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Rho kinases in cardiovascular physiology and pathophysiology: the effect of fasudil

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Summary

Rho kinase (ROCK) is a major downstream effector of the small GTPase RhoA. ROCK family, consisting of ROCK1 and ROCK2, plays central roles in the organization of actin cytoskeleton and is involved in a wide range of fundamental cellular functions such as contraction, adhesion, migration, proliferation, and apoptosis. Due to the discovery of effective inhibitors such as fasudil and Y27632, the biological roles of ROCK have been extensively explored with particular attention on the cardiovascular system. In many preclinical models of cardiovascular diseases including vasospasm, arteriosclerosis, hypertension, pulmonary hypertension, stroke, ischemia-reperfusion injury and heart failure, ROCK inhibitors have shown a remarkable efficacy in reducing vascular smooth muscle cell hypercontraction, endothelial dysfunction, inflammatory cell recruitment, vascular remodeling, and cardiac remodeling. Moreover, fasudil has been used in the clinical trials of several cardiovascular diseases. The continuing utilization of available pharmacological inhibitors and the development of more potent or isoform-selective inhibitors in ROCK signaling research and in treating human diseases are escalating. In this review, we discuss the recent molecular, cellular, animal and clinical studies with a focus on the current understanding of ROCK signaling in cardiovascular physiology and diseases. We particularly note that emerging evidence suggests that selective targeting ROCK isoform based on the disease pathophysiology may represent a novel therapeutic approach for the disease treatment including cardiovascular diseases.

Keywords

Rho kinase; ROCK; cardiovascular disease; fasudil; inhibitor

Introduction

Rho-kinase (Rho-associated coiled-coil-containing protein kinase, hereafter referred to as ROCK) is one of the best-characterized effectors of small GTPase RhoA and belongs to the AGC (protein kinases A, G and C) family of classical serine/threonine protein kinases.[1–4] As a major downstream effector of RhoA, ROCK is best known for regulating actin cytoskeleton organization and dynamics. ROCK promotes actin filament stabilization and generation of actin-myosin contractility by phosphorylating numerous downstream target proteins, including the myosin binding subunit of myosin light chain (MLC) phosphatase (MYPT1),[5–7] MLC2,[5,8] LIM kinases,[9–13] ezrin/radixin/moesin,[14] and adducin.[15] The

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ROCK family includes two members, ROCK1 (also called ROKβ or p160ROCK) and ROCK2 (also known as ROKα), that share 65% overall identity and 92% identity in the kinase domain. Both kinases contain a catalytic kinase domain at the N-terminus, followed by a central coiled-coil domain, including a Rho-binding domain (RBD) and a C-terminal pleckstrin-homology domain, with an internal cysteine-rich domain.[1–4] In human and mouse, both ROCK1 and ROCK2 are ubiquitously expressed across tissues.[3]

The Rho/ROCK family has been extensively studied, especially on its functions in the cardiovascular system; several recent excellent reviews are worth reading.[16–21] In addition, many publications have evaluated the potential therapeutic application of ROCK inhibitors in neurologic disorders, metabolic disorders, glaucoma and cancers.[22–26] ROCK-mediated signaling pathway was first identified in smooth muscle cells and connected to cardiovascular diseases where abnormal smooth muscle contraction in vascular bed was found.[5–8] The potential therapeutic applications of ROCK inhibitors were initially investigated for the treatment of cerebral vasospasm, hypertension and coronary artery spasm resulting in myocardial ischemic injury.[27–31] The recent progress in the translational research continuously supports the therapeutic importance of the ROCK pathway in cardiovascular pathophysiology. In this review, we focus on the current information derived from studies of cardiovascular diseases, mainly covering hypertension, arteriosclerosis, pulmonary hypertension, stroke, ischemia-reperfusion (I/R) injury and heart failure. Commonly used ROCK inhibitors, in particular fasudil[32] and Y27632,[27] which target the ATP-dependent kinase domain of ROCK1 and ROCK2, are discussed. Furthermore, we examine their application in dissecting the roles of ROCK in experimental animal models and clinical applications of cardiovascular diseases. Recent findings derived from targeting ROCK1 and ROCK2 by genetic approach, short interfering RNA (siRNA)-based gene silencing techniques and chemical inhibitors are also covered.

ROCK Isoform functions

Common and Selective Regulators of ROCK Isoform Activity

ROCK1 and ROCK2 are downstream targets of the small GTP-binding protein Rho including RhoA, RhoB and RhoC, working as a mediator in the Rho-dependent signaling pathway. Stimulation of tyrosine kinase and G-protein-coupled receptors leads to activation of Rho via the recruitment and activation of guanine nucleotide exchange factors (GEFs).[33,34] Activated Rho directly interacts with the RBD of ROCK and induces a conformational change, leading to activation of the serine/threonine kinase toward selective substrates.[1–4,35,36] ROCK activity can also be modulated through interaction of C-terminal pleckstrin-homology domain with lipid mediators such as arachidonic acid and sphingosylphosphorylcholine and with the plasma membrane,[37–40] auto-phosphorylation,[41,42] mechanical stress, and proteolytic cleavage of the inhibitory C-terminal domain.[43–45]

