

CONTEMPORARY REVIEW

Purinergic Dysfunction in Pulmonary Arterial Hypertension

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ABSTRACT: Pulmonary arterial hypertension (PAH) is a life-threatening disease characterized by increased pulmonary arterial pressure and pulmonary vascular resistance, which result in an increase in afterload imposed onto the right ventricle, leading to right heart failure. Current therapies are incapable of reversing the disease progression. Thus, the identification of novel and potential therapeutic targets is urgently needed. An alteration of nucleotide- and nucleoside-activated purinergic signaling has been proposed as a potential contributor in the pathogenesis of PAH. Adenosine-mediated purinergic 1 receptor activation, particularly A_{2A} R activation, reduces pulmonary vascular resistance and attenuates pulmonary vascular remodeling and right ventricle hypertrophy, thereby exerting a protective effect. Conversely, A_{2B} R activation induces pulmonary vascular remodeling, and is therefore deleterious. ATP-mediated $P2X_7$ R activation and ADP-mediated activation of $P2Y_1$ R and $P2Y_{12}$ R play a role in pulmonary vascular tone, vascular remodeling, and inflammation in PAH. Recent studies have revealed a role of ectonucleotidase nucleoside triphosphate diphosphohydrolase, that degrades ATP/ADP, in regulation of pulmonary vascular remodeling. Interestingly, existing evidence that adenosine activates erythrocyte A_{2B} R signaling, counteracting hypoxia-induced pulmonary injury, and that ATP release is impaired in erythrocyte in PAH implies erythrocyte dysfunction as an important trigger to affect purinergic signaling for pathogenesis of PAH. The present review focuses on current knowledge on alteration of nucleot(s)ide-mediated purinergic signaling as a potential disease mechanism underlying the development of PAH.

Key Words: adenosine ■ ATP ■ extracellular nucleotides ■ pulmonary arterial hypertension ■ purinergic receptor

Pulmonary arterial hypertension (PAH) is defined as an elevation of mean pulmonary arterial pressure (PAP) >20 mm Hg, with pulmonary arterial wedge pressure <15 mm Hg and pulmonary vascular resistance (PVR) >3 wood units at rest based on right heart catheterization at sea level.¹ PAH is a progressive disorder characterized by pulmonary endothelial dysfunction, increased pulmonary vascular tone, and pulmonary vascular remodeling, with muscularization, thickening, and occlusion of the pulmonary (micro)vasculature, leading to an increase in afterload of the right ventricle (RV), and eventually death caused by right heart failure.² Pulmonary vascular remodeling involves aberrant proliferation, hyperplasia, and/or hypertrophy of pulmonary artery endothelial cells (PAECs) and/or microvascular endothelial cells (ECs), pulmonary artery smooth muscle cells (PASMCs), and fibroblasts.

In addition, perivascular inflammatory foci, consisting of infiltrates with antigen-presenting cells and immune cells, such as macrophages and T and B lymphocytes, have been shown in PAH.³ Current therapies are principally aimed at reversing the increase in vascular tone and may reduce smooth muscle proliferation and muscularization of the distal vasculature.⁴ However, prognosis of PAH is still poor, with a 5-year survival of 59%.⁵ Therefore, more investigations are needed to identify novel underlying mechanisms in PAH to develop new therapies.

Recent findings have revealed that there is an alteration of nucleot(s)ide-mediated purinergic signaling in the pulmonary vasculature, which may contribute to the development and progression of P(A)H. Many cell types, such as ECs, immune cells, and erythrocytes, can produce nucleotides (such as ATP and UTP) and

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Nonstandard Abbreviations and Acronyms

CD39	nucleoside triphosphate diphosphohydrolase
EC	endothelial cell
iPAH	idiopathic PAH
PAP	pulmonary arterial pressure
NECA	nonselective adenosine receptor agonist 5'-(N-ethylcarboxamido) adenosine
P1R	purinergic receptor 1
P2R	purinergic receptor 2
PAEC	pulmonary artery endothelial cell
PAH	pulmonary arterial hypertension
PASMC	pulmonary artery smooth muscle cell
PH	pulmonary hypertension
PR	purinergic receptor
ROCK	Rho kinase
SMC	smooth muscle cell

nucleosides (such as adenosine), which activate purinergic receptors (PRs) to exert biological functions.^{6–8} Activation of purinergic signaling has been demonstrated to play an essential role in cardiovascular homeostasis,^{9–11} whereas there is increasing evidence to suggest that purinergic signaling may also play an important role in P(A)H by modulating vascular tone, remodeling, permeability, and inflammation.^{12–15} Therefore, nucleoti(s)ide-mediated purinergic signaling may serve as a potential target for the treatment of P(A)H. In the present review, we summarize available information on purinergic dysfunction in both patients with PAH and animal models of pulmonary hypertension (PH).

ACTIVATION AND REGULATION OF PURINERGIC SIGNALING IN PAH

Nucleotides (ATP, ADP, UTP, and UDP), nucleosides (adenosine), and even dinucleotides (eg, Up₄A) could be released directly or indirectly via ectonucleotidases from ECs, adventitial nerves, and circulating cells (including platelets, immune cells, and erythrocytes) in response to both physiological and pathological stimuli. The release of ATP from erythrocytes particularly occurs under hypoxic conditions and is increasingly recognized to play a role in regulation of tissue perfusion.^{16,17} Homeostasis between extracellular nucleotides and adenosine is governed by various ectonucleotidases. Ectonucleotidases are divided into 4 major families: (1) nucleoside triphosphate diphosphohydrolase (also known as CD39), (2) ecto-5'-nucleotidase, (3) nucleotide

pyrophosphatase/phosphodiesterase, and (4) alkaline phosphatases.⁹ For example, once ATP is released extracellularly, ATP is degraded to ADP and AMP through the continuous action of CD39. Ecto-5'-nucleotidase can further phosphohydrolyze AMP to adenosine (Figure 1).

