Rho-kinase: important new therapeutic target in cardiovascular diseases

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Submitted 1 April 2011; accepted in final form 21 May 2011


THE RHO FAMILY OF small G proteins comprises 20 members of ubiquitously expressed proteins in mammals, including RhoA, Rac1, and Cdc42 (25, 65, 122). Among them, RhoA is the best-characterized protein that acts as a molecular switch that cycles between an inactive GDP-bound and an active GTP-bound conformation, interacting with downstream targets to elicit a variety of cellular responses (Fig. 1) (23). The activity of RhoA is controlled by the guanine nucleotide exchange factors that catalyze the exchange of GDP for GTP (102). In contrast, GTPase-activating proteins stimulate the intrinsic GTPase activity and inactivate RhoA (12). It has been demonstrated that guanine nucleotide dissociation inhibitors block spontaneous RhoA activation (Fig. 1) (81).

In 1996, Rho-kinase (Rho-kinase-α/ROCK 2 and Rho-kinase-β/ROCK 1) was identified as the effector of Rho (Fig. 2) and has been extensively studied, especially on its functions in the cardiovascular system (6, 53). Phosphorylation of myosin light chain (MLC) is a key event in the regulation of vascular smooth muscle cell (VSMC) contraction. MLC is phosphorylated by Ca^{2+}-calmodulin-activated MLC kinase and dephosphorylated by MLC phosphatase. Agonists bind to G protein-coupled receptors and induce contraction by increasing both cytosolic Ca^{2+} concentration and Rho-kinase activity through mediating guanine nucleotide exchange factor. The substrates of Rho-kinase have been identified, including MLC, myosin-binding subunit or myosin phosphatase target subunit 1, ezrin/radixin/moesin family, adducin, phosphatase and tensin homolog on chromosome 10, and LIM-kinases (Fig. 1). Rho-kinase enhances MLC phosphorylation through the inhibition of myosin-binding subunit of myosin phosphatase and mediates agonists-induced VSMC contraction (Fig. 1).

The interaction between endothelial cells (ECs) and VSMCs plays an important role in regulating vascular integrity and vascular homeostasis. ECs release vasoactive factors, such as prostacyclin, nitric oxide (NO), and endothelium-derived hyperpolarizing factor, participating in the regulation of vascular tone and arterial resistance (110, 118, 135). It has been demonstrated that both endothelial NO production and NO-mediated signaling in VSMCs are targets and effectors of the RhoA/Rho-kinase pathway. In ECs, the RhoA/Rho-kinase pathway negatively regulates NO production. In contrast, VSMCs are among the most plastic of all cells in their ability to respond to different stimuli. Growth factors secreted from VSMCs play an important role in mediating various cellular responses in vascular remodeling (10, 11, 30). Recent evidence suggests that many other stimuli that modulate VSMC functions, including reactive oxygen species (ROS), promote VSMC growth by inducing auto/paracrine growth mechanisms (127). Among those auto/paracrine factors, cyclophilin A (CyPA) has been identified as a ROS-related protein that is secreted from VSMCs by RhoA/Rho-kinase activation (95, 120) (Figs. 3 and 4). We have recently demonstrated that the extracellular CyPA decreases endothelial NO synthase (eNOS) expression (78), suggesting the indirect role of RhoA/Rho-kinase for the negative regulation of endothelial NO production. The initial investigations in our laboratory on the therapeutic importance of Rho-kinase were previously summarized (117). Since then, significant progress has been made in our knowledge on the therapeutic importance of Rho-kinase in cardiovascular medicine. In this article, we will briefly review the recent progress in the translational research on the...
therapeutic importance of the Rho-kinase pathway in cardiovascular medicine.

**Role of Rho-Kinase in the Regulation of Cardiovascular Function**

Rho-kinase is a serine/threonine kinase with a molecular mass of ~160 kDa. Two isoforms of Rho-kinase encoded by two different genes have been identified (Fig. 2) (58, 69, 75). In humans, ROCK1 (Rho-kinase-α) and ROCK2 (Rho-kinase-β) genes are located separately on chromosome 18 and chromosome 2, respectively. They are ubiquitously expressed in invertebrates and vertebrates with ROCK1, especially in circulating inflammatory cells and ROCK2 in VSMCs. ROCKs consist of three major domains, including a kinase domain in its NH2-terminal domain, a coiled-coil domain with Rho-binding domain in its middle portion and a putative pleckstrin homology (PH) domain in its COOH-terminal domain (25) (Fig. 2). ROCKs activity is enhanced by binding of GTP-bound active form of RhoA (69) (Fig. 1). Rho-kinase inhibitors, fasudil (7) and Y-27632 (131), have been developed, and they inhibit Rho-kinase activity in a competitive manner with ATP at the Rho-binding site (19). It has been demonstrated that hydroxyfasudil, a major active metabolite of fasudil, exerts a more specific inhibitory effect on Rho-kinase (37, 116).

