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Rho-associated Coiled-coil Forming Kinase (ROCK): a potential target for the treatment of atherosclerosis and vascular disease

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Abstract

The Rho/Rho-associated coiled-coil forming kinases (ROCKs) are important regulators of the actin cytoskeleton. Because changes in the actin cytoskeleton underlie vascular contractility and remodeling, inflammatory cell recruitment, and cellular proliferation, it is likely that the Rho/ROCK pathway will play a central role in mediating vascular function. Indeed, increased ROCK activity is observed in cerebral and coronary vasospasm, hypertension, vascular inflammation, arteriosclerosis, and atherosclerosis. Recent experimental and clinical studies suggest that inhibition of ROCK could be a promising target for the treatment of cardiovascular disease. For example, inhibition of ROCK might be the underlying mechanism by which statins or HMG-CoA reductase inhibitors exert their therapeutic benefits beyond cholesterol reduction. In this review, we provide a current understanding of the critical role of RhoA/ROCK pathway in the regulation of vascular function and discuss its therapeutic potential in the treatment of atherosclerosis and vascular disease.

Keywords

Rho-associated coiled-coil forming kinase; atherosclerosis; inflammation; cardiovascular diseases; statins

Introduction

The Rho-associated coiled-coil forming kinases (ROCK1 and ROCK2) were initially discovered as downstream targets of the small GTP-binding protein RhoA. RhoA belongs to the small GTPases and is a major players in the regulation of cell motility, proliferation and apoptosis. ROCKs were characterized for their roles in mediating the formation of RhoA-induced stress fibres and focal adhesions^{1, 2}. However, there is increasing evidence suggesting a pivotal role of ROCKs in many aspects of vascular disorders such as abnormal vascular tone, endothelial dysfunction, inflammation, oxidative stress, and vascular remodelling³. Indeed, recent evidence suggests that ROCKs might play an important role in many cardiovascular diseases, e.g. systemic and pulmonary hypertension, atherosclerosis

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and cerebrovascular diseases⁴. However, it is not entirely clear how ROCKs are regulated, what some of their downstream targets are, and if ROCK1 and ROCK2 mediate different cellular functions.

Clinically, inhibition of the Rho/ROCK pathway is believed to contribute to some of the cardiovascular benefits of statin therapy that are independent of lipid lowering, i.e., pleiotropic effects. In particular, statins block the synthesis of isoprenoids, and therefore, the subsequent geranygeranylation of Rho GTPases. Through post-translational modifications, isoprenylation is critical for interacellular trafficking and function of small GTP-binding proteins. Thus, by inhibiting mevalonate synthesis, statins prevent membrane targeting of Rho and its subsequent activation of ROCK⁵. To what extent ROCK activity is inhibited in patients on statin therapy is not known, but this could have important clinical implications. Indeed, several pharmaceutical companies are already actively engaged in the development of ROCK inhibitors as the next generation of therapeutic agents for cardiovascular disease because evidence from animal studies suggests the potential involvement of ROCK in systemic and pulmonary hypertension, vascular inflammation, and atherosclerosis⁶. In this review, we discuss the role of ROCK inhibition as a therapeutic target in vascular diseases and atherosclerosis.

Structure of ROCK

The small GTP-binding proteins belonging to the Rho family regulate various aspects of cell shape, motility, proliferation and apoptosis⁷. ROCKs are downstream targets of RhoA^{8–10}, which mediate Rho-induced actin cytoskeletal changes through effects on myosin light chain phosphorylation^{1, 2}. They share 45–50% homology to other protein kinases such as myotonic dystrophy kinase (DMPK), myotonic dystrophy-related cdc42-binding kinase (MRCK), and citron kinase¹¹. ROCKs consist of an amino-terminal kinase domain, followed by a mid coiled-coil-forming region containing a Rho-binding domain (RBD), and carboxy-terminal cysteine-rich domain (CRD) located within the pleckstrin homology (PH) motif (Figure 1). In mammalian systems, two ROCK isoforms have been identified. *ROCK1*, which is also known as ROK β and p160ROCK, is located on chromosome 18, and encodes a 1354 amino acid protein^{1, 10}. *ROCK2*, which is also known as ROK α and sometimes referred to as Rho-kinase, is located on chromosome 2 and contains 1388 amino acids^{8, 9}. ROCK1 and ROCK2 share an overall 65% identity in amino-acid sequence and a 92% identity in their kinase domains¹¹.