Alterations in ectonucleotidases regulating the purinergic signaling have been reported in PAH. CD39, one of the ectonucleotidases hydrolyzing ATP and ADP to AMP, was found to be higher on circulating endothelial microparticles from patients with idiopathic PAH (iPAH).¹⁸ On the contrary, CD39 expression was significantly downregulated in the endothelium of pulmonary small arteries from patients with iPAH.^{13,19} Similarly, both expression and activity of CD39 were decreased in cultured PAECs derived from patients with PAH compared with healthy subjects.¹⁹ The downregulation of CD39 in the pulmonary vasculature may alter the balance between ATP and adenosine, thereby affecting purinergic signaling in PH. Indeed, the plasma adenosine concentration is lower in the pulmonary circulation in patients with PAH compared with the high plasma adenosine levels in healthy subjects (Table).^{20,21} Similarly, in newborn lambs with hypoxia-induced PH, baseline plasma adenosine levels in pulmonary circulation and left atrium were significantly lower than in normoxic controls (Table).²² Suppression of CD39 in cultured PAECs resulted in a phenotypic switch toward apoptosis-resistant PAECs¹⁹ and may thereby contribute to pulmonary vascular remodeling. Indeed, in addition to the markedly elevated ATP/adenosine ratio, hypoxic CD39 knockout mice demonstrated higher PAP, more pulmonary vascular remodeling, more RV hypertrophy, and a prothrombotic phenotype compared with normoxic controls (Table).¹³ Of note, systemic reconstitution of ATPase and ADPase enzymatic activities through continuous administration of apyrase dramatically decreased PAP in hypoxic CD39 knockout mice to levels found in hypoxic wild-type mice (Table).¹³ Altogether, these observations indicate that CD39 is an important enzyme regulating pulmonary vascular remodeling and suggest that therapeutic modulation of the balance between adenosine and ATP may directly affect pulmonary vascular remodeling (Figure 1). Interestingly, the activity of CD39 in both cultured PAECs and PAECs isolated from monocrotaline-induced PH rats can be potentiated by apelin (Table), a known regulator of pulmonary vascular homeostasis that is decreased in patients with PAH,^{23,24} suggesting that the therapeutic efficacy of apelin in animal models of PAH may be mediated, at least in part, through modulation of purinergic signaling.¹⁹

The biological effects of nucleot(s)ides are usually mediated via the activation of PRs, which consist of 2 subfamilies, purinergic receptor 1 (P1R) and purinergic receptor 2 (P2R).^{54,55} The P1R subfamily (all metabolic; also known as adenosine receptors) includes

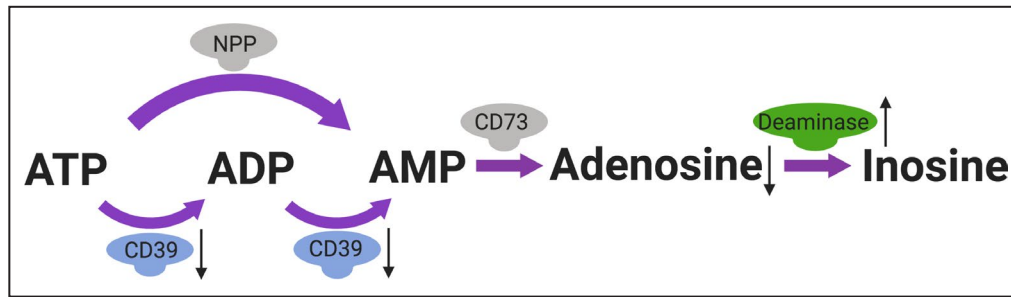


Figure 1. Nucleotides and adenosine regulation in pulmonary hypertension.

Homeostasis between extracellular nucleotides and adenosine is governed by ectonucleotidases, including nucleoside triphosphate diphosphohydrolase (CD39), ecto-5'-nucleotidase (CD73), nucleotide pyrophosphatase/phosphodiesterase (NPP), and adenosine deaminase. CD39 phosphohydrolyzes ATP and ADP to AMP, which is dephosphorylated to adenosine by CD73. Adenosine can be further degraded by adenosine deaminase to inosine. In pulmonary hypertension, CD39 is downregulated and adenosine deaminase is upregulated, resulting in low adenosine levels in the pulmonary vasculature (generated by Biorender).

4 subtypes: A₁R, A_{2A}R, A_{2B}R, and A₃R. The P₂R subfamily has 2 subgroups, P₂XRs and P₂YRs.^{55,56} The ionotropic P₂XRs include 7 P₂XRs (P₂X₁₋₇R), and the metabotropic P₂YRs contain 8 P₂YRs (ie, P₂Y₁R, P₂Y₂R, P₂Y₄R, P₂Y₆R, P₂Y₁₁R, P₂Y₁₂R, P₂Y₁₃R, and P₂Y₁₄R).⁵⁷ P₁R can be activated by adenosine, whereas P₂R are capable of mediating responses to several nucleotides and have overlapping ligand preferences. P₂X₁₋₇R and P₂Y₁₁R are mainly activated by ATP; P₂Y₁R, P₂Y₁₂R, and P₂Y₁₃R are activated by ADP, whereas P₂Y₁R is sensitive to both ATP and ADP. Moreover, P₂Y₂R and P₂Y₄R are preferably activated by UTP, whereas P₂Y₆R preferably responds to UDP (Figure 2).⁵⁵ On stimulation by different extracellular nucleotide(s)ides, PR-mediated signaling is initiated, which results in various responses, including platelet aggregation, cell proliferation, angiogenesis, immune responses, and vascular tone regulation.^{8,55,58-60} Although some heterogeneity of the PR expression and distribution was found between species,^{55,58,59} all 4 adenosine receptors were found in the lungs of mice and humans,⁶¹ and P₂R are located in the pulmonary arteries of various species. Thus, P₂X₁R, P₂X₂R, P₂X₄R, P₂X₅R, P₂X₇R, P₂Y₁R, P₂Y₂R, P₂Y₆R, and P₂Y₁₁R are expressed in isolated PAECs, whereas P₂X₁R, P₂X₃R, P₂X₄R, P₂X₇R, P₂Y₁R, P₂Y₂R, and P₂Y₁₂R have been found in the intact pulmonary vasculature (Figure 2).^{14,19,43,44,62}

