Rho Kinases and Cardiac Remodeling

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Hypertensive cardiac remodeling is characterized by left ventricular hypertrophy and interstitial fibrosis, which can lead to heart failure with preserved ejection fraction. The Rho-associated coiled-coil containing kinases (ROCKs) are members of the serine/threonine protein kinase family, which mediate the downstream effects of the small GTP-binding protein RhoA. There are 2 isoforms: ROCK1 and ROCK2. They have different functions in different types of cells and tissues. There is growing evidence that ROCKs contribute to the development of cardiovascular diseases, including cardiac fibrosis, hypertrophy, and subsequent heart failure. Recent experimental studies using ROCK inhibitors, such as fasudil, have shown the benefits of ROCK inhibition in cardiac remodeling. Mice lacking each ROCK isoform also exhibit reduced myocardial fibrosis in a variety of pathological models of cardiac remodeling. Indeed, clinical studies with fasudil have suggested that ROCKs could be potential novel therapeutic targets for cardiovascular diseases. In this review, we summarize the current understanding of the roles of ROCKs in the development of cardiac fibrosis and hypertrophy and discuss their therapeutic potential for deleterious cardiac remodeling. (Circ J 2016; 80: 1491–1498)

Key Words: Fibrosis; Heart diseases; Heart failure; Hypertrophy; Ventricular remodeling

The progression of cardiac remodeling, fibrosis and hypertrophy, is a pathological feature of many cardiac diseases that leads to heart failure (HF) and subsequent sudden death. Indeed, cardiac hypertrophy leading to HF is a serious and significant health problem with an estimated prevalence of 38 million patients worldwide. Previous clinical trials have demonstrated the efficiency of angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, mineralocorticoid-receptor antagonists, and β-blockers in reducing morbidity and mortality in patients with HF with reduced ejection fraction (HFrEF). In contrast to HFrEF, there is still no effective treatment for improving the prognosis of patients with HF with preserved EF (HFpEF). Interestingly, HFpEF currently consists of approximately half of HF cases and is characterized by excessive cardiomyocyte (CM) hypertrophy and interstitial fibrosis.

The Rho-associated coiled-coil containing kinases (ROCKs) are members of the serine/threonine protein kinase family, which were initially discovered as downstream targets of RhoA. RhoA is a small GTPase that plays an important role in the regulation of cell motility, proliferation, and apoptosis through effects on the actin cytoskeleton. Initially identified as downstream targets of RhoA, ROCKs mediate RhoA-induced stress fibers and focal adhesions. ROCKs have also been implicated in the regulation of vascular tone, cell proliferation, inflammation, and oxidative stress. Indeed, many animal and clinical studies suggest that ROCKs are important mediators of many cardiovascular diseases, including cardiac remodeling. Thus, ROCKs might be a potential therapeutic target for preventing or ameliorating the severity of cardiac hypertrophy and fibrosis, which could affect the development of HFpEF. Accordingly, a better understanding of ROCK pathway will provide greater insights into the pathogenesis of cardiac remodeling.

Structure and Function of ROCKs

ROCKs are downstream targets of RhoA and mediate Rho-induced actin cytoskeleton changes through effects on myosin light chain (MLC) phosphorylation. ROCKs consist of an N-terminal domain, followed by a mid-coiled-coil-forming region containing a Rho-binding domain (RBD), and a C-terminal cysteine-rich domain located within the pleckstrin homology (PH) motif domain (Figure 1). There are 2 isoforms: ROCK1 and ROCK2. The genes expressing human ROCK1 and ROCK2 are located on chromosome 18 (18q11.1) and chromosome 2 (2p24), respectively. ROCK1 and ROCK2 are highly homologous, sharing 65% homology in their amino acid sequence and 92% homology in their kinase domains. The ROCK C-terminus serves as an autoregulatory inhibitor of the N-terminal kinase domain in an inactive “closed” conformation under basal conditions. The activated GTP-bound RhoA binds to the RBD of ROCK, shifting the ROCK to an active “open” conformation and activating the N-terminal kinase domain. This open conformation can also be induced in a Rho-independent manner, by arachidonic acid binding to the PH domain or by caspase-3- and granzyme B-mediated cleavage of the C-terminus of ROCK1 and ROCK2, respectively.
in vivo, several groups successfully generated mutant mice with deletion of the \textit{ROCK1} and \textit{ROCK2} alleles. \textit{ROCK1}−/− mice exhibit eyelids open at birth (EOB) and die soon after birth with large omphalocele,\textsuperscript{16} whereas \textit{ROCK2}−/− mice die in utero because of placental dysfunction, intrauterine growth retardation, and fetal growth retardation.\textsuperscript{17} Interestingly, on a C57BL/6 genetic background, in addition to these phenotypes, EOB and omphalocele were also observed in \textit{ROCK2}−/− mice.\textsuperscript{18} These results indicate that both \textit{ROCK1} and \textit{ROCK2} have similar cytoskeletal targets and are necessary for eyelids and