The carboxy-terminus of ROCKs serves as an autoregulatory inhibitor of the amino-terminal kinase domain. The interaction of the active GTP-bound form of Rho and the Rho-binding domain of ROCK increases ROCK activity through *derepression* of the carboxyl-terminal RBD-pleckstrin homology (PH) domain on the amino-terminal kinase domain, leading to an active “open” kinase domain¹². The open conformation can also be induced by the binding of arachidonic acid to the PH domain¹³ or by cleavage of the carboxyl-terminus by caspase-3^{14, 15} or granzyme B¹⁶. This closed-to-open conformation of ROCK is similar to that of DMPK and MRCK activation¹⁷, and it is consistent with studies showing that over-expression of various carboxyl-terminal constructs of ROCK or kinase-defective forms of full-length ROCK, functions as dominant-negative ROCK mutants. ROCKs can also be activated independently of Rho through amino-terminal transphosphorylation¹⁷ or inhibited by other small GTP-binding proteins such as Gem and Rad¹⁸. However, recent findings from structural analysis indicate that phosphorylation at the activation loop and hydrophobic motif within the catalytic region (which is essential for the most of other AGC family kinase activation) is not necessary for ROCK activation¹⁹.

Despite having similar kinase domains, ROCK1 and ROCK2 might serve different functions and could have different downstream targets. Although ROCK1 and ROCK2 are ubiquitously expressed in mouse tissues from early embryonic development to adulthood, ROCK2 mRNA is highly expressed in cardiac muscle and vascular tissues, which indicates that ROCK2 might have a specialized role in these cell types¹¹. In contrast, ROCK1 is more abundantly expressed in immunological cells and has been shown to co-localize to centrosomes²⁰. But even in cells that contain both ROCK1 and ROCK2, recent findings suggest specific functions for both isoforms. Indeed, there is evidence that ROCK1 expression (rather than ROCK2) is upregulated upon macrophage adhesion²¹. At the same time, phagocytic uptake of fibronectin-coated beads is downregulated in ROCK2-depleted cells, but not in ROCK1-depleted cells²². These findings emphasize a distinct function for ROCK1 and ROCK2. Unfortunately, pharmacological inhibitors of ROCKs such as Y27632 and fasudil/hydroxyfasudil (HA1077), which target their ATP-dependent kinase domains, inhibit ROCK1 and ROCK2 at equimolar concentrations. Furthermore, at higher concentrations, Y27632 can also inhibit protein kinase C-related kinase (PRK)-2, protein kinase N, and citron kinase, whereas fasudil can inhibit protein kinase A (PKA) and protein kinase C (PKC)⁴. Therefore, it is difficult to ascribe specific function of ROCKs based upon studies with these ROCK inhibitors because they are nonselective for ROCK isoforms and can nonspecifically inhibit other protein kinases. Further studies such as gene targeting or silencing are necessary to unveil the precise mechanism(s) by which ROCK1 and ROCK2 regulate cellular function.