PURINERGIC DYSFUNCTION IN PULMONARY VASCULAR TONE AND REMODELING

Alterations in P₁R Signaling in PAH

Plasma adenosine concentrations are lower in patients with PAH than in healthy subjects,^{20,21} and the

response of pulmonary vasculature to adenosine and P₁R agonists is altered in PAH, indicating a potential deficiency in adenosine-mediated purinergic signaling in PAH. The pulmonary vasodilator effect of adenosine in patients with PH appears to be dependent on cause of the disease. Thus, in patients with PH after cardiac surgery, central venous infusion of adenosine not only induced pulmonary vasodilation with significant decreased PAP and PVR, but also induced an increase in cardiac output with mean arterial pressure being unaffected.²⁵ Intravenous infusion of a high dose of adenosine decreased PAP, PVR, and systemic arterial pressure in around 50% of patients with severe PAH secondary to a congenital heart defect.²⁶ Intravenous adenosine infusion decreased PAP in 6 of 9 neonates with persistent PH who received inhaled NO (Table).²⁷ Conversely, when adenosine was administered in patients with iPAH, only ~10% of patients exhibited significant decreases in mean PAP and PVR.⁶³ In children with iPAH, 3 of 15 cases did respond to adenosine with a reduction in PAP.⁶⁴

When testing a potential pulmonary vasodilator effect of adenosine, one should carefully consider dose and route of administration as adenosine has a short half-life of 5 to 10 seconds.⁶⁵ Hence, infusion into the pulmonary circulation is preferred as adenosine then likely has a (more) selective effect on pulmonary vasculature, because of its higher concentrations in the pulmonary circulation compared with the systemic circulation. Indeed, adenosine infusion via the right atrium (0.01–2.5 μmol/kg per minute) in hypoxic lambs has a pulmonary vasodilator effect, evidenced by a decrease in PAP and PVR at all doses tested.²² A similar effect was present in normoxic lambs, but required higher doses of 0.15 to 2.5 μmol/kg per minute, than in the hypoxic lambs,²² suggesting that hypoxia sensitizes the response of

Table. Purineric Dysfunction in Patients With PH and Various Animal Models

PH/PAH Models	Species/ Genotype	Lung	RV	Pulmonary Circulation	Systemic Circulation	Test/Treatment	Effects	References
PAH	Human	A _{2B} R↑ CD39↓		Adenosine↓ ADA activity↑	CD39↑	Adenosine Regadenoson ATP-MgCl ₂	Pulmonary vasoreactivity test Stress test mPAP↓, PVR↓	19–21, 25–30
PH attributable to lung diseases or hypoxia	Human	A _{2B} R↑		ATP release from RBCs↓		PGI ₂ /PDE5i	Vascular remodeling↑ ATP release↑	28, 31–33
Monocrotaline	Rat	CD39↓ P2X ₁ R↑				LASSBio-1386 LASSBio-1359 A-740003 Brilliant Blue G	Vascular remodeling↓ PVR↓, EC function↑, pulmonary vasodilation Vascular remodeling↓, RV remodeling↓, inflammation↓	15, 19, 34–36
Hypoxia	Rat		P2X ₁ R↑			Adenosine NECA N6-cyclopentyladenosine	mPAP↓, endothelin-1↓, NO↑, vascular remodeling↓ mPAP↓, endothelin-1↓, NO↑, vascular remodeling↓ mPAP↓, endothelin-1↓, NO↑ PAP↑	37, 38
Hypoxia	A _{2A} R knockout mouse						Spontaneous PH under normoxia RhoA/ROCK↑ Worse PH under hypoxia Vascular remodeling↑	39, 40
Hypoxia	A _{2B} R knockout mouse (RBCs)			Oxygen release↓			Lung injury	41, 42
Hypoxia	CD39 knockout mouse				ATP/ adenosine↑	ADPase	Prothrombotic phenotype↑, PAP↑, vascular remodeling↑, RV hypertrophy↑ mPAP↓	13
Hypoxia	Swine					ADP ATP ATP-MgCl ₂ MRS2500 Cangrelor	mPAP↑, PVR↑ Vasodilation at low dose, vasoconstriction at high dose mPAP↓, PVR↓ mPAP↓ mPAP↓	30, 43
Hypoxia	Lamb			Adenosine↓		Adenosine ATP	mPAP↓, PVR↓ at low dose Pulmonary vasodilation at low dose	22, 44–46
U46619	Young lamb Newborn lamb					ATP ATP Adenosine	mPAP↓, PVR↓ at low dose PAP↓ PAP↓	47, 48
Hemolysis associated	Rat			ADA from RBCs↑			PH phenotype	49

(Continues)

Table. Continued

PH/PAH Models	Species/Genotype	Lung	RV	Pulmonary Circulation	Systemic Circulation	Test/Treatment	Effects	References
Bleomycin	Mouse $A_{2B}R$ knockout mouse/SMC Guinea pig					GS-6201 PKT100 NECA/ N6-cyclopentyladenosine	PH↓, vascular remodeling↓ RV hypertrophy↓ Inflammation↓, endothelin-1↓, PH↓, vascular remodeling↓ Pulmonary contraction followed by relaxation	28, 50–52
Lung-injury PH	$A_{2B}R$ knockout mouse (myeloid cell)						Lung fibrosis/PH↓	53
SUGEN and hypoxia	$A_{2B}R$ knockout mouse (SMC)						PH↓, vascular remodeling↓, inflammation↓	28

The A1R agonist was N6-cyclopentyladenosine; the $A_{2A}R$ agonist, LASSBio-1386, LASSBio-1359, and regadenosin; the $A_{2B}R$ antagonist, GS-6201; the P1R agonist, NECA; the P2X₇R antagonist, A-740003, PKT100, and Brilliant Blue G; the P2Y₁₂R antagonist, cangrelor; and the P2Y₁₃R antagonist, MRS2500. ADA indicates adenosine deaminase; CD39, nucleoside triphosphate diphosphohydrolase; EC, endothelial cell; mPAP, mean PAP; NECA, nonselective adenosine receptor agonist 5'-(N-ethylcarboxamido) adenosine; PAH, pulmonary arterial hypertension; PAF, pulmonary arterial pressure; PDE5i, phosphodiesterase type 5 inhibitor; PGI₂, prostacyclin analogue; PH, pulmonary hypertension; PVR, pulmonary vascular resistance; RBC, red blood cell; RhoA, Ras homolog gene family member A; ROCK, Rho kinase; RV, right ventricle; and SMC, smooth muscle cell.

pulmonary vasculature to adenosine. The systemic effects of adenosine (decrease in aortic pressure and systemic vascular resistance, increase in heart rate and cardiac output) were observed in both normoxic and hypoxic lambs with doses $\geq 0.3 \mu\text{mol/kg}$ per minute (Table).^{22,45} These results suggest that lower doses of adenosine directly reduce the PAP by reducing PVR, whereas at higher doses the decrease in PAP is limited by the increase in cardiac output. This pulmonary vasodilator effect of adenosine in hypoxic lambs was attenuated by prior treatment of nonselective P1R antagonist aminophylline,²² indicating that the pulmonary vasodilator effect is mediated via P1R.