In addition to the common regulators such as RhoA/RhoB/RhoC, ROCK1 and ROCK2 can be individually activated or inhibited by a number of positive or negative regulators. The small GTP-binding protein RhoE interacts with the N-terminal region of ROCK1 (amino acids 1–420) and prevents Rho binding to RBD.[46–48] PDK1 selectively promotes ROCK1 membrane translocation and blocks its association with RhoE.[49] ROCK1 is cleaved by caspase 3 at the cleavage site DETD1113 during apoptosis.[43,44] This consensus sequence for caspase 3 cleavage is conserved in human, rat and mouse, but it is not present in ROCK2. On the other hand, during cytotoxic lymphocyte granule-induced cell death, human ROCK2 can be cleaved by the proapoptotic protease granzyme B at IGLD1131 site, and this site is not present in ROCK1.[45] Human ROCK2, but not ROCK1, can also be activated by caspase 2-dependent cleavage in endothelial cells in response to thrombin, but the cleavage site

remains to be identified.[50] Other studies have revealed that ROCK1 and ROCK2 are phosphorylated by other kinases at multiple sites which might differently influence their activities.[51–53]

Overlapping and Non-redundant ROCK Isoform Functions

ROCK1 and ROCK2 share more than 30 immediate downstream substrates due to the high degree homology in their kinase domains (reviewed in references 23,54–57). The majority of ROCK substrates have been identified from *in vitro* or cell culture experiments under overexpression conditions. Recent proteomic approach adds novel potential substrates.[58] In most cases, only ROCK2 was investigated. The consensus amino acid sequences which are phosphorylated on these substrates are R/KXS/T or R/KXXS/T.[7,11] However, these substrates can also be phosphorylated by other serine-threonine kinases such as protein MLC kinase, protein kinases A, C, and G.[59,60] Two well-established downstream signaling pathways of ROCK include MYPT1/MLC[5–8] and LIM kinase/cofilin[9–13] pathways. The ROCK/MYPT1/MLC2 pathway is extensively described in smooth muscle cells, where it mediates calcium sensitization and thereby enhances and sustains contraction in the vascular bed. On the other hand, ROCK/MYPT1/MLC and ROCK/LIM kinase/cofilin pathways are heavily involved in stress fiber formation. ROCK seems to induce and maintain stress fibers by increasing contractility via MLC phosphorylation and by stabilizing actin filaments through LIM kinase activation, resulting in cofilin phosphorylation and thereby inhibiting its actin-depolymerisation activity. The prominent effects of RhoA/ROCK on cytoskeletal dynamics not only control cell contraction, adhesion, morphology, and motility, but also influence other cellular processes including transcriptional regulation, proliferation, differentiation and apoptosis. In many instances, however, the molecular mechanisms have not been fully characterized.

A growing body of evidence indicates that endogenous ROCK1 and ROCK2 also have non-redundant functions. Recent studies with individual knockdowns of ROCK1 and ROCK2 using siRNA-based gene silencing or genetic approach have shown that these two isoforms have non-redundant *in vitro* functions. For instance, although both ROCK1 and ROCK2 control assembly of the actin cytoskeleton and cell contractility via phosphorylation of MYPT1, the mechanism may vary between the two isoforms. Only ROCK2 binds directly to and phosphorylates MYPT1,[61] suggesting that intermediate proteins are involved in ROCK1 binding to MYPT1. In addition, both ROCK1 and ROCK2 mediate insulin-stimulated insulin receptor substrate (IRS)-1 phosphorylation, but only ROCK2 binds directly to IRS-1.[62] Moreover, functional differences between ROCK1 and ROCK2 have been reported in fibroblasts,[63–66] smooth muscle cells,[61,67] endothelial cells,[68–72] keratinocytes,[73–75] adipocytes,[62,65] neurons[66] and cancer cells.[76,77] These studies reveal that ROCK1 and ROCK2 have functional differences in regulating actin cytoskeleton, but the underlying mechanisms are not fully understood, which could be explained by the facts that both isoforms are expressed at different levels, distributed at different subcellular locations, and/or they have different interaction partners in individual cell types.[43–46,49,57,61–64,78–82]

Genetic approach using ROCK1 and ROCK2 deficient mouse embryonic fibroblasts (MEFs) derived from ROCK1 and ROCK2 knockout mice further supports functional differences between ROCK isoforms in regulating actin cytoskeleton[65,83,84] These studies reveal that ROCK1 or ROCK2 deficiency has a minimal effect on the architecture of actin cytoskeleton in MEFs under baseline culture condition, but they have clearly distinct effects on the re-organization of actin cytoskeleton induced by differentiation or cytotoxic stimuli. Only ROCK2 deficiency enhances adipogenesis and insulin signaling, which is associated with actin cytoskeleton changes from long stress fibers to cortical actin rings.[65] We recently observed that only ROCK1 deficiency inhibits doxorubicin-induced disruption of central

stress fibers and formation of cortical contractile rings leading to reduced cell detachment.[83] The anti-detachment effects of ROCK1 deficiency in response to this cytotoxic stress is mediated through reduced MLC phosphorylation but preserved cofilin phosphorylation leading to the reduced actomyosin contraction and preserved actin polymerization. Moreover, only ROCK1 deficiency inhibits actin-cytoskeleton re-organization and cell detachment in response to serum starvation, an environmental stress.[84]

The *in vivo* functional similarity and differences of ROCK1 and ROCK2 have been shown by mouse genetic studies during development and under pathological conditions.[85] ROCK1 or ROCK2 knockout in C57BL/6 genetic background can result in mice born with eyes open at birth and an omphalocele phenotype due to disorganization of actin filaments in the epithelial cells of the eyelids and of the umbilical ring.[85–88] These phenotypes are absent in other genetic backgrounds.[89–92] For both genetic knockouts, the mice that survive perinatal period develop phenotypically normal and are fertile, supporting the idea that the two isoforms are mostly redundant. Homozygous and heterozygous ROCK1 and ROCK2 knockout mice have been used to examine their contributions to several pathological conditions. For some diseases, such as glaucoma, both ROCK isoforms contribute to the regulation of intraocular pressure.[93] Both ROCK1 and ROCK2 are important in regulating allergic airway responses.[94] On the other hand, ROCK1 appears to play a predominant role in vascular inflammation diseases.[95] Both ROCK isoforms, but to a greater extent ROCK1, are involved in diabetes-induced vascular endothelial dysfunction.[96] In many studies, only one isoform has been investigated[87,89,91,92,97–109] and future studies are required to determine the contribution of another isoform.

