

REVIEW

Therapeutic potential of RhoA/Rho kinase inhibitors in pulmonary hypertension

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A burgeoning body of evidence suggests that RhoA/Rho kinase (ROCK) signalling plays an important role in the pathogenesis of various experimental models of pulmonary hypertension (PH), including chronic hypoxia-, monocrotaline-, bleomycin-, shunt- and vascular endothelial growth factor receptor inhibition plus chronic hypoxia-induced PH. ROCK has been incriminated in pathophysiologic events ranging from mediation of sustained abnormal vasoconstriction to promotion of vascular inflammation and remodelling. In addition, the 3-hydroxy-3-methylglutaryl CoA reductase inhibitors, statins, which inhibit activation of RhoA by preventing post-translational isoprenylation of the protein and its translocation to the plasma membrane ameliorate PH in several different rat models, and may also be effective in PH patients. Also, phosphorylation of RhoA and prevention of its translocation to the plasma membrane are involved in the protective effect of the type 5-PDE inhibitor, sildenafil, against hypoxia- and bleomycin-induced PH. Collectively, these and other observations indicate that independent of the cause of PH, activation of the RhoA/ROCK pathway serves as a point of convergence of various signalling cascades in the pathogenesis of the disease. We propose that ROCK inhibitors and other drugs that inhibit this pathway might be useful in the treatment of various forms of PH.

British Journal of Pharmacology (2008) 155, 444–454; doi:10.1038/bjp.2008.239; published online 9 June 2008

Keywords: vasoconstriction; vascular remodelling; fasudil; Y-27632; Rac

Abbreviations: EC, endothelial cell; eNOS, endothelial NOS; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; NO, nitric oxide; PSMCs, pulmonary artery smooth muscle cells; PH, pulmonary hypertension; ROCK, Rho-associated kinase; SMC, smooth muscle cell

Introduction

The complex pathophysiology of pulmonary hypertension (PH) includes vasoconstriction, vascular remodelling and *in situ* thrombosis. The cellular/molecular signalling pathways underlying these components of the pulmonary arteriopathy are not well understood. More importantly, current pharmacological treatment of patients with severe, progressive PH improves symptoms but does not prevent the untimely death due to right heart failure (Macchia *et al.*, 2007), thus emphasizing the urgent need to identify more effective therapies. This overview summarizes current evidence that activation of the small GTPase RhoA and its downstream effector Rho-associated kinase (ROCK) is important in the pathogenesis of PH, and discusses the potential efficacy of RhoA/ROCK inhibitors in the treatment of PH. As most

studies of the roles of RhoA/ROCK signalling in cardiovascular diseases have been in the systemic circulation, we briefly review that work before addressing evidence of its involvement in PH. Although not yet studied in PH as extensively as RhoA/ROCK, we also discuss the possible importance of another Rho family GTPase, Rac1.

RhoA/ROCK signalling

Rho (Ras homologous) GTP-binding proteins, which comprise multiple members of the Rho, Rac and Cdc42 subfamilies, and their downstream effectors interact with each other and with other intracellular signalling pathways to regulate numerous cellular processes. These include gene transcription, differentiation, proliferation, hypertrophy, apoptosis, phagocytosis, adhesion, migration and contraction (Riento and Ridley, 2003; Jaffe and Hall, 2005). The prototypical mechanism of RhoA GTPase signalling is that various environmental cues, acting through G-protein-coupled receptors or receptor-dependent and -independent

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Received 3 March 2008; revised 28 April 2008; accepted 20 May 2008; published online 9 June 2008

TKs, activate guanine nucleotide exchange factors, which induce exchange of GDP for GTP binding and translocation of GTP-RhoA to the plasma membrane. Upon translocation to the plasma membrane, GTP-RhoA activates one or more of its effectors, including the two isoforms of ROCK, ROCK I (ROK β) and ROCK II (ROK α). Negative regulators of RhoA activation include guanine nucleotide dissociation inhibitors, which oppose the exchange of GTP for GDP; GTPase-activating proteins, which catalyse dephosphorylation and inactivation of membrane-bound GTP-RhoA; statins, which inhibit isoprenylation of RhoA and thereby prevent translocation of GTP-RhoA to the cell membrane (Cordle *et al.*, 2005; Rikitake and Liao, 2005) and PKA and PKG, which by phosphorylating RhoA also prevent membrane translocation of the GTP-bound protein (Sawada *et al.*, 2001; Guilluy *et al.*, 2005; Murthy, 2006). Following sustained activation, RhoA signalling can also be inhibited by transamidation, ubiquitylation and proteasomal degradation (Guilluy *et al.*, 2007).

