

Novel Therapeutic Targets of Pulmonary Hypertension

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Pulmonary hypertension (PH) is a fatal disease caused by small pulmonary artery obstruction by vascular proliferation and remodeling.¹ PH is characterized by elevated pulmonary arterial pressure and increased pulmonary vascular resistance, frequently leading to right heart failure and death. PH is defined as a mean pulmonary arterial pressure ≥ 25 mmHg at rest with right heart catheterization.²

The classification of PH includes 5 major categories of the disorder.² Group 1, which is called as pulmonary arterial hypertension (PAH), is a clinical condition defined as mean pulmonary arterial pressure ≥ 25 mmHg and pulmonary capillary wedge pressure ≤ 15 mmHg, which is characterized by the presence of precapillary PH in the absence of other causes of precapillary PH, such as PH caused by lung diseases, chronic thromboembolic PH (CTEPH), or other rare diseases. Pulmonary veno-occlusive diseases and pulmonary capillary hemangiomatosis (Group 1) are caused by occlusion of pulmonary vein and should be a distinct category. These diseases are not completely separated from PAH because they share similar characteristics with PAH. Group 2 PH is caused by left heart disease, which is characterized by the passive backward transmission of the pressure elevation (postcapillary PH). Group 3 PH is caused by lung diseases and hypoxia, which is caused by hypoxic vasoconstriction as a result of lung diseases. Group 4 PH is called as CTEPH, which is caused by chronic and mechanical obstruction of central and distal pulmonary arteries by organized thrombi. Group 5 PH is caused by rare diseases, such as sarcoidosis and aortitis.

Although the treatment of PH has been developed, including prostaglandin I_2 ,³ endothelin receptor antagonists,⁴ phosphodiesterase V inhibitor,⁵ and soluble guanylate cyclase,⁶ PH still remains a fatal disease. Thus, the novel therapeutic targets for PH remain to be elucidated. Indeed, significant progresses have been made in this research field. We briefly review the recent progress on the novel therapeutic targets of PH.

New Therapeutic Targets in Vascular Smooth Muscle Cells in PAH

PAH is characterized as remodeling of small pulmonary arteries, including proliferation of vascular smooth muscle cells (VSMCs) and endothelial cells (ECs), and microthrombus in small pulmonary arteries. Thus, the research for therapeutic

targets of PAH has been performed with VSMCs and ECs. First, we review the new therapeutic targets in VSMCs. Recently, some therapeutic targets of VSMCs in PAH have been identified, including Rho-kinase pathway, cyclophilin A (CyPA) pathway, NADPH (nicotinamide adenine dinucleotide phosphate) oxidase family of oxidases pathways (Nox-1 and Nox-4),^{7,8} platelet-derived growth factor–dependent signaling pathway,⁹ lysyl oxidases,¹⁰ and carbon monoxide–releasing molecules.¹¹ Among these new therapeutic targets, we review Rho-kinase pathways and CyPA pathways.

RhoA/Rho-Kinase Pathway

Rho-kinase (ROCKs) is an effector protein 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 ROCKs in PH. RhoA is activated by the guanine nucleotide exchange factors¹² that catalyze exchange of GDP for GTP and is inactivated by the GTPase-activating proteins.¹³ Under physiological conditions, there is a balance of the activate conformation and inactivate conformation in RhoA, and RhoA regulates the function of ROCKs.

There are 2 isoforms of ROCK, ROCK α /ROCK2 and ROCK β /ROCK1, which have been shown to play important roles in the regulation of vasoconstriction.^{14,15} Agonists bind to G-protein–coupled receptors and induce contraction by increasing both cytosolic Ca^{2+} concentration and Rho-kinase activity through guanine nucleotide exchange factors (Figure 1). Phosphorylation of myosin light chain (MLC) is crucial for VSMC contraction.¹⁶ MLC is phosphorylated by Ca^{2+} /calmodulin-activated MLC kinase and is dephosphorylated by MLC phosphatase. The substrates of ROCKs include MLC, myosin phosphatase target subunit-1, ezrin/radixin/ moesin family, adducin, phosphatase and tensin homolog, and LIM-kinases (Figure 1).