Development and therapeutic effects of ROCK inhibitors

Early drug discovery efforts concentrated on the development of non-isoform selective ROCK inhibitors. Fasudil (for an overview see Figure 1) and Y-27632 are among the most commonly used ROCK pan-inhibitors, all of which target the ATP-dependent kinase domain of ROCK with K_i values of 330 and 140 nM, respectively.[27,32,110] Hydroxyfasudil, the main metabolite of fasudil after oral administration, and H-1152P, another analogue of fasudil, are more potent than fasudil. These inhibitors have possible non-selective effects, and at higher concentrations they also inhibit other serine/threonine kinases such as protein kinases A and C.[111,112] Fasudil is the only ROCK inhibitor approved for human use, and was approved in Japan in 1995 for the prevention and treatment of cerebral vasospasm after surgery for subarachnoid hemorrhage (SAH).[113] Postmarketing surveillance studies have found that fasudil has exhibited no serious side effects.[31]

Based on the overall promising studies showing beneficial effects of fasudil and Y27632 in a variety of animal disease models, considerable interest and efforts have been devoted to the development of more potent and selective ROCK inhibitors. Most of these novel inhibitors, also targeting the ROCK ATP pocket, are non-isoform selective.[114–124] The resolution of crystal structure of ROCK1 complexes with inhibitors helps to improve the selectivity and potency of novel inhibitors.[125] Recent efforts have also been devoted to developing isoform-selective inhibitors. Both traditional high-throughput library screens and fragmented-based drug discovery approaches have yielded compounds that are reported to have significant selectivity for ROCK1 or ROCK2.[126,127] SLx-2119, which is also an ATP-competitive inhibitor, exhibits IC_{50} values of 24 μM for ROCK1 and 0.105 μM for ROCK2.[126] A combined approach using high concentration biochemical assays and fragment-based screening assisted by structure-guided design has yielded several compounds with selectivity for ROCK1 or ROCK2 respectively.[127] Future studies for specific targeting ROCK1 or ROCK2 are needed, using compounds with isoform selectivity or using systemic and conditional tissue-specific knockout mice, to determine whether

isoform-selective inhibition would be a more efficacious therapeutic strategy than non-selective ROCK inhibitors for the treatment of cardiovascular and other diseases.

An important puzzle for many clinicians is whether the efficacy and safety could be further improved by combining conventional drugs with ROCK inhibitors to enhance their effectiveness while minimizing adverse effects. An example is using statins in conjunction with ROCK specific inhibitors.[126,128] Statins, which inhibit HMG-CoA reductase and block the synthesis of cholesterol, are drugs for the treatment of hyperlipidemia to reduce the risk of adverse cardiovascular events. In addition to the cholesterol lowering effects, statins have also been found to reduce ROCK expression and activity.[129–132] The drug combination stratagem was found to be synergistic in some studies; therefore brings beneficial effect in reducing drug toxicity and enhancing outcome compared to monotherapy. These trials include adding fasudil with nitroglycerin, another vasodilator, causing further dilations;[133] fasudil with imidapril, an angiotensin-converting enzyme, further reducing renal interstitial fibrosis induced by unilateral ureteral obstruction;[134] fasudil with ozagrel, a thromboxane A(2) synthase inhibitor, further decreasing cerebral infarction induced by middle cerebral artery occlusion;[135] fasudil with tissue plasminogen activator preventing hemorrhagic transformation induced by this thrombolytic factor in an experimental stroke model in mice;[136] fasudil with prostacyclin or with sildenafil showing to be more effective for the treatment of pulmonary hypertension when compared with each monotherapy;[137,138] fasudil with FR167653, a p38 MAPK inhibitor, demonstrating to be more effective for the improvement of cardiovascular remodeling, inflammation, and oxidative stress in hypertensive rats.[139] In addition to cardiovascular diseases, the inclusion of fasudil in therapeutic strategies has been tested in the treatment of cancers[103,140] and in cell transplantation therapy.[141] A list of combination treatment is rapidly expanding, mainly attributable to the wide spectrum of biological processes influenced by ROCK and to the acceptable clinical safety of fasudil.

ROCK in Cardiovascular Diseases

The role of Rho/ROCK family in cardiovascular diseases has been extensively studied in animal experimental models.[16–21] Since this review is particularly focusing on fasudil application, this section mainly covers experimental studies using fasudil in several cardiovascular diseases as discussed below and some most recent studies are summarized in Table 1.