RhoA/ROCK-mediated vasoconstriction

It is now clear that activation of RhoA/ROCK signalling is a major regulator of vascular tone (Figure 1) (Somlyo and Somlyo, 2003; Ratz *et al.*, 2005). Smooth muscle cell (SMC) tension is determined primarily by phosphorylation (contraction) and dephosphorylation (relaxation) of the regulatory myosin light chain (MLC). Phosphorylation is catalysed by Ca²⁺/calmodulin-dependent MLC kinase (MLCK), and dephosphorylation by Ca²⁺-independent MLC phosphatase

(MLCP) that is targeted to myosin by its regulatory myosin-binding subunit. Thus, the balance in activities of MLCK and MLCP regulates contraction. At a given level of cytosolic Ca²⁺, second messenger-mediated pathways can modulate the activity of both enzymes to modify MLC phosphorylation and force, that is, to modify the Ca²⁺ sensitization, but two major pathways in vascular smooth muscle are inhibition of MLCP by ROCK-mediated phosphorylation of regulatory myosin-binding subunit, and PKC-mediated phosphorylation and activation of the MLCP-inhibitor protein CPI-17. ROCK can also phosphorylate CPI-17. Although RhoA/ROCK-mediated sustained vasoconstriction is generally attributable to inhibition of MLCP and Ca²⁺ sensitization, there are instances in which ROCK activity also induces Ca²⁺ signalling (Ghisal *et al.*, 2003; Luykenaar *et al.*, 2004), and Ca²⁺ signals can activate RhoA/ROCK (Sakurada *et al.*, 2003; Ratz *et al.*, 2005; Wang *et al.*, 2006). Thus, while considerable evidence now exists that regulating Ca²⁺ sensitivity is as important as regulating cytosolic [Ca²⁺] in the control of vascular tone, there can be agonist- and artery segment-dependent interplay between the two pathways (Ratz *et al.*, 2005).

Ca²⁺ desensitization is also a mechanism of vasodilation. Besides inducing SMC relaxation by desensitizing receptors and decreasing cytosolic [Ca²⁺] and MLCK activity, the nitric oxide (NO)/soluble guanylate cyclase/cGMP/PKG pathway also decreases Ca²⁺ sensitivity by phosphorylating and inactivating RhoA or by directly phosphorylating MLCP, which increases MLCP activity (Sawada *et al.*, 2001; Murthy, 2006). Similarly, vasodilation by stimuli that activate the adenylate kinase/cAMP/PKA pathway is also attributable partly to inhibition of RhoA/ROCK signalling (Murthy, 2006; Azam *et al.*, 2007).

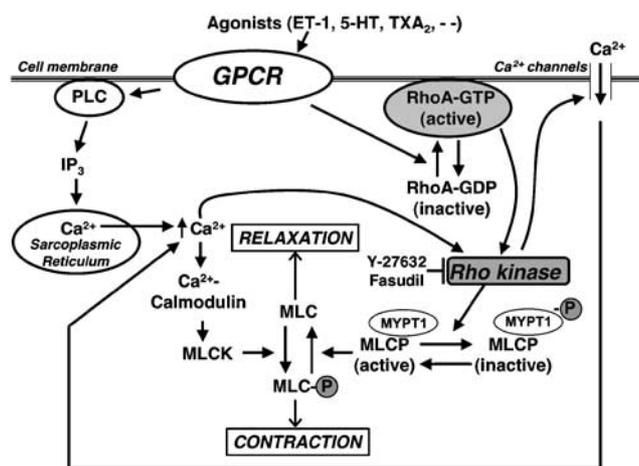


Figure 1 Regulation of smooth muscle cell contraction. Vascular smooth muscle contraction is determined primarily by the balance in activities of Ca²⁺/calmodulin-dependent myosin light chain kinase (MLCK, contraction) and Ca²⁺-independent MLC phosphatase (MLCP, relaxation). G-protein-coupled receptor (GPCR) agonists (ET-1, endothelin-1; 5-HT, serotonin; TXA₂, thromboxane A₂; and so on) not only activate MLCK, but also inactivate MLCP by activation of RhoA/Rho kinase pathway and/or activation of the MLCP-inhibitor protein CPI-17 (not shown) to cause smooth muscle cell contraction. In some instances, Rho kinase activation can also induce Ca²⁺ signalling, and Ca²⁺ signals can activate RhoA/Rho kinase. IP₃, inositol 1,4,5-triphosphate; PLC; MYPT1, regulatory myosin-binding subunit of MLCP (modified from Nagaoka *et al.* (2005) *Am J Respir Crit Care Med* 171: 494–499).

RhoA/ROCK in systemic vascular diseases

In addition to mediating sustained, abnormal vascular SMC contraction, RhoA/ROCK signalling has also been implicated in several other processes that contribute to systemic vascular diseases (Shimokawa and Takeshita, 2005; Budzyn *et al.*, 2006; Loirand *et al.*, 2006; Noma *et al.*, 2006). These include SMC proliferation and migration, increased matrix protein production, endothelial cell (EC) contraction and increased permeability, oxidative stress, adhesion molecule expression and monocyte/macrophage infiltration, platelet activation and thrombosis, and inflammation. Given the regulation of the above cellular pathophysiological events by RhoA/ROCK, it is not surprising that the pathway has also been incriminated in important systemic vasculopathies. For example, several studies show that RhoA/ROCK signalling is involved in the pathogenesis of systemic hypertension, cerebral and coronary vasospasm, and arteriosclerosis and restenosis. Various inhibitors of RhoA/ROCK activity, such as TAT-C3, statins, rapamycin, Rad GTPase, dominant-negative ROCK, and the ROCK inhibitors fasudil (HA-1077) and Y-27632, have been found in animal models to suppress systemic vascular neointimal formation and constrictive remodelling in vein grafts (Yamanouchi *et al.*, 2005;