ROCKs are involved in the pathogenesis of PH because it is associated with hypoxic exposure, endothelial dysfunction, VSMC proliferation, enhanced reactive oxygen species (ROS) production, inflammatory cell migration, and platelets activation.^{17–19} Pulmonary vascular remodeling is induced with chronic exposure to hypoxia in mice. We demonstrated that pulmonary vascular dysfunction plays a crucial role in the development of hypoxia-induced PH, for which ROCKs play crucial roles.^{20–24}

We recently demonstrated the crucial role of ROCK2 in the development of hypoxia-induced PH in mice.²⁴ We also observed that ROCKs are activated in neutrophils and pulmonary VSMCs of patients with PAH.²³ Moreover, both intravenous infusion and oral administration of fasudil, an inhibitor of ROCKs, significantly reduced pulmonary vascular resistance in patients with PAH.^{25,26} In the lung tissues from wild-type mice, ROCK2 expression and ROCK

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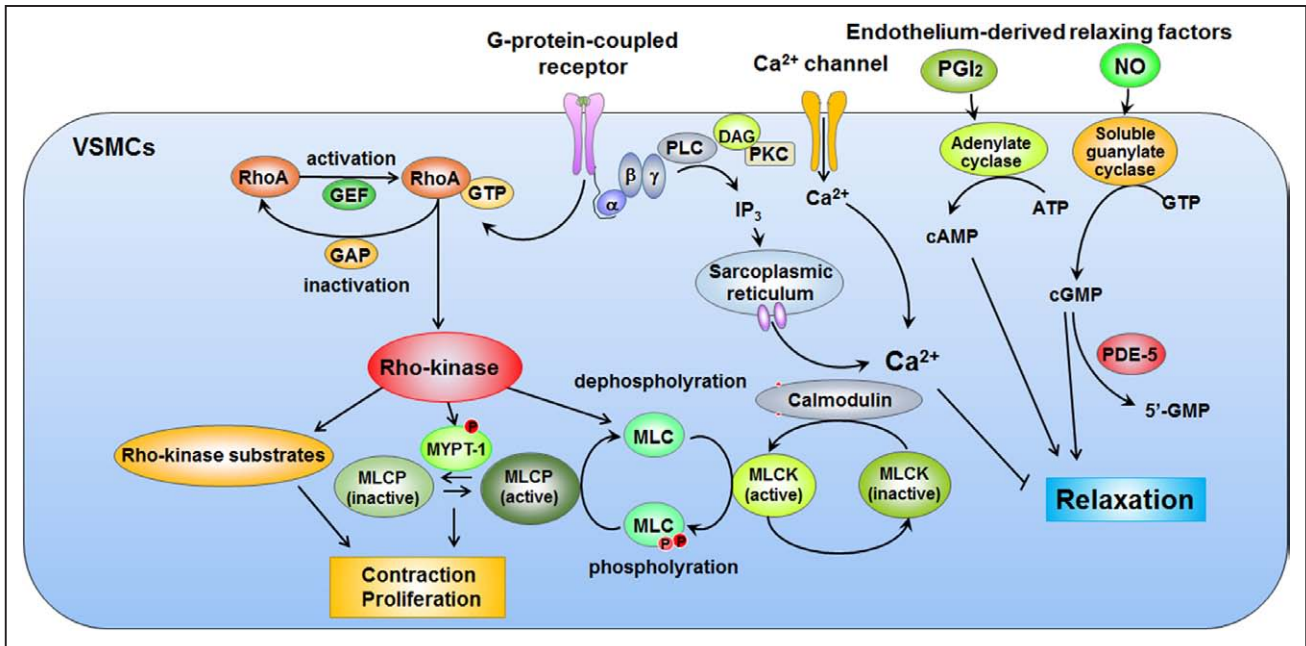


