VSMCs,[109,123] and pravastatin ameliorates hypoxia-induced PH in mice.[123] 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.[29] Consistently, we observed Rho-kinase activation in patients with pulmonary arterial hypertension (PAH).[178] Furthermore, fasudil significantly reduced pulmonary vascular resistance in patients with PAH.[179,180]

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.[29] Because CyPA secretion is regulated by Rho-kinase,[109,144] we further determined whether CyPA contributes to the development of PH in mice and humans.[156] Importantly, we demonstrated that extracellular CyPA and its receptor, basigin (Bsg, CD147), are crucial for hypoxia-induced PH.[156] 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).[156]

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.[181] 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.[172] Indeed, we obtained direct evidence of Rho-kinase activation in patients with PAH.[178] 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.[179,180]

Rho-Kinase in Vascular Diseases

Rho-kinase plays a crucial role in ROS augmentation and vascular inflammation.[3] ROS are involved in the pathogenesis

of neointima formation, in part, by promoting VSMC growth and stimulating proinflammatory events.[102,182] Arteriosclerosis is a slowly progressing process of inflammation of the arterial wall that involves the intima, media, and adventitia.[14,112] Accumulating evidence indicates that Rho-kinase–mediated pathway is substantially involved in EC dysfunction,[105,139] VSMC hypercontraction,[183] VSMC proliferation and migration in the media,[184] and accumulation of inflammatory cells in the adventitia.[130] These Rho-kinase–mediated cellular responses lead to the development of vascular disease.[185] In fact, mRNA expression of ROCKs is enhanced in the inflammatory and arteriosclerotic arterial lesions in animals[183] and humans.[186] In the context of atherosclerosis, Rho-kinase should be regarded as a proinflammatory and proatherogenic molecule.[45] Indeed, recent studies demonstrated that ROCK inhibition by statins could lead to improved endothelial function and decreased atherosclerosis.[187]

Rho-kinase plays a crucial role in the pathogenesis of coronary artery spasm.[2] Coronary spasm plays an important role in variant angina, myocardial infarction, and sudden death.[2,188] 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.[189] The activity and the expression of Rho-kinase are enhanced at the inflammatory/arteriosclerotic coronary lesions.[190] Intracoronary administration of fasudil[191] and hydroxyfasudil[88] inhibits coronary spasm in a porcine model.[131] 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.[190] 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.[190] 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.[190,191] Furthermore, another Rho-kinase inhibitor, Y-27632,[158] also inhibited not only serotonin-induced contractions in vivo and in vitro but also the increase in MYPT-1 phosphorylation.[190] 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.[190] 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).[111,190] 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.[88,192] 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.[193] Key mechanisms include VSMC senescence,[194] oxidative stress,[12,195] increased local production of proinflammatory cytokines, and increased MMPs activities that degrade the extracellular matrix.[196] Chronic AngII infusion into apolipoprotein E knockout mice promotes aortic aneurysm formation.[197,198] In animal models of aortic aneurysm, genetic and pharmacological inhibition of ROS production[199,200] and MMPs[201,202] suppressed the development of aneurysm. Chronic inhibition of Rho-kinase by fasudil reduces AngII-induced aortic aneurysm formation in mice.[203] Rho-kinase activation promotes CyPA secretion from VSMC, and extracellular CyPA stimulates VSMC migration and proliferation and MMP activation.[141,142] Extracellular CyPA is also a chemoattractant for inflammatory cells[109,141,175] 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.[204] 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.[144] 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.[157,199]

Rho-Kinase in Cardiac Hypertrophy and Failure

AngII plays a key role in many physiological and pathological processes in cardiac cells, including cardiac hypertrophy.[205] Understanding the molecular mechanisms of AngII-induced myocardial disorders is important to develop new therapies for cardiac dysfunction and failure.[206] ROS production is one important mechanism now recognized to be involved in AngII-induced cardiac hypertrophy is ROS production.[207,208] Cardiac troponin is a substrate of Rho-kinase (Figure 1).[209] 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.[210] Because ROS stimulates myocardial hypertrophy, matrix remodeling, and cellular dysfunction,[211] 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.[212] 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.[213] The beneficial effect of fasudil has also been demonstrated in a rabbit model of myocardial ischemia induced by intravenous administration of endothelin-1,[214] a canine model of pacing-induced myocardial ischemia,[215] and a rat model of vasopressin-induced chronic myocardial ischemia.[216]

Different Roles and Regulation of ROCKs in Cardiac Hypertrophy

The fundamental functional difference between RV and left ventricular (LV) failure remains unclear.[217] Thus, our knowledge and strategies for the treatment of RV failure are still limited.[218] We recently addressed this fundamental issue by comparing the responses of both ventricles to chronic pressure overload in mice.[173] 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.[173] Furthermore, ROCK2 was promptly upregulated in the RV after PAC and was colocalized with ROS induction.[173] 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 activates the RhoA/Rho-kinase pathway through Rho-GEFs.[219] Mechanotransduction through integrins leads to the activation of the RhoA/Rho-kinase pathway, which induces hypertrophic gene activation.[220,221] In contrast, mechanosensing by actin filaments causes actin cytoskeleton remodeling through small GTPases of the Rho/Rac/Cdc42 family.[220,221] 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.[173] 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.[173]

AngII plays a key role in many physiological and pathological processes in cardiac cells, including cardiac hypertrophy.[205] Understanding the molecular mechanisms involved in AngII-induced myocardial disorders is important to develop new therapies for cardiac dysfunction.[206] ROS production is involved in AngII-induced cardiac hypertrophy.[207,208] However, the precise mechanism by which ROS cause myocardial hypertrophy and dysfunction still remains to be fully elucidated.[222] In addition, our recent study demonstrated a synergy between CyPA and Rho-kinase to increase ROS generation.[126,143] Because ROS stimulate myocardial hypertrophy, matrix remodeling, and cellular dysfunction,[211] 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.[223] Thus, ARVC has been recognized as a disease of the desmosome.[224–226] We recently demonstrated that Rho-kinase inhibition during cardiac development causes ARVC in mice.[13] Rho-kinase regulates a wide range of cellular functions, including actin cytoskeleton assembly, cell contractility, proliferation, and differentiation, as well as gene expression.[44,227] In addition, the RhoA/Rho-kinase pathway plays an important role in the regulation of adipogenesis.[228] Indeed, the RhoA/Rho-kinase pathway negatively regulates adipogenesis through interacting with Wnt signaling.[229] Activation of the canonical Wnt/β-catenin signaling pathway is known to inhibit adipogenesis.[228] 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.[230] Interestingly, Wnt signaling downregulation has been recently implicated in the development of ARVC in mice.[231–233] Finally, we demonstrated that these Rho-kinase–deficient mice spontaneously developed unique phenotypes fulfilling the criteria of ARVC in humans,[234] including cardiac dilatation and dysfunction, myocardial fibrofatty changes, ventricular arrhythmias, and sudden death.[13]

Rho-Kinase as a Therapeutic Target

Fasudil[235] and Y-27632,[158] Rho-kinase inhibitors, have been shown to inhibit Rho-kinase activity by competing with ATP at the Rho-binding site.[236] Hydroxyfasudil, a major active metabolite of fasudil, exerts a more specific inhibitory effect on Rho-kinase.[88,104] The role of the Rho-kinase pathway has been emerging, and the indications of Rho-kinase inhibitors have been expanding in cardiovascular medicine.[2,3,14,125,126] 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.[3,112,125,237] Among them, Akama et al[238] 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.[238] 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,[13] which may warn against the use of Rho-kinase inhibitors during pregnancy as in the case of inhibitors of the renin–angiotensin system.[239] To date, we demonstrated that several medications, including statins, calcium channel blockers, and eicosapentaenoic acid, have an indirect inhibitory effect on Rho-kinase.[14,126] Thus, higher doses of these drugs during pregnancy might potentially cause the development of congenital heart diseases.[240]

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.

Acknowledgments

We are grateful to the laboratory members of the Department of Cardiovascular Medicine of Tohoku University Graduate School of Medicine, especially Hiromi Yamashita, Ai Nishihara, and Yumi Watanabe, for the valuable technical assistance.

Sources of Funding

This work was supported, in part, by a Grant-in-Aid for Tohoku University Global COE for Conquest of Signal Transduction Diseases with Network Medicine, the Takeda Science Foundation, and Grants-in-Aid for Scientific Research (21790698, 23659408, 24390193, 15H02535, 15H04816, and 15K15046), all of which were from the Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan, and Grants-in-Aid for Scientific Research from the Ministry of Health, Labour, and Welfare, Tokyo, Japan (10102895 and 15545346).

Disclosures

None.