Hypertension

Arterial hypertension is a major risk factor and one of the most common cardiovascular diseases. It is characterized by a high arterial pressure level resulting from increased peripheral vascular resistance attributable to increased vascular contractility and arterial wall remodeling. Many studies have found that the activity of Rho/ROCK pathway increases in experimental hypertension models[142–149] and hypertensive patients.[150–152] Increased RhoA/ROCK activities appear to be the consequence of the up-regulation of renin-angiotensin-aldosterone system[143,144,147,148] and the increased production of reactive oxygen species (ROS),[149] which have been implicated in the pathophysiology of hypertension.[153–157]

Additional evidence supporting the importance of the Rho/ROCK pathway in hypertensive humans comes from genetic studies showing that ROCK2 polymorphisms[158,159] are associated with changes in systemic blood pressure. Genetic variants in Arhgef11, an activator of the Rho/ROCK signaling pathway are associated with kidney injury in the Dahl salt-sensitive rat.[160] Limited studies using ROCK1 and ROCK2 knockout mice have been performed to examine their contributions to the regulation of blood pressure. ROCK1 haploinsufficiency had no effect on baseline blood pressure and angiotensin II-induced

hypertension.[87] In addition, we observed that ROCK1 homozygous knockout mice had normal blood pressure under baseline condition (unpublished observation). One recent study reported lower blood pressure levels at baseline and diabetes-induced hypertension in ROCK1 and ROCK2 heterozygous knockout mice compared with wild type mice.[96]

The role of ROCK signaling in arterial hypertension has also been extensively studied using ROCK inhibitors including fasudil[142,145,146] and other inhibitors[27,116] in a variety of experimental models. Although ROCK inhibitors reduce vascular bed remodeling in hypertensive models, they do not always lower blood pressure. ROCK inhibition reduces smooth muscle contractility through decreasing MLC phosphorylation in smooth muscle cells and improving endothelial function via restoring eNOS expression/activity and NO production.[27,144–146,161,162] ROCK inactivation also reduces inflammation and remodeling through 1) suppressing the expression of pro-inflammatory cytokines and adhesion molecules in endothelial and smooth muscle cells including plasminogen activator inhibitor-1, monocyte chemoattractant protein-1 and transforming growth factor-β1;[145,163,164] 2) inhibiting ROS production through down-regulation of NADPH oxidases in endothelial cells[149,165] and reducing the secretion of cyclophilin A from smooth muscle cells.[18] In addition, the administration of ROCK inhibitors in the brainstem lowered blood pressure and reduced sympathetic nerve activity in hypertensive animals.[166,167] Future studies with systemic and conditional deletion of ROCK1 and ROCK2 are necessary to validate ROCK as a crucial target for the treatment of hypertension.

Atherosclerosis

Atherosclerosis is characterized by progressive inflammation, lipid accumulation and fibrosis occurred in arterial wall. Rho/ROCK pathway is substantially involved in inflammatory and arteriosclerotic arterial lesions in animals and humans (reviewed in references 16,168,169). Multiple studies in experimental models involving fasudil[170–172] and other ROCK inhibitors[173,174] have demonstrated that ROCK is a critical contributor to the inflammatory atherosclerotic process. ROCK inhibitors lead to up-regulation of eNOS and reduced endothelial dysfunction,[174,175] decreased vascular cell contraction, proliferation and migration,[174,176,177] decreased vascular oxidative stress,[178] decreased vascular inflammation, macrophage infiltration and foam cell formation,[171,173] and reduced arterial intima-medial thickness and atherosclerosis plaque formation.[171,172] More support for a critical role of ROCK1 and ROCK2 in the development of atherosclerosis comes from experiments using ROCK1 and ROCK2 knockout mice. These studies suggest that ROCK1 in bone-marrow-derived macrophages mediates macrophage foam cell formation and macrophage chemotaxis,[179] and that ROCK2 in bone-marrow-derived macrophages mediates macrophage foam cell formation in part, through inhibiting cholesterol efflux.[108]

Pulmonary hypertension

Pulmonary hypertension is a general term comprising a spectrum of pulmonary hypertensive disorders which is characterized by elevated pulmonary arterial pressure and increased pulmonary vascular resistance, leading to right-sided heart failure and death. Multiple mechanisms contribute to pulmonary hypertension including prolonged vasoconstriction, increased smooth muscle cell proliferation and migration, inhibition of smooth muscle cell apoptosis, endothelial dysfunction, increased ROS production and inflammatory cell recruitment, and in situ thrombosis of pulmonary vessels, etc., all of which are involved in the pulmonary vascular remodeling leading to disease progression. Robustly raised Rho/ROCK signaling plays a substantial role in the pathogenesis of different experimental models of pulmonary hypertension (reviewed in references 19,180–182). Recent studies have shown that Rho/ROCK pathway is increased in pulmonary hypertension patients.[183,184] Numerous studies using fasudil[137,185–197] and other ROCK

inhibitors[117,196,198] in different experimental models have shown that ROCK is a critical contributor to pulmonary hypertension. ROCK inhibitors can reduce pulmonary arterial pressure, pulmonary vascular resistance and remodeling by reducing pulmonary vasoconstriction,[185–187,193] improving endothelial function,[188,191,193] decreasing smooth muscle cell proliferation and migration,[196,199,200] increasing smooth muscle cell apoptosis,[193,198] increasing myofibroblast apoptosis and reducing fibrosis,[194] decreasing vascular oxidative stress and inflammation.[193] ROCK activity has also been specifically linked to a number of known effectors of pulmonary hypertension, including endothelin-1,[198,201] serotonin,[183] and eNOS.[188] Because of non-isoform selectivity of employed inhibitors, the roles of the two ROCK isoforms in pulmonary hypertension pathogenesis remain largely unknown and need to be determined.