Figure 1. Signaling from endothelial cells (ECs) to vascular smooth muscle cells (VSMCs) and the function of Rho-kinase. Rho-kinase is a downstream effector of the active form of RhoA. Phosphorylation of myosin light chain (MLC) is a key event in the regulation of VSMCs contraction. MLC is phosphorylated by Ca²⁺/calmodulin-activated MLC kinase (MLCK) and dephosphorylated by MLC phosphatase (MLCP). Rho-kinase mediates agonist-induced VSMC contraction. DAG indicates diacylglycerol; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; IP₃, 1,4,5-triphosphate; MYPT, myosin phosphatase target subunit; NO, nitric oxide; PDE, phosphodiesterase; PGI₂, prostacyclin; PKC, protein kinase C; and PLC, phospholipase C.

activity were significantly increased in response to chronic hypoxia, and the development of PH and RV hypertrophy was suppressed in VSMC-specific ROCK2-deficient mice.²⁴ Furthermore, systemic and pulmonary arterial pressure was improved with intravenous injection of other Rho-kinase inhibitors in rats.^{27,28} Indeed, we obtained direct evidence for Rho-kinase activation with immunostaining of lung or evaluating the activity of ROCKs in neutrophil from patients with PAH.²³ Furthermore, we demonstrated that the combination therapy using fasudil and sildenafil showed additional effects through inhibition of ROCKs activity in monocrotaline-induced PH rats.²¹ These results indicate that RhoA–Rho-kinase signaling pathways are the novel therapeutic targets of PAH.

CyPA—Basigin Pathway

CyPA is a ubiquitously distributed protein belonging to the immunophilin family. CyPA is initially recognized as the intracellular receptor for cyclosporine, which is an immunosuppressive drug.²⁹ Although CyPA was thought to function primarily as an intracellular protein, it has been reported to be secreted from VSMCs through Rho-kinase activation (Figure 2).^{30,31} This extracellular CyPA binds to its receptor, basigin (Bsg, CD147), and regulates intracellular signaling pathways.³² Extracellular CyPA is also a chemoattractant for inflammatory cells.^{33,34} Furthermore, extracellular CyPA stimulates VSMCs proliferation and induces ECs adhesion molecule expression and apoptosis³⁵ and activation of platelets via Bsg.³⁶ Moreover, intracellular CyPA plays a

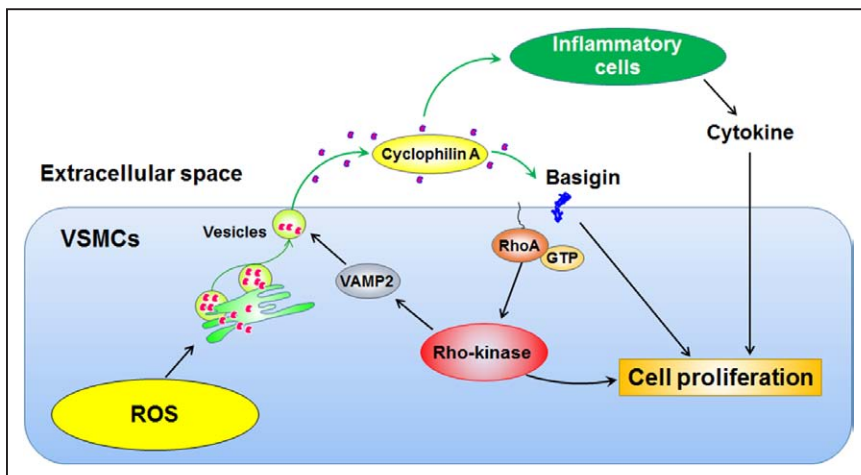


Figure 2. Cyclophilin A and basigin play a crucial role in cell proliferation in vascular smooth muscle cells (VSMCs). Hypoxia induces reactive oxygen species (ROS) in VSMCs, which promotes cyclophilin A (CyPA) secretion. Rho-kinase also promotes the secretion of CyPA. Extracellular CyPA recruits and stimulates inflammatory cells and promotes secretion of inflammatory cytokines. Extracellular CyPA directly stimulates proliferation of VSMCs through basigin, promoting additional secretion of cytokines/chemokines and growth factors. The interaction between extracellular CyPA and basigin in VSMCs may contribute to VSMC proliferation and pulmonary vascular remodeling. VAMP indicates vesicle-associated membrane protein.