\begin{figure}
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\includegraphics[width=\textwidth]{figure1}
\caption{Structure of Rho-associated coiled-coil containing kinases (ROCKs). ROCK1 and ROCK2 have 1,354 and 1,388 amino acids, respectively. Both consist of an N-terminal kinase domain, followed by a coiled-coil-forming region containing a Rho-binding domain (RBD) and a C-terminal cysteine-rich domain (CRD) located within the pleckstrin homology (PH) motif. The isoforms share an overall 65\% homology in amino acid sequence and 92\% homology in their kinase domains.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{figure2}
\caption{Closed-to-open conformation of Rho-associated coiled-coil containing kinase (ROCK) activation. In the inactive form, the pleckstrin homology and Rho-binding domain (RBD) domain bind to the N-terminus of the enzyme, forming an autoinhibitory loop. Binding of GTP-RhoA to the RBD opens the configuration, leading to the active kinase form of ROCK. ROCKs are also activated after the cleavage of the C-terminus of ROCK1 and ROCK2 by caspase-3 and granzyme B, respectively. CRD, C-terminal cysteine-rich domain.}
\end{figure}

\textsuperscript{11–13} Despite having similar kinase domains, ROCK1 and ROCK2 might have different functions through specific downstream targets. Although ROCK1 and ROCK2 are ubiquitously expressed in mouse tissues from early embryonic development to adulthood, mRNA coding ROCK1 is more abundantly expressed in immunological cells. In contrast, ROCK2 is highly expressed in cardiac muscle and vascular tissues, which indicates that ROCK2 might have a greater role in these cells.\textsuperscript{14,15}

To elucidate the specific functions of ROCK1 and ROCK2...
umbilical ring closure at a development stage without full compensation from the other isofrom. In addition, mice with mutated myosin phosphatase target subunit 1 (MYPT1) at either T694A or T852A, which are phosphorylation sites of ROCKs, also exhibited a similar phenotype of large omphalocele.19 These findings support the involvement of the ROCK pathway in the development of the ventral body wall. Taken together, these genetic studies using ROCK1−/− and ROCK2−/− mice have provided valuable insights into the role of both isoforms, which appear to be functionally necessary during embryonic development.