Furuyama *et al.*, 2006), cardiac allograft vasculopathy (Hattori *et al.*, 2004), balloon injury (Eto *et al.*, 2000; Sawada *et al.*, 2000; Shibata *et al.*, 2003; Fu *et al.*, 2005; Iso *et al.*, 2006) and in-stent restenosis (Matsumoto *et al.*, 2004; Guerin *et al.*, 2005). Collectively, these studies indicate that inhibition of RhoA/ROCK signalling is associated with increased expression of p27^{Kip1} and suppression of neointimal cell proliferation, downregulation of bcl-2 and increased neointimal cell apoptosis, decreased expression of TGF- β 1 and deposition of collagen, decreased expression of proinflammatory cytokines, such as monocyte chemoattractant protein-1, macrophage migration inhibitory factor and interferon- γ , and decreased vascular accumulation of macrophages and T cells. Similarly, ROCK inhibition also suppresses chronic NO synthase inhibitor- and angiotensin II-induced expression of connective tissue growth factor and monocyte chemoattractant protein-1, monocyte/macrophage infiltration, and medial thickening and perivascular fibrosis (Funakoshi *et al.*, 2001; Kataoka *et al.*, 2002; Kanda *et al.*, 2005; Ruperez *et al.*, 2005). Additional support for important links between RhoA/ROCK activation and vascular inflammation includes *in vitro* evidence that ROCK activity mediates, at least partly, adherence and transendothelial migration of monocytes and neutrophils (Ashida *et al.*, 2001; Saito *et al.*, 2002; Pizurki *et al.*, 2003; Honing *et al.*, 2004; Bolick *et al.*, 2005), inflammatory mediator production by vascular SMCs, cardiomyocytes and monocytes (Ito *et al.*, 2002; Segain *et al.*, 2003; Horton *et al.*, 2005), and T-cell activation (Bardi *et al.*, 2003; Tharaux *et al.*, 2003; Aihara *et al.*, 2004). Thus, RhoA/ROCK signalling is a major player in the pathogenesis of systemic arterial inflammation and neointimal formation, and it seems likely this signalling pathway also contributes significantly to the arteriopathy of PH.

RhoA/ROCK in acute pulmonary vasoconstriction

As of 23 February 2008, the search terms 'rho kinase and pulmonary hypertension' in Entrez PubMed captured 64 citations, 21 of them were reviews. The list did not include several additional reports on the function of ROCKs in acute pulmonary vasoconstriction. That the majority of these studies have been published in the past 2 years reflects the growing interest in the likelihood that RhoA/ROCK signalling plays important roles in mediating both vasoconstriction and vascular remodelling in PH, and that pharmacological inhibition of this signalling pathway might be efficacious in the treatment of the disease (Shimokawa and Takeshita, 2005; Fukumoto *et al.*, 2007).

An early report of RhoA/ROCK signalling in regulation of pulmonary vascular tone was by Robertson *et al.* (2000) who observed in perfused rat lungs and isolated pulmonary arteries that ROCK-mediated Ca²⁺ sensitization was necessary for the sustained phase of acute hypoxic pulmonary vasoconstriction. They subsequently reported that an unidentified endothelium-derived factor was responsible for the hypoxic activation of RhoA/ROCK (Robertson *et al.*, 2003). Similarly, Wang *et al.* (2001, 2003) observed activation of RhoA/ROCK in hypoxic pulmonary vasoconstriction,

except in this case hypoxia directly activated RhoA in cultured pulmonary artery SMCs (PASMCs). Several studies have demonstrated the participation of ROCK in acute pulmonary vasoconstriction to various stimuli, including KCl (Robertson *et al.*, 2000; Nagaoka *et al.*, 2004), endothelin-1 (Weigand *et al.*, 2006; Barman, 2007), thromboxane A₂ (Janssen *et al.*, 2001; Martin *et al.*, 2004), prostaglandin F_{2 α} (Robertson *et al.*, 2000), serotonin (Witzenrath *et al.*, 2006), isoprostanes (Janssen *et al.*, 2001), norepinephrine/phenylephrine (Janssen *et al.*, 2001; Boer *et al.*, 2002; Damron *et al.*, 2002), platelet-activating factor (Martin *et al.*, 2004), 20-hydroxyeicosatetraenoic acid (Yaghi and Sims, 2005), epoxyeicosatrienoic acids (Losapio *et al.*, 2005), sphingosylphosphorylcholine (Thomas *et al.*, 2005) and sphingosine-1-phosphate (Beutz *et al.*, 2005). Whether the ROCK-dependent component of the vasoconstriction occurs parallel to or in series with increases in SMC cytosolic [Ca²⁺] seems to vary with agonist and pulmonary artery preparation.