critical role in the translocation of NADPH oxidase family of oxidases (Nox), such as Nox-1, Nox-4, and p47phox, leading to VSMCs proliferation and various cardiovascular diseases. The interaction between CyPA and Nox amplifies ROS formation in a synergistic manner, leading to increase oxidative stress as ROS production by Nox via activation of other oxidase systems (Figure 2).^{37,38} Furthermore, secretion of CyPA requires ROS production in VSMCs, RhoA/Rho-kinase activation, and vesicle formation.³⁰ Thus, both intracellular and extracellular CyPA contribute to ROS production with Rho-kinase activation. We recently demonstrated that CyPA and Bsg play crucial roles in PH and pressure overload heart failure.^{32,39}

It has been reported that the secretion of CyPA from VSMCs is reduced with statins and Rho-kinase inhibitors.³¹ Indeed, pravastatin ameliorated hypoxia-induced PH in mice.^{20,40} In addition, extracellular CyPA was significantly higher in the serum from patients with PAH, and the serum concentration of extracellular CyPA was correlated to the prognosis of PH.³² Thus, the inhibition of extracellular CyPA may be a novel therapeutic target of PH.

We further examined whether CyPA contributes to the progression of PH in mice and humans. Importantly, we demonstrated that extracellular CyPA and Bsg were crucial for hypoxia-induced PH in mice.³² In addition, hypoxia-induced PH was ameliorated in Bsg^{+/-} mice compared with Bsg^{+/+}.³² Mechanistic studies demonstrated that Bsg^{+/-} VSMCs secreted less cytokines/chemokines and growth factors compared with Bsg^{+/+} VSMCs.³² On the basis of these findings, we proposed a novel mechanism for hypoxia-induced PH in which hypoxia induces growth-promoting genes in VSMCs through a CyPA/Bsg-dependent pathway.³² These results suggest that extracellular CyPA and vascular Bsg could be potential therapeutic targets of PH (Figure 2).

New Therapeutic Targets in ECs in PAH

Endothelial dysfunction is also considered as a key underlying mechanism in PAH, which is enhanced by inflammatory cytokines/chemokines and growth factors. Indeed, we often experience rapid progression and worsening of PAH during infectious diseases, leading to endothelial dysfunction. Pulmonary endothelial dysfunction in patients with PAH promotes pulmonary vascular remodeling through impaired release of vasodilators, such as nitric oxide and prostacyclin (Figure 1). Thus, the research for therapeutic targets in ECs is also important in PAH. Recently, several novel therapeutic targets, such as endothelial AMP-activated protein kinase (AMPK)⁴¹ and endothelial uncoupling protein 2,⁴² have been reported. Moreover, the cell-based therapy with pulmonary arterial ECs overexpressing interleukin-8 receptor has been reported in monocrotalin-induced PH rats.^{43,44} Then, we review endothelial AMPK as a new therapeutic target in ECs.

Endothelial AMPK

AMPK is a heterotrimeric complex consisting of a catalytic subunit α and 2 regulatory subunits β and γ , being expressed in various tissues and subcellular locations.⁴⁵ AMPK is a

serine/threonine kinase that functions as an important energy sensor⁴⁶ and is activated by inhibition of Rho-kinase.^{47,48} AMPK has an antiapoptotic effect in ECs^{49,50} and a proapoptotic effect in VSMCs⁵¹ because of the upregulation of endothelial NO synthase and vascular cell adhesion molecule-1. Endothelial dysfunction and interaction between ECs and VSMCs in the pulmonary artery play a crucial role for pulmonary vascular remodeling in PAH.