**Downstream Targets of ROCKs**

ROCKs are important regulators of cellular apoptosis, growth, metabolism, and migration via control of the actin cytoskeletal assembly and cell contraction.20 Stimulation of tyrosine kinase and G-protein-coupled receptors recruits and activates Rho guanine nucleotide exchange factors, leading to the activation of GTP-bound RhoA. ROCKs are pivotal downstream effectors of RhoA in the regulation of a broad range of cellular responses. Indeed, ROCKs have been shown to phosphorylate various substrates that are important in regulating actin cytoskeletal remodeling and focal adhesions, such as MLN, myosin-binding subunit (MBS, also named MYPT1) on MLCP, LIM kinase, ezrin-radixin-moesin (ERM) proteins, and adducin.5,6 Interestingly, ROCKs can also be auto-phosphorylated, which could modulate their function.7,8 MLN phosphorylation is one of the major downstream consequences of ROCKs. Although ROCK2 has been shown to directly phosphorylate Ser79 of MLN,7,8 the same residue that is phosphorylated by MLN kinase, ROCKs regulate the phosphorylation of MLN indirectly, mostly through the inhibition MLCP activity. Because this inhibition of MLCP activity leads to Ca2+ sensitization, ROCK2 can actually increase the sensitivity of smooth muscle cell (SMC) contraction in response to intracellular Ca2+ concentration.9 The MLCP holoenzyme is composed of 3 subunits: a catalytic subunit (PP1δ), a MBS composed of 58kD head and 32 kD tail region, and a small non-catalytic subunit, M21. In particular, MYPT1 on MLCP is an important downstream target of ROCKs. ROCK-mediated phosphorylation and inhibition of MYPT1 leads to persistent MLCP phosphorylation and subsequent SMC contraction. ROCK2 can phosphorylate MYPT1 at Thr697, Ser854, and Thr855. Indeed, phosphorylation of Thr697 and Thr855 has been shown to decrease MLCP activity,22 and in some instances, the dissociation of MLCP from myosin.23 ROCK2 also phosphorylates ERM proteins, namely Thr567 of ezrin, Thr564 of radixin, and Thr555 of moesin.24 However, the relative specificity of MYPT1 or ERM phosphorylation by ROCK isoforms is still not known.

**ROCKs and Cardiac Fibrosis and Hypertrophy**

ROCKs regulate various important cellular functions, including proliferation, migration, adhesion, and apoptosis/survival. Although these are critical functions for normal development and function, when in excess they can lead to the development of various human diseases, including cardiac remodeling and subsequent HF.7,8 Indeed, long-term treatment with ROCK inhibitors, such as fasudil and Y27632, has been shown to limit the progression of pathologic cardiac remodeling, fibrosis and hypertrophy, in mice and rats in response to angioten-sin II (AngII) and NG-nitro-L-arginine methyl ester treatment, transverse aortic constriction (TAC) operation, and myocardial infarction (MI).25−28 However, these ROCK inhibitors, which target the amino terminal ROCK kinase domain, are equimolar with respect to ROCK1 and ROCK2 inhibition, and therefore it is not possible to separate the specific effects of individual ROCK isoforms. Accordingly, the specific roles of ROCK1 and ROCK2 in cardiac fibrosis and hypertrophy have not been fully elucidated. Thus, ROCK1 and ROCK2 genetically-modified mouse models have been used to determine the isoform-specific and redundant roles of ROCKs in regulating cardiac remodeling. Global hemizygous ROCK1−/− and homozygous ROCK1−/− mice show decreased cardiac fibrosis, CM apoptosis, left ventricular (LV) dilatation, and contractile dysfunction, but not cardiac hypertrophy, in response to AngII and TAC. The decrease in perivascular fibrosis is partially caused by suppression of transforming growth factor-β1 and its downstream targets, such as connective tissue growth factor (CTGF) and collagen type I/III.29,30 In addition, in transgenic mice overexpressing an active form of ROCK1 in CMs, the development of fibrotic cardiomyopathy occurred spontane-ously, which was further augmented by AngII stimulation. This AngII-induced augmentation was attenuated by administration of fasudil.31 These findings provide strong evidence that ROCK1 is necessary for causing pathologic cardiac fibrosis and CM apoptosis, but not hypertrophy. Moreover, the absence of role of ROCK1 in the development of cardiac hypertrophy is also shown in another transgenic mouse model with CM-specific overexpression of Gaq, in which cardiac hypertrophy, dilation, and contractile dysfunction develop at older ages. Deletion of ROCK1 attenuated CM apoptosis and preserved cardiac structure and function in 1-year-old Gaq-
global hemizygous ROCK2−/− and CM-specific ROCK2-deficient mice. This mechanism is related, in part, to the upregulation of four-and-a-half LIM-only protein 2 (FHL2), which is unaltered in global ROCK1−/− mice. Subsequently, FHL2 was found to inhibit the expression of serum response factor (SRF) and extracellular signal-regulated kinase, both of which promote CM growth. Consistent with findings concerning ROCK2-mediated cardiac remodeling, the echocardiographic parameters of cardiac hypertrophy and diastolic dysfunction were also improved in global ROCK2−/− mice subjected to high-fat diet-induced obesity. Taken together, these results indicate that both ROCK1 and ROCK2 contribute to pathologic cardiac fibrosis and CM apoptosis. However, ROCK2, and not ROCK1, is important in mediating the cardiac prohypertrophic response (Figure 3).

