

## 2015 ATVB Plenary Lecture Translational Research on Rho-Kinase in Cardiovascular Medicine

Hiroaki Shimokawa, Kimio Satoh

**Abstract**—Rho-kinase (ROCKs) is an important downstream effector of the small GTP-binding protein Ras homolog gene family member A. There are 2 isoforms of ROCK, ROCK1 and ROCK2, and they have different functions in several vascular components. The Ras homolog gene family member A/ROCK pathway plays an important role in various fundamental cellular functions, including contraction, motility, proliferation, and apoptosis, whereas its excessive activity is involved in the pathogenesis of cardiovascular diseases. For the past 20 years, a series of translational research studies have demonstrated the important roles of ROCK in the pathogenesis of cardiovascular diseases. At the molecular and cellular levels, ROCK upregulates several molecules related to inflammation, thrombosis, and fibrosis. In animal experiments, ROCK plays an important role in the pathogenesis of vasospasm, arteriosclerosis, hypertension, pulmonary hypertension, and heart failure. Finally, at the human level, ROCK is substantially involved in the pathogenesis of coronary vasospasm, angina pectoris, hypertension, pulmonary hypertension, and heart failure. Furthermore, ROCK activity in circulating leukocytes is a useful biomarker for the assessment of disease severity and therapeutic responses in vasospastic angina, heart failure, and pulmonary hypertension. In addition to fasudil, many other ROCK inhibitors are currently under development for various indications. Thus, the ROCK pathway is an important novel therapeutic target in cardiovascular medicine. (*Arterioscler Thromb Vasc Biol.* 2015;35:1756-1769. DOI: 10.1161/ATVBAHA.115.305353.)

**Key Words:** cardiovascular disease ■ heart failure ■ hypertension ■ inflammation ■ rho-associated kinases

Rho-kinase (ROCKs) is an important downstream effector of the small GTP-binding protein Ras homolog gene family member A (RhoA). During the past 20 years, significant progress has been made in our knowledge on the molecular mechanisms and therapeutic importance of ROCK in cardiovascular medicine. The Rho family of small G proteins comprises 20 members of ubiquitously expressed proteins, including RhoA, Rac1, and Cdc42.<sup>1-6</sup> Among them, RhoA acts as a molecular switch that cycles between an inactive GDP-bound and an active GTP-bound conformation interacting with downstream targets (Figure 1).<sup>7</sup> RhoA is activated by the guanine nucleotide exchange factors (GEFs) that catalyze exchange of GDP for GTP<sup>8</sup> and is inactivated by the GTPase-activating proteins (GAPs).<sup>9</sup> Under physiological conditions, there is a balance of the positive and negative outcomes of Rho activation and there are signaling pathways that keep the negative pathways in check. There are 2 isoforms of ROCK, ROCK  $\alpha$ /ROCK2 and ROCK  $\beta$ /ROCK1, which were identified as the effector of Rho and have been shown to play important roles in the pathogenesis of cardiovascular diseases.<sup>10,11</sup> Phosphorylation of myosin light chain (MLC) is crucial for vascular smooth muscle cell (VSMC) contraction. MLC is phosphorylated by Ca<sup>2+</sup>/calmodulin-activated MLC kinase (MLCK) and is dephosphorylated by MLC phosphatase

(MLCP).<sup>12</sup> Agonists bind to G-protein-coupled receptors and induce contraction by increasing both cytosolic Ca<sup>2+</sup> concentration and ROCK activity<sup>13,14</sup> through mediating GEF.<sup>12,15</sup> The substrates of ROCK include MLC, myosin phosphatase target subunit (MYPT)-1, ezrin/radixin/moesin family, adducin, phosphatase and tensin homolog, and LIM-kinases (Figure 1).<sup>16</sup> In this review article, we will briefly review the recent progress in the translational research on the therapeutic importance of ROCK in cardiovascular medicine, ranging from molecular and cellular levels to animal and human levels (Figure 2).

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### Molecular and Cellular Levels

#### Interactions Between Endothelial Cells and VSMC for Vascular Homeostasis

The interactions between endothelial cells (EC) and VSMC play important roles in regulation of vascular integrity and homeostasis. EC release vasoactive factors, such as prostacyclin, nitric oxide (NO), and endothelium-derived hyperpolarizing factor (Figure 1).<sup>17-19</sup> We have previously demonstrated that endothelium-derived hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is an endothelium-derived hyperpolarizing factor in animals and in

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From the Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan.

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Correspondence to Hiroaki Shimokawa, MD, PhD, Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan. E-mail shimo@cardio.med.tohoku.ac.jp

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Nonstandard Abbreviations and Acronyms	
<b>Ang II</b>	angiotensin II
<b>CyPA</b>	cyclophilin A
<b>EC</b>	endothelial cells
<b>ERK1/2</b>	extracellular signal-regulated kinase 1/2
<b>GAPs</b>	GTPase-activating proteins
<b>GEFs</b>	guanine nucleotide exchange factors
<b>H<sub>2</sub>O<sub>2</sub></b>	hydrogen peroxide
<b>IL</b>	interleukin
<b>LV</b>	left ventricular
<b>MLC</b>	myosin light chain
<b>MLCK</b>	myosin light chain kinase
<b>MLCP</b>	myosin light chain phosphatase
<b>MYPT</b>	myosin phosphatase target subunit
<b>NO</b>	nitric oxide
<b>PAH</b>	pulmonary arterial hypertension
<b>PGI</b>	prostacyclin
<b>ROS</b>	reactive oxygen species
<b>RV</b>	right ventricular
<b>VSA</b>	vasospastic angina
<b>VSMC</b>	vascular smooth muscle cell

humans (Figure 1).<sup>20–23</sup> Both endothelial NO production and NO-mediated signaling in VSMC are targets and effectors of the RhoA/ROCK pathway.<sup>1,24,25</sup> In EC, the RhoA/ROCK pathway negatively regulates NO production, although in VSMC, the pathway enhances MLC phosphorylation through inhibition of MYPT-1 of MLCP and promotes VSMC contraction (Figure 1).<sup>26</sup>

ROCK is a serine/threonine kinase, which is encoded by 2 different genes. In humans, ROCK1 (ROCK $\beta$ ) and ROCK2 (ROCK $\alpha$ ) genes are located separately on chromosome 18 and 2, respectively.<sup>27,28</sup> ROCK consists of 3 major domains, including a kinase domain, a coiled-coil domain that include Rho-binding domain and a putative pleckstrin homology domain (Figure 3).<sup>4</sup> ROCK activity is enhanced by binding of GTP-bound active form of RhoA.<sup>28</sup> Several ROCK substrates have been identified<sup>27</sup> and phosphorylation of ROCK-mediated substrate causes actin filament formation, organization, and cytoskeleton rearrangement (Figure 1).<sup>29</sup> Nowadays, many ROCK inhibitors, such as fasudil<sup>30</sup> and Y-27632,<sup>31</sup> have been developed and they inhibit ROCK activity in a competitive manner with ATP at the Rho-binding site.<sup>32</sup> Among them, hydroxyfasudil, a major active metabolite of fasudil, exerts a more specific inhibitory effect on ROCK.<sup>33,34</sup>