AMPK positively regulates nitric oxide production via endothelial NO synthase in ECs, whereas it reduces intracellular signaling and secretion of many growth factors, promoting VSMC proliferation, and vascular remodeling in VSMCs.⁴⁷ Indeed, several drugs (eg, statins and metformin) and molecules (eg, apelin) activate AMPK, all of which could be potentially protective against PAH.^{20,52} We have recently demonstrated that endothelial-specific AMPK, as well as AMPK in VSMCs, plays a crucial role in PAH (Figure 3).⁴¹

We demonstrated that endothelial AMPK is downregulated in the pulmonary arteries from PAH patients and mice with hypoxia-induced PH.⁴¹ Furthermore, we demonstrated that hypoxia-induced PH is accelerated in endothelial-specific AMPK-knockout mice (eAMPK^{-/-}).⁴¹ Importantly, proliferation of VSMCs from the pulmonary artery was induced with conditioned medium coincubated with ECs, which was enhanced by the treatment with AMPK inhibitor.⁴¹ Consistently, the expression levels of AMPK in ECs were significantly reduced by the treatment with the serum from patients with PAH, which contained inflammatory cytokines, such as IFN γ and TNF α . Importantly, long-term treatment with metformin, which is an AMPK activator, significantly attenuated hypoxia-induced PH in mice.⁴¹ These results suggest that endothelial AMPK could be potential novel therapeutic targets of PH (Figure 3).

New Therapeutic Targets in CTEPH

CTEPH is one of the distinct disease entities of PH, characterized by the obstruction of major pulmonary artery by organized thrombus and pulmonary vascular remodeling.⁵³ The prognosis of CTEPH has been significantly improved with pulmonary endarterectomy, riociguat,⁶ a stimulator of soluble guanylate cyclase, and balloon pulmonary angioplasty.⁵⁴ The pathogenesis of CTEPH has not been fully elucidated, and thus the fundamental therapy remains to be developed. In residual thrombi after fibrinolysis with plasmin, thrombi is dissolved by angiogenesis and leukocyte recruitment with the expression of vascular endothelial growth factor and basic fibroblast growth factor.⁵⁵ Because organized thrombus in the central pulmonary artery is a key pathological feature of CTEPH, key mechanism(s) may underlie in the process of thrombus resolution. Indeed, thrombus resolution was delayed in CTEPH because of the reduction of angiogenic factors, such as vascular endothelial growth factor and kinase insert domain protein receptor.⁵⁶ However, the mechanism of residual thrombus remains to be elucidated. We found that thrombolysis is impaired in patients with CTEPH, for which thrombin-activatable fibrinolysis inhibitor (TAFI) is involved. Thus, we review TAFI as a new therapeutic target of CTEPH.⁵⁷