Downstream targets of ROCKs

In response to activators of Rho, such as lysophosphatidic acid (LPA) or sphingosine-1 phosphate (S1P), which stimulate RhoGEF and lead to the formation of active GTP-bound Rho, ROCKs mediate a broad range of cellular responses that involve the actin cytoskeleton^{11, 23}. For example, they control assembly of the actin cytoskeleton and cell contractility by phosphorylating a variety of proteins, such as myosin light chain (MLC) phosphatase, LIM kinases, adducin, and ezrin-radixin-moesin (ERM) proteins (Figure 3). The consensus amino acid sequences for phosphorylation are R/KXS/T or R/KXXS/T (R: arginine, K: lysine, X: any amino acid, S: serine, T: threonine)²⁴. ROCKs can also be auto-phosphorylated⁸, which might modulate their function. Specifically, ROCK2 phosphorylates Ser¹⁹ of MLC, the same residue that is phosphorylated by MLC kinase (MLCK). In addition, ROCKs regulate MLC phosphorylation indirectly through the inhibition of MLC phosphatase (MLCP) activity. Because inhibition of MLCP is believed to primarily contribute to Ca²⁺-sensitization, ROCK2 can also alter the sensitivity of SMC contraction to Ca²⁺²⁵. The MLCP holoenzyme is composed of three subunits: a catalytic subunit (PP1), a myosin-binding subunit (MBS) composed of a 58 kD head and 32 kD tail region, and a small non-catalytic subunit, M21. Depending upon the species, ROCK2 phosphorylates MBS at Thr⁶⁹⁷, Ser⁸⁵⁴, and Thr⁸⁵⁵. Phosphorylation of Thr⁶⁹⁷ or Thr⁸⁵⁵ attenuates MLCP activity¹³, and in some instances, the dissociation of MLCP from myosin²⁶. ROCK2 also phosphorylates ezrin-radixin-moesin (ERM) proteins, namely Thr⁵⁶⁷ of ezrin, Thr⁵⁶⁴ of radixin, and Thr⁵⁵⁸ of moesin⁴. ROCK-mediated phosphorylation leads to the disruption of the head-to-tail association of ERM proteins and actin cytoskeletal reorganization. By contrast, ROCK1 phosphorylates LIM kinase-1 at Thr⁵⁰⁸²⁷ and LIM kinase-2 at Thr⁵⁰⁵²⁴, which enhances the ability of LIM kinases to phosphorylate cofilin²⁸. Because cofilin is an actin-binding and -depolymerizing protein that regulates the turnover of actin filaments, the phosphorylation of LIM kinases by ROCKs inhibits cofilin-mediated actin filament disassembly and leads to an increase in the number of actin filaments.

Cellular functions of ROCKs

Stimulation of tyrosine kinase and G protein-coupled receptors leads to activation of Rho, the direct upstream activator of ROCKs, via recruitment and activation of RhoGEF^{29, 30}. Administration of Y27632 and fasudil, or overexpression of dominant-negative mutants of ROCKs, leads to the loss of stress fibers and focal adhesion complexes³¹. This is due predominantly to the phosphorylation and inhibition of MLCP by ROCK, which increases MLC phosphorylation and cellular contraction by facilitating interaction of myosin with F-actin. Thus, ROCKs regulate cell polarity and migration predominantly through enhancing actomyosin contraction and focal adhesions. In addition, ROCKs can also regulate macrophage phagocytic activity via actin cytoskeletal membrane protrusions and mediate endothelial cell permeability through effects on tight and adherens junctions³². ROCKs can inhibit insulin signaling via phosphorylation of IRS-1, which uncouples the insulin receptor to phosphatidylinositol-3 kinase³³. Conversely, ROCKs can also regulate cell size by enhancing IGF-induced CREB phosphorylation³⁴. Indeed, this might be the underlying mechanism by which ROCK inhibitors reduce cardiac hypertrophy. Finally, ROCKs might be involved in tissue differentiation from adipocytes to myocytes. In p190-B RhoGAP-deficient mice, which have high basal Rho/ROCK activity because there is no “off switch” for Rho, there is a defect in adipogenesis, with a predilection towards myogenesis^{34, 35}. Treatment of p190-B RhoGAP-deficient mice with Y27632 restores normal adipogenesis³⁵, suggesting that ROCKs are involved in the myogenesis differentiation program.

ROCKs and vascular disease

Large clinical trials suggest that 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors (also known as statins) reduce cardiovascular events, possibly by improving or restoring endothelial function⁵. Many of cholesterol-independent or so-called “pleiotropic” effects of statins are due to their ability to block the synthesis of isoprenoid intermediates, which serve as important lipid attachments for a variety of intracellular signaling molecules. In particular, the inhibition of small GTP-binding proteins Rho, Ras, and Rac, whose proper membrane localization and function are dependent upon isoprenylation³⁶, might play an important role in mediating the biological effects of statins. Statins increase the expression of endothelial nitric oxide synthase (eNOS) via inhibition of RhoA-mediated actin cytoskeletal changes, leading to the stabilization of eNOS mRNA³⁷. Indeed, a recent report suggests that binding of G-actin to the 3'-untranslated region of eNOS mRNA decreases eNOS mRNA expression³⁸. Furthermore, inhibition of the Rho/ROCK pathway leads to the rapid phosphorylation and activation of eNOS via the phosphatidylinositol (PI)-3 kinase/protein kinase Akt pathway³⁹. Thus, Rho/ROCKs negatively regulate endothelial function at the level of both eNOS expression and activation via two distinct mechanisms.

