

# RhoA/Rho-kinase signaling: a therapeutic target in pulmonary hypertension

Scott A Barman  
Shu Zhu  
Richard E White

Department of Pharmacology  
and Toxicology, Medical College  
of Georgia, Augusta, Georgia, USA

**Abstract:** Pulmonary arterial hypertension (PAH) is a devastating disease characterized by progressive elevation of pulmonary arterial pressure and vascular resistance due to pulmonary vasoconstriction and vessel remodeling as well as inflammation. Rho-kinases (ROCKs) are one of the best-described effectors of the small G-protein RhoA, and ROCKs are involved in a variety of cellular functions including muscle cell contraction, proliferation and vascular inflammation through inhibition of myosin light chain phosphatase and activation of downstream mediators. A plethora of evidence in animal models suggests that heightened RhoA/ROCK signaling is important in the pathogenesis of pulmonary hypertension by causing enhanced constriction and remodeling of the pulmonary vasculature. Both animal and clinical studies suggest that ROCK inhibitors are effective for treatment of severe PAH with minimal risk, which supports the premise that ROCKs are important therapeutic targets in pulmonary hypertension and that ROCK inhibitors are a promising new class of drugs for this devastating disease.

**Keywords:** pulmonary arterial hypertension, Rho-kinase, vasoconstriction, fasudil

## Pulmonary hypertension

Pulmonary arterial hypertension (PAH), characterized by an elevated, sustained increase in pulmonary artery pressure greater than 25 mmHg at rest or 30 mmHg upon exertion, is a progressive disease with poor prognosis and death usually occurring within 5 years if left untreated.<sup>1</sup> Further, primary or idiopathic pulmonary hypertension (IPAH) can result in death within a median of 3 years from right ventricular failure without treatment, with a 15% 1-year mortality rate despite current therapy.<sup>2</sup> Factors contributing to PAH include prolonged vasoconstriction, vascular remodeling, inflammatory cell migration, and in situ thrombosis which result in the formation of vascular lesions.<sup>3,4</sup> It is currently thought that the primary cause of the elevated pulmonary vascular resistance that occurs in PAH is due to mechanical obstruction from vascular remodeling.<sup>5,6</sup> In addition, pathologic findings show that PAH is associated with intimal and/or medial hypertrophy, intimal fibrosis, and plexiform lesions.<sup>7</sup>

## Animal models of pulmonary hypertension

Although the long-term prognosis for patients with PAH is rather poor, recent advances in the understanding of pathophysiological mechanisms underlying the progression of PAH have been made possible through the use of experimental animal models. The monocrotaline model of PAH, initially used over 40 years ago, is induced via a single injection of 60 mg/kg monocrotaline either intraperitoneally or subcutaneously.<sup>8</sup> Rapid and severe pulmonary vascular disease usually occurs within a few days (independent of

Correspondence: Scott A Barman  
Department of Pharmacology and  
Toxicology, Medical College of Georgia,  
Augusta, Georgia 30912, USA  
Tel +1 706-721-6352  
Fax +1 706-721-2347  
Email sbarman@mcg.edu

any cardiac or lung parenchymal disorders), suggesting that this model is an excellent choice to study IPAH. Although the basic underlying mechanism of monocrotaline-induced PAH is not well understood, it is known that the parent compound is not toxic, and must be activated to the reactive monocrotaline pyrrole by hepatic cytochrome P450 3A, which targets the pulmonary vascular endothelium.<sup>9-12</sup> A limitation of this experimental model is that differences exist in monocrotaline sensitivity between rat strains as well as individual variances in the pharmacokinetics of monocrotaline involving degradation and hepatic formation of the pyrrole or conjugation and excretion.<sup>13</sup>