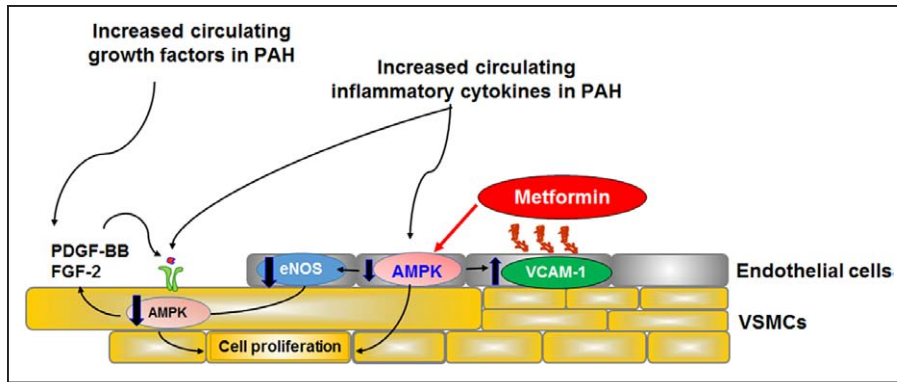


Figure 3. Endothelial AMP-activated protein kinase plays a crucial role in cell proliferation of vascular smooth muscle cells (VSMCs). Endothelial AMP-activated kinase (AMPK) promotes AMPK in VSMCs through endothelial NO synthase (eNOS). In pulmonary arterial hypertension (PAH) patients, the expression levels of endothelial AMPK are reduced because of cytokines in the serum. This downregulation of endothelial AMPK leads to downregulation of AMPK in VSMCs and accelerated proliferation of VSMCs. As metformin activates endothelial AMPK, it inhibits cell proliferation of VSMCs. FGF-2 indicates fibroblast growth factor-2; PDGF-BB, platelet-derived growth factor-BB; and VCAM-1, vascular cell adhesion molecule-1.

Thrombin-Activatable Fibrinolysis Inhibitor

TAFI is a plasma carboxypeptidase inhibitor produced by the liver. TAFI is activated by thrombin, thrombin/thrombomodulin complex, and plasmin, whereas activated form of TAFI removes the C-terminal lysines from fibrin and reduces the binding of tissue-type plasminogen activator and plasmin to fibrin.⁵⁸ Activated platelets promote thrombin generation with a resultant activation of TAFI.⁵⁹ Indeed, TAFI has been reported to be a risk factor for recurrent deep vein thrombosis.⁶⁰

It has been reported that fibrinolysis capacity is impaired in patients with CTEPH because of the mutation in fibrinogen.⁶¹ We further examined whether fibrinolysis capacity is impaired in CTEPH patients with a special reference to TAFI and whether TAFI is directly involved in the pathogenesis of CTEPH in humans.

We demonstrated that the clot from whole blood and plasma in patients with CTEPH is resistant to fibrinolysis *ex vivo* with whole-blood clot lysis assay and plasma clot lysis assay.⁵⁷ Importantly, plasma levels of TAFI were significantly higher in CTEPH patients and were significantly correlated with the extent of impairment of fibrinolysis.⁵⁷ Furthermore, we demonstrated that some patients with CTEPH had the single nucleotide polymorphisms, rs7999168, which has been reported that plasma levels of TAFI was increased.⁵⁷ Moreover, the impairment of fibrinolysis in patients with CTEPH was significantly improved with CPI-2KR, an inhibitor of activated TAFI.⁵⁷

Furthermore, we demonstrated that platelets from patients with CTEPH were highly activated with flow cytometric analysis.⁶² Especially, activation of P2Y₁, which is a small GTPase and plays a role in granule secretion in platelets, was significantly higher in platelets from patients with CTEPH.⁶² Indeed, platelets in patients with CTEPH were hyperresponsive to thrombin stimulation *ex vivo*. Furthermore, platelets in patients with CTEPH induced the expression levels of vascular cell adhesion molecule-1, indicating that platelets in patients with CTEPH are involved in the inflammation in ECs.⁶²

The possible correlation between TAFI and platelets has been reported^{59,63} because TAFI can be released from activated platelets and cause vascular damage and pathological

thrombus formation. It was also demonstrated that TAFI was more activated on the surface of aggregating platelets and that activated platelets inhibit fibrinolysis through the activation of TAFI and clot retraction.⁵⁹ Thus, we further examined the relation between TAFI and platelets in CTEPH. Indeed, TAFI levels released from platelets were significantly higher in CTEPH patients compared with controls, and the impairment of fibrinolysis in patients with CTEPH was improved with prostaglandin E₁, an inhibitor of platelet activation.⁵⁷ These results indicate that TAFI could be a potential therapeutic target of CTEPH.

Conclusions

PH remains a fatal disease, leading to right ventricular failure. Although significant research progress has been made on the pathogenesis, especially with regard to Rho-kinase, CyPA, endothelial AMPK, and TAFI, the detailed mechanisms of the disorder still remain to be elucidated. The usefulness of these new therapeutic targets remains to be fully examined in future studies.

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Disclosures

None.

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