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Review

Key role of the RhoA/Rho kinase system in pulmonary hypertension

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ABSTRACT

Pulmonary hypertension (PH) is a general term comprising a spectrum of pulmonary hypertensive disorders which have in common an elevation of mean pulmonary arterial pressure (mPAP). The prototypical form of the disease, termed pulmonary arterial hypertension (PAH), is a rare but lethal syndrome with a complex aetiology characterised by increased pulmonary vascular resistance (PVR) and progressive elevation of mPAP; patients generally die from heart failure. Current therapies are inadequate and median survival is less than three years. PH due to chronic hypoxia (CH) is a condition separate from PAH and is strongly associated with chronic obstructive pulmonary disease (COPD). An early event in the pathogenesis of this form of PH is hypoxic pulmonary vasoconstriction (HPV), an acute homeostatic process that maintains the ventilation–perfusion ratio during alveolar hypoxia. The mechanisms underlying HPV remain controversial, but RhoA/Rho kinase (ROK)-mediated Ca^{2+}-sensitisation is considered important. Increasing evidence also implicates RhoA/ROK in PASMC proliferation, inflammatory cell recruitment and the regulation of cell motility, all of which are involved in the pulmonary vascular remodelling occurring in all forms of PH. ROK is therefore a potential therapeutic target in treating PH of various aetiologies. Here, we examine current concepts regarding the aetiology of PAH and also PH due to CH, focusing on the contribution that RhoA/ROK-mediated processes may make to their development and on ROK inhibitors as potential therapies.

1. Introduction

The term pulmonary hypertension (PH) is a general one, applying to a wide spectrum of rare (~15 cases per million [1]) but lethal pulmonary hypertensive conditions that have different aetiologies but may have a similar clinical presentation. PH is defined as a mean pulmonary arterial pressure (mPAP) of >25 mmHg at rest or >30 mmHg during exertion. PH is currently classified into five categories based on pathological, functional and clinical characteristics [2,3]. Group 1 PH, also termed pulmonary arterial hypertension (PAH), includes heritable and idiopathic PAH (hPAH, iPAH), which are associated with mutations in the bone morphogenetic receptor type II (BMPR-II) in ~80 and ~20% of cases, respectively. Group 1 also includes persistent pulmonary hypertension of the newborn (PPHN) and PAH associated with the use of certain drugs (notably amphetamine-related appetite suppressants such as dexfenfluramine) and with a group of conditions including connective tissue diseases such as systemic sclerosis, HIV infection, portal hypertension, and congenital heart diseases characterised by a systemic to pulmonary shunt. Groups 2 to 5 comprise forms of PH distinct from PAH, and are therefore also referred to as 'non-PAH PH'. Group 2 PH is associated with left ventricular failure or mitral/aortic valve disease, resulting in increased left atrial pressure which is transmitted to the pulmonary artery. The principle aetiology of group 3 PH is chronic alveolar hypoxia, which is commonly associated with lung diseases such as chronic obstructive pulmonary disease (COPD). For example, as COPD progresses, right ventricular hypertrophy (RVH) and dilatation secondary to PH – *cor pulmonale* – may develop, depending on the elevation in mPAP [4]. In most cases, PH is mild to moderate [5], but it may be severe and observed in the absence of major airflow obstruction, a condition which has been termed 'out of proportion' PH [6] and often leads to right heart failure and death. Considering,

Abbreviations: BMPR-II, bone morphogenetic receptor type II; CH, chronic hypoxia; EC, endothelial cell; hPAH, heritable pulmonary arterial hypertension; HPV, hypoxic pulmonary vasoconstriction; iPAH, idiopathic pulmonary arterial hypertension; Kv, voltage-gated potassium channel; NFAT, nuclear factor of activated T cells; PA, pulmonary artery; PAEC, pulmonary arterial endothelial cell; PAH, pulmonary arterial hypertension; PASMC, pulmonary arterial smooth muscle cell; PH, pulmonary hypertension; PPHN, persistent pulmonary hypertension of the newborn; ROK, Rho kinase; SMA, smooth muscle α-actin; SMC, smooth muscle cell; VOCC, voltage-operated calcium channel.