A second widely employed model of PAH is the use of chronic hypoxia. Studies show that decreasing the alveolar oxygen pressure to <70 mmHg elicits a strong pulmonary vasoconstrictor response; however, the hypoxic-induced effect varies among animal species.<sup>14</sup> For example, rabbits show very little response to alveolar hypoxia, but cattle exhibit the greatest vasoconstriction, and hypoxic pulmonary vasoconstriction is milder in humans than in rats.<sup>15</sup> Further, the hypoxic pulmonary vasoconstrictor response varies among humans.<sup>16</sup> The time of exposure to hypoxia appears to be critical as short exposure causes acute pulmonary vasoconstriction, while prolonged hypoxia results in remodeling of the distal pulmonary arterial branches. It has also been observed that endothelial and smooth muscle hyperplasia occurs in the walls of pulmonary arteries in rats during the first days of hypoxic exposure.<sup>17,18</sup> In animal models, intermittent severe hypoxia leads to the development of PAH, independent of the duration of the hypoxia to normoxia intervals. However, in humans, intermittent hypoxia elicits only a small clinically irrelevant effect on pulmonary hemodynamics.<sup>19</sup> Thus, caution must be exercised when extrapolating animal models of chronic hypoxic-induced PAH to the human setting. Another documented animal model of PAH involves the formation of chronic emboli in pulmonary vessels. Shelub et al induced chronic embolic PAH through repeated microembolizations with the injection of Sephadex® microspheres.<sup>20</sup> The utilization of this approach allows different-sized vessels to be targeted depending on the diameter size of the microspheres that are injected, and vascular obstruction and vasoconstriction are the primary mechanisms of the high pulmonary vascular resistance that occurs.<sup>20,21</sup> More recently, repeated embolizations with polydextran microspheres were used in pigs to elicit a sustained elevation in pulmonary arterial pressure.<sup>22</sup>

A recent rat animal model of experimental PAH described by Taraseviciene-Stewart and colleagues involves the

combination of vascular endothelial growth factor receptor blockade with SUGEN (SU) 5416 and chronic hypoxia exposure.<sup>23</sup> A severe, progressive PAH occurs which is accompanied by precapillary arterial occlusion by proliferating factor VIII-positive endothelial cells. In addition, the PAH in these animals is resistant to treatment with drugs that are commonly used to treat human PAH.<sup>24</sup> Thus, it appears that this particular model of PAH more closely mimics human severe PAH than the monocrotaline and chronic hypoxia models of PAH which can be successfully treated with a variety of agents.<sup>25</sup> Finally, the newest animal models of PAH involve the use of genetically modified animals as genetic screening has identified a number of potentially important gene variants that may contribute towards the development of PAH. For example, since a heterozygous mutation of the BMPR2 gene which encodes for the bone morphogenetic protein receptor-II is present in a large portion of patients with IPAH, heterozygous BMPR2-deficient mice have been used to mimic the human condition with limited success.<sup>26-28</sup> Further, serotonin (5-HT), and its plasma membrane transporter (5-TTT) has been reported to be involved in the pathogenesis of PAH in humans, and genetically engineered mice lacking the 5-HTTT exhibit attenuated hypoxia-induced PAH, and mice overexpressing 5-HTTT can cause PAH.<sup>29,30</sup>

## Current therapies for pulmonary hypertension

Although there is a variety of drugs that are currently used or under investigation for the treatment of PAH, there is no specific drug class that is completely efficacious in reversing the deleterious effects of this disease. Nitric oxide (NO) has been used with mixed success as a therapy for PAH. Exogenous administration of the inhaled preparation of NO reduces pulmonary vascular resistance without having an effect on systemic vascular resistance or cardiac function.<sup>31,32</sup> Inhaled NO has been used to treat primary pulmonary hypertension, secondary pulmonary hypertension in association with congenital or acquired heart disease, chronic obstructive pulmonary disease (COPD), and Adult Respiratory Distress Syndrome (ARDS).<sup>33-35</sup> Evidence suggests that the efficacy of NO is dependent on the inhaled concentration. For example, it has been observed that 40 ppm inhaled NO will reverse hypoxic pulmonary vasoconstriction in healthy individuals independent of systemic effects, and initial results showed improvement in ARDS patients.<sup>36,37</sup> However, it was also reported that inhaled NO in doses greater than 10 ppm worsen arterial oxygenation and therefore, it was suggested that lower doses be used to treat ARDS.<sup>38</sup> The primary limiting

factor in the global use of inhaled NO is the potential toxicity that may occur if high concentrations are used. Chemical reactions with oxygen and reactive oxygen species yield toxic nitrogen oxides and hydroxyl radicals. Subsequently, nitrogen oxide reacts with superoxide to form peroxynitrite, which can elicit pulmonary cellular injury.<sup>39</sup>