### Roles of ROCK in EC

The RhoA/ROCK pathway is critically involved in actin dynamics.<sup>35</sup> Cyclic strain stimulates RhoA activation and enhances cell contractility. Mechanical activation of the RhoA/ROCK system makes cells more sensitive to external stimuli.<sup>36</sup> Thus, RhoA/Rho-kinase-mediated actin contractility may contribute as a mechanosensor (Figure 1). However, disruption of endothelial barrier can lead to increased endothelial permeability,<sup>37</sup> promoting organ damage in various diseases. The quantity of pinocytotic vesicles and permeability

in EC is regulated by the expression and phosphorylation of caveolin-1 and caveolin-2 in EC as well as the levels of p-Src and the activity of RhoA/ROCK signaling.<sup>37</sup> Thus, the RhoA/ROCK signaling pathway is involved in the mechanotransduction mechanism based on the adherens junction strengthening at EC-cell contacts.<sup>37</sup> These endothelial mechanosensing is required for EC alignment along flow direction, which contributes to vascular homeostasis (Figure 1).

Several reports have demonstrated that NO and ROCK have opposing effects on each other.<sup>38,39</sup> Partial deletion of either ROCK isoform, but to a greater extent ROCK1, attenuated diabetes mellitus-induced vascular endothelial dysfunction by reduction of NO production.<sup>39</sup> However, ROCK-deficient mice revealed preserved EC function in diabetic model.<sup>39</sup> Moreover, we demonstrated that a ROCK inhibitor, fasudil, significantly enhanced phosphorylation of AMP-activated protein kinase in the liver and skeletal muscle, suggesting that NO and ROCK play opposing roles for lipid metabolism.<sup>40</sup> It has been previously shown that statins enhance endothelial NO synthase mRNA by cholesterol-independent mechanisms involving inhibition of Rho geranyl-geranylation.<sup>41</sup> Moreover, statins and ROCK inhibitors completely block the secretion of cyclophilin A (CyPA), a novel mediator of ROCK, from VSMC.<sup>42,43</sup> We also have recently demonstrated that small GTP-binding protein dissociation stimulator plays a central role of the pleiotropic effects of statins independent of the ROCK pathway.<sup>44</sup>

### Roles of ROCK in VSMC

When agonists bind to their receptors, phospholipase C is activated, leading to the formation of inositol 1,4,5-triphosphate and diacylglycerol by the hydrolysis of phosphatidylinositol 4,5-bis-phosphate (Figure 1).<sup>45</sup> 1,4,5-triphosphate then binds to an 1,4,5-triphosphate receptor on the membrane of the sarcoplasmic reticulum to mobilize the stored calcium ions (Ca<sup>2+</sup>) from the sarcoplasmic reticulum into the cytosol. Diacylglycerol activates protein kinase C, which causes vasoconstriction and augments the Ca<sup>2+</sup> sensitivity of contractile proteins.<sup>46</sup> It has been demonstrated that several mechanisms are involved in the Ca<sup>2+</sup> sensitivity of myosin filaments, including myosin phosphatase<sup>12</sup> and the small GTPase Rho and its target, ROCK (Figure 1).<sup>10,13</sup>

MLC phosphorylation is one of the most important steps for VSMC contraction.<sup>12</sup> VSMC contraction is initiated by Ca<sup>2+</sup>/calmodulin-activated MLCK with subsequent phosphorylation of the 20-kDa regulatory MLC.<sup>12</sup> Phosphorylation of the regulatory MLC then activates myosin Mg<sup>2+</sup>-ATPase and permits cross-bridge cycling, which leads to force generation and contraction.<sup>12</sup> The level of MLC phosphorylation is determined by a balance between MLC phosphorylation by MLCK and dephosphorylation by MLCP.<sup>12</sup> Phosphorylation of the second site of MLC is known to further increase the actin-activated Mg<sup>2+</sup>-ATPase activity of myosin *in vitro*.<sup>47,48</sup> These results indicate that enhanced MLC phosphorylations play a central role in the augmentation of vascular tone. The phosphorylated site of MLC is MLCK-dependent Ser19 for MLC monophosphorylation and MLCK-dependent Ser19/Thr18 for MLC diphosphorylation.<sup>33</sup> Phenotype modulation

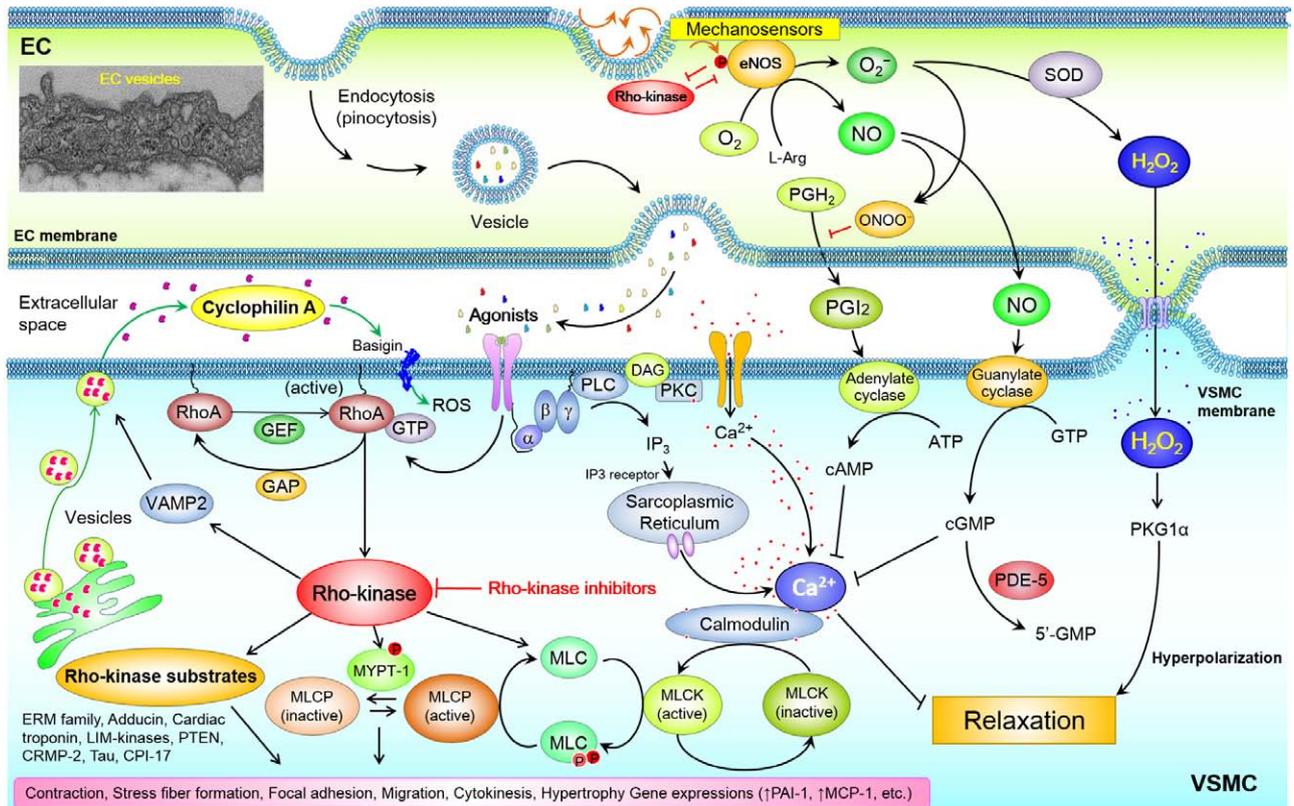
of VSMC (from contractile type to synthetic type) was noted in the neointimal regions of the atherosclerotic artery.<sup>49,50</sup> In cultured VSMC, MLC diphosphorylation is augmented in actively growing cells compared with growth-arrested cells.<sup>48</sup> Phenotype change of arterial VSMC may thus be one of the important mechanisms of cardiovascular diseases.