References

1. Vanhoutte PM. Endothelium-derived free radicals: for worse and for better. *J Clin Invest*. 2001;107:23–25. doi: 10.1172/JCI11832.
2. Shimokawa H. 2014 Williams Harvey Lecture: importance of coronary vasomotion abnormalities-from bench to bedside. *Eur Heart J*. 2014;35:3180–3193. doi: 10.1093/eurheartj/ehu427.
3. Shimokawa H, Satoh K. 2015 ATVB Plenary Lecture: translational research on Rho-kinase in cardiovascular medicine. *Arterioscler Thromb Vasc Biol*. 2015;35:1756–1769. doi: 10.1161/ATVBAHA.115.305353.
4. Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J*. 1996;15:2208–2216.
5. Leung T, Chen XQ, Manser E, Lim L. The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. *Mol Cell Biol*. 1996;16:5313–5327.
6. Ishizaki T, Maekawa M, Fujisawa K, Okawa K, Iwamatsu A, Fujita A, Watanabe N, Saito Y, Kakizuka A, Morii N, Narumiya S. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *EMBO J*. 1996;15:1885–1893.
7. Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science*. 1996;273:245–248.
8. Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem*. 1996;271:20246–20249.
9. Berk BC, Alexander RW, Brock TA, Gimbrone MA Jr, Webb RC. Vasoconstriction: a new activity for platelet-derived growth factor. *Science*. 1986;232:87–90.
10. Griendling KK, Berk BC, Ganz P, Gimbrone MA Jr, Alexander RW. Angiotensin II stimulation of vascular smooth muscle phosphoinositide metabolism. State of the art lecture. *Hypertension*. 1987;9:III181–III185.
11. Berk BC. Vascular smooth muscle growth: autocrine growth mechanisms. *Physiol Rev*. 2001;81:999–1030.
12. Taniyama Y, Griendling KK. Reactive oxygen species in the vasculature: molecular and cellular mechanisms. *Hypertension*. 2003;42:1075–1081. doi: 10.1161/01.HYP.0000100443.09293.4F.
13. Ellawindy A, Satoh K, Sunamura S, et al. Rho-kinase Inhibition During Early Cardiac Development Causes Arrhythmogenic Right Ventricular Cardiomyopathy in Mice. *Arterioscler Thromb Vasc Biol*. 2015;35:2172–2184. doi: 10.1161/ATVBAHA.115.305872.
14. Shimokawa H, Takeshita A. Rho-kinase is an important therapeutic target in cardiovascular medicine. *Arterioscler Thromb Vasc Biol*. 2005;25:1767–1775. doi: 10.1161/01.ATV.0000176193.83629.c8.
15. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. *Nature*. 2002;420:629–635. doi: 10.1038/nature01148.
16. Schmidt A, Hall A. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev*. 2002;16:1587–1609. doi: 10.1101/gad.1003302.
17. Bernards A. GAPs galore! A survey of putative Ras superfamily GTPase activating proteins in man and Drosophila. *Biochim Biophys Acta*. 2003;1603:47–82.
18. Olofsson B. Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling. *Cell Signal*. 1999;11:545–554.
19. Hirata K, Kikuchi A, Sasaki T, Kuroda S, Kaibuchi K, Matsuura Y, Seki H, Saida K, Takai Y. Involvement of rho p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction. *J Biol Chem*. 1992;267:8719–8722.
20. Wirth A, Benyó Z, Lukasova M, Leutgeb B, Wettschureck N, Gorbey S, Orsy P, Horváth B, Maser-Gluth C, Greiner E, Lemmer B, Schütz G, Gutkind JS, Offermanns S. G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. *Nat Med*. 2008;14:64–68. doi: 10.1038/nm1666.
21. Nishioka T, Shohag MH, Amano M, Kaibuchi K. Developing novel methods to search for substrates of protein kinases such as Rho-kinase. *Biochim Biophys Acta*. 2015;1854:1663–1666. doi: 10.1016/j.bbapap.2015.03.001.
22. Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. *Nature*. 1994;372:231–236. doi: 10.1038/372231a0.
23. Leung T, Manser E, Tan L, Lim L. A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J Biol Chem*. 1995;270:29051–29054.
24. Nakagawa O, Fujisawa K, Ishizaki T, Saito Y, Nakao K, Narumiya S. ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS Lett*. 1996;392:189–193.
25. Fukata Y, Amano M, Kaibuchi K. Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. *Trends Pharmacol Sci*. 2001;22:32–39.
26. Shimizu Y, Thumkeo D, Keel J, Ishizaki T, Oshima H, Oshima M, Noda Y, Matsumura F, Taketo MM, Narumiya S. ROCK-I regulates closure of the eyelids and ventral body wall by inducing assembly of actomyosin bundles. *J Cell Biol*. 2005;168:941–953. doi: 10.1083/jcb.200411179.
27. Thumkeo D, Keel J, Ishizaki T, Hirose M, Nonomura K, Oshima H, Oshima M, Taketo MM, Narumiya S. Targeted disruption of the mouse rho-associated kinase 2 gene results in intrauterine growth retardation and fetal death. *Mol Cell Biol*. 2003;23:5043–5055.
28. Noma K, Rikitake Y, Oyama N, Yan G, Alcaide P, Liu PY, Wang H, Ahl D, Sawada N, Okamoto R, Hiroi Y, Shimizu K, Luscinskas FW, Sun J, Liao JK. ROCK1 mediates leukocyte recruitment and neointima formation following vascular injury. *J Clin Invest*. 2008;118:1632–1644. doi: 10.1172/JCI29226.
29. Shimizu T, Fukumoto Y, Tanaka S, Satoh K, Ikeda S, Shimokawa H. Crucial role of ROCK2 in vascular smooth muscle cells for hypoxia-induced pulmonary hypertension in mice. *Arterioscler Thromb Vasc Biol*. 2013;33:2780–2791. doi: 10.1161/ATVBAHA.113.301357.
30. Hiroki J, Shimokawa H, Higashi M, Morikawa K, Kandabashi T, Kawamura N, Kubota T, Ichiki T, Amano M, Kaibuchi K, Takeshita A. Inflammatory stimuli upregulate Rho-kinase in human coronary vascular smooth muscle cells. *J Mol Cell Cardiol*. 2004;37:537–546. doi: 10.1016/j.yjmcc.2004.05.008.
31. Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A, Olson MF. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. *Nat Cell Biol*. 2001;3:339–345. doi: 10.1038/35070009.
32. Sebbagh M, Hamelin J, Bertoglio J, Solary E, Bréard J. Direct cleavage of ROCK II by granzyme B induces target cell membrane blebbing in a caspase-independent manner. *J Exp Med*. 2005;201:465–471. doi: 10.1084/jem.20031877.
33. Gabet AS, Coulon S, Fricot A, Vandekerckhove J, Chang Y, Ribeil JA, Lordier L, Zermati Y, Asnafi V, Belaid Z, Debili N, Vainchenker W, Varet B, Hermine O, Courtois G. Caspase-activated ROCK-1 allows erythroblast terminal maturation independently of cytokine-induced Rho signaling. *Cell Death Differ*. 2011;18:678–689. doi: 10.1038/cdd.2010.140.
34. Afonina IS, Cullen SP, Martin SJ. Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B. *Immunol Rev*. 2010;235:105–116. doi: 10.1111/j.0105-2896.2010.00908.x.
35. Hansen SH, Zegers MM, Woodrow M, Rodriguez-Viciana P, Chardin P, Mostov KE, McMahon M. Induced expression of Rnd3 is associated with