Although the regulation of ROCK expression has not been fully elucidated, some studies have reported changes in ROCK expression. Functional differences between ROCK1 and ROCK2 have been reported; ROCK1 is specifically cleaved by caspase-3, whereas ROCK2 is cleaved by granzyme B (15, 104). The small G protein RhoE specially binds to the NH2-terminal region of ROCK1 at the kinase domain (Fig. 2), whereas the myosin phosphatase target subunit 1 binds specifically to ROCK2 (56, 139). RhoE binding to ROCK1 inhibits its activity and prevents RhoA binding to Rho-binding domain (85). Both ROCK1 and ROCK2 mRNAs and proteins are upregulated by angiotensin II (ANG II) via ANG II type 1 receptor stimulation and by interleukin-1/β (IL-1β) (38). A number of Rho-kinase substrates have been identified (64) (Fig. 1), and Rho-kinase-mediated substrate phosphorylation causes actin filament formation, organization, and cytoskeleton rearrangement (Fig. 1) (86). The NH2-terminal regions, upstream of the kinase domains of ROCKs, may play a role in determining substrate specificity of the two Rho-kinase isoforms (Fig. 2) (86).
The majority of Rho-kinase substrates have been identified in vitro. Thus ROCK1- and ROCK2-deficient mice have been generated to further elucidate the functions of the ROCK isoforms (108, 130). Importantly, ROCK1-deficient mice showed the eyelids opened at birth (108), whereas ROCK2-deficient mice showed placental dysfunction and fetal death (61, 79, 130, 146). Thus the role of ROCK2, the main isoform in the cardiovascular system, remains to be fully elucidated in vivo. To address this point, we have recently developed VSMC-specific ROCK2-deficient mice and found the crucial role of ROCK2 in the development of hypoxia-induced pulmonary hypertension (107). These mutant mice revealed normal growth and body weight under physiological conditions. However, chronic hypoxia significantly increased ROCK2 expression and ROCK activity in lung tissues from littermates, and the development of right ventricular systolic pressure and right ventricular hypertrophy induced by chronic hypoxia in vivo was evident in littermates but was suppressed in the VSMC-specific ROCK2-deficient mice. In vitro, the growth and migration of VSMCs were significantly reduced in ROCK2-deficient VSMCs compared with control VSMCs.

Rho-Kinase and Vascular Function

Rho-kinase has been implicated in the pathogenesis of cardiovascular disease, in part by promoting VSMC proliferation (4, 8, 82). Changes in the vascular redox state are a common pathway involved in the pathogenesis of atherosclerosis, aortic aneurysms, and vascular stenosis. Vascular ROS formation can be stimulated by mechanical stretch, pressure, shear stress, environmental factors (e.g., hypoxia), and growth factors (e.g., ANG II) (32). Importantly, Rho-kinase is substantially involved in the vascular effects of various vasoactive factors, including ANG II (28, 33, 123), thrombin (103, 134), platelet-derived growth factor (54), extracellular nucleotides (99), and urotensin (100) (Fig. 1). It has been previously shown that statins enhance eNOS mRNA by cholesterol-independent mechanisms involving the inhibition of Rho geranylgeranylation (124). We have also demonstrated that statins and Rho-kinase inhibitors completely block the secretion of CyPA from VSMCs (93, 120). Rho-kinase plays an important role in mediating various cellular functions, not only VSMC contraction (109, 111) but also actin cytoskeleton organization (5, 34), adhesion, and cytokinesis (117). Thus Rho-kinase plays a crucial role for the development of cardiovascular disease through ROS production, inflammation, EC damage, and VSMC contraction and proliferation. Rho-kinase inhibitors have excellent vasodilator activity and can induce vasodilation when vasoconstrictor tone is increased by a variety of mechanisms, including the activation of G-coupled receptors-enhanced calcium entry, ventilatory hypoxia, NOS inhibition, and other mechanisms (14, 20, 21, 137).