The generation of diphosphorylated MLC is caused, in part, by inhibition of MLCP in VSMC.<sup>51</sup> Studies in vitro demonstrated that a GTP-binding protein regulates the receptor-mediated sensitization of MLC phosphorylation<sup>52</sup> and that small GTPase Rho is involved in GTP-enhanced Ca<sup>2+</sup> sensitivity of VSMC contraction.<sup>13,14,47</sup> Recent studies further demonstrated that Rho regulates MLC phosphorylation through its target, ROCK, and the MYPT-1 of MLCP.<sup>10,11</sup> Smooth muscle MLCP consists of 38-kDa catalytic subunit, 130-kDa MYPT-1, and 20-kDa subunit.<sup>53,54</sup> Activated ROCK subsequently phosphorylates MYPT-1, thereby inactivating MLCP.<sup>10</sup> ROCK itself might also phosphorylate MLC at the same site that is phosphorylated by MLCK and activate myosin ATPase

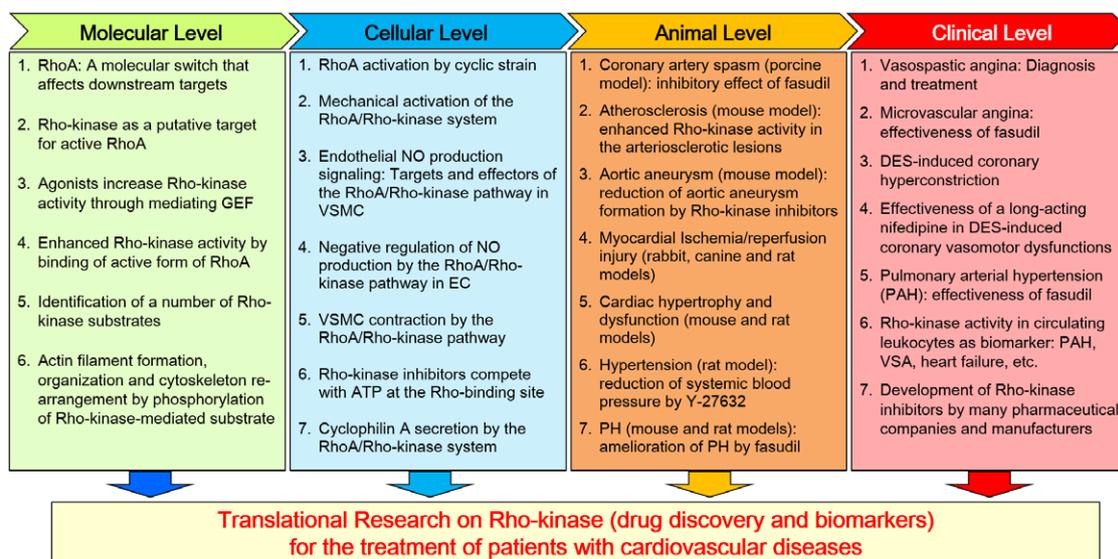
in vitro.<sup>11</sup> Activated form of ROCK enhances transcriptional regulation of serum response factor (SRF)<sup>55</sup> and induces VSMC contraction<sup>56</sup> and stress fiber formation (Figure 1).<sup>57</sup> Some studies suggest that both pathways, inhibition of MLCP and direct phosphorylation of MLC, contribute to the increase in MLC phosphorylations.<sup>56</sup> In contrast, H<sub>2</sub>O<sub>2</sub> causes VSMC dilatation through several mechanisms, including cGMP, cAMP, cyclooxygenase, and several K channels. Importantly, H<sub>2</sub>O<sub>2</sub> rapidly reaches VSMC, stimulates the 1- $\alpha$  isoform of cGMP-dependent protein kinase to form disulfide form, and opens Ca-activated K channels (K<sub>Ca</sub>) with subsequent VSMC hyperpolarization and relaxation (Figure 1).<sup>17-19</sup>

**A Novel Mediator of ROCK: CyPA**

Growth factors secreted from VSMC play an important role in mediating various cellular responses in the development of cardiovascular diseases.<sup>58-60</sup> Recent evidence suggests that many other stimuli that modulate VSMC functions, including reactive oxygen species (ROS), promote VSMC proliferation