Further evidence for alterations of P1R in the adenosine-mediated regulation of vascular function in pulmonary vasculature has been shown in endothelium-denuded pulmonary arteries isolated from guinea pigs, in which the nonselective adenosine receptor agonist 5'-(N-ethylcarboxamido) adenosine (NECA) produced concentration-dependent contraction.⁵⁰ The vasoconstrictor response to NECA was converted to relaxation in the presence of cyclooxygenase inhibition.⁵⁰ Interestingly, although exposure of these vessels to hypoxia did not alter the contractile response to NECA under baseline conditions, a biphasic response, contraction followed by relaxation, was observed in response to NECA in the presence of cyclooxygenase inhibition. This initial vasoconstrictor effect was prevented by A1R blockade (Table).⁵⁰ This study indicates that, in addition to the cyclooxygenase-dependent pulmonary vasoconstrictor effect of adenosine, exposure to hypoxia alters adenosine signaling and induces an A1R-mediated vasoconstrictor effect.

Long-term subcutaneous infusion of adenosine, the nonselective P1R agonist NECA, or the selective A1R agonist N6-cyclopentyladenosine into rats with chronic hypoxia-induced PH significantly reduced PAP, plasma renin activity, and angiotensin II levels, as well as endothelin-1 levels, and increased NO levels, thereby counteracting the effects of chronic hypoxia.³⁷ Of note, adenosine and NECA but not N6-cyclopentyladenosine also significantly attenuated the pulmonary vascular remodeling induced by chronic hypoxia (Table).³⁷ These data, showing that A1R agonism only partially mimicked the beneficial effects of P1R agonism, are consistent with a low A1R expression in the (healthy) human pulmonary vasculature,⁶⁶ and suggest that other P1Rs than the A1R are also involved.

The involvement of $A_{2A}R$ in the development of PH has been characterized in $A_{2A}R$ knockout mice. The $A_{2A}R$ knockout mice already show hemodynamic and histological characteristics of PAH, as evidenced by increased pulmonary vascular remodeling with

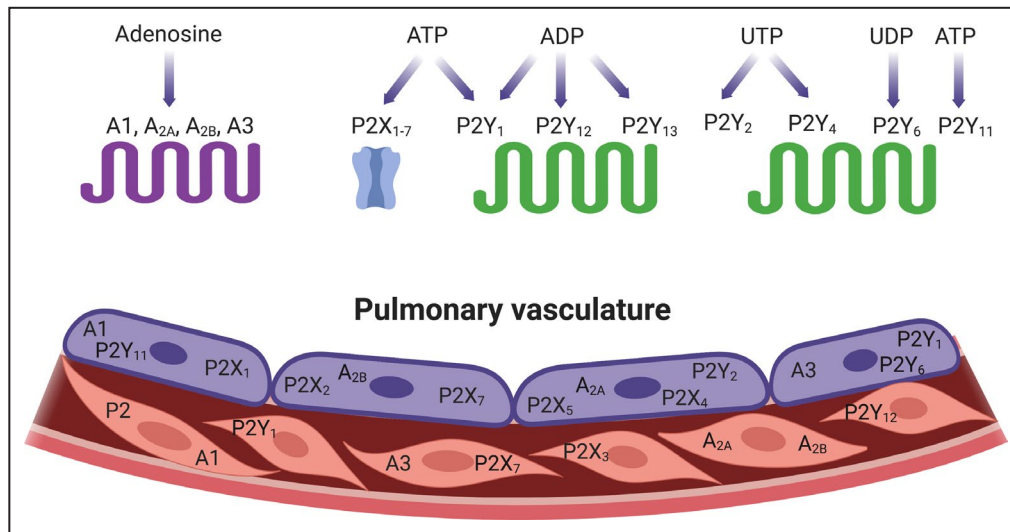


Figure 2. The ligand, expression and location of purinergic receptors.

Different nucleos(t)ides activate their preferable purinergic receptors to exert vascular biological influence (top panel). Distribution and location of key purinergic receptors in pulmonary vasculature (bottom panel). In pulmonary vasculature, all 4 adenosine receptors are present. P2X₁, P2X₂, P2X₄, P2X₅, P2X₇, P2Y₁, P2Y₂, P2Y₆, and P2Y₁₁ are expressed in endothelial cells, whereas P2X₁, P2X₃, P2X₄, P2X₇, P2Y₁, P2Y₂, and P2Y₁₂ have been found in the intact pulmonary vasculature (generated by Biorender).

excessive vascular cell (PAEC and PASMC) proliferation and hypertrophy in pulmonary resistance vessels, as well as increased collagen deposition and an increased RV pressure even in the absence of other triggers of PAH.³⁹ Exposure of these mice to chronic hypoxia exacerbates hypertrophy and increased cell proliferation in pulmonary resistance vessels in the A_{2A}R knockout mice, resulting in further elevations in RV pressure and RV hypertrophy (Table).³⁹ Pulmonary vascular remodeling in A_{2A}R knockout mice was accompanied by increased mRNA and protein expression for Ras homolog gene family member A and Rho kinase (ROCK) 1 (Table), with localization of ROCK1 protein in PAECs and PASMCs, bronchial, and alveolar epithelial cells.⁴⁰ Activation of the Ras homolog gene family member A/ROCK pathway has been proposed to play a key role in regulation of smooth muscle contraction and proliferation in PH.⁶⁷ Once activated, these pathways not only activate Ca²⁺/calmodulin-dependent myosin light chain kinase (contraction) but also inactivate Ca²⁺-independent myosin light chain phosphatase (relaxation). As these 2 components balance each other in the healthy vasculature to maintain a low level of pulmonary vascular tone, an imbalance induces vasoconstriction. In PH, activation of Rho kinase induces Ca²⁺ signaling, which further activates Ras homolog gene family member A/Rho kinase, leading to a vicious circle of vascular contraction and remodeling.⁶⁸ Taken together, these observations suggest that the loss of adenosine in PAH and consequent reduction in A_{2A}R activation may trigger the Rho/ROCK signaling