transformation of polarized epithelial cells by the Raf-MEK-extracellular signal-regulated kinase pathway. *Mol Cell Biol*. 2000;20:9364–9375.
36. Nobes CD, Lauritzen I, Mattei MG, Paris S, Hall A, Chardin P. A new member of the Rho family, Rnd1, promotes disassembly of actin filament structures and loss of cell adhesion. *J Cell Biol*. 1998;141:187–197.
37. Riento K, Guasch RM, Garg R, Jin B, Ridley AJ. RhoE binds to ROCK I and inhibits downstream signaling. *Mol Cell Biol*. 2003;23:4219–4229.
38. Riento K, Totty N, Villalonga P, Garg R, Guasch R, Ridley AJ. RhoE function is regulated by ROCK I-mediated phosphorylation. *EMBO J*. 2005;24:1170–1180. doi: 10.1038/sj.emboj.7600612.
39. Komander D, Garg R, Wan PT, Ridley AJ, Barford D. Mechanism of multi-site phosphorylation from a ROCK-I:RhoE complex structure. *EMBO J*. 2008;27:3175–3185. doi: 10.1038/emboj.2008.226.
40. Wang Y, Zheng XR, Riddick N, Bryden M, Baur W, Zhang X, Surks HK. ROCK isoform regulation of myosin phosphatase and contractility in vascular smooth muscle cells. *Circ Res*. 2009;104:531–540. doi: 10.1161/CIRCRESAHA.108.188524.
41. Miyazaki K, Komatsu S, Ikebe M. Dynamics of RhoA and ROKα translocation in single living cells. *Cell Biochem Biophys*. 2006;45:243–254. doi: 10.1385/CBB:45:3:243.
42. Pinner S, Sahai E. PDK1 regulates cancer cell motility by antagonising inhibition of ROCK1 by RhoE. *Nat Cell Biol*. 2008;10:127–137. doi: 10.1038/ncb1675.
43. Loirand G, Guérin P, Pacaud P. Rho-kinases in cardiovascular physiology and pathophysiology. *Circ Res*. 2006;98:322–334. doi: 10.1161/01.RES.0000201960.04223.3c.
44. Riento K, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol*. 2003;4:446–456. doi: 10.1038/nrm1128.
45. Huveneers S, Daemen MJ, Hordijk PL. Between Rho(k) and a hard place: the relation between vessel wall stiffness, endothelial contractility, and cardiovascular disease. *Circ Res*. 2015;116:895–908. doi: 10.1161/CIRCRESAHA.116.305720.
46. Chapados R, Abe K, Ihida-Stansbury K, McKean D, Gates AT, Kern M, Merklinger S, Elliott J, Plant A, Shimokawa H, Jones PL. ROCK controls matrix synthesis in vascular smooth muscle cells: coupling vasoconstriction to vascular remodeling. *Circ Res*. 2006;99:837–844. doi: 10.1161/01.RES.0000246172.77441.f1.
47. van Nieuw Amerongen GP, Beckers CM, Achekar ID, Zeeman S, Musters RJ, van Hinsbergh VW. Involvement of Rho-kinases in endothelial barrier maintenance. *Arterioscler Thromb Vasc Biol*. 2007;27:2332–2339. doi: 10.1161/ATVBAHA.107.152322.
48. Bell RD, Winkler EA, Singh I, Sagare AP, Deane R, Wu Z, Holtzman DM, Betsholtz C, Armulik A, Sallstrom J, Berk BC, Zlokovic BV. Apolipoprotein E controls cerebrovascular integrity via cyclophilin A. *Nature*. 2012;485:512–516. doi: 10.1038/nature11087.
49. Shimokawa H, Tomoike H, Nabeyama S, Yamamoto H, Araki H, Nakamura M, Ishii Y, Tanaka K. Coronary artery spasm induced in atherosclerotic miniature swine. *Science*. 1983;221:560–562.
50. Shimokawa H. Primary endothelial dysfunction: atherosclerosis. *J Mol Cell Cardiol*. 1999;31:23–37. doi: 10.1006/jmcc.1998.0841.
51. Berk BC. Atheroprotective signaling mechanisms activated by steady laminar flow in endothelial cells. *Circulation*. 2008;117:1082–1089. doi: 10.1161/CIRCULATIONAHA.107.720730.
52. Nigro P, Abe J, Woo CH, Satoh K, McClain C, O'Dell MR, Lee H, Lim JH, Li JD, Heo KS, Fujiwara K, Berk BC. PKCzeta decreases eNOS protein stability via inhibitory phosphorylation of ERK5. *Blood*. 2010;116:1971–1979. doi: 10.1182/blood-2010-02-269134.
53. Abe J, Berk BC. Atheroprone flow activation of the sterol regulatory element binding protein 2 and nod-like receptor protein 3 inflammasome mediates focal atherosclerosis. *Circulation*. 2013;128:579–582. doi: 10.1161/CIRCULATIONAHA.113.004390.
54. Nigro P, Abe J, Berk BC. Flow shear stress and atherosclerosis: a matter of site specificity. *Antioxid Redox Signal*. 2011;15:1405–1414. doi: 10.1089/ars.2010.3679.
55. Zhou Q, Liao JK. Rho-kinases: an important mediator of atherosclerosis and vascular disease. *Curr Pharm Des*. 2009;15:3108–3115.
56. Yao L, Chandra S, Toque HA, Bhatta A, Rojas M, Caldwell RB, Caldwell RW. Prevention of diabetes-induced arginase activation and vascular dysfunction by Rho-kinases (ROCK) knockout. *Cardiovasc Res*. 2013;97:509–519. doi: 10.1093/cvr/cvs371.
57. Noda K, Godo S, Saito H, Tsutsui M, Shimokawa H. Opposing roles of nitric oxide and Rho-kinase in lipid metabolism in mice. *Tohoku J Exp Med*. 2015;235:171–183. doi: 10.1620/tjem.235.171.
58. Noda K, Nakajima S, Godo S, Saito H, Ikeda S, Shimizu T, Enkhjargal B, Fukumoto Y, Tsukita S, Yamada T, Katagiri H, Shimokawa H. Rho-kinase inhibition ameliorates metabolic disorders through activation of AMPK pathway in mice. *PLoS One*. 2014;9:e110446. doi: 10.1371/journal.pone.0110446.
59. Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. *Arterioscler Thromb Vasc Biol*. 2001;21:1712–1719.
60. Tanaka S, Fukumoto Y, Nochioka K, Minami T, Kudo S, Shiba N, Takai Y, Williams CL, Liao JK, Shimokawa H. Statins exert the pleiotropic effects through small GTP-binding protein dissociation stimulator upregulation with a resultant Rac1 degradation. *Arterioscler Thromb Vasc Biol*. 2013;33:1591–1600. doi: 10.1161/ATVBAHA.112.300922.
61. Shao D, Oka S, Brady CD, Haendeler J, Eaton P, Sadoshima J. Redox modification of cell signaling in the cardiovascular system. *J Mol Cell Cardiol*. 2012;52:550–558. doi: 10.1016/j.yjmcc.2011.09.009.
62. Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, Kanaide H, Takeshita A. Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest*. 2000;106:1521–1530. doi: 10.1172/JCI10506.
63. Morikawa K, Shimokawa H, Matoba T, Kubota H, Akaike T, Talukder MA, Hatanaka M, Fujiki T, Maeda H, Takahashi S, Takeshita A. Pivotal role of Cu,Zn-superoxide dismutase in endothelium-dependent hyperpolarization. *J Clin Invest*. 2003;112:1871–1879. doi: 10.1172/JCI19351.
64. Takaki A, Morikawa K, Tsutsui M, Murayama Y, Tekes E, Yamagishi H, Ohashi J, Yada T, Yanagihara N, Shimokawa H. Crucial role of nitric oxide synthases system in endothelium-dependent hyperpolarization in mice. *J Exp Med*. 2008;205:2053–2063. doi: 10.1084/jem.20080106.
65. Takaki A, Morikawa K, Murayama Y, Yamagishi H, Hosoya M, Ohashi J, Shimokawa H. Roles of endothelial oxidases in endothelium-derived hyperpolarizing factor responses in mice. *J Cardiovasc Pharmacol*. 2008;52:510–517. doi: 10.1097/FJC.0b013e318190358b.
66. Cohen RA, Adachi T. Nitric-oxide-induced vasodilatation: regulation by physiologic s-glutathiolation and pathologic oxidation of the sarcoplasmic endoplasmic reticulum calcium ATPase. *Trends Cardiovasc Med*. 2006;16:109–114. doi: 10.1016/j.tcm.2006.02.001.
67. Wu C, Parrott AM, Fu C, Liu T, Marino SM, Gladyshev VN, Jain MR, Baykal AT, Li Q, Oka S, Sadoshima J, Beuve A, Simmons WJ, Li H. Thioredoxin 1-mediated post-translational modifications: reduction, transnitrosylation, denitrosylation, and related proteomics methodologies. *Antioxid Redox Signal*. 2011;15:2565–2604. doi: 10.1089/ars.2010.3831.
68. Spindel ON, Burke RM, Yan C, Berk BC. Thioredoxin-interacting protein is a biomechanical regulator of Src activity: key role in endothelial cell stress fiber formation. *Circ Res*. 2014;114:1125–1132. doi: 10.1161/CIRCRESAHA.114.301315.
69. Park SY, Shi X, Pang J, Yan C, Berk BC. Thioredoxin-interacting protein mediates sustained VEGFR2 signaling in endothelial cells required for angiogenesis. *Arterioscler Thromb Vasc Biol*. 2013;33:737–743. doi: 10.1161/ATVBAHA.112.300386.
70. Yamawaki H, Pan S, Lee RT, Berk BC. Fluid shear stress inhibits vascular inflammation by decreasing thioredoxin-interacting protein in endothelial cells. *J Clin Invest*. 2005;115:733–738. doi: 10.1172/JCI23001.
71. Wang XQ, Nigro P, World C, Fujiwara K, Yan C, Berk BC. Thioredoxin interacting protein promotes endothelial cell inflammation in response to disturbed flow by increasing leukocyte adhesion and repressing Kruppel-like factor 2. *Circ Res*. 2012;110:560–568. doi: 10.1161/CIRCRESAHA.111.256362.
72. Ishida T, Ishida M, Suero J, Takahashi M, Berk BC. Agonist-stimulated cytoskeletal reorganization and signal transduction at focal adhesions in vascular smooth muscle cells require c-Src. *J Clin Invest*. 1999;103:789–797. doi: 10.1172/JCI4189.
73. Haendeler J, Hoffmann J, Tischler V, Berk BC, Zeiher AM, Dimmeler S. Redox regulatory and anti-apoptotic functions of thioredoxin depend on S-nitrosylation at cysteine 69. *Nat Cell Biol*. 2002;4:743–749. doi: 10.1038/ncb851.
74. Shimokawa H, Satoh K. Light and dark of reactive oxygen species for vascular function: 2014 ASVB (Asian Society of Vascular Biology). *J Cardiovasc Pharmacol*. 2015;65:412–418. doi: 10.1097/FJC.0000000000000159.
75. Vanhoutte PM, Shimokawa H, Tang EH, Feletou M. Endothelial dysfunction and vascular disease. *Acta Physiol (Oxf)*. 2009;196:193–222. doi: 10.1111/j.1748-1716.2009.01964.x.
76. Shimokawa H. Hydrogen peroxide as an endothelium-derived hyperpolarizing factor. *Pflugers Arch*. 2010;459:915–922. doi: 10.1007/s00424-010-0790-8.
77. 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.