**Figure 1.** Interactions between endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) through multiple intercellular signaling pathways. Rho GTPases, including Ras homolog gene family member A (RhoA), are small GTP-binding proteins acting as a molecular switch that cycle between an inactive GDP-bound and an active GTP-bound conformation, interacting with downstream targets to elicit a variety of cellular responses. RhoA is activated by the guanine nucleotide exchange factors (GEFs) that catalyze exchange of GDP for GTP and inactivated by the GTPase-activating proteins (GAPs). Rho-kinase was identified as the effector of Rho. Phosphorylation of myosin light chain (MLC) is a key event in the regulation of VSMC contraction. MLC is phosphorylated by Ca<sup>2+</sup>-calmodulin-activated MLC kinase (MLCK) and dephosphorylated by MLC phosphatase (MLCP). Several substrates of Rho-kinase have been identified, including LIM-kinase, myosin phosphatase target subunit (MYPT)-1, ezrin/radixin/moesin family, adducin, phosphatase and tensin homolog (PTEN), and LIM-kinases. Rho-kinase mediates agonists-induced VSMC contraction. Intracellular signaling pathways for Rho-kinase activation, reactive oxygen species (ROS) production, cyclophilin A secretion are closely linked through vesicle-associated membrane protein (VAMP)-2 vesicle formation. H<sub>2</sub>O<sub>2</sub> has been reported to cause vasodilatation through several mechanisms, including cGMP, cAMP, cyclooxygenase, and several K channels. Importantly, H<sub>2</sub>O<sub>2</sub> rapidly reaches VSMC, stimulates the 1- $\alpha$  isoform of cGMP-dependent protein kinase (PKG<sub>1 $\alpha$</sub> ) to form disulfide form, and opens Ca-activated K channels (K<sub>Ca</sub>) with subsequent VSMC hyperpolarization and relaxation. ERK1/2 indicates extracellular signal-regulated kinase 1/2; GDIs, guanine nucleotide dissociation inhibitors; LRP1, low-density lipoprotein receptor-related protein 1; and PKA, cAMP-dependent protein kinase.



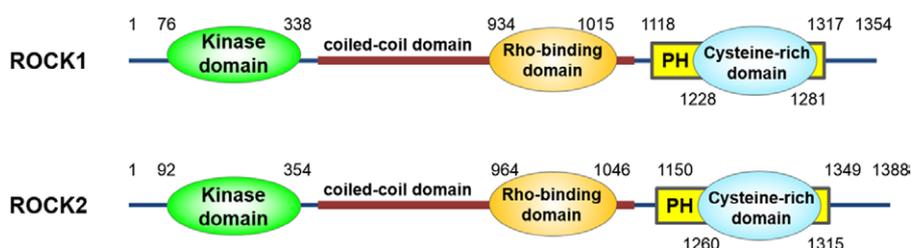
**Figure 2.** Translational research on Rho-kinase. Translation research in the authors' and other laboratories has elucidated the roles of Rho-kinase in cardiovascular medicine, ranging from molecular/cellular levels to animal and human levels. DES indicates drug-eluting stents; GEF, guanine nucleotide exchange factors; PH, pulmonary hypertension; RhoA, Ras homolog gene family member A; VSA, vasospastic angina; and VSMC, vascular smooth muscle cell.

by inducing auto/paracrine growth mechanisms.<sup>61</sup> One of the recent topics is that the secretion of CyPA, an important novel mediator of oxidative stress, is regulated by the RhoA/ROCK system (Figure 1).<sup>42,62</sup> It has been recently demonstrated that ROS activates a pathway containing vesicles with resultant secretion of CyPA.<sup>42,63</sup> We have demonstrated that secreted extracellular CyPA stimulates ERK1/2, Akt and JAK in VSMC that contribute to ROS production and constitute a vicious cycle for ROS augmentation.<sup>64,65</sup> CyPA is secreted from VSMC via a highly regulated pathway that involves vesicle transport and plasma membrane binding (Figure 1).<sup>42</sup> In the vesicular trafficking pathways, Rho GTPases including RhoA play a central role by organization of actin cytoskeleton and are thus required for secretion.<sup>66</sup> Indeed, dominant-negative mutants of RhoA inhibited oxidative stress-induced CyPA secretion, suggesting that RhoA-dependent signaling events regulate CyPA secretion.<sup>42</sup> ROCK inactivates MLCP through altering cytoskeletal dynamics.<sup>10</sup> Myosin II is involved in secretory mechanisms as a motor for vesicle transport.<sup>67</sup> Consistently, ROCK inhibitor reduced ROS-induced CyPA secretion.<sup>42,68</sup> These results suggest that ROCK-mediated myosin II activation promotes vesicle transport, which is required for CyPA secretion from VSMC. CyPA is transported into the plasma membrane and colocalized with

vesicle-associated membrane protein (VAMP)-2 in response to ROCK activation (Figure 1). Moreover, extracellular CyPA stimulates proinflammatory signals in EC, including expression of E-selectin and vascular cell adhesion molecule-1.<sup>69</sup> In addition, extracellular CyPA decreases endothelial endothelial NO synthase expression, suggesting the indirect role of RhoA/ROCK for negative regulation of endothelial NO production. CyPA is also a direct chemoattractant for inflammatory cells,<sup>70,71</sup> promoting matrix metalloproteinases (MMPs) activation.<sup>72,73</sup> CyPA plays an important role as a Ca<sup>2+</sup> regulator in platelets.<sup>74</sup> Moreover, extracellular CyPA activates platelets via Basigin (CD147)-mediated phosphoinositid-3-kinase/Akt-signaling, leading to enhanced adhesion and thrombus formation.<sup>75,76</sup> It is also known that thrombin suppresses endothelial NO synthase in EC via ROCK pathway.<sup>77</sup> Thus, CyPA and ROCK work in concert to develop vascular diseases. Indeed, CyPA may be a key mediator of ROCK that generates a vicious cycle for ROS augmentation, affecting EC, VSMC, and inflammatory cells (Figure 1).<sup>64</sup>

### Clinical Implications

Physiological level of ROCK activity is important for vascular homeostasis. In contrast, excessive ROCK activity



**Figure 3.** Molecular structures of Rho-kinase (ROCK) isoforms. There are 2 isoforms of ROCK, ROCK1 and ROCK2, which consist of 3 major domains, including a kinase domain in the N-terminal domain, a coiled-coil domain with Rho-binding domain in the middle portion and a putative pleckstrin homology (PH) domain in the C-terminal domain. ROCK1 and ROCK2 are highly homologous with an overall amino acid sequence identity of 65%.