So far, most of the previous animal studies related to the ROCK pathway have focused on LV systolic dysfunction, cardiac hypertrophy, and HF induced by agonist infusion, chronic pressure overload, and MI. However, there is a close relationship between the ROCK pathway and right ventricular (RV) failure. One recent study reported that compared with wild-type mice, transgenic mice with CM-specific overexpression of dominant negative ROCK (DN-ROCK), which suppresses the function of both ROCK1 and ROCK2, exhibited less cardiac fibrosis and hypertrophy and improved survival in response to both TAC and pulmonary artery constriction (PAC). In addition, that study reported that immunostaining of ROCKs showed that the expression of both ROCK1 and ROCK2 was increased in wild-type mice, 1 week after TAC, whereas the expression of ROCK2, but not ROCK1, was increased in the RV after PAC. These findings suggest that ROCK2 might be a better therapeutic target for RV remodeling and failure. In agreement with the many putative beneficial effects of pharmacological ROCK inhibition, these genetic mouse studies indicate that both ROCK1 and ROCK2 in CMs could contribute to the pathogenesis of LV and RV failure. In contrast, another recent study demonstrated that transgenic mice with overexpression of DN-ROCK driven by the SM22α promoter, which is expressed in the developing embryonic heart at E7.5 to E12.5, spontaneously exhibited histological and functional phenotypes of arrhythmogenic RV
cardiomyopathy (ARVC). Thus, through the critical role of the ROCK pathway in cardiac development, administration of ROCK inhibitors, as well as medications with inhibitory effects on the ROCK pathway such as statins, during pregnancy might contribute to the development of ARVC.

As mentioned, the small G-protein RhoA is the direct upstream activator of ROCKs. Because of its central role in several signaling pathways, RhoA appears to be intricately involved in the pathophysiology of cardiac diseases. Several studies have addressed the role of RhoA using mouse models. Overexpression (~20-fold increase) of constitutively active RhoA in CMs leads to the spontaneous development of dilated cardiomyopathy, HF, and bradycardia. However, conditional moderate overexpression (~2- to 5-fold increase) of this protein in CMs does not lead to such a phenotype, but instead, exerts a cardioprotective effect against ischemic-reperfusion (I/R) injury. In contrast, CM-specific RhoA-deficient mice, which do not show an apparent abnormality under physiological conditions, exhibit an increase in MI size when using the same I/R injury model. This finding, however, is not consistent with previous studies showing that ROCK inhibitors decreased infarct size and apoptosis in the murine heart after I/R injury, although ROCK inhibitors when given systemically are not cardiac-specific. By using a pressure-overload model, another study demonstrated that conditional deletion of RhoA in CMs caused accelerated dilation of the heart, but reduced cardiac fibrosis in mice after TAC, suggesting that RhoA is not required for cardiac development, but for adaptive compensatory hypertrophy under stress to prevent HF. In addition, conditional deletion of Rho guanine nucleotide exchange factor 12 (RhoGEF12), which leads to inhibition of RhoA and presumably ROCKs in CMs, protected mice from TAC-induced cardiac fibrosis, hypertrophy, and HF. Taken together, these studies indicate a critical role of ROCKs in mediating cardiac function and remodeling. However, further investigation is needed to determine the relative roles of the ROCK isoforms in the heart under different experimental conditions and models.