RhoA/ROCK in rodent models of PH

Studies in chronically hypoxic, monocrotaline-injected and bleomycin-injected rats indicate that sustained ROCK-mediated vasoconstriction contributes substantially to the increased pulmonary vascular resistance in all three models based on the acute *in vivo* effects of selective ROCK inhibitors. Although Y-27632 and fasudil (or HA-1077), the two most frequently used ROCK inhibitors, are relatively selective for ROCK up to 10 μ M (IC₅₀ values of Y-27632 and fasudil for ROCK II are 0.162 and 0.158 μ M, respectively), their higher concentrations also inhibit other kinases, such as PKC (IC₅₀ values are 25.8 and 12.3 μ M, respectively) and G (IC₅₀ values are 3.27 and 1.65 μ M, respectively) (Tamura *et al.*, 2005). Nagaoka *et al.* (2004) found that acute administration of Y-27632 and fasudil, but not normoxic ventilation or the Ca²⁺ channel blocker nifedipine, to chronically hypoxic rats and perfused hypertensive lungs elicited considerable vasodilation and nearly normalized the high vascular resistance. Hyvelin *et al.* (2005) and McNamara *et al.* (2008) subsequently confirmed that sustained ROCK-mediated vasoconstriction played a central role in the PH of chronically hypoxic adult and neonatal rats, and similar observations have been made in rats with monocrotaline- (Nagaoka *et al.*, 2005; Jiang *et al.*, 2007) and bleomycin-induced PH (McNamara *et al.*, 2008). These findings suggest that the PH in these models is due largely to sustained ROCK-mediated vasoconstriction, rather than simply to the pulmonary artery medial and adventitial thickening and arteriolar muscularization that accompanies the hypertension.

The above pharmacological observations agree with histological evidence in the vasodilated hypertensive lungs of hypoxic and monocrotaline-injected rats that the arterial wall thickening itself causes little inward remodelling (van Suylen *et al.*, 1998; Howell *et al.*, 2004). In this regard, Crossno *et al.* (2007) recently found that treatment of hypoxic rats with the PPAR γ agonist rosiglitazone inhibited pulmonary vascular remodelling but not the development of PH. The hypertension was immediately reversed by acute i.v. fasudil. Interestingly, acute fasudil has also been observed to

elicit substantial pulmonary vasodilation, albeit not to near-normal levels, in VEGF receptor blocker (Sugen 5416)-injected/chronic hypoxia-exposed rats (Oka *et al.*, 2007a) and left-pneumonectomized plus monocrotaline-injected rats (M Oka and IF McMurtry, unpublished). In addition to increased pulmonary arterial muscularization, these latter two models also develop obstructive neointimal lesions in distal pulmonary arteries (Taraseviciene-Stewart *et al.*, 2001; Nishimura *et al.*, 2002). It is presumably these fixed neointimal lesions that prevent near-normalization of the high vascular resistance by acute inhibition of ROCK. Whether or not chronic treatment with ROCK inhibitors will reverse the neointimal lesions is unknown (see below).

Additional evidence of involvement of RhoA/ROCK signalling in rat models of PH include reports that acute NO-induced vasodilation of hypoxic hypertensive pulmonary arteries is due to inhibition of RhoA/ROCK-mediated Ca^{2+} sensitization rather than to decreased cytosolic $[Ca^{2+}]$ (Jernigan *et al.*, 2004), and acute ET-1-induced constriction is due largely to ROCK-mediated Ca^{2+} sensitization rather than to increased cytosolic $[Ca^{2+}]$ (Weigand *et al.*, 2006). Furthermore, it has recently been found that chronic hypoxia-induced PH in rats is associated with induction of ROCK-dependent myogenic tone in small pulmonary arteries (Broughton *et al.*, 2008). Also, treatment of chronically hypoxic rats with Y-27632 inhibits development of PH (McMurtry *et al.*, 2003; Hyvelin *et al.*, 2005), and treatment with the type PDE5 inhibitor sildenafil, which promotes phosphorylation of RhoA and thereby prevents translocation of GTP-RhoA to the PSMC plasma membrane, mimics the inhibition of hypoxia-induced PH by fasudil (Guilluy *et al.*, 2005), and also attenuates bleomycin-induced activation of RhoA/ROCK and development of PH in mice (Hemnes *et al.*, 2008). Acute administration of fasudil to Denver-raised fawn-hooded rats, which have lung dysplasia and severe PH, elicits marked pulmonary vasodilation and chronic treatment from birth to 10 weeks of age ameliorates the lung dysplasia and PH (Nagaoka *et al.*, 2005, 2006). Similarly, treatment with fasudil inhibits and also reverses monocrotaline-induced PH (Abe *et al.*, 2004). The latter inhibitory effects of fasudil were associated with improvement of endothelium-dependent relaxation and inhibition of vascular hypercontraction to serotonin, and with suppression of pulmonary artery macrophage infiltration, inhibition of vascular SMC proliferation and enhanced SMC apoptosis. In addition, Chapados *et al.* (2006) found in monocrotaline-injected rats that fasudil-induced inhibition of pulmonary artery medial thickening was associated with reduced expression of the matrix protein, tenascin-C. Finally, treatment with fasudil has been reported to inhibit activation of ROCK and development of shunt-induced PH in rats (Li *et al.*, 2007a). The inhibition of pulmonary vascular remodelling by fasudil was associated with decreased proliferation and increased apoptosis of PSMCs. It is noteworthy that a recent report suggests transglutaminase-dependent activation of RhoA by serotonin (Walther *et al.*, 2003) may be involved in chronic hypoxia-induced pulmonary artery remodelling and hypertension (Guilluy *et al.*, 2007).