Rho-Kinase, Inflammation, and Oxidative Stress

Rho-kinase augments inflammation by inducing proinflammatory molecules, including IL-6 (83), monocyte chemoattractant protein-1 (28), macrophage migration inhibitory factor (MIF) (35, 36), and sphingosine-1-phosphate (136). In ECs, Rho-kinase downregulates eNOS (125) and substantially activates proinflammatory pathways, including an enhanced expression of adhesion molecules. The expression of Rho-kinase is accelerated by inflammatory stimuli, such as ANG II and IL-1β (38), and by a remnant lipoproteins in human coronary VSMCs (80). Rho-kinase also upregulates NAD(P)H oxidases (nox1, nox4, gp91phox, and p22phox) and augments ANG II-induced ROS production (37). Several growth factors are known to be secreted from VSMCs in response to oxidative stress. We have recently demonstrated that ROS activate a pathway containing vesicles that results in the secretion of CyPA (45, 120). Secreted extracellular CyPA stimulates ERK1/2, Akt, and JAK in VSMCs that contribute to ROS production and compose a vicious cycle for ROS augmentation (94, 97). CyPA is secreted from VSMCs via a highly regulated pathway that involves vesicle transport and plasma membrane binding (Fig. 3) (120). Rho GTPases, including RhoA, are key regulators in signaling pathways linked to actin cytoskeletal...
Rho-A plays a central role in vesicular trafficking pathways by controlling the organization of actin cytoskeleton. It has been reported that active participation of Rho GTPases is required for secretion. We showed that the expression of dominant-negative mutants of RhoA inhibited ROS-induced CyPA secretion, suggesting that RhoA-dependent signaling events regulate CyPA secretion (120). Myosin II is involved in the secretory mechanisms as a motor for vesicle transport (77). ROCKs, downstream effectors of RhoA, mediate myosin II activation via phosphorylation and inactivation of myosin II light chain phosphatase (53). We have also recently demonstrated that Rho-kinase inhibitor reduced ROS-induced CyPA secretion (95, 120) (Fig. 4). These results suggest that myosin II-mediated vesicle transport is required for CyPA secretion from VSMCs. CyPA is transported to the plasma membrane and colocalized with vesicle-associated membrane protein 2 in response to ROS stimulation (Fig. 3).

We demonstrated that extracellular CyPA stimulates proinflammatory signals in ECs, including the expression of E-selectin and vascular cell adhesion molecule-1 (44). In addition to the effects on vascular cells, CyPA has been shown to be a direct chemoattractant for inflammatory cells (16, 52), promoting matrix metalloproteinases (MMPs) activation (138, 144). All of these roles of CyPA derive from the activation of Rho-kinase in the cardiovascular system (Fig. 4). Recently, we have demonstrated that the extracellular CyPA activates Rho-kinase in human pulmonary VSMCs from patients with pulmonary hypertension (91). Thus CyPA may be a key mediator of Rho-kinase that generates a vicious cycle for ROS augmentation, affecting ECs, VSMCs, and inflammatory cell functions (Fig. 4) (94, 97).

Rho-Kinase and Arteriosclerosis/Restenosis

As mentioned above, Rho-kinase plays a crucial role in the ROS augmentation and vascular inflammation. ROS have been implicated in the pathogenesis of neointima formation in part by promoting VSMC growth (8, 84) and by stimulating pro-inflammatory events (40, 59, 62, 87). Accumulating evidence indicates that Rho-kinase inhibitors have broad pharmacological properties (111, 115, 117). The beneficial effects of the long-term inhibition of Rho-kinase for the treatment of cardiovascular disease have been demonstrated in various animal models, such as coronary vasospasm, arteriosclerosis, restenosis, ischemia-reperfusion injury, hypertension, pulmonary hypertension, stroke, and cardiac hypertrophy/heart failure (111, 115, 117). Gene transfer of dominant-negative Rho-kinase reduced the neointimal formation in pigs (24). Long-term treatment with a Rho-kinase inhibitor suppressed neointima formation after vascular injury in vivo (101, 105), monocyte chemotactic protein-1-induced vascular lesion formation (72), constrictive remodeling (113, 114), in-stent restenosis (70) and the development of cardiac allograft vasculopathy (36).

Arteriosclerosis is a slowly progressing inflammatory process of the arterial wall that involves the intima, media, and adventitia (111, 117). Accumulating evidence indicates that Rho-kinase-mediated pathway is substantially involved in EC dysfunction (125, 134), VSMC contraction (46), VSMC proliferation and migration in the media (143), and accumulation of inflammatory cells in the adventitia (72). Those Rho-kinase-mediated cellular responses led to the development of vascular disease. In fact, the mRNA expression of ROCKs is enhanced in the inflammatory and arteriosclerotic arterial lesions in animals (46) and humans (48). In the context of atherosclerosis, Rho-kinase should be regarded as a proinflammatory and proatherogenic molecule. Thus Rho-kinase is an important new therapeutic target for the treatment of atherosclerosis.