78. Prysyazhna 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. doi: 10.1038/nm.2603.
79. Félétou M, Vanhoutte PM. Endothelium-dependent hyperpolarizations: past beliefs and present facts. *Ann Med*. 2007;39:495–516. doi: 10.1080/07853890701491000.
80. Burgoyne JR, Madhani M, Cuello F, Charles RL, Brennan JP, Schröder E, Browning DD, Eaton P. Cysteine redox sensor in PKGIa enables oxidant-induced activation. *Science*. 2007;317:1393–1397. doi: 10.1126/science.1144318.
81. Burgoyne JR, Prysyazhna O, Rudyk O, Eaton P. cGMP-dependent activation of protein kinase G precludes disulfide activation: implications for blood pressure control. *Hypertension*. 2012;60:1301–1308. doi: 10.1161/HYPERTENSIONAHA.112.198754.
82. Ohashi J, Sawada A, Nakajima S, Noda K, Takaki A, Shimokawa H. Mechanisms for enhanced endothelium-derived hyperpolarizing factor-mediated responses in microvessels in mice. *Circ J*. 2012;76:1768–1779.
83. Nakajima S, Ohashi J, Sawada A, Noda K, Fukumoto Y, Shimokawa H. Essential role of bone marrow for microvascular endothelial and metabolic functions in mice. *Circ Res*. 2012;111:87–96. doi: 10.1161/CIRCRESAHA.112.270215.
84. Streb H, Irvine RF, Berridge MJ, Schulz I. Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature*. 1983;306:67–69.
85. Berridge MJ. Smooth muscle cell calcium activation mechanisms. *J Physiol*. 2008;586:5047–5061. doi: 10.1113/jphysiol.2008.160440.
86. Ikebe M, Hartshorne DJ. Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. *J Biol Chem*. 1985;260:10027–10031.
87. Seto M, Sasaki Y, Sasaki Y. Stimulus-specific patterns of myosin light chain phosphorylation in smooth muscle of rabbit thoracic artery. *Pflugers Arch*. 1990;415:484–489.
88. Shimokawa H, Seto M, Katsumata N, Amano M, Kozai T, Yamawaki T, Kuwata K, Kandabashi T, Egashira K, Ikegaki I, Asano T, Kaibuchi K, Takeshita A. Rho-kinase-mediated pathway induces enhanced myosin light chain phosphorylations in a swine model of coronary artery spasm. *Cardiovasc Res*. 1999;43:1029–1039.
89. Cai Y, Nagel DJ, Zhou Q, Cygnar KD, Zhao H, Li F, Pi X, Knight PA, Yan C. Role of cAMP-phosphodiesterase 1C signaling in regulating growth factor receptor stability, vascular smooth muscle cell growth, migration, and neointimal hyperplasia. *Circ Res*. 2015;116:1120–1132. doi: 10.1161/CIRCRESAHA.116.304408.
90. Satoh K, Kikuchi N, Kurosawa R, Shimokawa H. PDE1C negatively regulates growth factor receptor degradation and promotes VSMC proliferation. *Circ Res*. 2015;116:1098–1100. doi: 10.1161/CIRCRESAHA.115.306139.
91. Fukumoto Y, Shimokawa H, Ito A, Kadokami T, Yonemitsu Y, Aikawa M, Owada MK, Egashira K, Sueishi K, Nagai R, Yazaki Y, Takeshita A. Inflammatory cytokines cause coronary arteriosclerosis-like changes and alterations in the smooth-muscle phenotypes in pigs. *J Cardiovasc Pharmacol*. 1997;29:222–231.
92. Seto M, Yano K, Sasaki Y, Azuma H. Intimal hyperplasia enhances myosin phosphorylation in rabbit carotid artery. *Exp Mol Pathol*. 1993;58:1–13. doi: 10.1006/exmp.1993.1001.
93. Kitazawa T, Masuo M, Somlyo AP. G protein-mediated inhibition of myosin light-chain phosphatase in vascular smooth muscle. *Proc Natl Acad Sci U S A*. 1991;88:9307–9310.
94. Gong MC, Iizuka K, Nixon G, Browne JP, Hall A, Eccleston JF, Sugai M, Kobayashi S, Somlyo AV, Somlyo AP. Role of guanine nucleotide-binding proteins–ras-family or trimeric proteins or both–in Ca^{2+} sensitization of smooth muscle. *Proc Natl Acad Sci U S A*. 1996;93:1340–1345.
95. Alessi D, MacDougall LK, Sola MM, Ikebe M, Cohen P. The control of protein phosphatase-1 by targetting subunits. The major myosin phosphatase in avian smooth muscle is a novel form of protein phosphatase-1. *Eur J Biochem*. 1992;210:1023–1035.
96. Shimizu H, Ito M, Miyahara M, Ichikawa K, Okubo S, Konishi T, Naka M, Tanaka T, Hirano K, Hartshorne DJ. Characterization of the myosin-binding subunit of smooth muscle myosin phosphatase. *J Biol Chem*. 1994;269:30407–30411.
97. Chihara K, Amano M, Nakamura N, Yano T, Shibata M, Tokui T, Ichikawa H, Ikebe R, Ikebe M, Kaibuchi K. Cytoskeletal rearrangements and transcriptional activation of c-fos serum response element by Rho-kinase. *J Biol Chem*. 1997;272:25121–25127.
98. Kureishi Y, Kobayashi S, Amano M, Kimura K, Kanaide H, Nakano T, Kaibuchi K, Ito M. Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *J Biol Chem*. 1997;272:12257–12260.
99. Amano M, Chihara K, Nakamura N, Fukata Y, Yano T, Shibata M, Ikebe M, Kaibuchi K. Myosin II activation promotes neurite retraction during the action of Rho and Rho-kinase. *Genes Cells*. 1998;3:177–188.
100. Alexander RW. Theodore Cooper Memorial Lecture. Hypertension and the pathogenesis of atherosclerosis. Oxidative stress and the mediation of arterial inflammatory response: a new perspective. *Hypertension*. 1995;25:155–161.
101. Omar HA, Cherry PD, Mortelliti MP, Burke-Wolin T, Wolin MS. Inhibition of coronary artery superoxide dismutase attenuates endothelium-dependent and -independent nitrovasodilator relaxation. *Circ Res*. 1991;69:601–608.
102. Baas AS, Berk BC. Differential activation of mitogen-activated protein kinases by H_2O_2 and O_2- in vascular smooth muscle cells. *Circ Res*. 1995;77:29–36.
103. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res*. 1994;74:1141–1148.
104. Higashi M, Shimokawa H, Hattori T, Hiroki J, Mukai Y, Morikawa K, Ichiki T, Takahashi S, Takeshita A. Long-term inhibition of Rho-kinase suppresses angiotensin II-induced cardiovascular hypertrophy in rats in vivo: effect on endothelial NAD(P)H oxidase system. *Circ Res*. 2003;93:767–775. doi: 10.1161/01.RES.0000096650.91688.28.
105. van Nieuw Amerongen GP, van Delft S, Vermeer MA, Collard JG, van Hinsbergh VW. Activation of RhoA by thrombin in endothelial hyperpermeability: role of Rho-kinase and protein tyrosine kinases. *Circ Res*. 2000;87:335–340.
106. Kishi H, Bao J, Kohama K. Inhibitory effects of ML-9, wortmannin, and Y-27632 on the chemotaxis of vascular smooth muscle cells in response to platelet-derived growth factor-BB. *J Biochem*. 2000;128:719–722.
107. Sauzeau V, Le Jeune H, Cario-Toumaniantz C, Vaillant N, Gadeau AP, Desgranges C, Scalbert E, Chardin P, Pacaud P, Loirand G. P2Y(1), P2Y(2), P2Y(4), and P2Y(6) receptors are coupled to Rho and Rho-kinase activation in vascular myocytes. *Am J Physiol Heart Circ Physiol*. 2000;278:H1751–H1761.
108. Sauzeau V, Le Mellionnec E, Bertoglio J, Scalbert E, Pacaud P, Loirand G. Human urotensin II-induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-kinase. *Circ Res*. 2001;88:1102–1104.
109. Suzuki J, Jin ZG, Meoli DF, Matoba T, Berk BC. Cyclophilin A is secreted by a vesicular pathway in vascular smooth muscle cells. *Circ Res*. 2006;98:811–817. doi: 10.1161/01.RES.0000216405.85080.a6.
110. Satoh K, Matoba T, Suzuki J, O'Dell MR, Nigro P, Cui Z, Mohan A, Pan S, Li L, Jin ZG, Yan C, Abe J, Berk BC. Cyclophilin A mediates vascular remodeling by promoting inflammation and vascular smooth muscle cell proliferation. *Circulation*. 2008;117:3088–3098. doi: 10.1161/CIRCULATIONAHA.107.756106.
111. Shimokawa H. Cellular and molecular mechanisms of coronary artery spasm: lessons from animal models. *Jpn Circ J*. 2000;64:1–12.
112. Shimokawa H. Rho-kinase as a novel therapeutic target in treatment of cardiovascular diseases. *J Cardiovasc Pharmacol*. 2002;39:319–327.
113. Amano M, Chihara K, Kimura K, Fukata Y, Nakamura N, Matsuura Y, Kaibuchi K. Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science*. 1997;275:1308–1311.
114. Wang J, Weigand L, Foxson J, Shimoda LA, Sylvester JT. Ca^{2+} signaling in hypoxic pulmonary vasoconstriction: effects of myosin light chain and Rho-kinase antagonists. *Am J Physiol Lung Cell Mol Physiol*. 2007;293:L674–L685. doi: 10.1152/ajplung.00141.2007.
115. Satoh K, Godo S, Saito H, Enkhjargal B, Shimokawa H. 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. doi: 10.1016/j.yjmcc.2013.12.022.
116. Fleming I, Michaelis UR, Bredenkötter D, Fisslthaler B, Dehghani F, Brandes RP, Busse R. Endothelium-derived hyperpolarizing factor synthase (Cytochrome P450 2C9) is a functionally significant source of reactive oxygen species in coronary arteries. *Circ Res*. 2001;88:44–51.
117. Lassègue B, San Martín A, Griendling KK. Biochemistry, physiology, and pathophysiology of NADPH oxidases in the cardiovascular system. *Circ Res*. 2012;110:1364–1390. doi: 10.1161/CIRCRESAHA.111.243972.
118. Nishida M, Hara Y, Yoshida T, Inoue R, Mori Y. TRP channels: molecular diversity and physiological function. *Microcirculation*. 2006;13:535–550. doi: 10.1080/10739680600885111.