**ROCKs as Profibrotic Mediators**

CMs have been extensively studied for their roles in the pathogenesis of cardiac disease, including cardiac fibrosis and hypertrophy. However, there is increasing evidence that cardiac fibroblasts (CFs), the most abundant cell type in the mammalian heart, are also critically important for cardiac fibrosis and hypertrophy, in part through the enhanced synthesis of extracellular matrix proteins and the paracrine regulation of hypertrophic growth of CMs. Fibroblasts are also observed in multiple organs and tissues and substantially contribute to the pathogenesis of fibrotic diseases.

ROCK activation is required for the development of fibrosis in animal models of cardiac fibrosis, as well as fibrosis of multiple other organs such as lung, liver, kidney, and peritoneum (Figure 4). These findings strongly suggest the involvement of ROCKs in the pathogenesis of a broad array of fibrotic diseases. Indeed, ROCK inhibitors have been shown to suppress the extent of fibrosis in different fibrotic disease models in animals, such as pulmonary fibrosis induced by bleomycin, hepatic fibrosis induced by dimethylnitrosamine and diabetes, renal fibrosis induced by unilateral ureteral obstruction (UUO) and diabetes, and peritoneal fibrosis induced by dialysate and chlorhexidine. Although the isoform-specific role of ROCKs in the profibrotic process is unknown, some investigations using global ROCK1 and ROCK2 knockout mice have been reported to determine their respective roles in regulating renal fibrosis. The progression of renal fibrosis was attenuated in ROCK1−/− mice compared with littermate controls after streptozotocin-induced diabetic kidney injury. However, the extent of renal fibrosis was not affected after UUO. In addition, no protection against renal fibrosis after UUO was also observed in ROCK2−/− mice. These results are in contrast to the protective effects of ROCK inhibitors in a UUO model. It is possible that partial deletion of ROCK2 may not be sufficient to protect UUO-induced renal fibrosis.

Using ROCK inhibitors in mouse models of cardiac and pulmonary fibrosis to elucidate the downstream profibrotic pathway of ROCKs, it has been shown that ROCKs activate myocardin-related transcription factor (MRTF)/SRF-mediated profibrotic gene transcription, targeting several genes, such as profibrotic cytokine, CTGF, and the fibroblast-myofibroblast differentiation marker, α-SMA (Figure 5). Thus, the ROCK/MRTF/SRF pathway could account for the transition of fibroblast to myofibroblast. This could be a therapeutic target for various fibrotic diseases, including cardiac fibrosis (Figure 6).
Further studies are needed to determine the relative contributions of ROCK1 and ROCK2 and their downstream targets to the pathogenesis of fibrosis.

**ROCK Activity in Human Cardiac Hypertrophy and Heart Failure**

Because many animal studies have demonstrated the involvement of ROCKs in cardiovascular diseases, the circulating leukocyte ROCK activity, as determined by phosphorylation of MBS/MYPT1, has been reported to be a useful clinical biomarker for various cardiovascular diseases. Increased leukocyte ROCK activity has been observed in patients with hypertension,

60 pulmonary hypertension,

61 metabolic syndrome,

62 dyslipidemia,

63 coronary artery diseases,

64 coronary vasospasm,

65 and atherosclerosis.

66 Focusing on LV hypertrophy (LVH), leukocyte ROCK activity was higher in hypertensive patients with LVH compared with those without LVH.

67 Patients with dialysis-dependent renal failure and stage 3–4 chronic kidney disease, who are at high risk for cardiovascular events, exhibited enhanced leukocyte ROCK activity, correlating with the extent of LVH.

68 In addition, leukocyte ROCK activity was increased in patients with HF.

69–71 This higher leukocyte ROCK activity was associated with systolic dysfunction

72 and death. The elevated leukocyte ROCK activity in the acute phase of HF was decreased after treatment to the stable chronic phase.

73 Taken together, leukocyte ROCK activity could be a potential and novel biomarker of cardiovascular diseases, including cardiac hypertrophy and HF, despite the unknown functional relationship between leukocyte ROCK activity and the development of cardiovascular diseases.

**Clinical Implications of ROCK Inhibitors**

Until recently, only one ROCK inhibitor has been approved for clinical use, in Japan and China. Fasudil (intravenous administration) was approved in 1995 for the prevention and treatment of cerebral vasospasm after surgery for subarachnoid hemorrhage.