Stroke

ROCK has a role in the pathogenesis of several cerebral vascular diseases, such as ischemic stroke and cerebral vasospasm (reviewed in references. 16,21,202). Fasudil was shown highly effective in reducing cerebral vasospasm and beneficial to stroke prevention, acute neuroprotection and chronic stroke recovery in experimental models.[135,136,203–211]. Published studies have shown that ROCK inhibitors were able to reduce cerebral vasoconstriction,[205,212] up-regulate eNOS and decrease the inflammatory response,[136,203,205,206,208,209] reduce neuron degeneration,[204,213] stimulate proliferation and differentiation of adult neural stem cells.[207] Further investigations are desired to evaluate the function of each ROCK isoform in stroke and stroke recovery.

Myocardial Ischemic Injury

ROCK was found to have a role in cardiac I/R injuries, where blood flow is restricted or cut off and then is reintroduced into the area. I/R provokes tissue damage due to augmented oxidative stress, mitochondrial dysfunction and inflammation. A deleterious role of RhoA/ROCK signaling in I/R injury has been demonstrated in several *in vivo* models including mouse,[214] rat,[215–217] dog[218] and swine.[219] In these models ROCK inhibition with fasudil[215–220] or Y27632[214,215,221] achieved smaller infarct size, less inflammation, attenuated apoptosis, and enhanced cardiac contractile function. The proposed cardioprotective mechanisms of ROCK inhibition include 1) activation of the PI3K/Akt/eNOS signaling pathway[215,216,218,220] and the JAK2/STAT3 signaling pathway;[220] 2) reduction of endothelial-leukocyte interaction during ischemia-reperfusion injury by preserving endothelial function[222] and suppression of inflammatory responses;[214–216] 3) reduction of endothelial cell shape changes and apoptosis through reducing actin cytoskeleton re-organization;[223,224] 4) inhibition of myocyte apoptosis through increasing the expression of antiapoptotic Bcl-2 protein,[214] decreasing mitochondria-nuclear translocation of apoptotic-inducing factor through the inhibition of c-Jun NH2-terminal kinase,[217] and reducing endoplasmic reticulum stress through the elevation of sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity;[220] 5) improving energy production through increasing the levels of lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, normalizing creatine kinase levels, and inhibiting ATP synthase degradation.[221]

The beneficial effects of ROCK inhibition by fasudil[225–227] or Y27632[228] in ischemic preconditioning have also been observed in several animal models, which showed reduced infarct size, oxidative stress and apoptosis. Although the studies cited above overwhelmingly support a deleterious role for Rho/ROCK pathway in cardiac I/R, Rho/ROCK pathway can also have cardioprotective activities as reported by other studies. For example, it is involved in the protective effects of early ischemic preconditioning in the rat heart[229] and in KATP channel-induced improvement of postischemic functional

recovery.[230] Limited studies using ROCK1 and ROCK2 knockout mice have examined their contributions to I/R injuries. In a model for repetitive ischemia/reperfusion injury, an increase of fibrosis but not apoptosis was induced, and ROCK1 deletion significantly reduced cardiac fibrosis through inhibiting cardiac fibroblast differentiation and the monocyte-to-fibroblast transition of transmigrated monocytes.[109] Further investigations should evaluate the function of each ROCK isoform in acute I/R injuries.

Pathological Cardiac Hypertrophy and Heart Failure

Pathological cardiac hypertrophy is defined by the augmentation of ventricular mass induced by pathological stimuli such as hypertension, valvular insufficiency and stenosis, myocardial infarction or ischemia associated with coronary artery disease, etc. Cardiac hypertrophy is initially beneficial to compensating elevated hemodynamic load in order to maintain cardiac output, which is an adaptive response and characterized by increased ventricular wall thickness. However, persistent overloading stress can eventually lead to decompensation and consequently congestive heart failure, in which heart chambers become markedly enlarged and the cardiac contractile function is compromised. Significant amount of evidence indicates that RhoA/ROCK signaling mediates a hypertrophic response (reviewed in references 20,231). Recent studies have shown that Rho/ROCK pathway is increased in heart failure patients.[232,233]*In vivo* studies using pharmacological inhibitors, including fasudil[139,165,234–240] and other inhibitors,[241–243] support an *in vivo* role for ROCK in the pathogenesis of cardiac hypertrophy and remodeling in various animal models. The proposed cardioprotective mechanisms of ROCK inhibition include 1) inhibition of cardiomyocyte hypertrophy due to mechanic stretch and G-protein-coupled-receptor agonists such as angiotensin II, α-adrenergic agonists, and endothelin-1;[244–250] 2) inhibition of cardiac fibrosis and inflammation through suppressing expression of fibrogenic/inflammatory cytokines and NADPH oxidase components in part by inhibiting cytoskeleton re-organization and NF-kappaB activation;[89,251,252] 3) inhibition of cardiomyocyte apoptosis through activation of the ERK/MAPK and/or PI3K/Akt survival pathways[99,106] and suppression of Bax expression;[253] 4) improving cardiac contraction through inhibiting phosphorylation of cardiac troponin I/T[254] and preserving SERCA2a expression;[255] 5) reduction of vascular resistance through decreasing MLC phosphorylation;[256,257] 6) restoration of baroreflex sensitivity in the brainstem and reducing sympathetic nerve activity.[258,259]