119. Shimizu S, Takahashi N, Mori Y. TRPs as chemosensors (ROS, RNS, RCS, gasotransmitters). *Handb Exp Pharmacol*. 2014;223:767–794. doi: 10.1007/978-3-319-05161-1_3.
120. Vanhoutte PM. Say NO to ET. *J Auton Nerv Syst*. 2000;81:271–277.
121. Yang D, Félétou M, Boulanger CM, Wu HF, Levens N, Zhang JN, Vanhoutte PM. Oxygen-derived free radicals mediate endothelium-dependent contractions to acetylcholine in aortas from spontaneously hypertensive rats. *Br J Pharmacol*. 2002;136:104–110. doi: 10.1038/sj.bjp.0704669.
122. Shimokawa H. Reactive oxygen species promote vascular smooth muscle cell proliferation. *Circ Res*. 2013;113:1040–1042. doi: 10.1161/CIRCRESAHA.113.302049.
123. Satoh K, Fukumoto Y, Nakano M, Sugimura K, Nawata J, Demachi J, Karibe A, Kagaya Y, Ishii N, Sugamura K, Shimokawa H. Statin ameliorates hypoxia-induced pulmonary hypertension associated with down-regulated stromal cell-derived factor-1. *Cardiovasc Res*. 2009;81:226–234. doi: 10.1093/cvr/cvn244.
124. Nigro P, Satoh K, O'Dell MR, Soe NN, Cui Z, Mohan A, Abe J, Alexis JD, Sparks JD, Berk BC. Cyclophilin A is an inflammatory mediator that promotes atherosclerosis in apolipoprotein E-deficient mice. *J Exp Med*. 2011;208:53–66. doi: 10.1084/jem.20101174.
125. Shimokawa H, Rashid M. Development of Rho-kinase inhibitors for cardiovascular medicine. *Trends Pharmacol Sci*. 2007;28:296–302. doi: 10.1016/j.tips.2007.04.006.
126. Satoh K, Fukumoto Y, Shimokawa H. Rho-kinase: important new therapeutic target in cardiovascular diseases. *Am J Physiol Heart Circ Physiol*. 2011;301:H287–H296. doi: 10.1152/ajpheart.00327.2011.
127. Eto Y, Shimokawa H, Hiroki J, Morishige K, Kandabashi T, Matsumoto Y, Amano M, Hoshijima M, Kaibuchi K, Takeshita A. Gene transfer of dominant negative Rho-kinase suppresses neointimal formation after balloon injury in pigs. *Am J Physiol Heart Circ Physiol*. 2000;278:H1744–H1750.
128. Sawada N, Itoh H, Ueyama K, Yamashita J, Doi K, Chun TH, Inoue M, Masatsugu K, Saito T, Fukunaga Y, Sakaguchi S, Arai H, Ohno N, Komeda M, Nakao K. Inhibition of rho-associated kinase results in suppression of neointimal formation of balloon-injured arteries. *Circulation*. 2000;101:2030–2033.
129. Shibata R, Kai H, Seki Y, Kato S, Morimatsu M, Kaibuchi K, Imaizumi T. Role of Rho-associated kinase in neointima formation after vascular injury. *Circulation*. 2001;103:284–289.
130. Miyata K, Shimokawa H, Kandabashi T, Higo T, Morishige K, Eto Y, Egashira K, Kaibuchi K, Takeshita A. Rho-kinase is involved in macrophage-mediated formation of coronary vascular lesions in pigs in vivo. *Arterioscler Thromb Vasc Biol*. 2000;20:2351–2358.
131. Shimokawa H, Ito A, Fukumoto Y, Kadokami T, Nakaike R, Sakata M, Takayanagi T, Egashira K, Takeshita A. Chronic treatment with interleukin-1 beta induces coronary intimal lesions and vasospastic responses in pigs in vivo. The role of platelet-derived growth factor. *J Clin Invest*. 1996;97:769–776. doi: 10.1172/JCI118476.
132. Shimokawa H, Morishige K, Miyata K, Kandabashi T, Eto Y, Ikegaki I, Asano T, Kaibuchi K, Takeshita A. Long-term inhibition of Rho-kinase induces a regression of arteriosclerotic coronary lesions in a porcine model in vivo. *Cardiovasc Res*. 2001;51:169–177.
133. Matsumoto Y, Uwatoku T, Oi K, Abe K, Hattori T, Morishige K, Eto Y, Fukumoto Y, Nakamura K, Shibata Y, Matsuda T, Takeshita A, Shimokawa H. Long-term inhibition of Rho-kinase suppresses neointimal formation after stent implantation in porcine coronary arteries: involvement of multiple mechanisms. *Arterioscler Thromb Vasc Biol*. 2004;24:181–186. doi: 10.1161/01.ATV.0000105053.46994.5B.
134. Hattori T, Shimokawa H, Higashi M, Hiroki J, Mukai Y, Tsutsui H, Kaibuchi K, Takeshita A. Long-term inhibition of Rho-kinase suppresses left ventricular remodeling after myocardial infarction in mice. *Circulation*. 2004;109:2234–2239. doi: 10.1161/01.CIR.0000127939.16111.58.
135. Radeff JM, Nagy Z, Stern PH. Rho and Rho-kinase are involved in parathyroid hormone-stimulated protein kinase C alpha translocation and IL-6 promoter activity in osteoblastic cells. *J Bone Miner Res*. 2004;19:1882–1891. doi: 10.1359/JBMR.040806.
136. Funakoshi Y, Ichiki T, Shimokawa H, Egashira K, Takeda K, Kaibuchi K, Takeya M, Yoshimura T, Takeshita A. Rho-kinase mediates angiotensin II-induced monocyte chemoattractant protein-1 expression in rat vascular smooth muscle cells. *Hypertension*. 2001;38:100–104.
137. Hattori T, Shimokawa H, Higashi M, Hiroki J, Mukai Y, Kaibuchi K, Takeshita A. Long-term treatment with a specific Rho-kinase inhibitor suppresses cardiac allograft vasculopathy in mice. *Circ Res*. 2004;94:46–52. doi: 10.1161/01.RES.0000107196.21335.2B.