promotes vascular diseases, in part, by promoting EC dysfunction, VSMC contraction/proliferation, and inflammatory cell migration.<sup>1,2,78–80</sup> Vascular ROCK is augmented by mechanical stretch, pressure, shear stress, hypoxia, and growth factors<sup>81</sup> and is extensively involved in the intracellular signaling initiated by many vasoactive agonists, including angiotensin II (Ang II),<sup>34,82–84</sup> thrombin,<sup>85,86</sup> platelet-derived growth factor,<sup>87</sup> thromboxanes,<sup>88</sup> extracellular nucleotides,<sup>89</sup> and urotensin (Figure 1).<sup>83</sup> ROCK downregulates endothelial NO synthase in EC and promotes proinflammatory pathways including enhanced expression of adhesion molecules.<sup>90</sup> Enhanced ROCK activity augments inflammation by inducing proinflammatory molecules, including interleukin (IL)-6 in osteoblasts,<sup>91</sup> monocyte chemoattractant protein-1,<sup>82</sup> macrophage migration inhibitory factor,<sup>92,93</sup> and sphingosine-1-phosphate.<sup>94</sup> In contrast, ROCK expression is accelerated by inflammatory stimuli, such as Ang II and IL-1 $\beta$ ,<sup>95</sup> and by remnant lipoproteins in human coronary VSMC.<sup>96</sup> ROCK upregulates NAD(P)H oxidases and augments Ang II–induced ROS production, which also contribute to the secretion of growth factors from VSMC.<sup>34</sup> Thus, enhanced ROCK activity substantially contributes to vascular inflammation. As a result, ROCK activation causes vascular diseases through EC damage, VSMC hypercontraction/proliferation, and inflammation and can be a common pathway involved in the pathogenesis of vascular diseases.<sup>1,2</sup>

### Experimental Studies

Accumulating evidence has indicated that ROCK plays important roles in the pathogenesis of a wide range of cardiovascular diseases.<sup>1,2,97,98</sup> Indeed, the RhoA/ROCK pathway not only mediates VSMC hypercontraction through inhibition of MLCP but also promotes cardiovascular diseases through enhancing ROS production.<sup>2,97,98</sup> The beneficial effects of long-term inhibition of ROCK for the treatment of cardiovascular disease have been demonstrated in various animal models, such as coronary artery spasm, arteriosclerosis, restenosis, ischemia/reperfusion injury, hypertension, pulmonary hypertension, stroke, and cardiac hypertrophy/heart failure (Figure 3).<sup>2,97,99</sup> Gene transfer of dominant-negative ROCK reduced neointimal formation of the coronary artery in pigs.<sup>100</sup> Long-term treatment with a ROCK inhibitor suppressed neointima formation after vascular injury in vivo,<sup>101,102</sup> Monocyte chemoattractant protein-1–induced vascular lesion formation,<sup>103</sup> constrictive remodeling,<sup>104,105</sup> in-stent restenosis,<sup>106</sup> and the development of cardiac allograft vasculopathy.<sup>92</sup>

### Coronary Artery Spasm

Accumulating evidence indicates that ROCK plays a crucial role in the pathogenesis of coronary artery spasm. Coronary spasm plays an important role in variant angina, myocardial infarction, and sudden death.<sup>1,107</sup> It was demonstrated that long-term treatment with cortisol, one of the important stress hormones, causes coronary hyperreactivity through activation of ROCK in pigs in vivo.<sup>108</sup> The activity and the expression of ROCK are enhanced at the inflammatory/arteriosclerotic coronary lesions.<sup>109</sup> Intracoronary administration of fasudil<sup>110</sup> and of hydroxyfasudil<sup>33</sup> inhibit coronary spasm in pigs.<sup>104</sup> To further elucidate the molecular mechanism of coronary spasm

in our porcine model, experiments were performed to examine whether ROCK is upregulated at the spastic site and if so, how it induces VSMC hypercontraction.<sup>109</sup> Reverse transcription polymerase chain reaction analysis demonstrated that the expression of ROCK mRNA and, to a lesser extent, that of RhoA mRNA were significantly upregulated in the spastic than in the control coronary segment.<sup>109</sup> Western blot analysis showed that during the serotonin-induced contractions, the extent of MYPT-1 phosphorylation was significantly greater in the spastic than in the control segment.<sup>109,110</sup> Furthermore, another ROCK inhibitor, Y-27632,<sup>31</sup> also inhibited not only serotonin-induced contractions in vivo and in vitro but also the increase in MYPT-1 phosphorylations.<sup>109</sup> Importantly, there was a highly significant positive correlation between the extent of MYPT-1 phosphorylations and that of contractions in the spastic but not in the control segments.<sup>109</sup> These results indicate that ROCK is upregulated at the spastic site and plays a key role in inducing VSMC hypercontraction by inhibiting MLCP through MYPT-1 phosphorylation (Figure 1).<sup>109,111</sup> Hydroxyfasudil causes dose-dependent inhibition of serotonin-induced coronary spasm both in vitro and in vivo in the porcine model with chronic adventitial treatment with IL-1 $\beta$  through suppression of serotonin-induced increases in MLC mono- and diphosphorylations.<sup>33,104,109,110,112</sup> Thus, the hydroxyfasudil-sensitive ROCK-mediated pathway plays an important role in the enhanced MLC phosphorylations in the spastic coronary artery (Figure 1).

### Atherosclerosis

Arteriosclerosis is a slowly progressive inflammatory process of the arterial wall that involves all the 3 layers, such as the intima, media, and adventitia.<sup>2,25</sup> In the context of atherosclerosis, ROCK should be regarded as a proinflammatory and proatherogenic molecule. ROCK-mediated pathway is substantially involved in EC dysfunction,<sup>85,90</sup> VSMC contraction,<sup>113</sup> VSMC proliferation and migration in the media<sup>114</sup> and accumulation of inflammatory cells in the adventitia.<sup>103</sup> Those ROCK-mediated cellular responses lead to the development of vascular diseases. In fact, mRNA expression of ROCK is enhanced in the inflammatory and arteriosclerotic arterial lesions in animals<sup>113</sup> and in humans.<sup>115</sup> Taken together, ROCK may be an important novel therapeutic target for atherosclerosis.

### Aortic Aneurysm

Aortic aneurysm is formed by chronic inflammation of the aortic wall, associated with medial VSMC loss and progressive destruction of structural components, particularly the elastic lamina.<sup>116</sup> Key mechanisms include VSMC senescence,<sup>117</sup> oxidative stress,<sup>61,118</sup> increased local production of proinflammatory cytokines,<sup>119</sup> and increased activities of MMPs that degrade extracellular matrix.<sup>120,121</sup> Chronic Ang II infusion into apolipoprotein E-KO mice promotes aortic aneurysm formation.<sup>122,123</sup> In animal models of aortic aneurysm, genetic and pharmacological inhibition of ROS production<sup>124,125</sup> and MMPs<sup>126,127</sup> suppressed development of aneurysms. Chronic inhibition of ROCK by fasudil has been demonstrated to reduce Ang II–induced aortic aneurysm formation in apolipoprotein E-KO mice.<sup>128</sup> Activation of ROCK

promotes CyPA secretion from VSMC and extracellular CyPA stimulates VSMC migration, proliferation and MMPs activation (Figure 1).<sup>63,129</sup> Extracellular CyPA is also a chemoattractant for inflammatory cells<sup>42,63,130</sup> and further activates vascular ROCK. Recently, we have demonstrated that ROCK-mediated CyPA augments Ang II-induced ROS production, MMP activation, and inflammatory cell recruitment into the aortic VSMC, contributing to the aortic aneurysm formation, in these animal models.<sup>68</sup> Our findings suggest that ROCK/CyPA signaling pathway is a novel therapeutic target for aortic aneurysm. Ang II induces ROCK activation and promotes CyPA secretion. Secreted extracellular CyPA augments ROCK activity in a synergistic manner.<sup>65</sup> Thus, secreted CyPA, acting as a proinflammatory cytokine, synergistically augments Ang II-mediated ROS production, contributing to the onset of vascular inflammatory cell migration and aortic aneurysm formation.<sup>124,131</sup>