Rho-Kinase and Coronary Vasospasm

It has been demonstrated that Rho-kinase is substantially involved in the pathogenesis of coronary vasospasm. Coronary vasospasm plays an important role in variant angina, myocardial infarction, and sudden death (121). It was demonstrated that the serum level of cortisol, one of the important stress hormones,
Rho-kinase/CyPA signaling pathway is a novel therapeutic formation (95). CyPA is highly expressed in the aorta of mice into the aortic VSMCs, contributing to the aortic aneurysm formation in patients with vasospastic angina (68). Thus fasudil is useful for the treatment of ischemic coronary syndromes caused by coronary artery spasm. Fasudil is also effective in treating patients with microvascular angina (73). The clinical trials of the effects of fasudil in Japanese patients with stable effort angina demonstrated that the long-term oral treatment with the Rho-kinase inhibitor is effective in ameliorating exercise tolerance in those patients (112). We also have recently demonstrated that Rho-kinase activity in circulating neutrophils is a useful biomarker for the diagnosis and disease activity assessment in patients with vasospastic angina (51).

Rho-Kinase and Myocardial Ischemia-Reperfusion Injury

ROS production and Rho-kinase activation play a crucial role in myocardial damage after ischemia-reperfusion. We have demonstrated that pretreatment with fasudil before reperfusion prevents endothelial dysfunction and reduces the extent of myocardial infarction in dogs in vivo (142). The beneficial effect of fasudil has been also demonstrated in a rabbit model of myocardial ischemia induced by an intravenous administration of endothelin-1 (89), a canine model of pacing-induced myocardial ischemia (132), and a rat model of vasopressin-induced chronic myocardial ischemia (98).

Rho-Kinase and Aortic Aneurysms

Aortic aneurysm is formed by chronic inflammation of the aortic wall, associated with decreased medial VSMCs and progressive destruction of structural components, particularly the elastic lamina (63). Key mechanisms include VSMC senescence (57), oxidative stress (31, 127), increased local production of proinflammatory cytokines (13), and increased activities of MMPs that degrade extracellular matrix (119, 145). Chronic ANG II infusion into apolipoprotein E-deficient mice promotes aortic aneurysm formation (17, 18). In animal models of aortic aneurysm, the genetic and pharmacological inhibition of ROS production (29, 128) and MMPs (67, 129) suppressed the development of aneurysms. A chronic inhibition of Rho-kinase by fasudil has been reported to reduce ANG II-induced aortic aneurysm formation (140). The activation of Rho-kinase promotes CyPA secretion from VSMCs and extracellular CyPA stimulates VSMC migration, proliferation, and MMP activation (45, 60) (Fig. 4). Extracellular CyPA is also a chemoattractant for inflammatory cells (45, 50, 120) and further activates vascular Rho-kinase (Fig. 4). Recently, we have demonstrated that CyPA augments ANG II-induced ROS production, MMP activation, and inflammatory cell recruitment into the aortic VSMCs, contributing to the aortic aneurysm formation (95). CyPA is highly expressed in the aorta of patients with aortic aneurysm (95). Our findings suggest that Rho-kinase/CyPA signaling pathway is a novel therapeutic target for aortic aneurysm. All these data are a proof of concept that both Rho-kinase and CyPA play a crucial role in VSMCs by augmenting ROS generation. ANG II induces Rho-kinase activation and promotes CyPA secretion (Fig. 3). Secreted extracellular CyPA augments Rho-kinase activity in a synergistic manner (91) (Fig. 4). Secreted CyPA, acting as a proinflammatory cytokine, then synergistically augments ANG II-mediated ROS production, contributing to the onset of vascular inflammatory cell migration and aortic aneurysm formation (128, 141).

Rho-Kinase, Cardiac Hypertrophy, and Heart Failure

ANG II plays a key role in many physiological and pathological processes in cardiac cells, including cardiac hypertrophy (71). Understanding the molecular mechanisms for ANG II-induced myocardial disorders is important to develop new therapies for cardiac dysfunction (88). One important mechanism now recognized to be involved in ANG II-induced cardiac hypertrophy is ROS production (3, 76); however, the precise mechanism by which ROS cause myocardial hypertrophy and dysfunction still remains to be fully elucidated (106). It has been demonstrated that cardiac troponin is a substrate of Rho-kinase (133). Rho-kinase phosphorylates troponin and inhibits tension generation in cardiac myocytes. We have recently demonstrated that Rho-kinase inhibition with fasudil suppresses the development of cardiac hypertrophy and diastolic heart failure in Dahl salt-sensitive rats (26). Furthermore, our recent study provides strong mechanistic evidence of synergy between CyPA and Rho-kinase to increase ROS generation (95). Since ROS stimulates myocardial hypertrophy, matrix remodeling, and cellular dysfunction (126), Rho-kinase and CyPA may work together to promote ROS production and ANG II-induced cardiac hypertrophy (Fig. 4). In fact, CyPA was required for ANG II-mediated cardiac hypertrophy by directly potentiating ROS production, stimulating proliferation and migration of cardiac fibroblasts, and promoting cardiac myocyte hypertrophy in mice (96). In patients with heart failure, intra-arterial infusion of fasudil caused a preferential increase in forearm blood flow compared with that in control subjects, suggesting an involvement of Rho-kinase in the increased peripheral vascular resistance in patients with heart failure (55).