138. Wang F, Okamoto Y, Inoki I, et al. Sphingosine-1-phosphate receptor-2 deficiency leads to inhibition of macrophage proinflammatory activities and atherosclerosis in apoE-deficient mice. *J Clin Invest*. 2010;120:3979–3995. doi: 10.1172/JCI42315.
139. Takemoto M, Sun J, Hiroki J, Shimokawa H, Liao JK. Rho-kinase mediates hypoxia-induced downregulation of endothelial nitric oxide synthase. *Circulation*. 2002;106:57–62.
140. Oi K, Shimokawa H, Hiroki J, Uwatoku T, Abe K, Matsumoto Y, Nakajima Y, Nakajima K, Takeichi S, Takeshita A. Remnant lipoproteins from patients with sudden cardiac death enhance coronary vasospastic activity through upregulation of Rho-kinase. *Arterioscler Thromb Vasc Biol*. 2004;24:918–922. doi: 10.1161/01.ATV.0000126678.93747.80.
141. Jin ZG, Melaragno MG, Liao DF, Yan C, Haendeler J, Suh YA, Lambeth JD, Berk BC. Cyclophilin A is a secreted growth factor induced by oxidative stress. *Circ Res*. 2000;87:789–796.
142. Liao DF, Jin ZG, Baas AS, Daum G, Gygi SP, Aebersold R, Berk BC. Purification and identification of secreted oxidative stress-induced factors from vascular smooth muscle cells. *J Biol Chem*. 2000;275:189–196.
143. Satoh K. Cyclophilin A in cardiovascular homeostasis and diseases. *Tohoku J Exp Med*. 2015;235:1–15. doi: 10.1620/tjem.235.1.
144. Satoh K, Nigro P, Berk BC. Oxidative stress and vascular smooth muscle cell growth: a mechanistic linkage by cyclophilin A. *Antioxid Redox Signal*. 2010;12:675–682. doi: 10.1089/ars.2009.2875.
145. Satoh K, Shimokawa H, Berk BC. Cyclophilin A: promising new target in cardiovascular therapy. *Circ J*. 2010;74:2249–2256.
146. Mackay DJ, Hall A. Rho GTPases. *J Biol Chem*. 1998;273:20685–20688.
147. Neco P, Giner D, Viniegra S, Borges R, Villarroel A, Gutiérrez LM. New roles of myosin II during vesicle transport and fusion in chromaffin cells. *J Biol Chem*. 2004;279:27450–27457. doi: 10.1074/jbc.M311462200.
148. Damsker JM, Bukrinsky MI, Constant SL. Preferential chemotaxis of activated human $CD4^+$ T cells by extracellular cyclophilin A. *J Leukoc Biol*. 2007;82:613–618. doi: 10.1189/jlb.0506317.
149. Yang Y, Lu N, Zhou J, Chen ZN, Zhu P. Cyclophilin A up-regulates MMP-9 expression and adhesion of monocytes/macrophages via CD147 signalling pathway in rheumatoid arthritis. *Rheumatology (Oxford)*. 2008;47:1299–1310. doi: 10.1093/rheumatology/ken225.
150. Elvers M, Herrmann A, Seizer P, Münzer P, Beck S, Schönberger T, Borst O, Martin-Romero FJ, Lang F, May AE, Gawaz M. Intracellular cyclophilin A is an important Ca^{2+} regulator in platelets and critically involved in arterial thrombus formation. *Blood*. 2012;120:1317–1326. doi: 10.1182/blood-2011-12-398438.
151. Seizer P, Ungern-Sternberg SN, Schönberger T, Borst O, Münzer P, Schmidt EM, Mack AF, Heinzmann D, Chatterjee M, Langer H, Malešević M, Lang F, Gawaz M, Fischer G, May AE. Extracellular cyclophilin A activates platelets via EMMPRIN (CD147) and PI3K/Akt signaling, which promotes platelet adhesion and thrombus formation in vitro and in vivo. *Arterioscler Thromb Vasc Biol*. 2015;35:655–663. doi: 10.1161/ATVBAHA.114.305112.
152. Seizer P, Gawaz M, May AE. Cyclophilin A and EMMPRIN (CD147) in cardiovascular diseases. *Cardiovasc Res*. 2014;102:17–23. doi: 10.1093/cvr/cvu035.
153. Eto M, Barandiér C, Rathgeb L, Kozai T, Joch H, Yang Z, Lüscher TF. Thrombin suppresses endothelial nitric oxide synthase and upregulates endothelin-converting enzyme-1 expression by distinct pathways: role of Rho/ROCK and mitogen-activated protein kinase. *Circ Res*. 2001;89:583–590.
154. Soe NN, Sowden M, Baskaran P, Smolock EM, Kim Y, Nigro P, Berk BC. Cyclophilin A is required for angiotensin II-induced p47phox translocation to caveolae in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2013;33:2147–2153. doi: 10.1161/ATVBAHA.113.301894.
155. Pushkarsky T, Zybarth G, Dubrovsky L, Yurchenko V, Tang H, Guo H, Toole B, Sherry B, Bukrinsky M. CD147 facilitates HIV-1 infection by interacting with virus-associated cyclophilin A. *Proc Natl Acad Sci U S A*. 2001;98:6360–6365. doi: 10.1073/pnas.111583198.
156. Satoh K, Satoh T, Kikuchi N, et al. Basigin mediates pulmonary hypertension by promoting inflammation and vascular smooth muscle cell proliferation. *Circ Res*. 2014;115:738–750. doi: 10.1161/CIRCRESAHA.115.304563.
157. Weintraub NL. Understanding abdominal aortic aneurysm. *N Engl J Med*. 2009;361:1114–1116. doi: 10.1056/NEJMcibr0905244.
158. Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature*. 1997;389:990–994. doi: 10.1038/40187.
159. Hirooka Y, Shimokawa H, Takeshita A. Rho-kinase, a potential therapeutic target for the treatment of hypertension. *Drug News Perspect*. 2004;17:523–527.
160. Mukai Y, Shimokawa H, Matoba T, Kandabashi T, Satoh S, Hiroki J, Kaibuchi K, Takeshita A. Involvement of Rho-kinase in hypertensive vascular disease: a novel therapeutic target in hypertension. *FASEB J*. 2001;15:1062–1064.