### Myocardial Ischemia/Reperfusion Injury

ROS production and ROCK 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*.<sup>132</sup> The beneficial effect of fasudil has also been demonstrated in a rabbit model of myocardial ischemia induced by intravenous administration of endothelin-1,<sup>133</sup> a canine model of pacing-induced myocardial ischemia,<sup>134</sup> and a rat model of vasopressin-induced chronic myocardial ischemia.<sup>135</sup>

### Cardiac Hypertrophy and Heart Failure

Although the structural difference between the 2 ventricles is obvious, the fundamental functional difference between right ventricular (RV) failure and left ventricular (LV) failure remains unclear. Thus, our knowledge and strategy for the treatment of RV failure are still limited. We have recently addressed this fundamental issue by comparing the responses of both ventricles to chronic pressure-overload.<sup>136</sup> Interestingly, there were significant differences in the induction pattern and localization of oxidative stress at 24 hours after pressure-overload; pulmonary artery constriction rapidly induced oxidative stress in the RV without significant change in the LV, whereas transverse aortic constriction slowly induced oxidative stress in the LV without significant change in the RV.<sup>136</sup> Furthermore, ROCK2 was promptly upregulated in the RV after pulmonary artery constriction and was colocalized with ROS induction.<sup>136</sup> Thus, it is conceivable that the increased ROCK2 expression in the RV after pulmonary artery constriction contributes, at least in part, to the vulnerability of the RV to pressure-overload and the characteristic difference between the 2 ventricles. At present, we still have limited knowledge on the roles of ROCK1 and ROCK2 in the pathogenesis of RV and LV failure. Mechanical stretch stimulates integrins, which activates the RhoA/ROCK pathway through RhoGEFs.<sup>137</sup> Mechanotransduction through integrins leads to the activation of RhoA/ROCK pathway, which induces hypertrophic gene activation.<sup>138,139</sup> In contrast, mechanosensing by actin filaments causes actin cytoskeleton remodeling through

small GTPases of the Rho/Rac/Cdc42 family.<sup>138,139</sup> However, the detailed mechanisms are not fully elucidated as to the mechanoresponses and the link among the integrin, RhoGEFs and the downstream targets of the RhoA/ROCK pathway. In the downstream mechanotransduction through integrin- $\beta$  by pressure-overload, adhesion of  $\alpha$ -actinin, talin, and vinculin to actin filaments, may potentially contribute to the activation of FGD2 (FYVE, RhoGEF and PH domain-containing protein 2; RhoGEF) preferentially in the RV after pulmonary artery constriction.<sup>136</sup> Our microarray analysis suggested that there is a special signaling cascade in the RV that connects the FGD2 and RhoA/ROCK2 signaling to the downstream of integrin- $\beta$ , which may be the difference between the RV and the LV in response to mechanical stretch.<sup>136</sup>

Next, Ang II plays a key role in many physiological and pathological processes in cardiac cells, including cardiac hypertrophy.<sup>140</sup> Understanding of the molecular mechanisms for Ang II-induced myocardial disorders is important to develop new therapies for cardiac dysfunction.<sup>141</sup> ROS production is recognized to be involved in Ang II-induced cardiac hypertrophy,<sup>142,143</sup> however, the precise mechanism by which ROS cause myocardial hypertrophy and dysfunction still remains to be fully elucidated.<sup>144</sup> It has been demonstrated that cardiac troponin is a substrate of ROCK.<sup>145</sup> ROCK phosphorylates troponin and inhibits tension generation in cardiac myocytes. We have previously demonstrated that ROCK inhibition with fasudil suppresses the development of cardiac hypertrophy and diastolic heart failure in Dahl salt-sensitive rats.<sup>146</sup> In addition, our recent study demonstrated a synergy between CyPA and ROCK to increase ROS generation.<sup>68</sup> Because ROS stimulate myocardial hypertrophy, matrix remodeling, and cellular dysfunction,<sup>147</sup> ROCK and CyPA may work together to promote ROS production and Ang II-induced cardiac hypertrophy. 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.<sup>148</sup>

### Hypertension

Uehata et al<sup>31</sup> demonstrated that ROCK-mediated Ca<sup>2+</sup> sensitization is involved in the pathophysiology of hypertension. Short-term administration of Y-27632, another ROCK inhibitor, preferentially reduces systemic blood pressure in a dose-dependent manner in rat models of systemic hypertension, suggesting an involvement of ROCK in the pathogenesis of increased systemic vascular resistance in hypertension.<sup>31</sup> The expression of ROCK is significantly increased in resistance vessels of spontaneously hypertensive rats.<sup>149</sup> ROCK is also involved in the central mechanisms of sympathetic nerve activity.<sup>150-153</sup>

### Pulmonary Hypertension

Pulmonary hypertension (PH) is associated with hypoxic exposure, endothelial dysfunction, VSMC hypercontraction and proliferation, enhanced ROS production, and inflammatory cell migration, for which ROCK seems to be substantially involved. Indeed, long-term treatment with fasudil suppresses the development of monocrotaline-induced PH in rats<sup>154</sup> and

hypoxia-induced PH in mice.<sup>155</sup> We also have recently developed VSMC-specific ROCK2-KO mice and demonstrated the specific role of ROCK2 in the development of hypoxia-induced PH.<sup>156</sup> These mutant mice revealed normal growth and body weight gain under physiological conditions. However, chronic hypoxia significantly increased ROCK2 expression and ROCK activity in the lung tissues from wild-type littermates and the development of pulmonary hypertension and RV hypertrophy caused by chronic hypoxia *in vivo* was evident in littermates but was suppressed in VSMC-specific ROCK2-KO mice.<sup>156</sup> *In vitro*, the growth and migration of VSMC were significantly reduced in ROCK2-KO VSMC, compared with control VSMC.<sup>156</sup>