The prostanoids are another class of agents used to treat PAH as these substances have been successfully tested in animal models of PAH, and prostacyclin and its analogs have been extensively studied in the treatment of human PAH.<sup>40,41</sup> Mechanistically, prostacyclin exerts vasoprotective effects through pulmonary vasodilatation, inhibition of platelet aggregation, and pulmonary arterial smooth muscle proliferation.<sup>42</sup> Because of prostacyclin's short half-life, several analogs have been developed which appear to exhibit long-term beneficial vasodilatory and antithrombotic effects in patients with PAH when given by daily inhalation.<sup>43</sup> Specifically, aerosolized iloprost causes selective pulmonary vasodilation in patients with either primary or secondary pulmonary hypertension.<sup>43</sup> In contrast, beraprost has limited effectiveness in treating both primary and secondary PAH.<sup>44,45</sup> Most recently, it was found that intravenous epoprostenol improves survival in patients with PAH.<sup>46</sup>

Endothelin (ET) receptor antagonists represent some of the newest class of agents available for treatment of PAH. The selective ET<sub>A</sub> receptor antagonist BQ-123 was the first ET receptor antagonist to exhibit beneficial effects in animal models of PAH, and bosentan, an antagonist of both ET<sub>A</sub> and ET<sub>B</sub> receptors reduces the medial thickening and neomuscularization of pulmonary arteries as well as lowering pulmonary arterial pressure in rat animal models of PAH.<sup>47-49</sup> Studies also show that bosentan is clinically effective in patients with PAH, which included significant improvement in exercise capacity, functional class, and pulmonary hemodynamics.<sup>50,51</sup>

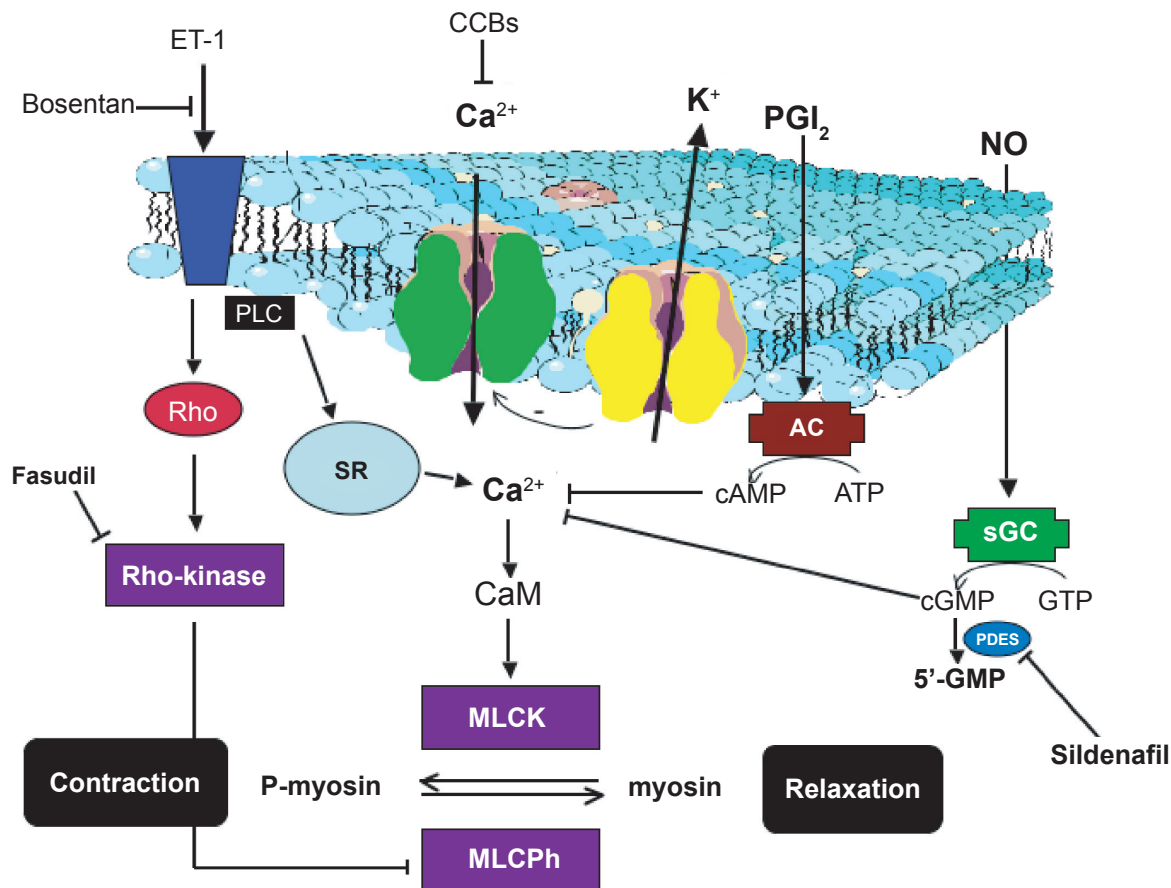
The newest and most potentially efficacious therapeutic agents to date are the phosphodiesterase (PDE) 5 inhibitors, which were discovered incidentally to have pulmonary vasodilating effects in addition to being used to treat erectile dysfunction. Sildenafil attenuates the acute pulmonary vasoconstrictor response to hypoxia in humans as well as lowering high-altitude-induced temporary PAH in healthy volunteers.<sup>52,53</sup> In addition, sildenafil decreases RV mass in patients with PAH, suggesting that PDE5 inhibitors may be able to reverse the RV remodeling that occurs in PAH.<sup>54</sup> Further, the combination of sildenafil and beraprost may be efficacious in treating PAH.<sup>55</sup> Other therapies include adrenomedullin, an endogenously produced vasodilator originally