161. Haack KK, Gao L, Schiller AM, Curry PL, Pellegrino PR, Zucker IH. Central Rho-kinase inhibition restores baroreflex sensitivity and angiotensin II type 1 receptor protein imbalance in conscious rabbits with chronic heart failure. *Hypertension*. 2013;61:723–729. doi: 10.1161/HYPERTENSIONAHA.111.00396.
162. Ito K, Hirooka Y, Sakai K, Kishi T, Kaibuchi K, Shimokawa H, Takeshita A. Rho/Rho-kinase pathway in brain stem contributes to blood pressure regulation via sympathetic nervous system: possible involvement in neural mechanisms of hypertension. *Circ Res*. 2003;92:1337–1343. doi: 10.1161/01.RES.0000079941.59846.D4.
163. Zamanian RT, Kudelko KT, Sung YK, de Jesus Perez V, Liu J, Spiekerkoetter E. Current clinical management of pulmonary arterial hypertension. *Circ Res*. 2014;115:131–147. doi: 10.1161/CIRCRESAHA.115.303827.
164. Rabinovitch M, Guignabert C, Humbert M, Nicolls MR. Inflammation and immunity in the pathogenesis of pulmonary arterial hypertension. *Circ Res*. 2014;115:165–175. doi: 10.1161/CIRCRESAHA.113.301141.
165. Paulin R, Michelakis ED. The metabolic theory of pulmonary arterial hypertension. *Circ Res*. 2014;115:148–164. doi: 10.1161/CIRCRESAHA.115.301130.
166. Michelakis ED. Pulmonary arterial hypertension: yesterday, today, tomorrow. *Circ Res*. 2014;115:109–114. doi: 10.1161/CIRCRESAHA.115.301132.
167. Lai YC, Potoka KC, Champion HC, Mora AL, Gladwin MT. Pulmonary arterial hypertension: the clinical syndrome. *Circ Res*. 2014;115:115–130. doi: 10.1161/CIRCRESAHA.115.301146.
168. Austin ED, Loyd JE. The genetics of pulmonary arterial hypertension. *Circ Res*. 2014;115:189–202. doi: 10.1161/CIRCRESAHA.115.303404.
169. Fukumoto Y, Shimokawa H. Rho-kinase inhibitors. *Handb Exp Pharmacol*. 2013;218:351–363. doi: 10.1007/978-3-642-38664-0_14.
170. Stenmark KR, Fagan KA, Frid MG. Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms. *Circ Res*. 2006;99:675–691. doi: 10.1161/01.RES.0000243584.45145.3f.
171. Satoh K, Kagaya Y, Nakano M, et al. Important role of endogenous erythropoietin system in recruitment of endothelial progenitor cells in hypoxia-induced pulmonary hypertension in mice. *Circulation*. 2006;113:1442–1450. doi: 10.1161/CIRCULATIONAHA.105.583732.
172. Elias-Al-Mamun M, Satoh K, Tanaka S, Shimizu T, Nergui S, Miyata S, Fukumoto Y, Shimokawa H. Combination therapy with fasudil and sildenafil ameliorates monocrotaline-induced pulmonary hypertension and survival in rats. *Circ J*. 2014;78:967–976.
173. Ikeda S, Satoh K, Kikuchi N, Miyata S, Suzuki K, Omura J, Shimizu T, Kobayashi K, Kobayashi K, Fukumoto Y, Sakata Y, Shimokawa H. Crucial role of Rho-kinase in pressure overload-induced right ventricular hypertrophy and dysfunction in mice. *Arterioscler Thromb Vasc Biol*. 2014;34:1260–1271. doi: 10.1161/ATVBAHA.114.303320.
174. Jin ZG, Lungu AO, Xie L, Wang M, Wong C, Berk BC. Cyclophilin A is a proinflammatory cytokine that activates endothelial cells. *Arterioscler Thromb Vasc Biol*. 2004;24:1186–1191. doi: 10.1161/01.ATV.0000130664.51010.28.
175. Khromykh LM, Kulikova NL, Anfalova TV, Muranova TA, Abramov VM, Vasiliev AM, Khlebnikov VS, Kazansky DB. Cyclophilin A produced by thymocytes regulates the migration of murine bone marrow cells. *Cell Immunol*. 2007;249:46–53. doi: 10.1016/j.cellimm.2007.11.002.
176. Abe K, Shimokawa H, Morikawa K, Uwatoku T, Oi K, Matsumoto Y, Hattori T, Nakashima Y, Kaibuchi K, Sueishi K, Takeshit A. Long-term treatment with a Rho-kinase inhibitor improves monocrotaline-induced fatal pulmonary hypertension in rats. *Circ Res*. 2004;94:385–393. doi: 10.1161/01.RES.0000111804.34509.94.
177. Abe K, Tawara S, Oi K, Hizume T, Uwatoku T, Fukumoto Y, Kaibuchi K, Shimokawa H. Long-term inhibition of Rho-kinase ameliorates hypoxia-induced pulmonary hypertension in mice. *J Cardiovasc Pharmacol*. 2006;48:280–285. doi: 10.1097/01.fjc.0000248244.64430.4a.
178. Do e Z, Fukumoto Y, Takaki A, Tawara S, Ohashi J, Nakano M, Tada T, Saji K, Sugimura K, Fujita H, Hoshikawa Y, Nawata J, Kondo T, Shimokawa H. Evidence for Rho-kinase activation in patients with pulmonary arterial hypertension. *Circ J*. 2009;73:1731–1739.
179. Fukumoto Y, Matoba T, Ito A, Tanaka H, Kishi T, Hayashidani S, Abe K, Takeshita A, Shimokawa H. Acute vasodilator effects of a Rho-kinase inhibitor, fasudil, in patients with severe pulmonary hypertension. *Heart*. 2005;91:391–392. doi: 10.1136/hrt.2003.029470.
180. Fukumoto Y, Yamada N, Matsubara H, et al. Double-blind, placebo-controlled clinical trial with a Rho-kinase inhibitor in pulmonary arterial hypertension. *Circ J*. 2013;77:2619–2625.
181. Dhaliwal JS, Casey DB, Greco AJ, Badejo AM Jr, Gallen TB, Murthy SN, Nossaman BD, Hyman AL, Kadowitz PJ. Rho-kinase and Ca^{2+} entry mediate increased pulmonary and systemic vascular resistance in L-NAME-treated rats. *Am J Physiol Lung Cell Mol Physiol*. 2007;293:L1306–L1313. doi: 10.1152/ajplung.00189.2007.
182. Rao GN, Berk BC. Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression. *Circ Res*. 1992;70:593–599.
183. Kandabashi T, Shimokawa H, Miyata K, Kunihiro I, Eto Y, Morishige K, Matsumoto Y, Obara K, Nakayama K, Takahashi S, Takeshita A. Evidence for protein kinase C-mediated activation of Rho-kinase in a porcine model of coronary artery spasm. *Arterioscler Thromb Vasc Biol*. 2003;23:2209–2214. doi: 10.1161/01.ATV.0000104010.87348.26.
184. Yamakawa T, Tanaka S, Numaguchi K, Yamakawa Y, Motley ED, Ichihara S, Inagami T. Involvement of Rho-kinase in angiotensin II-induced hypertrophy of rat vascular smooth muscle cells. *Hypertension*. 2000;35:313–318.
185. Shimokawa H. Bone-marrow-derived matrix metalloproteinase-14: a novel target for plaque stability. *Circulation*. 2008;117:863–865. doi: 10.1161/CIRCULATIONAHA.107.756346.
186. Kandabashi T, Shimokawa H, Mukai Y, Matoba T, Kunihiro I, Morikawa K, Ito M, Takahashi S, Kaibuchi K, Takeshita A. Involvement of Rho-kinase in agonists-induced contractions of arteriosclerotic human arteries. *Arterioscler Thromb Vasc Biol*. 2002;22:243–248.
187. Sawada N, Liao JK. Rho/Rho-associated coiled-coil forming kinase pathway as therapeutic targets for statins in atherosclerosis. *Antioxid Redox Signal*. 2014;20:1251–1267. doi: 10.1089/ars.2013.5524.
188. Takagi Y, Yasuda S, Takahashi J, Takeda M, Nakayama M, Ito K, Hirose M, Wakayama Y, Fukuda K, Shimokawa H. Importance of dual induction tests for coronary vasospasm and ventricular fibrillation in patients surviving out-of-hospital cardiac arrest. *Circ J*. 2009;73:767–769.
189. Hizume T, Morikawa K, Takaki A, Abe K, Sunagawa K, Amano M, Kaibuchi K, Kubo C, Shimokawa H. Sustained elevation of serum cortisol level causes sensitization of coronary vasoconstricting responses in pigs in vivo: a possible link between stress and coronary vasospasm. *Circ Res*. 2006;99:767–775. doi: 10.1161/01.RES.0000244093.69985.2f.
190. Kandabashi T, Shimokawa H, Miyata K, Kunihiro I, Kawano Y, Fukata Y, Higo T, Egashira K, Takahashi S, Kaibuchi K, Takeshita A. Inhibition of myosin phosphatase by upregulated Rho-kinase plays a key role for coronary artery spasm in a porcine model with interleukin-1beta. *Circulation*. 2000;101:1319–1323.
191. Katsumata N, Shimokawa H, Seto M, Kozai T, Yamawaki T, Kuwata K, Egashira K, Ikegaki I, Asano T, Sasaki Y, Takeshita A. Enhanced myosin light chain phosphorylations as a central mechanism for coronary artery spasm in a swine model with interleukin-1β. *Circulation*. 1997;96:4357–4363.
192. Ito A, Shimokawa H, Kadokami T, Fukumoto Y, Owada MK, Shiraishi T, Nakaike R, Takayanagi T, Egashira K, Takeshita A. Tyrosine kinase inhibitor suppresses coronary arteriosclerotic changes and vasospastic responses induced by chronic treatment with interleukin-1β in pigs in vivo. *J Clin Invest*. 1995;96:1288–1294. doi: 10.1172/JCI118163.
193. Libby P. Inflammation in atherosclerosis. *Nature*. 2002;420:868–874. doi: 10.1038/nature01323.
194. Kunieda T, Minamino T, Nishi J, Tateno K, Oyama T, Katsuno T, Miyauchi H, Orimo M, Okada S, Takamura M, Nagai T, Kaneko S, Komuro I. Angiotensin II induces premature senescence of vascular smooth muscle cells and accelerates the development of atherosclerosis via a p21-dependent pathway. *Circulation*. 2006;114:953–960. doi: 10.1161/CIRCULATIONAHA.106.626606.
195. Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part II: animal and human studies. *Circulation*. 2003;108:2034–2040. doi: 10.1161/01.CIR.0000093661.90582.c4.
196. Bruemmer D, Collins AR, Noh G, Wang W, Territo M, Arias-Magallona S, Fishbein MC, Blaschke F, Kintscher U, Graf K, Law RE, Hsueh WA. Angiotensin II-accelerated atherosclerosis and aneurysm formation is attenuated in osteopontin-deficient mice. *J Clin Invest*. 2003;112:1318–1331. doi: 10.1172/JCI18141.
197. Daugherty A, Manning MW, Cassis LA. Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice. *J Clin Invest*. 2000;105:1605–1612. doi: 10.1172/JCI7818.
198. Daugherty A, Cassis L. Angiotensin II-mediated development of vascular diseases. *Trends Cardiovasc Med*. 2004;14:117–120. doi: 10.1016/j.tcm.2004.01.002.
199. Thomas M, Gavrila D, McCormick ML, Miller FJ Jr, Daugherty A, Cassis LA, Dellsperger KC, Weintraub NL. Deletion of p47phox attenuates angiotensin II-induced abdominal aortic aneurysm formation in apolipoprotein E-deficient mice. *Circulation*. 2006;114:404–413. doi: 10.1161/CIRCULATIONAHA.105.607168.
200. Gavazzi G, Deffert C, Trocme C, Schäppi M, Herrmann FR, Krause KH. NOX1 deficiency protects from aortic dissection in response