Studies in chronically hypoxic mice also implicate involvement of RhoA/ROCK signalling in the pathogenesis of

PH. Fagan *et al.* (2004) observed that inhibition of PH by treatment of hypoxic mice with Y-27632 was associated with decreased muscularization of distal pulmonary arteries and an upregulation of lung tissue endothelial NOS (eNOS) expression. Similarly, Abe *et al.* (2006) reported that inhibition of hypoxic PH in wild-type mice by fasudil was accompanied by increased lung eNOS expression and Akt phosphorylation, and that the ROCK inhibitor was less effective in blunting the hypertension in eNOS-deficient mice.

The HMG-CoA reductase inhibitors, statins, can interfere with RhoA/ROCK signalling by blocking synthesis of mevalonate and its isoprenoid intermediate geranylgeranylpyrophosphate, which in turn prevents isoprenylation of RhoA and its translocation to the plasma membrane (Noma *et al.*, 2006). Although not a universal finding (McMurtry *et al.*, 2007), numerous studies report that treatment with statins attenuates, and in some cases reverses, PH in various rat models (Nishimura *et al.*, 2002, 2003; Girgis *et al.*, 2003, 2007; Lee *et al.*, 2005; Murata *et al.*, 2005; Guerard *et al.*, 2006; Rakotoniaina *et al.*, 2006; Taraseviciene-Stewart *et al.*, 2006; Laudi *et al.*, 2007). A recent study by Girgis *et al.* (2007) provides evidence that both attenuation and reversal of hypoxic PH by simvastatin are associated with decreases in lung tissue expression and activity of both ROCK I and II. Interestingly, dehydroepiandrosterone, another agent reported to block mevalonate synthesis and protein isoprenylation (Schulz and Nyce, 1991; Pascale *et al.*, 1995), is a highly effective inhibitor of hypoxic PH in rats (Bonnet *et al.*, 2003; Hampl *et al.*, 2003; Oka *et al.*, 2007b). A preliminary report indicates that dehydroepiandrosterone also inhibits formation of distal pulmonary artery neointimal lesions and development of PH in left-pneumonectomized/monocrotaline-injected rats (Homma *et al.*, 2008). The inhibition of PH was associated with decreases in lung tissue RhoA, ROCK, and caspase-3 activities and decreased expression of cleaved ROCK I. The latter is noteworthy because cleaved ROCKs have lost their auto-inhibitory domain and are constitutively active, that is, are active independently of the status of RhoA (Sebbagh *et al.*, 2001, 2005; Chang *et al.*, 2006; Sapet *et al.*, 2006).

RhoA/ROCK in human PH

Studies of RhoA/ROCK signalling in human PH are limited. A preliminary report by Hemnes *et al.* (2005) suggests that the blunted vasodilator responsiveness of hypertensive pulmonary arteries and perfused lung lobes isolated from patients undergoing lung transplantation for severe PH is associated with high RhoA/ROCK activity. The RhoA/ROCK activation was reduced by exposure of the isolated hypertensive pulmonary arteries to sildenafil. Also, low i.v. doses of fasudil acutely cause modest decreases in pulmonary vascular resistance in patients with PH (Fukumoto *et al.*, 2005; Ishikura *et al.*, 2006). Chronic simvastatin treatment has improved symptoms in patients with PH (Kao, 2005), but it is unknown if the effects are related to inhibition of RhoA/ROCK signalling (Xing *et al.*, 2007).

Mechanisms of suppression of PH by ROCK inhibitors

Collectively, the *in vivo* studies reviewed above (summarized in the Table 1) suggest that the activation of RhoA/ROCK signalling is significantly involved in the pathogenesis of PH in several different rodent models, and probably also in humans, and that this involvement ranges from mediation of sustained abnormal vasoconstriction to promotion of vascular inflammation and remodelling (illustrated in Figure 2). Some of these *in vivo* studies have found that suppression of PH by ROCK inhibitors is associated with decreased pulmonary artery expression of growth factors and of markers of cell proliferation, matrix protein production, and inflammatory cell infiltration, and with increased markers of apoptosis. However, it is difficult in these studies to interpret whether the antivasular remodelling effects of ROCK inhibitors are due to direct inhibition of molecular signalling pathways or to indirect consequences of vasodilation

and lower pulmonary arterial pressure. There are a few *in vitro* studies supporting the possible direct involvement of RhoA/ROCK activation in mediating pulmonary vascular cell growth. For example, Liu *et al.* (2004) report that serotonin-induced proliferation of bovine PASMCs depends on multiple serotonin receptors and the serotonin transporter and involves several intracellular signalling pathways, including activation of RhoA and ROCK, and ROCK-mediated translocation of phosphorylated extracellular signal-regulated kinase to the nucleus (Liu and Fanburg, 2006). Similarly, inhibition of serotonin-induced PASMC proliferation and migration by atorvastatin has been attributed to prevention of RhoA membrane translocation and lack of activation of ROCK (Li *et al.*, 2007b).

Chapados *et al.* (2006) have observed that Y-27632-induced inhibition of ROCK not only prevents stress fiber formation and the spreading of vascular SMC cultured on denatured collagen but also reduces nuclear extracellular signal-regulated kinase and tenascin-C expression.