pathway. Considering a potential role of A_{2A}R in suppressing P(A)H, 2 new A_{2A}R agonists have been developed and tested in animal models of PH. The A_{2A}R agonist LASSBio-1386, administered 2 weeks after monocrotaline treatment, significantly reduced the proliferative changes in the pulmonary arterioles in a rat model of monocrotaline-induced PH.³⁴ Similarly, another A_{2A}R agonist, LASSBio-1359, abolished the increased RV overload and reduced vessel wall hypertrophy, demonstrating satisfactory efficacy through long-term oral administration in monocrotaline-induced PH, with no adverse effect on the systemic vasculature.³⁵ Of note, the impaired pulmonary endothelial function reflected by the reduced acetylcholine-induced NO-dependent relaxation in isolated pulmonary arteries was markedly attenuated in rats with PH treated with LASSBio-1386 or LASSBio-1359 (Table).^{34,35} More recently, the same group showed that combination therapy with a 5 times lower dosage of LASSBio-1359 than previously used and the phosphodiesterase 5 inhibitor sildenafil, but not the monotherapy with either of them, ameliorated all the PH-associated abnormalities, as observed in their previous studies.³¹ Therefore, activation of A_{2A}R is a promising additional tool for the treatment of PAH.

Although activation of A_{2A}R may be beneficial in PAH, activation of the A_{2B}R may result in pulmonary vascular remodeling in P(A)H in both human and animal studies. An upregulation of the A_{2B}R in human lungs was found in patients with various forms of PH, including PAH,²⁸ PH secondary to idiopathic pulmonary fibrosis compared with those without PH,⁶⁹ and

PH secondary to chronic obstructive pulmonary disease.⁷⁰ $A_{2B}R$ expression was increased specifically in PASMCs from patients with iPAH compared with controls (Figure 3).²⁸ A functional role for the $A_{2B}R$ was shown by pharmacological inhibition of $A_{2B}R$ using the antagonist GS-6201 as well as genetic deletion of $A_{2B}R$, which attenuated the fibroproliferative and vascular remodeling processes that contribute to PH in the lung of mice with bleomycin-induced PH and lung fibrosis.⁵¹ The detrimental role of $A_{2B}R$ activation was narrowed down to the smooth muscle cell (SMC) by specific depletion of the $A_{2B}R$ in SMCs in mice, which protected these mice from the development of PH and pulmonary vascular remodeling in response to exposure to bleomycin or SUGEN with hypoxia.²⁸ Specific $A_{2B}R$ depletion in SMCs also inhibited the production of several proremodeling mediators in these PH models, including interleukin-6, hyaluronan synthase 2, and transglutaminase 2.²⁸ Elevation of interleukin-6 and endothelin-1, which are implicated as important mediators in pulmonary vascular remodeling in PH, was also attenuated by pharmacological inhibition and genetic deletion of $A_{2B}R$.⁵¹ A direct role for $A_{2B}R$ activation in these processes was confirmed by experiments showing that adenosine receptor stimulation promoted interleukin-6 and endothelin-1 release from both PAECs and PASMCs, which could be inhibited

by a selective $A_{2B}R$ antagonist.⁵¹ Furthermore, the culture medium from $A_{2B}R$ -activated PAECs was able to promote proliferation of PASMCs.⁵¹ Taken together, these findings suggest a crucial role of $A_{2B}R$ in pulmonary vascular remodeling in PAH (Table). Targeting $A_{2B}R$ may serve as potential therapeutic strategy for the abnormal remodeling of the pulmonary vasculature associated with PAH.

Altogether, these findings support the potential of targeting P1R signaling to ameliorate PAH. To our knowledge, P1R subtype distribution in the pulmonary vasculature in patients with PAH is unclear. To date, most research has focused on A_2R , whereas the role of A_1R and A_3R in pulmonary vascular remodeling is not fully understood. As the receptor expression and distribution may change in pathological conditions, further characterization of the P1R subtypes in the pulmonary vasculature from patients with PAH is required. Cross talk among PRs has been reported in other vascular beds. For instance, there is an upregulation of $A_{2B}R$ in coronary arteries of $A_{2A}R$ knockout mice.⁷¹ However, in these global knockout mice, the adenosine receptor expression and function were not examined in pulmonary tissue. It could be speculated that the pulmonary pathological features observed in $A_{2A}R$ knockout mice may partially be attributed to the upregulation of $A_{2B}R$.³⁹ On the