to angiotensin II. *Hypertension*. 2007;50:189–196. doi: 10.1161/HYPERTENSIONAHA.107.089706.
201. Thompson RW, Baxter BT. MMP inhibition in abdominal aortic aneurysms. Rationale for a prospective randomized clinical trial. *Ann N Y Acad Sci*. 1999;878:159–178.
202. Manning MW, Cassis LA, Daugherty A. Differential effects of doxycycline, a broad-spectrum matrix metalloproteinase inhibitor, on angiotensin II-induced atherosclerosis and abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol*. 2003;23:483–488. doi: 10.1161/01.ATV.0000058404.92759.32.
203. Wang YX, Martin-McNulty B, da Cunha V, et al. Fasudil, a Rho-kinase inhibitor, attenuates angiotensin II-induced abdominal aortic aneurysm in apolipoprotein E-deficient mice by inhibiting apoptosis and proteolysis. *Circulation*. 2005;111:2219–2226. doi: 10.1161/01.CIR.0000163544.17221.BE.
204. Satoh K, Nigro P, Matoba T, O'Dell MR, Cui Z, Shi X, Mohan A, Yan C, Abe J, Illig KA, Berk BC. Cyclophilin A enhances vascular oxidative stress and the development of angiotensin II-induced aortic aneurysms. *Nat Med*. 2009;15:649–656. doi: 10.1038/nm.1958.
205. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol Cell Physiol*. 2007;292:C82–C97. doi: 10.1152/ajpcell.00287.2006.
206. Sadoshima J, Xu Y, Slayter HS, Izumo S. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell*. 1993;75:977–984.
207. Nakamura K, Fushimi K, Kouchi H, Mihara K, Miyazaki M, Ohe T, Namba M. Inhibitory effects of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor-α and angiotensin II. *Circulation*. 1998;98:794–799.
208. Akki A, Zhang M, Murdoch C, Brewer A, Shah AM. NADPH oxidase signaling and cardiac myocyte function. *J Mol Cell Cardiol*. 2009;47:15–22. doi: 10.1016/j.yjmcc.2009.04.004.
209. Vahebi S, Kobayashi T, Warren CM, de Tombe PP, Solaro RJ. Functional effects of Rho-kinase-dependent phosphorylation of specific sites on cardiac troponin. *Circ Res*. 2005;96:740–747. doi: 10.1161/01.RES.0000162457.56568.7d.
210. Fukui S, Fukumoto Y, Suzuki J, Saji K, Nawata J, Tawara S, Shinozaki T, Kagaya Y, Shimokawa H. Long-term inhibition of Rho-kinase ameliorates diastolic heart failure in hypertensive rats. *J Cardiovasc Pharmacol*. 2008;51:317–326. doi: 10.1097/FJC.0b013e31816533b7.
211. Takimoto E, Kass DA. Role of oxidative stress in cardiac hypertrophy and remodeling. *Hypertension*. 2007;49:241–248. doi: 10.1161/01.HYP.0000254415.31362.a7.
212. Satoh K, Nigro P, Zeidan A, Soe NN, Jaffré F, Oikawa M, O'Dell MR, Cui Z, Menon P, Lu Y, Mohan A, Yan C, Blaxall BC, Berk BC. Cyclophilin A promotes cardiac hypertrophy in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. 2011;31:1116–1123. doi: 10.1161/ATVBAHA.110.214601.
213. Yada T, Shimokawa H, Hiramatsu O, Kajita T, Shigeto F, Tanaka E, Shinozaki Y, Mori H, Kiyooka T, Katsura M, Ohkuma S, Goto M, Ogasawara Y, Kajiya F. Beneficial effect of hydroxyfasudil, a specific Rho-kinase inhibitor, on ischemia/reperfusion injury in canine coronary microcirculation in vivo. *J Am Coll Cardiol*. 2005;45:599–607. doi: 10.1016/j.jacc.2004.10.053.
214. Sato S, Ikegaki I, Asano T, Shimokawa H. Antiischemic properties of fasudil in experimental models of vasospastic angina. *Jpn J Pharmacol*. 2001;87:34–40.
215. Utsunomiya T, Satoh S, Ikegaki I, Toshima Y, Asano T, Shimokawa H. Antianginal effects of hydroxyfasudil, a Rho-kinase inhibitor, in a canine model of effort angina. *Br J Pharmacol*. 2001;134:1724–1730. doi: 10.1038/sj.bjp.0704410.
216. Satoh Si, Ikegaki I, Toshima Y, Watanabe A, Asano T, Shimokawa H. Effects of Rho-kinase inhibitor on vasopressin-induced chronic myocardial damage in rats. *Life Sci*. 2002;72:103–112.
217. Zoccarato A, Surdo NC, Aronsen JM, et al. Cardiac hypertrophy is inhibited by a local pool of cAMP regulated by phosphodiesterase 2. *Circ Res*. 2015;117:707–719. doi: 10.1161/CIRCRESAHA.114.305892.
218. Ryan JJ, Archer SL. The right ventricle in pulmonary arterial hypertension: disorders of metabolism, angiogenesis and adrenergic signaling in right ventricular failure. *Circ Res*. 2014;115:176–188. doi: 10.1161/CIRCRESAHA.113.301129.
219. Takefuji M, Krüger M, Sivaraj KK, Kaibuchi K, Offermanns S, Wettschureck N. RhoGEF12 controls cardiac remodeling by integrating G protein- and integrin-dependent signaling cascades. *J Exp Med*. 2013;210:665–673. doi: 10.1084/jem.20122126.
220. Brakebusch C, Fässler R. The integrin-actin connection, an eternal love affair. *EMBO J*. 2003;22:2324–2333. doi: 10.1093/emboj/cdg245.
221. Zhao XH, Laschinger C, Arora P, Szászi K, Kapus A, McCulloch CA. Force activates smooth muscle α-actin promoter activity through the Rho signaling pathway. *J Cell Sci*. 2007;120:1801–1809. doi: 10.1242/jcs.001586.
222. Shibata R, Ouchi N, Murohara T. Adiponectin and cardiovascular disease. *Circ J*. 2009;73:608–614.
223. Gillers BS, Chiplunkar A, Aly H, Valenta T, Basler K, Christoffels VM, Efimov IR, Boukens BJ, Rentschler S. Canonical Wnt signaling regulates atrioventricular junction programming and electrophysiological properties. *Circ Res*. 2015;116:398–406. doi: 10.1161/CIRCRESAHA.116.304731.
224. Delmar M, McKenna WJ. The cardiac desmosome and arrhythmogenic cardiomyopathies: from gene to disease. *Circ Res*. 2010;107:700–714. doi: 10.1161/CIRCRESAHA.110.223412.
225. Awad MM, Calkins H, Judge DP. Mechanisms of disease: molecular genetics of arrhythmogenic right ventricular dysplasia/cardiomyopathy. *Nat Clin Pract Cardiovasc Med*. 2008;5:258–267. doi: 10.1038/ncpcardio1182.
226. Corrado D, Basso C, Pilichou K, Thiene G. Molecular biology and clinical management of arrhythmogenic right ventricular cardiomyopathy/dysplasia. *Heart*. 2011;97:530–539. doi: 10.1136/hrt.2010.193276.
227. Amano M, Fukata Y, Kaibuchi K. Regulation and functions of Rho-associated kinase. *Exp Cell Res*. 2000;261:44–51. doi: 10.1006/excr.2000.5046.
228. Cristancho AG, Lazar MA. Forming functional fat: a growing understanding of adipocyte differentiation. *Nat Rev Mol Cell Biol*. 2011;12:722–734. doi: 10.1038/nrm3198.
229. Schlessinger K, Hall A, Tolwinski N. Wnt signaling pathways meet Rho GTPases. *Genes Dev*. 2009;23:265–277. doi: 10.1101/gad.1760809.
230. Angers S, Moon RT. Proximal events in Wnt signal transduction. *Nat Rev Mol Cell Biol*. 2009;10:468–477. doi: 10.1038/nrm2717.
231. Garcia-Gras E, Lombardi R, Giocondo MJ, Willerson JT, Schneider MD, Khoury DS, Marian AJ. Suppression of canonical Wnt/β-catenin signaling by nuclear plakoglobin recapitulates phenotype of arrhythmogenic right ventricular cardiomyopathy. *J Clin Invest*. 2006;116:2012–2021. doi: 10.1172/JCI27751.
232. Lombardi R, Dong J, Rodriguez G, Bell A, Leung TK, Schwartz RJ, Willerson JT, Brugada R, Marian AJ. Genetic fate mapping identifies second heart field progenitor cells as a source of adipocytes in arrhythmogenic right ventricular cardiomyopathy. *Circ Res*. 2009;104:1076–1084. doi: 10.1161/CIRCRESAHA.109.196899.
233. Lombardi R, da Graca Cabreira-Hansen M, Bell A, Fromm RR, Willerson JT, Marian AJ. Nuclear plakoglobin is essential for differentiation of cardiac progenitor cells to adipocytes in arrhythmogenic right ventricular cardiomyopathy. *Circ Res*. 2011;109:1342–1353. doi: 10.1161/CIRCRESAHA.111.255075.
234. Marcus FI, McKenna WJ, Sherrill D, et al. Diagnosis of arrhythmogenic right ventricular cardiomyopathy/dysplasia: proposed modification of the task force criteria. *Circulation*. 2010;121:1533–1541. doi: 10.1161/CIRCULATIONAHA.108.840827.
235. Asano T, Ikegaki I, Satoh S, Suzuki Y, Shibuya M, Takayasu M, Hidaka H. Mechanism of action of a novel antivasospasm drug, HA1077. *J Pharmacol Exp Ther*. 1987;241:1033–1040.
236. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J*. 2000;351:95–105.
237. Akama T, Dong C, Virtucio C, Freund YR, Chen D, Orr MD, Jacobs RT, Zhang YK, Hernandez V, Liu Y, Wu A, Bu W, Liu L, Jarnagin K, Plattner JJ. Discovery and structure-activity relationships of 6-(benzoylamino) benzoxaboroles as orally active anti-inflammatory agents. *Bioorg Med Chem Lett*. 2013;23:5870–5873. doi: 10.1016/j.bmcl.2013.08.096.
238. Akama T, Dong C, Virtucio C, Sullivan D, Zhou Y, Zhang YK, Rock F, Freund Y, Liu L, Bu W, Wu A, Fan XQ, Jarnagin K. Linking phenotype to kinase: identification of a novel benzoxaborole hinge-binding motif for kinase inhibition and development of high-potency Rho-kinase inhibitors. *J Pharmacol Exp Ther*. 2013;347:615–625. doi: 10.1124/jpet.113.207662.
239. Irani RA, Xia Y. The functional role of the renin-angiotensin system in pregnancy and preeclampsia. *Placenta*. 2008;29:763–771. doi: 10.1016/j.placenta.2008.06.011.
240. Wei L, Roberts W, Wang L, Yamada M, Zhang S, Zhao Z, Rivkees SA, Schwartz RJ, Imanaka-Yoshida K. Rho-kinase play an obligatory role in vertebrate embryonic organogenesis. *Development*. 2001;128:2953–2962.

RhoA/Rho-Kinase in the Cardiovascular System

Hiroaki Shimokawa, Shinichiro Sunamura and Kimio Satoh

Circ Res. 2016;118:352-366
doi: 10.1161/CIRCRESAHA.115.306532
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571