Although the studies described above largely support that Rho/ROCK pathway contributes to maladaptive responses in pathological cardiac remodeling, Rho/ROCK pathway may also contribute beneficially to adaptive responses. ROCK was reported to mediate agonist-stimulated contraction in the hearts through MYPT/MLC pathway.[260–263] In addition, RhoA/ROCK is involved in cardiomyocyte protection through activation of focal adhesion kinase/PI3K/Akt survival signaling.[264] Genetic studies using ROCK1 deficient[89,99,100] and haploinsufficient mice[87] have demonstrated a critical role for this isoform in pathological remodeling. Partial or full ROCK1 deletion did not block the development of cardiomyocyte hypertrophy,[87,89,99,100] but significantly reduced a number of structural and functional alterations attributable to pathological hypertrophic remodeling including cardiac fibrosis,[87,89] cardiomyocyte apoptosis,[99,106] cardiac dilation and contractile dysfunction.[99,100] Genetic studies using cardiac-specific ROCK2 knockout mice have demonstrated that ROCK2 is involved in cardiomyocyte hypertrophy and apoptosis, cardiac fibrosis during compensatory cardiac hypertrophy.[107] Further studies are needed to determine the contribution of ROCK2 to cardiac decompensation and heart failure.

Clinical Implications of Fasudil

In addition to the extensive preclinical data accumulated from experimental model systems (Table 1), some clinical benefits of fasudil can be derived from large scale clinical treatment for vasospasm after SAH and also from small clinical studies for the treatment of cardiovascular diseases (Table 2). These clinical applications include 1) essential hypertension,[150,151] 2) coronary vasospasm and atherosclerosis,[133,175,265–273] 3) pulmonary hypertension,[274–278] 4) aortic stiffness,[178] 5) heart failure associated vascular resistance and contraction,[257] 6) cerebral vasospasm[30,31,279–282] and ischemic stroke,[29,283] 7) kidney transplantation.[284]

Although fasudil is in general well tolerated without serious adverse reactions and there are no statistically significant differences in fasudil vs. placebo group (Table 2), a serious complication after intra-arterial administration for the treatment of cerebral vasospasm was recently reported.[285] Other reported mild side effects include convulsion,[286] temporary systemic hypotension and a disturbance in consciousness.[287] In these clinical studies, the underlying mechanism of the beneficial effects of fasudil has been attributable to the inhibition of ROCK in the vascular system resulting in the attenuation of smooth muscle hypercontraction, reduction of endothelial dysfunction and inflammatory response. However, the clinical effects of fasudil may also result from inhibition of other kinases because of the possible non-selective effects. Along with the development of ROCK isoform-selective inhibitors, more clinical studies will be needed to further validate ROCK as the crucial target of fasudil in the treatment of cardiovascular diseases and other diseases.

Conclusions and Future Directions

The research in RhoA/ROCK pathway has attracted much attention for more than a decade since the discovery of ROCK in 1996. Our rapidly accumulated knowledge on ROCK cellular functions, substrates, isoform functions and dynamic cross talks with other signaling pathways are mainly derived from ROCK inhibitor studies ranging from *in vitro* studies using various cell culture systems, *in vivo* studies in numerous animal models, and an increasing number of clinical studies. Although the two isoforms are largely assumed to be functionally redundant mainly based on that they are highly homologous within the kinase domain and sharing major activators and substrates, recent studies with individual knockdowns of ROCK1 and ROCK2 using siRNA-based gene silencing or a genetic approach have shown that the two isoforms have non-redundant *in vitro* and *in vivo* functions.

ROCK has been confirmed to be involved in various cardiovascular disease pathologies with increased ROCK activity mediating vascular smooth muscle cell hypercontraction, endothelial dysfunction, inflammatory cell recruitment, vascular and cardiac remodeling. The obvious beneficial effects from applications of ROCK inhibitors have been demonstrated by a significant amount of animal studies and human clinical trials, which support the notion that ROCKs are promising therapeutic targets for broad spectra of human diseases, including all the cardiovascular diseases discussed in this review.

Questions which remain to be answered are emerging. Although the effects of ROCK inhibitors in animal models of cardiac diseases are getting clear, the cellular sites of inhibitory action are still largely unsolved, in particular in the myocardium and brain. Meanwhile, it remains to be determined whether the observed beneficial effects are mediated by inhibiting ROCK1, ROCK2, or both. Moreover, most studies and trials were done by using fasudil and/or Y-27632 to inhibit ROCK and they are known to have non-specific effects, therefore the connections between the observed beneficial effects and other co-inhibited kinases are not profoundly investigated by far, especially when ROCK

inhibitors were used at higher concentrations. Prospectively, we will see more development and application of isoform-specific ROCK inhibitors in animal studies and clinical trials. Finally, we expect to see more fundamental studies with tissue-specific and conditional ROCK isoform knockout animal models. Determining the specific functions of each isoform in different organs and tissues will help to generate refined treatments for specific diseases of cardiovascular and other body systems.

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Abbreviations

GEF	guanine nucleotide exchange factor
eNOS	endothelial nitric oxide synthase
MEF	mouse embryonic fibroblast
I/R	ischemia-reperfusion
IRS	insulin receptor substrate
MLC2	myosin light chain 2
MYPT1	myosin light chain phosphatase 1
RBD	Rho-binding domain
ROCK	Rho-associated coiled-coil-containing protein kinase
siRNA	small interfering ribonucleic acid
ROS	reactive oxygen species
SAH	subarachnoid hemorrhage
SERCA	sarco-endoplasmic reticulum Ca^{2+}-ATPase