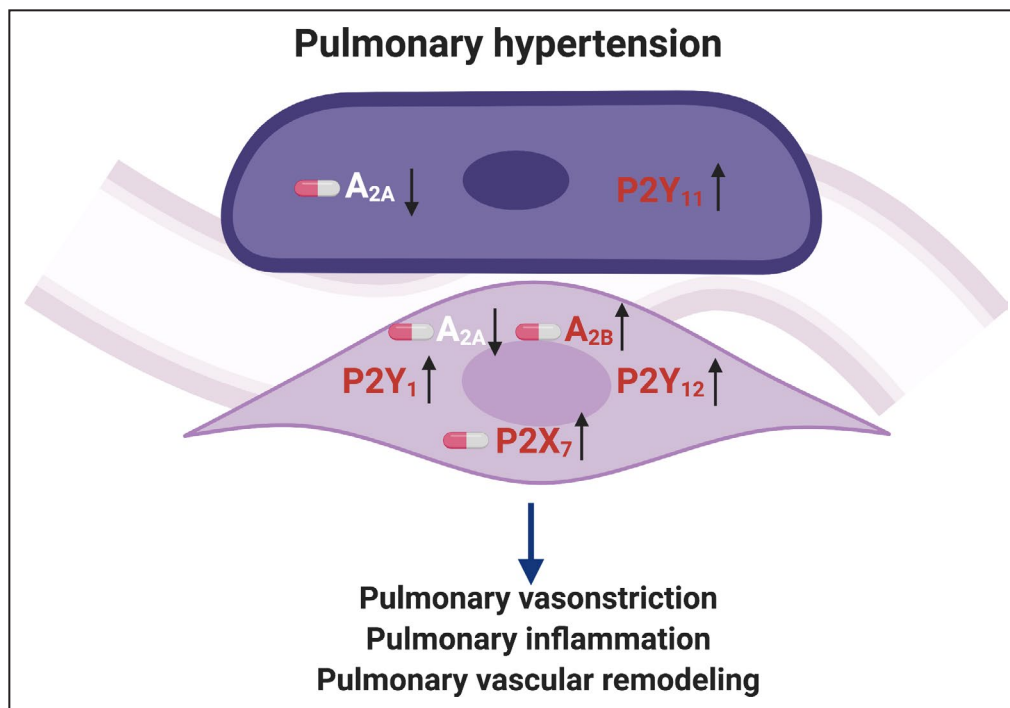


Figure 3. Purinergic receptor as the potential therapeutic target for the treatment of pulmonary hypertension.

In pulmonary hypertension, A_{2B} , $P2X_7$, $P2Y_1$, $P2Y_{11}$, and $P2Y_{12}$ receptors are upregulated (red) and A_{2A} receptors are downregulated (white) in the pulmonary vasculature. The potential receptors (A_{2A} , A_{2B} , and $P2X_7$) as therapeutic targets are highlighted with the capsule symbols, whereas the $A_{2A}R$ may be involved in initiation of the disease (generated by Biorender).

basis of experimental animal studies, A_{2A}R activation and A_{2B}R inhibition both seem to be promising strategies for reversing pulmonary vasoconstriction in the treatment of PAH.¹² Selective A_{2A}R agonism has been used in clinical studies mainly for the evaluation of cardiac function and detection of coronary artery disease.^{72,73} Stress tests have also been performed in patients with PAH using the A_{2A}R agonist regadenoson and showed no major adverse effects (Table), suggesting that such intervention is safe.²⁹ However, clinical trials are needed in the future to further characterize the therapeutic role of A_{2A}R activation in the treatment of patients with PAH.

Alterations in P2R Signaling in PAH

The role of P2R activation in the pulmonary vasculature has been addressed in different P(A)H models. Depending on the agonist and, most likely, the P2R involved, P2R activation can cause either pulmonary vasoconstriction or vasodilation. In swine with acute hypoxia-induced PH, blockade of P2Y₁R with the selective antagonist MRS2500 decreased PAP and PVR, whereas cardiac output was unchanged, while targeting P2Y₁₂R with cangrelor reduced PAP secondary to a decrease in cardiac output, with no change in PVR.⁴³ Infusion of ADP increased both PAP and PVR, which was abolished by P2Y₁R blockade and P2Y₁₂R blockade (Figure 3).⁴³ In contrast to the vasoconstriction induced by ADP, ATP induced pulmonary vasodilation, as evidenced by significant decreases in PAP and PVR in newborn lambs. The pulmonary vasodilator effect was not affected by the nonselective P1R antagonist theophylline, suggesting involvement of P2Rs.⁴⁶ Furthermore, the sensitivity of pulmonary vasculature from hypoxic newborn lambs to ATP infusion (0.01–0.3 μmol/kg per minute via right atrium) was greater than that from normoxic newborn lambs⁴⁶ as vasodilation occurred at a lower dose of ATP. In both normoxic and hypoxic lambs, the systemic effect of ATP only occurred at dosages >0.3 μmol/kg per minute.^{45,46} ATP infused into the pulmonary artery also significantly reduced mean PAP and PVR in young lambs, with PH induced by the thromboxane analogue U46619 without affecting mean arterial pressure.⁷⁴ In swine with meconium aspiration-induced PH, ATP infusion at low dose (0.02–0.08 μmol/kg per minute) selectively decreased PAP and PVR; a reduction in systemic resistance was only observed with higher ATP dosage (0.32–0.8 μmol/kg per minute).⁴⁷ Similarly, ATP-MgCl₂ infusion (at the optimal dose of 0.1 mg/kg per minute) reduced mean PAP and PVR without affecting mean arterial pressure in piglets with hypoxia-induced PH.³⁰ ATP-MgCl₂ was also shown to be a safe and effective vasodilator in children with PAH associated with congenital heart disease, as evidenced by a reduction in

PAP and PVR without major systemic adverse effects (Table).⁷⁵

Interestingly, in isolated intrapulmonary arteries, neither ATP-induced relaxation⁷⁶ nor UTP-induced vasoconstriction was different between healthy swine and PH swine exposed to hypoxia for 3 days.⁷⁷ Surprisingly, ATP induced vasoconstriction in both pulmonary arteries from broiler chicken with PH induced by excess tryptophan and control.⁷⁸ The dinucleotide Up₄A, which contains both a purine and a pyrimidine, induced contraction in healthy rat pulmonary arteries via P2Y_R,^{79,80} but the effect of Up₄A on pulmonary vasculature in PH has not been investigated to date.

Few studies actually investigated the functional role of different P2R subtypes in pulmonary vascular remodeling in PH. It has been reported that ATP or the P2Y₁₁R agonist β-NAD induced a survival response and increased cell viability in healthy ECs, whereas the ATP-induced effect on survival and viability was not present in ECs when P2Y₁₁R was knocked down.¹⁹ Furthermore, knockdown of P2Y₁₁R in itself also decreased the survival capacity of healthy ECs and increased vulnerability to serum starvation-induced apoptosis.¹⁹ More important, knockdown of P2Y₁₁R in ECs derived from patients with PAH also sensitized the cells to apoptosis and decreased the cell viability.¹⁹

In a rat model of monocrotaline-induced PH, P2X₇R expression in the pulmonary arterial smooth muscle layer was markedly increased (Figure 3).¹⁵ Long-term treatment with the selective P2X₇R antagonist A-740003 reversed pulmonary vascular remodeling in this PH model (Table).¹⁵ Although the mechanisms were not investigated in this study, impaired vasomotor function was restored in a model of subfailure overstretch injury in rat aorta by treatment with P2X₇R antagonists or P2X₇R/pannexin complex antagonists. In this study, P2X₇R antagonism reduced stretch-induced ATP release, decreased p38 mitogen-activated protein kinase phosphorylation, increased phosphorylation of the antiapoptotic protein Akt in aorta, and reduced tumor necrosis factor-α-stimulated caspase 3/7 activity in cultured rat vascular SMCs.⁸¹

Taken together, both vasodilator (to ATP) and vasoconstrictor (to ADP and UTP) responses to nucleotide-induced P2R activation have been observed in the pulmonary vasculature of healthy subjects as well as subjects with PH. On the basis of the existing data on the involvement of P2Rs in pulmonary vasculature (Figure 2), ADP-mediated vasoconstriction is likely through activation of P2Y₁R and P2Y₁₂R, whereas UTP-induced vascular contraction is via P2Y₂R. The P2R subtypes activated by ATP, as well as whether their expression patterns change in PH, remain to be determined, although the presence of PH seems to sensitize to pulmonary vasculature to the vasoactive effects of ATP.