Table 1 Summary of *in vivo* studies of RhoA/ROCK signaling and of acute and chronic effects of ROCK inhibitors in animal models and patients with PH

PH models	RhoA/ROCK signaling	Treatments	Pulmonary effects	References
<i>Chronic hypoxia</i>				
Rat	?	Acute Y-27632	Vasodilation	(Nagaoka <i>et al.</i> , 2004)
Rat	?	Acute fasudil	Vasodilation	(Nagaoka <i>et al.</i> , 2005)
Rat	?	Acute fasudil	Vasodilation	(Crossno <i>et al.</i> , 2007)
Rat	?	Chronic Y-27632	↓ Hypertension and RVH	(McMurtry <i>et al.</i> , 2003)
Rat	↑ ROCK I and II expression	Acute and chronic Y-27632	Vasodilation, ↓ hypertension, wall thickness and RVH	(Hyvelin <i>et al.</i> , 2005)
Rat	↑ RhoA and ROCK activity	Chronic sildenafil and fasudil	↓ Hypertension, wall thickness and RVH	(Guilluy <i>et al.</i> , 2005)
Rat	↑ ROCK activity	Chronic simvastatin	↓ Hypertension, wall thickness and RVH	(Girgis <i>et al.</i> , 2007)
Neonatal rat	↑ ROCK expression and activity	Acute Y-27632 and fasudil	Vasodilation	(McNamara <i>et al.</i> , 2008)
FHR	?	Acute fasudil	Vasodilation	(Nagaoka <i>et al.</i> , 2005)
Neonatal FHR	↑ RhoA activity and ROCK I expression	Chronic fasudil	↓ Hypertension, wall thickness and RVH	(Nagaoka <i>et al.</i> , 2006)
Mouse	↑ ROCK activity	Chronic Y-27632	↓ Hypertension, neomuscularization and RVH ↑ eNOS	(Fagan <i>et al.</i> , 2004)
Mouse	?	Chronic fasudil	↓ Hypertension and RVH ↑ eNOS	(Abe <i>et al.</i> , 2006)
<i>Monocrotaline</i>				
Rat	?	Acute fasudil	Vasodilation	(Nagaoka <i>et al.</i> , 2005)
Rat	↑ ROCK activity	Acute fasudil	Vasodilation	(Jiang <i>et al.</i> , 2007)
Rat	↑ ROCK activity	Chronic fasudil	↓ Hypertension, wall thickness and RVH	(Abe <i>et al.</i> , 2004)
Rat	?	Chronic fasudil	↓ Wall thickness and TN-C expression	(Chapados <i>et al.</i> , 2006)
Rat	?	Chronic fasudil + beraprost	↓ Hypertension, wall thickness and RVH	(Tawara <i>et al.</i> , 2007)
<i>SU5416/hypoxia</i>				
Rat	↑ ROCK activity	Acute fasudil	Vasodilation	(Oka <i>et al.</i> , 2007a)
<i>Shunt</i>				
Rat	↑ RhoA and ROCK activity	Chronic fasudil	↓ Hypertension, wall thickness and RVH	(Li <i>et al.</i> , 2007a)
<i>Bleomycin</i>				
Neonatal rat	↑ ROCK expression and activity	Acute Y-27632 and fasudil	Vasodilation	(McNamara <i>et al.</i> , 2008)
Mouse	↑ RhoA and ROCK activity	Chronic sildenafil	↓ Hypertension, wall thickness and RVH	(Hemnes <i>et al.</i> , 2008)
<i>Human PH</i>				
PH patients	?	Acute fasudil	Vasodilation	(Fukumoto <i>et al.</i> , 2005)
PH patients	?	Acute fasudil	Vasodilation	(Ishikura <i>et al.</i> , 2006)

Abbreviations: eNOS, endothelial NOS; FHR, foetal heart rate; PH, pulmonary hypertension; ROCK, Rho-associated kinase; RVH, right ventricular hypertrophy.