Because the secretion of CyPA is regulated by ROCK,<sup>42,65</sup> we further tested the hypothesis that CyPA contributes to the development of PH in mice and in humans.<sup>157</sup> Importantly, we demonstrated that extracellular CyPA and its receptor, Basigin (CD147), are crucial for hypoxia-induced PH by inducing growth factor secretion, inflammatory cell recruitment, and VSMC proliferation.<sup>157</sup> These results suggest that extracellular CyPA and vascular Basigin are crucial for PH development and could be potential therapeutic targets for the disorder. Statins and ROCK inhibitor reduce the secretion of CyPA from VSMC<sup>42,65</sup> and pravastatin ameliorates hypoxia-induced PH in mice.<sup>158,159</sup> Thus, it is possible that inhibition of CyPA secretion by statins<sup>159</sup> or ROCK inhibitors<sup>154,160</sup> could contribute to the therapeutic effects of these drugs on PH. It has been reported that intravenous injection of several chemically different ROCK inhibitors reduces systemic and pulmonary arterial pressures even under resting conditions.<sup>161–164</sup> These results suggest that ROCK plays a physiological role in the maintenance of baseline vasoconstrictor tone in the pulmonary and systemic vascular beds and is involved in the development of PH.

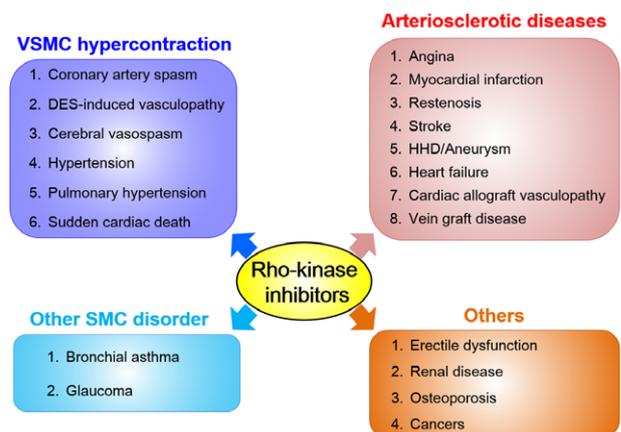
### Clinical Studies

Vasospastic angina (VSA) is known to exhibit circadian variation with an early morning peak. We have recently demonstrated that ROCK activity in circulating neutrophils is a useful biomarker for diagnosis and disease activity assessment in patients with VSA.<sup>165</sup> Furthermore, we also have recently demonstrated that ROCK activity shows a significant circadian variation with a peak at 6:00 AM in patients with VSA, whereas no such variation was noted in non-VSA patients.<sup>166</sup> Importantly, ROCK activity at spasm provocation test was significantly correlated with basal coronary tone evaluated by vasodilating responses to intracoronary nitrate and coronary vasoconstricting responses to acetylcholine in patients with VSA.<sup>166</sup> Furthermore, their ROCK activity at 6:00 AM was positively correlated with nocturnal parasympathetic activity as evaluated by heart rate variability in Holter monitoring.<sup>166</sup> Interestingly, some recent studies revealed that ROCK plays a critical role in determining the intrinsic circadian rhythm of vascular contractility and blood pressure.<sup>167,168</sup> Thus, ROCK activity exhibits distinct circadian variation associated with alterations in coronary vasomotor responses and autonomic activity in patients with VSA.<sup>166</sup> Others also suggested that cardiovascular risk may enhance ROCK activity and endothelial dysfunction, leading to progression of cardiovascular

diseases.<sup>169</sup> Next, we have demonstrated that intracoronary administration of fasudil is effective in preventing coronary spasm and resultant myocardial ischemia in patients with VSA.<sup>170</sup> Thus, fasudil is useful for diagnosis and treatment of ischemic coronary syndromes caused by the spasm.<sup>165,166,170</sup> Fasudil is also effective in treating patients with microvascular angina.<sup>171</sup> Hydroxyfasudil, an active metabolite of fasudil after oral absorption, selectively inhibits ROCK.<sup>33</sup> The clinical trials of the effects of fasudil in Japanese patients with stable effort angina demonstrated that the long-term oral treatment with the ROCK inhibitor is effective in ameliorating exercise intolerance in those patients.<sup>99</sup> Indeed, subsequent clinical studies also showed that intracoronary fasudil is effective in almost all patients with epicardial coronary spasm<sup>170</sup> and approximately two thirds of patients with microvascular angina.<sup>171</sup> These results indicate the usefulness of ROCK inhibitors for the treatment of coronary vasospastic disorders.<sup>1,2,97,98</sup>

Recently, we further demonstrated that the ROCK pathway plays a crucial role in the pathogenesis of coronary hyperconstricting responses induced by drug-eluting stents in pigs<sup>172</sup> and in humans<sup>173</sup> and that long-term treatment with a long-acting nifedipine suppresses drug-eluting stents-induced coronary vasomotor dysfunctions through indirect inhibition of ROCK pathway.<sup>174</sup> Indeed, the role of the ROCK pathway has been emerging and the indications of ROCK inhibitors have been expanding in cardiovascular medicine (Figure 4).<sup>1,2,97,98</sup> In addition, we recently demonstrated that the ROCK mediator, plasma CyPA, is a novel biomarker for coronary artery disease.<sup>175</sup> Multivariable analysis demonstrated that in addition to the established risk factors (eg, age, sex, smoking, hypertension, and diabetes mellitus), CyPA >15 ng/mL was significantly correlated with coronary artery disease.<sup>175</sup>

Next, we were able to obtain direct evidence for ROCK activation in circulating leukocytes in patients with pulmonary arterial hypertension (PAH).<sup>176</sup> Indeed, intravenous infusion of fasudil significantly reduced pulmonary vascular resistance in patients with PAH, indicating an involvement of ROCK in the pathogenesis of PAH in humans.<sup>177</sup> Fasudil decreases



**Figure 4.** Possible indications of Rho-kinase inhibitors. Selective Rho-kinase inhibitors could have a wide range of indications, including not only cardiovascular diseases caused by vascular smooth muscle (VSMC) hypercontraction but also those by atherosclerosis in general, those by smooth muscle hypercontraction, and others. DES indicates drug-eluting stents.