73 In the post-marketing surveillance studies involving 1,462 patients with fasudil treatment after surgery for ruptured aneurysms, the occurrence of adverse events was only 3.8%. In particular, the incidence of intracranial bleeding and hypotension was only 1.6% and 0.1%, respectively.

74 In a recent meta-analysis of the efficacy and safety of fasudil for the treatment of patients with subarachnoid hemorrhage, fasudil decreased the incidence of symptomatic and angiographic cerebral vasospasm without increased adverse events.

75 Therefore, fasudil appears to be a safe, effective, and well-tolerated treatment.

Despite robust animal studies with ROCK inhibitors, such as fasudil and Y27632, in order to translate the therapeutic benefits of ROCK inhibition to humans, clinical trials will need to be performed to document their beneficial effects. Currently, small clinical trials have shown that treatment with fasudil leads to improvements of symptoms and outcomes in patients with cardiovascular diseases, such as systemic hypertension,

76 pulmonary hypertension,

77,78 vasospastic angina,

79 stable effort angina,

80 and ischemic stroke.

81 Of note, many of the so-called “pleotropic” effects of statins in clinical trials may actually be mediated by ROCK inhibition.

82 However, the extent of the clinical benefits obtained by ROCK inhibition with statin therapy remains to be determined. Although the ROCK pathway also contributes to the development of cardiac remodeling and HF, based on the many previous animal studies, no prospective clinical trials using ROCK inhibitors have been conducted. One clinical study has demonstrated that intra-arterial infusion of fasudil caused a preferential increase in forearm blood flow in patients with HF compared with control subjects, suggesting that ROCK could be involved in the pathogenesis of underlying disorders leading to HF.

83 Furthermore, patients with coronary artery disease showed improved endothelial function with oral fasudil.

84 In addition, inhibition of ROCK has recently been suggested as a new target for RV HF. Indeed, fasudil improves both in-hospital mortality and re-hospitalization in patients with severe pulmonary hypertension and RV HF.

85 However, the long-term effects of fasudil administration on the RV are still unknown.

Besides fasudil, more than 170 different ROCK inhibitors have been developed.

86 More recently, ripasudil (eye drops), a novel ROCK inhibitor, was approved in Japan in 2014 for the treatment of glaucoma and ocular hypertension.

87 Furthermore, unlike previously tested nonselective ROCK inhibitors, an isoform-specific ROCK2 inhibitor, SLx-2119, appears to be 100-fold more selective towards ROCK2 than ROCK1 and could have a more favorable safety profile than dual ROCK inhibitors under certain conditions. SLx-2119 has been demonstrated to be a potential treatment for ischemic stroke

88 and autoimmune disease,

89 and is currently in Phase 2 clinical trials in patients with psoriasis. However, further clinical studies with this compound are warranted to determine its efficacy and safety in patients with cardiovascular diseases.

**Conclusions**

There is growing evidence from animal and clinical studies suggesting a critical role of the ROCK pathway in the pathogenesis of cardiovascular diseases, including cardiac remodeling and HF. ROCKs are known to mediate a large number of cellular and physiological functions. Importantly, ROCK activation in human circulating leukocytes correlates with various cardiovascular diseases and could potentially be used as a clinical biomarker for the diagnosis and monitoring of these disorders. Thus, inhibition of the ROCK pathway could be a novel and promising therapeutic target for attenuating the extent of cardiac remodeling and LV and RV HF, as demonstrated in studies to date. To further elucidate the redundant and non-redundant roles of ROCK1 and ROCK2 in these cardiac disorders, it will be necessary to generate mice with conditional and inducible deletion of each isoform in the specific cell types of the heart, such as CFs, vascular cells, and inflammatory cells, in addition to CMs. Based on these animal studies, further clinical trials could be performed to determine whether isoform-selective or nonselective ROCK inhibitors could be clinically effective and safe in the treatment of patients with cardiovascular disorders.

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**References**


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