discovered in human pheochromocytoma, and levosimendan, a dual calcium-sensitizing positive inotropic agent and potassium channel activator. Inhaled adrenomedullin may improve exercise capacity and selectively decrease pulmonary vascular pressure in patients with primary pulmonary hypertension, and intravenous levosimendan significantly lowers pulmonary vascular resistance after heart transplant procedure.<sup>56,57</sup> Finally, calcium channel blockers have been used with limited success as less than 10% of patients with PAH have a beneficial acute pulmonary vasodilatory response to long-term treatment.<sup>58</sup> Mechanisms by which currently used drugs elicit pulmonary vasodilatation for treatment of pulmonary hypertension are shown in Figure 1, although the limited success of these current therapeutic regimens has been the impetus for investigating the feasibility of therapeutically targeting Rho-kinase signaling mechanisms in PAH.

## Rho-kinase

There is convincing evidence that Rho-kinases are involved in a variety of cardiovascular diseases including pulmonary hypertension.<sup>59-64</sup> Approximately seventeen years ago it was revealed that a small monomeric GTPase called Rho induced the formation of stress fibers and focal adhesions in 3T3 cells.<sup>65</sup> Subsequently, a number of laboratories demonstrated that Rho was expressed in smooth muscle and could be activated by a plethora of contractile agonists.<sup>66</sup> Although Rho was shown to increase Ca<sup>2+</sup> sensitivity and phosphorylate myosin light chain in intact smooth muscle, these events could not occur in permeabilized smooth muscle cells, suggesting that activation required interaction of Rho with a second component of the plasma membrane.<sup>67,68</sup>

In the mid 1990s a number of investigators working independently identified one of the effectors of Rho and termed it Rho-kinase, and it was postulated that this was the component necessary for Rho activation.<sup>69,70</sup> In 1996, it was reported that Rho-kinase (ROCK) was activated by Rho, which subsequently phosphorylated and inhibited myosin light chain phosphatase.<sup>71</sup> One year later it was found that translocation of Rho to the plasma membrane was necessary for increasing Ca<sup>2+</sup> sensitivity.<sup>72</sup> Further, it was widely documented that Rho and/or ROCK antagonists inhibited the effect of contractile agonists indicating that both Rho and ROCK were major effectors of agonist-induced Ca<sup>2+</sup> sensitization in smooth muscle.<sup>67,73-75</sup> ROCKs are serine/threonine kinases with a molecular mass of approximately 160 kDa.<sup>1,61</sup> These kinases are expressed in invertebrates such as *Caenorhabditis elegans*, *Drosophila*, and mosquito, and in vertebrates such as zebrafish, *Xenopus*, chicken, mouse,