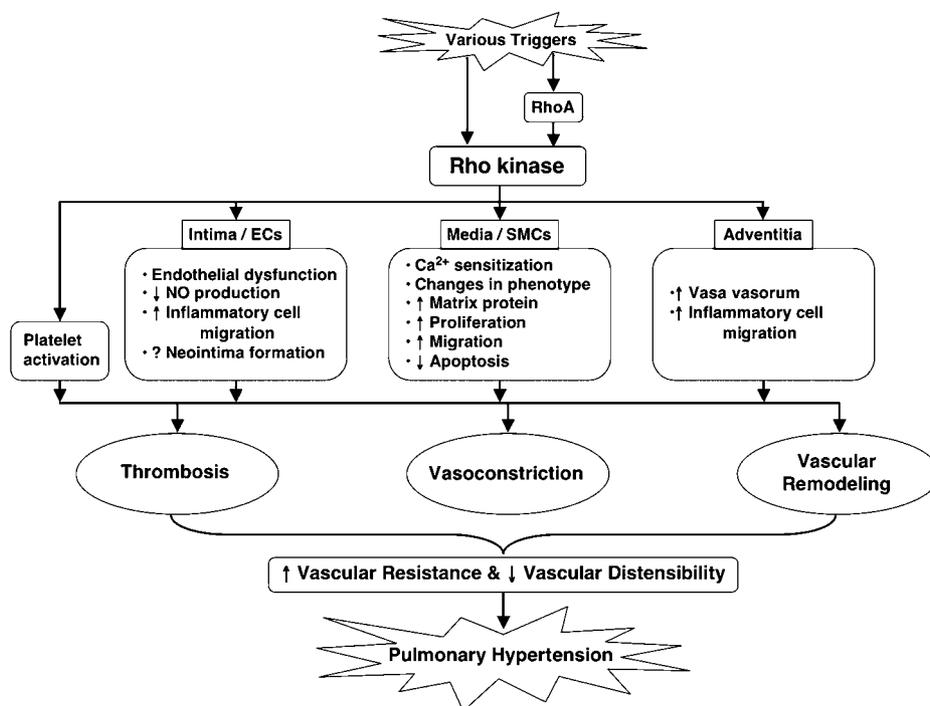


Figure 2 Schematic illustration of possible pathways and effects of RhoA/Rho kinase signalling in the pathogenesis of pulmonary hypertension (PH) (modified from Fukumoto *et al.* (2007) *Tohoku J Exp Med* **211**: 309–320).

Importantly, they propose a scheme whereby signals arising from changes in medial collagen biochemistry can activate SMC RhoA/ROCK signalling, presumably through integrins, to mediate both sustained vasoconstriction and vascular remodelling. RhoA activation of downstream effectors such as ROCK, PKN and diaphanous-related formins 1 and 2 is important in regulating myocardin/serum response factor-dependent transcription of contractile proteins and thereby maintaining SMC contractile phenotype (Deaton *et al.*, 2005; Hinson *et al.*, 2007; Staus *et al.*, 2007), and what remains to be sorted out is exactly how activation of RhoA/ROCK signalling can mediate cell contraction on the one hand and proliferation, migration and matrix protein synthesis on the other. Perhaps RhoA/ROCK signalling is differentially regulated among subsets of SMCs within a given segment of pulmonary artery (Bailey *et al.*, 2004). RhoA/ROCK signalling is apparently activated in at least some SMCs of the hypertensive pulmonary arteries of various rodent models, but we do not yet know the status of this signalling cascade in the ECs and adventitial fibroblasts or in the cells of pulmonary artery neointimal lesions.

Negative results with ROCK inhibitors in rodent models of PH

In contrast to the considerable evidence for important involvement of RhoA/ROCK signalling in the pathophysiology of PH, there have been some negative results. For example, we observed that while administration of the ROCK inhibitor Y-27632 by subcutaneous osmotic minipump to chronically hypoxic mice ($30 \text{ mg kg}^{-1} \text{ day}^{-1}$) and rats ($40 \text{ mg kg}^{-1} \text{ day}^{-1}$) attenuated the PH (McMurtry *et al.*,

2003; Fagan *et al.*, 2004), treatment with fasudil in the drinking water (30 and $40 \text{ mg kg}^{-1} \text{ day}^{-1}$, respectively) did not (unpublished). On the other hand, fasudil administered to mice in the drinking water at a higher dose ($100 \text{ mg kg}^{-1} \text{ day}^{-1}$) (Abe *et al.*, 2006), and to rats by gavage ($30 \text{ mg kg}^{-1} \text{ day}^{-1}$) (Guilluy *et al.*, 2005), did inhibit development of hypoxic PH. Also, Shimokawa's group originally observed marked inhibition of monocrotaline-induced PH in rats by $30 \text{ mg kg}^{-1} \text{ day}^{-1}$ fasudil in the drinking water (Abe *et al.*, 2004), but more recently found only modest effects of this dose when administered by i.p. minipump (Tawara *et al.*, 2007). Finally, we observed in SU5416-injected/hypoxia-exposed rats that fasudil in the drinking water at $30 \text{ mg kg}^{-1} \text{ day}^{-1}$ attenuated the initial lung inflammation and development of PH, but administration of this dose twice daily by gavage for 3 weeks did not reverse the pathology once it had become established (unpublished). Although the gavage administration of fasudil acutely caused pulmonary vasodilation in these rats, the twice-daily dosing protocol apparently did not lead to persistent effects. It remains to be determined if higher or more frequent dosing will be effective.

Thus, not surprisingly, the effectiveness of ROCK inhibitors, and especially of fasudil, against PH seems to depend on dose, route of administration and animal model. We speculate that if given at sufficiently high doses, ROCK inhibitors will unmask some degree of vasoconstriction in most forms of PH, including severe PH in humans. However, ROCK activity contributes to basal systemic vascular tone (Bussemaker *et al.*, 2007), and such high doses would likely have to be given through inhalation to avoid systemic hypotension (Nagaoka *et al.*, 2005). Fasudil has been reported to have beneficial effects in patients with systemic

hypertension, stroke, vasospastic angina, stable effort angina and chronic heart failure (Liao *et al.*, 2007; Shimokawa and Rashid, 2007), but whether long-term oral treatment with the drug at doses that do not cause systemic hypotension will reverse severe, established PH remains to be determined. We will hopefully know this soon, as clinical trials with oral fasudil in patients with PH are currently being planned in Japan (Fukumoto *et al.*, 2007). As is seemingly the case with the currently approved PH therapeutic agents, it may be more efficacious to use fasudil or some other ROCK inhibitor, in combination with another type of drug (Tawara *et al.*, 2007). Fasudil is currently the only ROCK inhibitor approved for human use, but several biotechnology and pharmaceutical companies are investigating numerous other ROCK isoform-selective and -nonselective compounds (Liao *et al.*, 2007; Shimokawa and Rashid, 2007).