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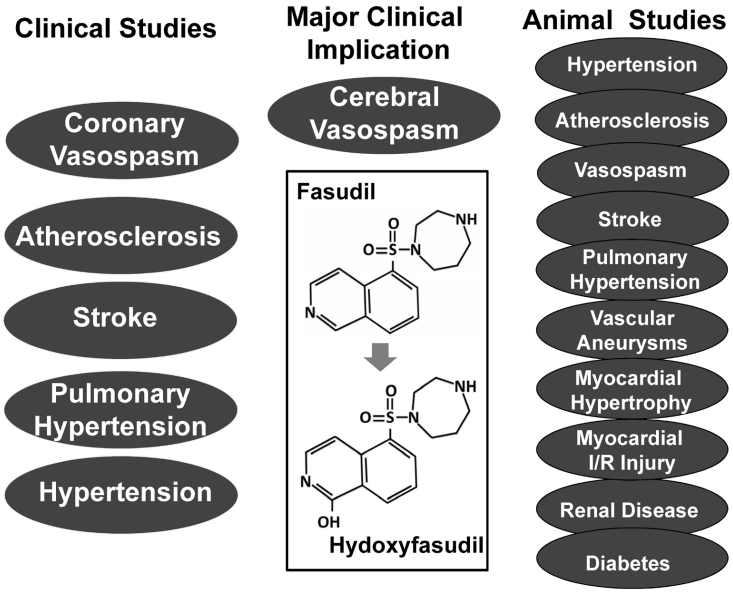

Figure 1. Overview of fasudil on cardiovascular diseases

Studies using fasudil indicate that ROCK is a promising therapeutic target for cardiovascular diseases. Fasudil is the only clinically approved ROCK inhibitor for the treatment of cerebral vasospasm following subarachnoid hemorrhage. Fasudil has also been used in the experimental and/or clinical studies for the treatment of other cardiovascular diseases as indicated. Major beneficial effects of fasudil on cardiovascular diseases and the associated cellular and molecular events are summarized in the Tables 1 and 2.

Table 1

Beneficial effects of fasudil in recent experimental studies of cardiovascular diseases

Therapeutic area	Species/Models	Application	Effects	Mechanisms	References
Hypertension	Rat SHR	10 mg/kg/day IP, 6 weeks	Decreasing blood pressure	Decreasing MLC phosphorylation, increasing eNOS expression and activity	146
	Rat DOCA-salt	100 mg/kg/day OG, 3 weeks	Decreasing blood pressure	Increasing ACE2 and eNOS, reducing PAI-1, MCP-1 and TGF-β1 expression	145
Atherosclerosis	Mouse ApoE$^{-/-}$ high fat diet	100 mg/kg/day DW, 8 or 12 weeks for early or delayed study	Reducing both the early development and later progression of atherosclerotic plaques	Reducing macrophage accumulation	171
	Mouse ApoE$^{-/-}$	100 mg/kg/day DW, 8 weeks	Decreasing atherosclerotic lesions and intima/media ratio, increasing SMC and macrophage density in plaque	Similar to the protective effects of exercise associated with decreased RhoA/ROCK activity	172
Pulmonary Hypertension	Transgenic Mouse	100 mg/kg/day DW, 14 days	Decreasing PH, RVH, and muscularization of pulmonary arteries	BMPRII mutation-induced Smad-independent pathway	190
	Rat MCT-induced PH	100 mg/kg/day DW, 14 days	Decreasing RV systolic pressure and mean pulmonary arterial pressure	Reducing pulmonary arterial remodeling	195
	Rat MCT-induced PH	10 mg/kg IV, 20 min	Decreasing PH, improving pulmonary blood flow distribution	Reducing SMC contraction, improving endothelial-mediated vasodilation	191
	Rat hypoxia neonatal	20 mg/kg/day IP, 14 days	Decreasing PH, RVH and arterial wall remodeling, but adverse effects on somatic growth	Decreasing SMC proliferation associated with reduced PDGFs and their receptors	196
Stroke	Mouse H/R injury	10 mg/kg/day IP, 3 days	Promoting neurogenesis especially in the subventricular zone after H/R	Increasing G-CSF level and astrocytes expressing G-CSF	207
	Mouse Photothrombotic cortical stroke	60 mg/kg/day OG, 3 days after stroke for 5 weeks	Improving motor function and recovery after stroke, no change on infarct size	Similar to the protective effects of ephrin receptor inhibition	211
	Rat MCAO tPA treatment	3 mg/kg IP, before reperfusion	Preventing tPA-induced hemorrhagic transformation, reducing mortality, increasing locomotor activity	Reducing endothelial cell damage, inhibiting MMP-9 activity	136
Myocardial I/R	Rat I/R	10 mg/kg IV, 15 min before reperfusion	Decreasing infarct size and ER stress	Increasing JAK2/STAT3 and PI3K/Akt signaling, and SERCA expression	220
	Rat IPC	10 mg/kg IV, before IPC	Decreasing infarct size due to inhibition of ERK/MAPK in IPC	Decreasing apoptosis, and caspase 3 activation	226
Cardiac Remodeling	Mouse MI or TAC	10 mg/kg/day IP, 3 weeks	Reducing fibrosis in TAC and MI mice; reducing LV cavity dilatation and dysfunction in MI mice	Decreasing profibrotic gene expression and TGF-β1-TAK1 pathway	239

Therapeutic area	Species/Models	Application	Effects	Mechanisms	References
	Rat Doxorubicin cardiotoxicity	2 or 10 mg/kg/day IP, 7 days	Improving cardiac function, decreasing loss of myofibrils	Decreasing apoptosis, Bax expression, and NF-κB activity, increasing c-FLIP (L) and Bcl-2 expression	240
	Rabbit Rapid ventricular pacing	1.5 mg/kg/day ICV, osmotic minipump, 4 days	Decreasing resting heart rate, restoring baroreflex sensitivity in the brainstem	Decreasing AT1R expression, increasing eNOS expression in the brain stem	258