There is growing evidence that abnormal ROCK function contributes to vascular disease. In the vascular wall, ROCK mediates vascular smooth muscle contraction, actin cytoskeleton organization, cell adhesion and motility. Thus, abnormal ROCK activity might contribute to the abnormal smooth muscle contraction observed in cerebral and coronary vasospasm^{40–42}, hypertension, and pulmonary hypertension^{43, 44}. In addition, ROCK might also regulate vascular tone and blood flow indirectly through negative effects on eNOS expression and activity³⁹ or through direct effects on the central nervous system⁴⁵. Indeed, inhibition of ROCK leads to increased cerebral blood flow and decreased cerebral infarct size via upregulation of eNOS⁴⁶. ROCK is also involved in vascular inflammation and remodeling, restenosis after balloon injury⁴⁷, ischemia-reperfusion injury^{39, 48} and atherosclerosis^{49, 50}. Recent studies also suggest that long-term treatment with fasudil improves monocrotaline-induced fatal pulmonary hypertension in rats⁴⁴ and suppresses cardiac allograft vasculopathy in mice⁵¹. ROCK has also been implicated in the expression of a

variety of genes that are pertinent to vascular function, such as monocyte chemoattractant protein-1 (*MCP-1*), plasminogen activator inhibitor-1 (*PAI-1*), and osteopontin³. Indeed, ROCK is upregulated by inflammatory stimuli, such as angiotensin II and interleukin-1 β , in cultured cells⁵², and by lipopolysaccharides (*LPS*) *in vivo*⁵³.

Because ROCK is involved in various aspects of vascular function and disease, understanding the role of ROCKs in the vascular wall could provide key insights into how the vasculature as a whole is regulated under normal and pathophysiological conditions. However, despite increasing number of reports showing that ROCK activity is increased under a variety of pathological conditions, little is known about the molecular mechanisms, which contribute to increased ROCK activity or what the downstream targets for ROCK are. As mentioned above, determining the precise role of ROCKs in the vascular wall is difficult because of the non-selectivity of the pharmacological inhibitors. Further studies using genetic approaches with tissue-specific gene targeting of specific ROCK deletion to individual components of the vascular wall offers the greatest likelihood of success in dissecting the role of ROCKs in vascular disease.

ROCK and atherosclerosis

Atherosclerosis is a complex pathophysiological procedure characterized by progressive inflammation, lipid accumulation and arterial wall fibrosis. The pathophysiological process usually starts with endothelial dysfunction in the vessel wall leading to the activation of endothelial cells and recruitment of pro-inflammatory cells. The ensuing local inflammation then promotes the chemotaxis of leukocytes adhesion and recruitment of activated platelets to the damaged endothelium. This leads to increased permeability of the vessel wall for lipid components in the plasma. Lipid-rich monocytes then accumulate in the arterial intima, differentiating into macrophage-derived foam cells⁵⁴. Following the accumulation of additional inflammatory cell subsets and extracellular lipids, these early plaques (also known as fatty streaks) progress into mature atherosclerotic plaques. By secreting cytokines and growth factors these “early plaques” stimulate their own growth, resulting in further deposition of extracellular matrix components and progression of plaques and stenosis. The thinning of the fibrous cap (with possible consecutive plaque erosion) is caused by matrix degrading proteases and cytokines secreted by the plaque cells⁵⁵.