**Figure 1** Mechanisms through which current drugs elicit pulmonary vasodilatation to treat pulmonary hypertension.

**Abbreviations:** ET-1, endothelin-1; CCBs, calcium channel blockers; PGI<sub>2</sub>, prostacyclin; NO, nitric oxide; CaM, calmodulin; SR, sarcoplasmic reticulum; AC, adenylate cyclase; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PDE5, phosphodiesterase 5; PLC, phospholipase C; sGC, soluble guanylate cyclase.

rat, and human.<sup>61</sup> ROCK's catalytic site is adjacent to its NH<sub>2</sub> terminus, whereas the Rho binding site is located at the COOH-terminal portion of its coiled-coil domain.<sup>66</sup> ROCKs regulate a variety of cellular functions including motility, proliferation, apoptosis, contraction, and gene expression, and are believed to be the most important regulators of Ca<sup>2+</sup> sensitivity in smooth muscle.<sup>61,66,76,77</sup> ROCK-mediated pathophysiological mechanisms that may contribute to the development of pulmonary hypertension are summarized in Figure 2.

Currently, two isoforms (ROCK-1 and ROCK-2) have been identified, which are expressed in vascular smooth muscle.<sup>61</sup> ROCK activation increases the Ca<sup>2+</sup> sensitivity of contraction in vascular smooth muscle via inhibition of myosin light chain phosphatase, which increases the phosphorylation of myosin light chain and augments contraction at any given level of cytosolic Ca<sup>2+</sup> and activity of myosin light chain kinase.<sup>78</sup> ROCK inhibits myosin light chain phosphatase by phosphorylating the 130 kDa

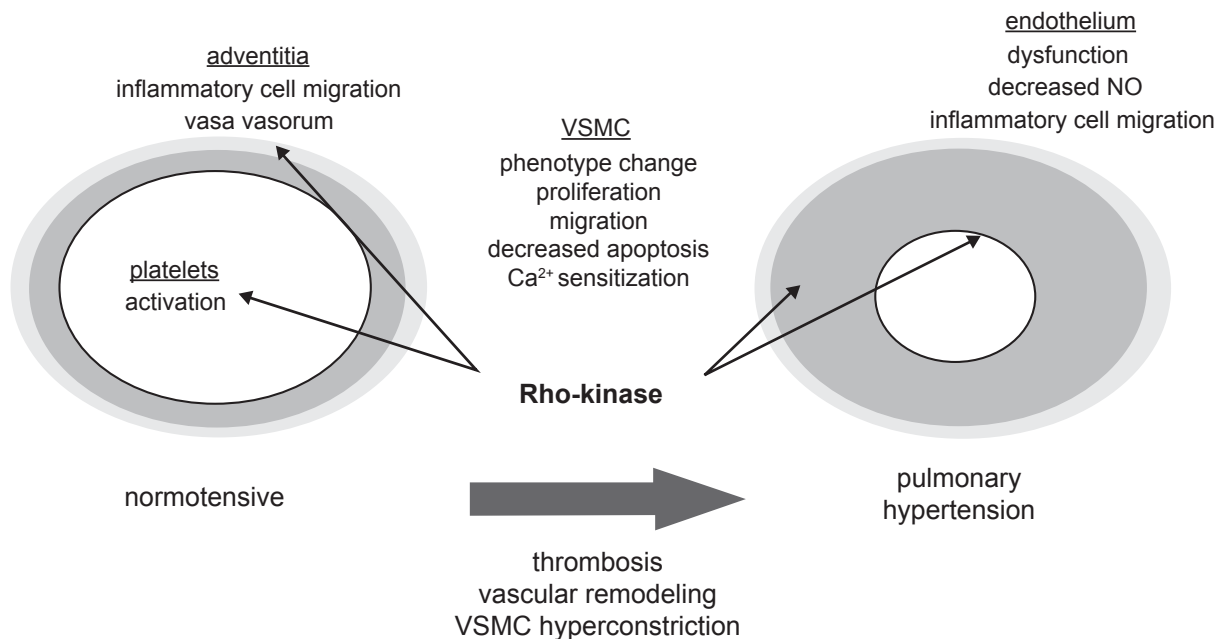
myosin-binding subunit myosin light chain phosphatase (MYPT-1) and/or the myosin light chain phosphatase inhibitor protein CPI-17.<sup>66</sup> Further, ROCKs target other substrates that are important for smooth muscle contraction such as calponin.<sup>61</sup>