Rho-Kinase and Hypertension

Short-term administration of Y-27632, another Rho-kinase inhibitor, preferentially reduces systemic blood pressure in a dose-dependent manner in rat models of systemic hypertension, suggesting an involvement of Rho-kinase in the pathogenesis of hypertension (131). The expression of Rho-kinase was significantly increased in spontaneously hypertensive rats (74). Rho-kinase may be also involved in the central mechanisms of sympathetic nerve activity (41, 42).

Rho-Kinase and Pulmonary Hypertension

Pulmonary hypertension is associated with hypoxic exposure, endothelial dysfunction, VSMC hypercontraction and proliferation, enhanced ROS production, and inflammatory cell migration, for which Rho-kinase may also be substantially involved. Indeed, a long-term treatment with fasudil suppresses the development of monocrotaline-induced pulmonary hyper-
tension in rats (1) and of hypoxia-induced pulmonary hypertension in mice (2). Recently, we were able to obtain direct evidence for Rho-kinase activation in patients with pulmonary arterial hypertension (PAH) (22). Because the secretion of CyPA is regulated by Rho-kinase (95, 120), we tested the hypothesis that CyPA contributes to Rho-kinase activation and pulmonary vascular remodeling in PAH patients and noted enhanced CyPA expression on the α-smooth muscle actin-positive cells in the lung from patients with PAH (91). Additionally, statins and Rho-kinase inhibitor reduced the secretion of CyPA from VSMCs (95, 120) and pravastatin ameliorated hypoxia-induced pulmonary hypertension in mice (90, 92). Thus it is possible that the inhibition of CyPA secretion by statins (90) or Rho-kinase inhibitors (1, 43) may contribute to the therapeutic effects of these drugs on pulmonary hypertension. It has been reported that an intravenous injection of a number of chemically different Rho-kinase inhibitors reduces systemic and pulmonary arterial pressures under resting baseline tone conditions (9, 14, 20, 21). These data suggest that Rho-kinase plays a physiological role in the maintenance of baseline vasoconstrictor tone in the pulmonary and systemic vascular beds. Furthermore, intravenous infusion of fasudil significantly reduced pulmonary vascular resistance in patients with PAH, indicating an involvement of Rho-kinase in the pathogenesis of PAH in humans (27). Therefore, fasudil will decrease pulmonary arterial pressure in any situation in which vasoconstrictor tone is increased in the pulmonary vascular bed. A most important point in clinical settings is the chronic effects of the drugs (Fig. 4). The effects of long-acting fasudil in patients with PAH are now under investigation.

Conclusion

The identification of Rho-kinase as a mediator of cardiovascular diseases associated with inflammation and oxidative stress provides insight into the development of new therapies. Accumulating evidence suggests that Rho-kinase is substantially involved in the pathogenesis of a variety of cardiovascular diseases and that Rho-kinase inhibitors are useful for the treatment of those cardiovascular diseases. Clinical studies with fasudil have suggested that the Rho-kinase inhibitor may be useful for the treatment of a wide range of cardiovascular diseases, as mentioned in this article. Importantly, Rho-kinase inhibitors and statins significantly reduce CyPA secretion from VSMCs in animals. Blocking the malignant cycle that augments ROS production through CyPA secretion may be partially involved in the beneficial effect of Rho-kinase inhibitors. In conclusion, accumulating experimental and clinical evidence indicates that Rho-kinase is an important new target for the treatment of cardiovascular disease.

GRANTS

This work was supported by Japanese Ministry of Education, Culture, Sports, Science, and Technology Grants-in-Aid 08107961, 08107961, 08101562, 09006924, and 10002019 and Japanese Ministry of Health, Labour, and Welfare Grants-in-Aid for Scientific Research (Tokyo, Japan) 08005713, 09158526, and 10102895 and grants-in-aid for scientific research on innovative areas (research in a proposed research area), MEXT, Japan.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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