Cumulative evidence suggests that ROCK pathway is involved in many steps of the inflammatory atherosclerotic process. ROCK activation downregulates eNOS expression and inhibition of ROCK prevents hypoxia-induced downregulation of endothelial nitric oxide synthase³. In addition, it has been shown that LPA-induced endothelial hyperpermeability requires RhoA/ROCK activation⁵⁶. Long-term inhibition of ROCK induces a regression of arteriosclerotic coronary lesions in a porcine model *in vivo*⁵⁷. Inhibition of ROCK with Y-27632 limits early atherosclerotic plaque development in mutant mice with LDL receptor deficiency fed with high cholesterol diet. This was associated with a significant reduction in T-lymphocyte accumulation⁴⁹.

Another study analyzed the distribution and phosphorylation of target proteins of ROCK, such as myosin light chain (MLC) and ezrin-radixin-moesin (ERM) proteins, in the apolipoprotein E (ApoE)-deficient mouse model of atherosclerosis. Results showed that treatment with the ROCK inhibitor Y-27632 inhibited ERM phosphorylation in the atherosclerotic plaques⁵⁸. Indeed, mutant mice with ROCK1-deficiency in bone marrow-derived cells exhibit decreased atherosclerosis on LDL receptor-deficient background⁵⁰. This was due, in part, to decreased chemotaxis, cholesterol uptake, and foam cell formation in ROCK1-deficient macrophages. Indeed, ROCK1 is predominantly upregulated in the process of macrophage adherence²¹. These findings indicate that ROCK1 plays a critical

part in the development of atherosclerosis and suggest potential therapeutic benefits of ROCK1 inhibition in atherosclerotic vascular disease. However, it remains to be determined whether inhibition of ROCK 2 could also be beneficial in inhibiting atherosclerosis.

Findings from *in vitro* experiments and animal studies in the past years have provided significant evidence for the importance for ROCK as a potential target for the treatment of endothelial dysfunction and atherosclerosis. Indeed, numerous clinical studies have demonstrated a link between ROCK and endothelial dysfunction and metabolic syndromes in humans^{59, 60}. In particular, our research team has demonstrated a correlation between elevated ROCK activity and impaired endothelial function in coronary artery disease (CAD) patients⁶¹. Furthermore, treatment with the ROCK inhibitor fasudil reduced the over-activity of ROCK in patients with atherosclerosis and improved endothelium-dependent vasodilation as well as flow-mediated, endothelium-dependent dilation. Importantly, this finding was only present in patients with CAD, but not in healthy individuals, in whom ROCK is presumably not “overactive”. Most interestingly, endothelium-dependent vasodilation in healthy subjects tended to worsen with fasudil therapy compared with placebo. This finding might be explained by the fact that inhibition of ROCK in healthy individuals could lead to a negative feedback loop with increased transcription of Rho. This would in turn lead to a compensatory increase in the downstream effects of Rho, including suppression of eNOS production. Also, ROCK inhibition in healthy individuals might lead to an excess of NO production, resulting in the formation of peroxynitrite, which could lead to eNOS uncoupling and worsening endothelial function⁶². These results suggest that some basal ROCK activity is probably required for the maintenance of vascular homeostasis and emphasizes the importance of selective ROCK inhibitors for clinical usage.

Future directions: ROCKs as therapeutic targets in cardiovascular disease

ROCK inhibitors such as fasudil have been shown to prevent cerebral vasospasm after subarachnoid hemorrhage^{63, 64}. Similarly, animal studies with Y-27632 showed that it could inhibit the development of atherosclerosis and arterial remodeling following vascular injury⁶. ROCK activity is involved in the expression of PAI-1 mediated by hyperglycemia, indicating that ROCK may function as a key regulator of cardiovascular injury in patients with diabetes mellitus⁶⁵. RhoA/ROCK pathway has been reported to be involved in angiogenesis⁶⁶, cerebral ischemia^{46, 67}, erectile dysfunction⁶⁸, hypertension³¹, myocardial hypertrophy, myocardial ischemia-reperfusion injury⁴⁸, neointima formation⁴⁷, pulmonary hypertension⁴⁴, and vascular remodelling⁵¹. In addition, ROCK inhibitors have shown benefits in animal models of Alzheimer’s disease, bronchial asthma, cancers, demyelinating diseases, glaucoma, and osteoporosis^{3, 4, 69}. Although a majority of the previous studies have shown that inhibition of both isoforms by ROCK inhibitors results in beneficial effects, whether the effects are mediated by inhibition of ROCK1, ROCK2, or both, remains to be determined.