ACE, angiotensin-converting enzyme; AT1R, angiotensin II type 1 receptor; DOCA, deoxycorticosterone acetate; DW, drinking water; ER, endoplasmic reticulum; G-CSF, granulocyte colony-stimulating factor; H/R, hypoxia/reoxygenation; ICV, intracerebroventricular cannula; IP, intraperitoneal; IPC, ischemic preconditioning; I/R, ischemia/reperfusion injury; IV, intravenous infusion; MCAO, middle cerebral artery occlusion; MCT, monocrotaline; MMP, matrix metalloproteinase; OG, oral gavaging; PH, pulmonary hypertension; RVH, right ventricular hypertrophy; SHR, spontaneously hypertensive rat; SMC, smooth muscle cell; TAC, transverse aortic constriction; tPA, tissue plasminogen activator.

Table 2

Beneficial effects of fasudil in human studies

Study	Patient population	Application	Beneficial Effects	Adverse Effects	References
SAH PMS (1995–2000)	1462 patients Age 16–69 years Mean age 54.0 ± 9.9 years	30 mg IV over 30 min 3 times daily 14 days	Preventing cerebral ischemic injury and improving clinical outcome	[a]Mild to moderate in 3.8% patients, similar to phase 3 trial: 3.5% in fasudil vs. 5.7% in placebo group	31
SAH PMS (1995–2000)	3690 patients for fasudil; 1138 patients for fasudil plus ozagrel	30 mg IV over 30 min 3 times daily 14 days	Fasudil plus ozagrel was well tolerated, but did not result in better efficacy than fasudil only	Mild to moderate in 5.2% patients, similar to phase 3 trial: 3.5% in fasudil vs. 5.7% in placebo group	279
SAH Database search meta-analysis (1994–2010)	8 randomized and controlled clinical studies	Variable	Reducing the occurrence of CVS and cerebral infarction (40–50% of the placebo group); improving the clinical outcomes of the patients		282
Acute ischemic stroke Multicenter Phase 3 trial	160 patients age ≥ 20 years mean age 68 years	60 mg IV over 60 min twice daily 14 days	Improving neurological functions and clinical outcome	Mild to moderate, no statistically significant differences in fasudil vs. placebo group	29
Stable angina Multicenter Phase 2 trial	84 patients age 30–80 years	20 to 80 mg PO 3 times daily 8 weeks	Increasing the ischemic threshold of angina patients during exercise and exercise duration	[b]Mild to moderate, 63% in fasudil vs. 53% in placebo group	267
Stable angina Multicenter Phase 2 trial	125 patients age 37–81 years mean age 62 years	5 to 40 mg PO 3 times daily 2–6 weeks	Increasing the maximum exercise time, decreasing the number of anginal attacks	Mild to moderate in <10% patients, including transient headache	266
Stable angina	Study 1: 6 patients age 66.3+/−5 years Study 2: 10 patients age 68.7+/−3.5 years	300 μg/min IC 15 min	Increasing oxygen saturation in coronary sinus vein in study 1; improving pacing-induced myocardial ischemia in study 2		268
Vasospastic angina	20 patients age 49–74 years	300 μg/min IC 15 min	Decreasing ACh-induced coronary constriction, preventing chest pain and ischemic ECG changes		270
Vasospastic angina	26 patients age 61+/−11 years	30 mg, IV following IC 300 μg nitroglycerin	Further dilating ACh-induced coronary spasm in addition to IC nitroglycerin treatment		133
Coronary artery disease	13 patient with confirmed ≥50% stenosis	40 mg, PO 3 times daily 1 month	Improving flow-mediated, endothelium-dependent vasodilation		175
PAH	15 patients age 45+/−4 years	30 mg inhalation over 10 min	Reducing mean PAP and tending to decrease PVR		275
PAH Congenital heart disease	12 pediatric patients age 9.4–16.5 yeras mean age 12.3 years	30 mg/kg IV over 30 min	Decreasing PASP, PVR and SVR, increasing cardiac input and blood oxygen saturation		276

Study	Patient population	Application	Beneficial Effects	Adverse Effects	References
PAH High-altitude	19 patients residents of the Tien-Shan Mountains (altitude 3,200-3,600 m)	1 mg/min IV over 30 min	Increasing pulmonary artery flow, decreasing PASP and PVR	Mild, one patient had facial flushing and four patients had feelings of dryness of the mouth.	274

ACh, acetylcholine; CVS, cerebral vasospasm; IC, intracoronary; IV, intravenous; PAP, pulmonary arterial pressure; PASP, pulmonary artery systolic pressure; PAH, pulmonary arterial hypertension; PMS, post-marketing surveillance; PO, orally; PVR, pulmonary vascular resistance; SAH, subarachnoid hemorrhage; SVR, systemic vascular resistance

[a] Mild to moderate adverse effects: hemorrhage, cardiovascular system disorders, blood and lymphatic system disorders, hepatic and hepatobiliary disorders, urinary system disorders, hypersensitivity, gastrointestinal system disorders.

[b] The skin and vascular disorders are apparently more frequent in the fasudil group than in the placebo group; skin disorders: allergic dermatitis, benign keratosis, bruise, erythematous rash, hive; vascular disorders: ecchymosis, face flushing, hypotension, hypertension, Raynaud-like phenomenon.