RhoA activates ROCK after extracellular G-protein coupled receptor binding.<sup>72</sup> Depending on the agonist stimulation, ROCK may increase Ca<sup>2+</sup> sensitivity of the contractile apparatus via phosphorylation of MYPT-1 at threonine (Thr)-696 and Thr-853, and phosphorylation of CPI-17 at Thr-38.<sup>66</sup> More recently, it was shown that ROCK also phosphorylates Thr-855 on MYPT-1.<sup>79,80</sup>

### Rho-kinases as targets for pulmonary hypertension

Evidence strongly suggests that RhoA/ROCK is an attractive target for the development of therapeutics to treat PAH as many studies have shown that ROCK signaling is involved in the sustained vasoconstriction and vascular remodeling and

## Multiple targets of Rho-kinase in pulmonary arteries



**Figure 2** Schematic illustration of possible Rho-kinase mediated pathophysiological mechanisms that may contribute to the development of pulmonary hypertension. **Abbreviation:** VSMC, vascular smooth muscle cells.

inflammation that occurs in this disease. Oka et al reported that ROCK signaling mediated vasoconstriction in severe occlusive pulmonary hypertension in rats, and other studies have shown that ROCKs are involved in hypoxic pulmonary vasoconstriction, hypoxic pulmonary hypertension, and monocrotaline-induced pulmonary hypertension.<sup>3,81–83</sup> More recently, studies confirmed that ROCK-mediated prolonged vasoconstriction was inherently involved in chronic hypoxic pulmonary hypertension in both neonatal and adult rats as well as in bleomycin-induced pulmonary hypertension.<sup>84,85</sup> Endothelin-1 increases pulmonary vasoconstriction via ROCK signaling, and small pulmonary arteries exhibit ROCK-dependent increases in myogenic tone in chronic hypoxic pulmonary hypertension.<sup>86–88</sup> Further, ROCKs have been implicated in the vascular remodeling associated with experimental models of PAH, and a recent report by Guilluy and colleagues suggests that transglutaminase-mediated activation of RhoA by serotonin may be involved in pulmonary vascular remodeling induced by chronic hypoxia.<sup>78,89–91</sup> In humans, preliminary studies show that the pulmonary vasodilatory response in hypertensive pulmonary arteries and isolated perfused lung lobes removed from lung

transplantation patients for severe PAH is associated with high RhoA/ROCK activity.<sup>92</sup>

Studies involving ROCK inhibitors have been primarily done with either fasudil or Y-27632. Both compounds are cell-permeable and potently inhibit ROCK in vascular smooth muscle.<sup>93,94</sup> Fasudil, initially described as an intracellular calcium antagonist, is a selective inhibitor of ROCK.<sup>61,74,95</sup> Specifically, fasudil is metabolized in the liver to the active compound hydroxyfasudil, which is a specific ROCK inhibitor because its efficacy for ROCK is 100-fold higher than for protein kinase C (PKC), and 1000-fold higher than for myosin light chain kinase.<sup>93,96</sup> Similarly, Y-27632, a pyridine derivative, binds to and inhibits p160ROCK up to 200-fold higher than PKC or PKA.<sup>74</sup>

The mechanisms by which ROCK antagonists attenuate PAH are widespread. Evidence from *in vivo* studies suggests that the effect of ROCK inhibitors is associated with decreased pulmonary artery expression of growth factors and markers of cell proliferation, matrix protein production, and inflammatory cell infiltration as well as an increase in signals for apoptosis.<sup>97</sup> *In vitro* studies also suggest a negating effect of ROCK inhibitors on pulmonary vascular cell growth.