Rac in pathogenesis of PH

Rac is another ubiquitously expressed small GTPase that contributes to regulation of a wide variety of cellular functions. With respect to vascular diseases, Rac activation has most often been linked to increased oxidative stress by its role in assembly and activation of the NADPH oxidase complex and resultant increase in production of superoxide anion (Hordijk, 2006). Mice engineered to overexpress a constitutively active mutant of Rac1 in SMCs develop moderate systemic hypertension associated with increased vascular levels of superoxide and peroxynitrite (Hassanain *et al.*, 2007). Black and his colleagues found in lambs with a surgically created left to right shunt that PH was associated with increased superoxide-dependent oxidative stress in hypertensive pulmonary arteries, and the increased superoxide production was attributed to combined effects of activation of NADPH oxidase and an uncoupling of eNOS (Grobe *et al.*, 2006). The same group also previously reported that serum-induced proliferation of lamb PSMCs is dependent on activation of Rac1 and NADPH-mediated ROS production (Patil *et al.*, 2004). This is similar to the observations of Fanburg's laboratory that serotonin-induced growth of bovine PSMCs involves Rac1/NADPH oxidase/ROS-dependent activation of extracellular signal-regulated kinase and stimulation of c-fos and cyclin D1 expression (Lee *et al.*, 2001; Simon *et al.*, 2005). Carlin *et al.* (2007) recently observed that hypoxia-induced activation of p38 mitogen-activated protein kinase and proliferation of rat pulmonary artery adventitial fibroblasts were inhibited by statins and the Rac1 inhibitor NSC23766, but not by the ROCK-inhibitor hydroxyfasudil. In contrast, ECs cultured from chronically hypoxic piglets exhibited decreased activities of Rac1 and its downstream effector p21 activated kinase and increased activity of RhoA (Wojciak-Stothard *et al.*, 2006).

Conclusion

Although numerous studies collectively suggest that RhoA/ROCK signalling is involved significantly in the pathogenesis of experimental as well as human PH, and that ROCK

inhibitors show promise in the treatment of PH, several questions regarding the roles and mechanisms of RhoA/ROCK signalling in the development and progression of the disease remain unanswered. For example, it remains unknown what upstream signals are responsible for the activation of RhoA/ROCK signalling. The relative roles of various GPCRs, reactive oxygen species, extracellular matrix proteins and integrins, RhoA-independent signals, cleaved and constitutively active ROCKs, and increased transmural pressure *per se* in different segments of the pulmonary arterial tree in different forms of PH are unclear. The relative roles of ROCK I and II are also uncertain. Although ROCKs are undoubtedly involved, do any of the other downstream effectors or RhoA, including diaphanous-related formins 1 and 2, PKN and citron kinase, play important roles in the pathogenesis of PH? ROCK-mediated constriction of pulmonary resistance arteries clearly contributes to increased pulmonary vascular resistance in several experimental models of PH, but whether this acts parallel to (Ca^{2+} sensitization) or in series with Ca^{2+} signalling has not been established. Is the high vascular tone due only to increased ROCK-dependent MLC phosphorylation or is increased actin polymerization through RhoA-, ROCK- or c-Abl-dependent pathways (Anfinogenova *et al.*, 2007; Zhang and Gunst, 2008) also involved? Similarly, it would be interesting to know if SMC contraction, by either MLC phosphorylation or actin polymerization, contributes to the decreased distensibility of hypertensive conduit pulmonary arteries. The hallmark of severe progressive PH in humans is the formation of obstructive neointimal lesions in small pulmonary arteries and a poor pulmonary vascular response to acute vasodilator testing. Thus, it will be informative to test if ROCK-mediated vasoconstriction contributes to the increased pulmonary vascular resistance in patients with severe PH, even in those unresponsive to conventional vasodilators such as inhaled NO, inhaled iloprost and i.v. epoprostenol, and if chronic administration of ROCK inhibitors effectively reverses the obstructive neointimal lesions. In this regard, there is a need to define the differential effects on pulmonary vascular function and structure of activation RhoA/ROCK (and Rac1) in SMCs, ECs, fibroblasts and perivascular inflammatory cells. Finally, several recent studies implicate ROCK activity in the left ventricular fibrosis and contractile dysfunction (Balakumar and Singh, 2006; Brown *et al.*, 2006; Chang *et al.*, 2006; Peters and Michel, 2007; Shi *et al.*, 2008), and it will be important to define the roles of Rac1, RhoA and ROCKs in the right ventricular failure of severe PH.

Acknowledgements

The writing of this review was supported by grants from the National Institute of Health (HL 14985 and HL 07171) and the American Heart Association (SDG 0335208N and BGIA 0765477Z), and by the University of South Alabama salary support for IF McMurtry. We thank Asahi Kasei Corporation (Tokyo, Japan) for providing fasudil and MN Gillespie for helpful editorial comments.

Conflict of interest

The authors state no conflict of interest.

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