Changes in P2R in PH are not limited to the pulmonary vasculature. In a rat model of hypobaric hypoxia-induced PH, P2X₄R mRNA levels were exclusively increased in RV of the heart compared with other tissues, including left ventricles of the heart, lung, liver, kidney, and brain.³⁸ The increased mRNA levels of P2X₄R were followed by increases in P2X₄R protein in the RV and were larger after more prolonged exposure of animals to hypobaric hypoxia.³⁸ These changes in P2X₄R in the RV occurred simultaneously with the increase in PAP and the development of the RV hypertrophy (Table).³⁸ However, the functional implications of the increase in P2X₄R in the RV require further investigations. A recent study investigating the therapeutic effect of P2X₇R antagonism for PH showed that the novel P2X₇R antagonist PKT100 attenuated RV hypertrophy and improved RV contractility and survival in a mouse model of PH induced by bleomycin independently of effects on the pulmonary vasculature (Table).⁵² This new finding may add new information on the current treatment option, in which the significant improvement in pulmonary pressure does not affect mortality caused by RV failure.

Possible Purinergic Dysfunction in Pulmonary Venous Remodeling?

Patients with PH, including PAH with scleroderma, chronic thromboembolic PH, and pulmonary venous occlusive disease, often display pulmonary venous and venular remodeling.^{82–84} The walls of septal veins and preseptal venules also show SMC hyperplasia with abnormal contraction, which may contribute to the elevated PVR. Contrary to the vasodilator effect of ATP in the pulmonary arteries, ATP induces a concentration-dependent contraction in pulmonary veins, which is inhibited by the nonselective P2R antagonist suramin and the P2Y₂R antagonist AR-C118925 but not by the P2Y₁R antagonist MRS2179.⁸⁵ The functional evidence is in accordance with the exclusive expression of P2Y₂R in SMCs within pulmonary veins.⁸⁵ Activation of phospholipase C-β and generation of intracellular Ca²⁺ oscillations may serve as post-PR mechanisms for ATP-induced contraction in pulmonary veins.⁸⁵ Therefore, it is of interest to study whether P2YR signaling in pulmonary veins plays a significant role in the development of PH.

PURINERGIC DYSFUNCTION IN INFLAMMATION AND IMMUNITY

Inflammation and maladapted immune responses have been reported to be involved in the pathogenesis of P(A)H, as evidenced by histological studies of the lung, the presence of circulating autoantibodies, and

high plasma levels of cytokines.^{3,86} Proinflammatory cytokines, such as interleukin-6, interleukin-13, tumor necrosis factor-α, and interleukin-1β, are independently associated with survival rate in patients with PAH, indicating that therapeutic drugs targeting inflammation would be beneficial to patients with PAH.⁸⁷

The expression and function of PRs on inflammatory and immune cells have been extensively characterized.^{88,89} Activation of many PRs, including A1R, P2X₇R, P2Y₁R, and P2Y₆R, generally results in proinflammatory responses.^{90–94} In a rat model of monocrotaline-induced PH, long-term treatment with P2X₇R antagonist Brilliant Blue G effectively attenuated inflammation by reducing the proinflammatory cytokines interleukin-1β and tumor necrosis factor-α through the p38/mitogen-activated protein kinase pathway, thereby reducing cell infiltration in the alveolar space.³⁶ In another study using the same model, the selective P2X₇R antagonist A-740003 reduced macrophage numbers and proinflammatory cytokine levels in bronchoalveolar lavage fluid through suppression of the upregulation of NLRP3 inflammasome (Table).¹⁵ Recent findings demonstrated that conditional deletion of A_{2B}R in myeloid cells altered the phenotype of macrophages and attenuated the development of fibrosis and inflammation (decreased interleukin-6 concentrations in bronchoalveolar lavage fluid) in a mouse model of lung injury-induced PH.⁵³ Taken together, purinergic signaling might contribute to the proinflammatory phenotype in P(A)H, and inhibition of these effects might contribute to therapeutic efficacy (Table).

POTENTIAL ROLE OF ERYTHROCYTE-MEDIATED PURINERGIC SIGNALING IN PAH

In addition to immune cells, emerging data suggest that erythrocytes not only act as regulators of normal physiological function to maintain cardiovascular homeostasis and integrity but also act as important triggers for the development of various cardiovascular diseases.^{95–97} It is well known that erythrocytes deliver oxygen to body tissues, and interestingly, erythrocytes also serve as ATP pool. The 2 functions are interdependent as ATP release from erythrocytes has been observed particularly under hypoxia, to precisely regulate tissue perfusion and vascular tone in both experimental animals and humans.^{16,17} This regulation of vascular function by erythrocyte-derived ATP has been proposed to be mediated via activation of vasodilator P2Y₁R on the vasculature.^{9,16} In the pulmonary vasculature, oxygen acts as an important vasodilator, which is likely to be mediated via ATP and subsequent