Despite robust animal data with ROCK inhibitors, in order to move the therapeutic benefits of ROCK inhibitors to humans, clinical trials will need to be performed to document their benefits in patients. Currently, small clinical trials have shown some benefits of ROCK inhibitors in cardiovascular diseases. For example, treatment with fasudil leads to improvements of symptoms and outcomes in patients with systemic hypertension^{31, 70}, pulmonary hypertension⁷¹, vasospastic angina⁴⁰, stable effort angina⁷², stroke⁶⁷, and chronic heart failure⁷³. Indeed, many of the so-called “pleiotropic” effects of statins might be mediated by ROCK inhibition⁵. However, the extent of clinical benefits obtained by ROCK inhibition with statin therapy remains to be determined. Currently, fasudil is the only ROCK inhibitor approved for human use. Fasudil was approved in Japan and China for prevention and treatment of cerebral vasospasm following surgery for subarachnoid

hemorrhage. Although several adverse effects have been reported (such as abnormal hepatic function, intracranial hemorrhage, and hypotension), fasudil appears to be relatively safe in patients with hemorrhagic stroke ⁶⁷. Therefore, fasudil may be one of the first promising ROCK inhibitors to be used for cardiovascular conditions such as acute stroke and pulmonary artery hypertension. Interestingly, another ROCK inhibitor, SAR407899, has been shown to be 8-fold more active than fasudil. In animal models, the antihypertensive effect of this novel ROCK inhibitor has been shown to be superior to that of fasudil and Y-27632 ⁷⁴. Thus, SAR407899 might represent a novel potent ROCK inhibitor for the treatment of cardiovascular disease. Finally, an isoform-selective ROCK2 inhibitor, SLx-2119 (Surface Logix), appears to be 100-fold more selective towards ROCK2 than ROCK1, and could have more favourable safety profile than dual ROCK inhibitors. However, further clinical studies with this compound need to be performed to determine its efficacy and safety in patients with cardiovascular disease. Because ROCK1 and ROCK2 both mediate various aspects of cardiovascular disease, there may not be an advantage of selective ROCK isoform inhibition in terms of efficacy, although such strategy might prove to be safer than dual ROCK isoform inhibition.

Concluding remarks

There is growing evidence that RhoA/ROCK pathway plays an important pathophysiological role in cardiovascular diseases. To date, a great number of cellular and physiological functions are mediated by ROCK, and ROCK activity is often elevated in disorders of the cardiovascular system. Thus, inhibition of ROCK might be an attractive therapeutic target in reducing cardiovascular disease. However, a greater understanding of the physiological role of each ROCK isoforms in the cardiovascular system and further clinical trials are needed to determine whether selective or non-selective ROCK inhibitors could be clinically usefully in treating patients with atherosclerosis and vascular disease.

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Figure 1. Structure of ROCK isoforms

Both of the isoforms (ROCK1 and ROCK 2) consist of an amino-terminal kinase domain followed by a coiled-coil forming region containing a Rho-binding (RB) domain and a carboxy-terminal cysteine-rich domain (CRD) located within the pleckstrin-homology (PH). The isoforms share overall 65% homology in amino acid sequence and 92% homology in their kinase domains. (adapted with permission from Rikitake R. and Liao J.K. (2005) *Expert Rev Cardiovasc Ther* 3:441–451, Expert Reviews Ltd.)

Figure 2. Activation of ROCK

Open configuration of active ROCK is mediated by binding of RhoA to the Rho binding domain (RBD), cleavage of the carboxy pleckstrin homology (PH) domain by caspase 3, or binding of arachidonic acid to the PH domain.

Figure 3. ROCK mediates RhoA-induced actin cytoskeletal changes

Activation of ROCK by GTP-bound RhoA inhibits myosin light chain phosphatase (MLCP) leading to increased MLC phosphorylation and stress fiber formation. ROCK can also activate LIM kinase, leading to phosphorylation of cofilin, actin nucleation and polymerization. GEF, guanine exchange factor; GAP, GTPase-activating protein; MLC, myosin light chain; MLCK, MLC kinase; p, phosphorylation.