Chapados and colleagues reported that Y-27632 prevented stress fiber formation and reduced nuclear extracellular signal-regulated kinase and tenascin-C expression.<sup>98</sup> In animal models of PAH, fasudil or Y-27632 decreases the sustained vasoconstrictor response and vascular remodeling that occurs during exposure to monocrotaline or hypoxia.<sup>78,99</sup> Further, inhibition of ROCK attenuates pulmonary arterial pressure and reverses pulmonary vasoconstriction caused by NO synthase (NOS) antagonists in chronically hypoxic lungs, and ROCK antagonists also lower acute hypoxia-mediated contraction of isolated rat pulmonary artery segments and isolated mouse lungs.<sup>77,78,83</sup> Oka et al also showed that this same compound acutely lowered right ventricular systolic pressure in SU5416/hypoxia-exposed rat lungs.<sup>3</sup> In mouse models of PAH, treatment with Y-27632 decreased the muscularization of distal pulmonary arteries and upregulated eNOS expression, and Abe and colleagues observed that inhibiting chronic hypoxic PAH in mice increased lung eNOS expression and Akt phosphorylation.<sup>78,100</sup>

Finally, evidence suggests that statins may also be effective inhibitors of ROCK. Statins (HMG-CoA reductase inhibitors) block the synthesis of mevalonate and its isoprenoid intermediate compound geranylgeranylpyrophosphate, which subsequently inhibits isoprenylation of RhoA and its translocation to the plasma membrane.<sup>101</sup> Statins ameliorate PAH in a variety of rat models, and a recent report by Girgis et al provides evidence that reversal of hypoxic PAH by simvastatin is coupled to decreased lung expression and activity of both ROCK I and II.<sup>24,102–107</sup> In addition, Li et al observed that atorvastatin blocked serotonergic-mediated pulmonary artery smooth muscle proliferation and migration by inhibiting membrane translocation of RhoA.<sup>108</sup>

## Rho-kinase inhibitors as therapies for pulmonary hypertension

ROCK inhibitors have been used for approximately 15 years to treat cardiovascular disorders including vasospasm after subarachnoid hemorrhage and stable effort angina pectoris with no adverse effects.<sup>61,95</sup> Although relatively few, clinical studies with ROCK inhibitors also show that fasudil is an effective treatment for severe pulmonary hypertension. In PAH patients that did not respond to oxygen inhalation, NO inhalation, or nifedipine, 30-minute intravenous fasudil treatment significantly decreased elevated pulmonary vascular resistance without causing systemic hypotension.<sup>63</sup> Further, Ishikura et al confirmed that fasudil had acute beneficial pulmonary hemodynamic effects in patients with PAH.<sup>109</sup> Collectively, these clinical studies suggest that ROCK

signaling is a viable therapeutic target as a treatment for pulmonary hypertension with ROCK inhibitors (Figure 1). However, although fasudil appears to be a promising agent to use for PAH, long-term effects of fasudil administration in patients with severe pulmonary hypertension need to be evaluated before more definitive conclusions can be made as to the efficacy of this type of agent. Studies also suggest that this drug can be used for other cardiovascular diseases with minimal side effects. Oral treatment with fasudil for at least 4 weeks significantly lengthened maximum exercise time without any effect on blood pressure and heart rate during the exercise independent of any serious unwanted side effects from the drug.<sup>61</sup> In addition, fasudil improved myocardial ischemia in patients with microvascular angina caused by coronary microvascular vasoconstriction.<sup>110</sup>

## Conclusions

One of the biggest obstacles in treating pulmonary hypertension is identifying effective therapeutic agents that selectively target the pulmonary vasculature with minimal side effects. The RhoA/ROCK signaling pathway is an important regulator of pulmonary vascular function and animal studies have shown that this pathway is important in the pathogenesis of major disease states such as pulmonary hypertension. Further studies should be done to determine how ROCKs mediate pulmonary vascular smooth muscle cell physiology and how these moieties are involved in vascular disease states. Recent clinical studies in humans show promise in that acute treatment with ROCK inhibitors in patients with severe pulmonary hypertension exhibited signs of improved pulmonary vascular function by attenuating the ROCK-mediated increase in pulmonary vascular resistance. Towards this end, large clinical trials are the logical next step in demonstrating that long-term treatment with ROCK inhibitors is both safe and efficacious in patients with pulmonary hypertension.

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