pulmonary arterial pressure in various situations, in which vascular tone is increased in the coronary and pulmonary vascular beds. Most important point in the clinical settings is the chronic effects of the drugs. Long-acting fasudil has recently been demonstrated to exert beneficial effects in patients with PAH.<sup>178</sup> We further confirmed the role of extracellular CyPA, which is a novel mediator of ROCK, in the pathogenesis of PAH in humans.<sup>157</sup> We examined human recombinant CyPA-induced secretion of growth factors from VSMCs harvested from the pulmonary arteries of patients with PAH. Extracellular CyPA induced secretion of growth factors and chemokines (eg, platelet-derived growth factor-BB, SDF-1 [stromal cell derived factor], and FGF-2 [fibroblast growth factor]) and inflammatory cytokines (eg, IL-1 $\beta$ , IL-2, and tumor necrosis factor- $\alpha$ ) and this effect was enhanced by hypoxia.<sup>157</sup> These results support the notion that ROCK-mediated extracellular CyPA promotes the secretion of growth factors from VSMCs in patients with PAH. Thus, we measured plasma levels of CyPA in patients with PAH. As expected, plasma CyPA levels were elevated in patients with PAH as compared with those without PAH or healthy controls.<sup>157</sup> Moreover, the event-free curve revealed that high plasma CyPA levels (>22 ng/mL) were associated with poor outcome (death or lung transplantation), suggesting plasma CyPA is a novel biomarker of disease severity, therapeutic efficacy, and prognosis in patients with PAH. We have previously reported that statins and ROCK inhibitors reduce CyPA secretion from VSMCs.<sup>42,68</sup> ROCK is an important therapeutic target in cardiovascular diseases<sup>2</sup> and ROCK inhibition ameliorates PH in animals and in humans.<sup>154,175,176,178</sup> Indeed, the secretion of a variety of cytokines/chemokines and growth factors was significantly reduced by treatment with fasudil (K. Satoh and H. Shimokawa, unpublished data, 2014). On the basis of our study, inhibition of CyPA secretion by ROCK inhibitors may contribute to the therapeutic efficacy of these drugs in PAH.<sup>154,176</sup> The identification of CyPA as a novel mediator of ROCK associated with inflammation provides insight into the mechanisms of several therapies.

Currently, many pharmaceutical companies and manufacturers have strong interests on the RhoA/ROCK signaling and the development of its inhibitors (Figure 4).<sup>179</sup> Among them, Akama et al<sup>180</sup> performed a kinome-wide screen to investigate the members of the benzoxaborole family and identified ROCK as a target. They showed competitive behavior, with respect to ATP, and determined the ROCK2-drug cocrystal structure.<sup>180</sup> They exhibited oral availability and 1 member reduced rat blood pressure, consistent with ROCK's role in smooth muscle contraction. Thus, the benzoxaborole moiety may possess a novel hinge-binding kinase scaffold that may have potential for therapeutic use.<sup>179,180</sup> On the basis of the role of ROCK in disease processes that include smooth muscle contraction, fibrosis, and inflammation, the target and therapeutic applications for ROCK inhibitors are mainly in the field of cardiovascular diseases, such as VSA, cardiac hypertrophy, and PH. Indeed, the role of the ROCK pathway has been emerging and the indications of ROCK inhibitors have been expanding especially in cardiovascular medicine (Figure 4).

## Conclusions

The identification of ROCK as a mediator of cardiovascular diseases associated with inflammation and oxidative stress provides insight into the development of new therapies. Indeed, accumulating evidence suggests that ROCK is substantially involved in the pathogenesis of a wide variety of cardiovascular diseases.

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## Disclosures

None.

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### Significance

The RhoA/Rho-kinase (ROCK) pathway plays an important role in various fundamental cellular functions, including contraction, motility, proliferation, and apoptosis, whereas its excessive activity is involved in the pathogenesis of cardiovascular diseases. A series of translational research studies have demonstrated the important roles of ROCK in the pathogenesis of cardiovascular diseases. At the molecular and cellular levels, ROCK upregulates several molecules related to inflammation, thrombosis, and fibrosis. In animal experiments, ROCK plays an important role in the pathogenesis of vasospasm, arteriosclerosis, hypertension, pulmonary hypertension, and heart failure. Finally, at the human level, ROCK is substantially involved in the pathogenesis of coronary vasospasm, angina pectoris, hypertension, pulmonary hypertension, and heart failure. Furthermore, ROCK activity in circulating leukocytes is a useful biomarker for the assessment of disease severity and therapeutic responses in vasospastic angina, heart failure, and pulmonary hypertension. Thus, the ROCK pathway is an important novel therapeutic target in cardiovascular medicine.

## ATVB Named Lecture Reviews— Insight Into Author



### ATVB Named Lecture Reviews—Plenary Lecture

**Insight Into the Author: Hiroaki Shimokawa, MD, PhD,  
Tohoku University Graduate School of Medicine, Sendai, Japan**

#### **Why did you choose the profession of scientific investigation?**

I chose the profession of scientific investigation because I thought (and think) that science is one of the most creative and stimulating fields and that medical research is one of the most important activities among scientific investigations.

#### **Who have been your role model(s) in your scientific and professional life?**

- (1) Professor Akira Takeshita, an emeritus professor of Kyushu University, who served as an Asian Associate Editor of ATVB and unfortunately passed away in 2009. I worked with him at the Kyushu University as an assistant professor (1991–1994) and then as an associate professor (1995–2005), when I studied molecular mechanisms of coronary artery spasm and arteriosclerosis with a special reference to Rho-kinase.
- (2) Professor Paul Vanhoutte, a professor and chairman of the University of Hong Kong. I worked in his laboratory at Mayo Clinic from 1985–1988, where I studied endothelial functions with a special reference to endothelium-derived relaxing factors.

#### **What have been important influences on your professional life?**

Interactions with young medical students, doctors (graduate students and postgraduate fellows), and staff in my department.

#### **What are your scientific inspirations?**

It is very stimulating and inspiring to elucidate unknown facts and to develop new diagnostic and therapeutic strategies, all of which are useful to advance medicine.

#### **How have mentors contributed to your professional development?**

Professor Vanhoutte taught me the pleasure of research where we can discover unknown facts and contribute to society. I have learned from Professor Takeshita about the importance of continuation of research despite any difficult situations.

#### **If you knew then what you know now, would you do anything different?**

No. I only could have been able to save some time, if any, even if I knew then what I know now.

#### **What wisdom do you impart on new investigators?**

Three points: (1) the theme of research should be original; (2) should have good mentors; and (3) should continue research despite any difficult situations (there always are good solutions to overcome them).

#### **If you were not a scientist, which profession would you pick?**

I would like to work for the country as a government official, like finance or diplomacy field.

#### **Which direction do you envisage your science taking?**

My research is directed to develop noninvasive diagnostic and therapeutic strategies to achieve a healthy society.

#### **What are your nonscientific activities?**

Reading books, music (classic), and sports.

#### **What sports do you follow?**

Football and baseball. I used to play football at high school and university.

#### **What are your favorite books, movies, music (pick one or all)?**

Ryotaro Shiba (Japanese novelist), human dramas (movies), and Beethoven (music).

#### **What are your favorite foods and are they heart healthy?**

Sushi and sashimi, definitely good for your heart!