activation of PRs, as both P1R and P2YR contribute to the oxygen-induced decrease in PAP in newborn lambs with PH.⁴⁸ Further evidence for an interaction between oxygen and release of purines is that young healthy humans exposed to high altitude exhibited increased circulating adenosine levels and ecto-5'-nucleotidase activity, suggesting that ATP is increased at high altitude and ATP breakdown to adenosine is subsequently enhanced via ecto-5'-nucleotidase.⁴¹ The increase in adenosine was associated with increased erythrocyte 2,3-bisphosphoglycerate and oxygen releasing capacity.⁴¹ Mechanistic studies using A_{2B}R knockout mice demonstrated that increased adenosine activates the erythrocyte A_{2B}R-AMP kinase axis, resulting in increased 2,3-bisphosphoglycerate production and a shift in the relation between oxygen tension and oxygen saturation, thereby enhancing oxygen release to reduce the hypoxic burden. This subsequently reduced inflammation and lung injury, reflected by decreased cell counts, albumin, and interleukin-6 concentrations in bronchoalveolar lavage fluid.⁴¹ The same group further found that adenosine-A_{2B}R-protein kinase A-induced proteasome-mediated degradation of the equilibrative nucleoside transporter 1 on erythrocytes is an important cellular purinergic signaling regulatory component to counteract hypoxic pulmonary vascular leakage and inflammation, and that erythrocytes with reduced equilibrative nucleoside transporter 1 retain a "hypoxic purinergic memory" for quicker adaptation to subsequent hypoxia (Table).⁴²

ATP release from erythrocytes was significantly reduced in patients with PAH compared with healthy subjects when erythrocytes were challenged with either mechanical (passing erythrocytes through filters) or pharmacological stimulation (incubation with cAMP analogue to increase cAMP level, a required process for ATP release from erythrocytes).³² Of interest, a prostacyclin analogue increases cAMP and ATP to a greater extent in erythrocytes of patients with PAH than in healthy subjects.³³ Additional phosphodiesterase-5 inhibition further increased ATP release from erythrocytes, indicating that common PAH therapeutic strategies synergistically induce release of this potent vasodilator from erythrocytes.³³ The impaired release of ATP from erythrocytes of PAH may alter vascular PR signaling and result in vascular dysfunction. It is also worth mentioning that oxygen concentrations are likely to be higher in pulmonary veins than in arteries. Thus, ATP released from erythrocytes may be lower in the vein. This potential difference may also have impact on pulmonary venous function.

In addition to the reduced ATP release from erythrocytes, higher adenosine deaminase activity in pulmonary circulation of patients with PAH may account for the lower levels of adenosine in pulmonary compared with systemic circulation (Figure 1).²⁰

Of interest, erythrocytes from a rat model of hemolysis-associated PH released more adenosine deaminase into the pulmonary circulation to hydrolyze extracellular adenosine, suggesting that erythrocytes may be a source of adenosine deaminase (Table).⁴⁹ Altogether, these findings suggest that erythrocytes are important intermediaries to initiate/activate purinergic signaling.

POTENTIAL INTERACTION WITH AIRWAY DISEASE

PRs are not only present in the vasculature but also on the airways, where their activation can induce bronchoconstriction and exert a proinflammatory effect. Hence, P2X₇R activation not only exerts a proinflammatory effect on the vasculature but also on the airways as its activation results in upregulation of ROCK1 in bronchial and alveolar epithelial cells.^{40,98} Antagonists of this receptor may therefore have a dual beneficial effect on both airways and vasculature in PAH. Conversely, there is evidence to suggest that activation of P1R and P2R by adenosine and ATP plays a detrimental role in airway diseases, including asthma, allergy, and chronic obstructive pulmonary disease.^{98,99} Particularly, activation of the A_{2A}R has been shown to induce bronchoconstriction in patients with asthma and chronic lung diseases.^{100,101} Furthermore, activation of this receptor may worsen the inflammation in patients with asthma.¹⁰⁰ Hence, when evaluating therapeutic effects of adenosine and A_{2A}R agonism in PH, potential detrimental effects on the airways should be carefully monitored, particularly in patients with chronic lung diseases, such as asthma and chronic obstructive pulmonary disease.

CONCLUSIONS AND PERSPECTIVES

Emerging observations have suggested a role of nucleot(s)ide-mediated purinergic signaling in the development and progression of P(A)H. The ectonucleotidase CD39 is an important enzyme regulating purine and pyrimidine degradation, thereby modulating P2R signaling. CD39 is downregulated on the pulmonary endothelium of patients with PAH, which may promote pulmonary vascular remodeling. Furthermore, erythrocyte dysfunction in these patients decreases ATP release and consequent dysregulation of the hypoxia-induced adenosine response may further contribute to the development of PAH and lung injury.

A_{2A}R and A_{2B}R, both members of the P1R family, play a role in regulation of pulmonary vascular tone, vascular remodeling, inflammation, and immunity, and have shown potential as therapeutic targets in experimental models (Figure 3). A_{2A}R knockout mice

exhibited a PH phenotype, suggesting loss of function in this receptor may initiate the disease. New A_{2A}R agonists have been developed and tested in an animal model of PH with promising outcome. Although the A_{2A}R agonist regadenoson has been applied in patients with PAH for a cardiac stress test, future clinical trials are needed to investigate a potential therapeutic role of A_{2A}R activation in the treatment of patients with PAH. These trials need to be carefully monitored as A_{2A}R activation may result in a proinflammatory effect and may induce dyspnea with bronchoconstriction.

From the P2R subtypes, P2X₇R, P2Y₁R, P2Y₁₁R, and P2Y₁₂R have been shown to play a role in pulmonary vascular remodeling, inflammation, and/or vascular tone regulation in PAH (Figure 3). Given the multitude of receptor subtypes expressed in different cells of the pulmonary arteries and lung, it remains to be investigated which receptors play a key role in the development of PAH, and whether single receptor or multiple receptors need to be targeted at the same time for the most effective therapy. This far, P2X₇R inhibition has been shown to exert anti-inflammatory and antiremodeling effects on both the vasculature and the airways and to reduce RV hypertrophy, suggesting that the P2X₇R may serve as a therapeutic target (Figure 3). Another important limitation to date is that there is limited knowledge about the molecular mechanisms involved in the P2R signaling. Future studies in specific P2R knockout mice can be useful to elucidate these molecular mechanisms.

Altogether, existing evidence suggests that a role of altered purinergic signaling in the development and progression of P(A)H is plausible. Targeting nucleod(s) ides and their regulated purinergic signaling may provide valuable possibilities for the treatment of PAH.

ARTICLE INFORMATION

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Disclosures

The authors declare that there is no conflict of interest.

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