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for example, that the prevalence of COPD in eight European cities is as high as 6.2% [7], and of these, approximately 6% have severe or very severe disease [6], PH associated with COPD confers a significant disease burden on the population. Exposure to high altitude, pneumonia, interstitial lung disease and cystic fibrosis can also cause PH associated with hypoxia. Group 4 includes chronic thromboembolic PH, and is mainly caused by pulmonary embolism. Group 5 PH is associated with a diverse group of conditions including sarcoidosis, certain metabolic conditions such as thyroid disease, and several haematologic disorders. Several animal models of PH have been developed to study its pathobiology and assess possible treatments; the most widely used of these utilise chronic hypoxia (CH) or monocrotaline injection to induce over a period of weeks a rise in pulmonary vascular resistance (PVR), with pulmonary vasoconstriction, vascular remodelling, and neomuscularisation. However these models do not demonstrate complex vascular lesions, a hallmark feature of human PAH in which the lumen of small pulmonary arteries (PA) is occluded by endothelial hyperproliferation. Therefore attempts to develop animal models which more closely resemble human PAH are underway [3,8]. Conversely, animal models appear to more closely resemble PH secondary to hypoxia.

The discovery that contraction of vascular smooth muscle, in addition to being regulated by the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), is also under the control of the monomeric G-protein RhoA and its downstream substrate Rho kinase (ROK) [9–11] initiated enormous interest in the effects of this pathway on vascular function. It has become clear that RhoA/ROK, in addition to regulating the contractile responses of the vasculature to diverse stimuli, also exerts important effects on smooth muscle differentiation, proliferation and migration as well as on endothelium-dependent vasodilatation. As dysfunction of each of these processes is thought to contribute to both PAH and non-PAH PH, ROK inhibitors have emerged as possible treatments for these conditions. One such drug, fasudil, has been available in Japan since 1995 for the prevention of cerebral vasospasm associated with subarachnoid haemorrhage. Although the possible adverse effects associated with long-term fasudil treatment have not been exhaustively assessed, one study where stepped doses of fasudil up to 240 mg per day were administered to patients with stable angina for 8 weeks reported few serious side effects [12]. In this review, we briefly describe the RhoA/ROK pathway and its functions, discuss aspects of the pathobiology of PH which may involve this system, and summarise studies in both human and animal models of PH which indicate that inhibitors of this system may be of use in treating these conditions.

2. RhoA/ROK and vascular function (Fig. 1)

In all vascular smooth muscle cells (SMC), whether systemic or pulmonary, contraction and tone are determined by the level of phosphorylation of Ser^{19} of the 20 kDa regulatory myosin light chain (MLC_{20}). This level is set by the balance between phosphorylation of Ser^{19} by the Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) and its dephosphorylation by myosin light chain phosphatase (MLCP), a type 1 protein phosphatase [13]. Decreases and increases in the activity of MLCP are able to evoke vascular contraction and relaxation independently of any changes in $[Ca^{2+}]_i$; these processes are termed Ca^{2+}-sensitisation and -desensitisation, respectively.

The RhoA/ROK system is the major mediator of Ca^{2+}-sensitisation/-desensitisation across most vascular beds [11] and hence is a key regulator of vascular tone [14]. RhoA (*R*as *h*omologue gene family, member *A*) is a small monomeric G-protein encoded by the gene *RHOA*, and belongs to the Ras superfamily of proteins which help regulate cell growth, differentiation and survival. In PA smooth muscle cells (PASMC), RhoA is activated following stimulation of G-protein coupled receptors by vasoconstrictors, an event which triggers the activation of guanine nucleotide exchange factors (GEFs). The mechanism by which this occurs has not been well characterised, but GEF activation facilitates the exchange of GDP-bound RhoA for GTP-bound RhoA, rendering RhoA active. GTP-bound RhoA then translocates from the cytosol to the membrane to interact with downstream targets [15], thus eliciting cellular responses. There is also evidence that Ca^{2+}, acting though calmodulin, can activate RhoA [16]. Although other proteins, including protein kinase N, rhophilin, rhotekin, citron, p140mDia and citron kinase [17,18] are also activated by RhoA, ROKs are the best characterised effectors of RhoA (and also of the related G-proteins RhoB and RhoC). ROKs are serine/threonine kinases with a molecular weight of ~160 kDa. Two isoforms ROK1 (ROKβ) and ROK2 (ROKα) have been identified [19,20]. Although both are ubiquitous, the latter is preferentially expressed in brain and skeletal muscle. Hereafter, ROKα/ROK2 and ROKβ/ROK1 are collectively referred to as 'ROK'. ROK can be activated independently of RhoA, *e.g.* by lipid messengers such as arachidonic acid [21] or sphingosine phosphorylcholine [22] and by an increase in $[Ca^{2+}]_i$ [14]. The targets for ROK include the ezrin, radixin, moesin family (ERM), adducin, intermediate filament, Na^+/H^+ exchanger, LIM kinase, protein kinase C (PKC)-potentiated myosin phosphatase inhibitor (CPI-17), calponin and MLC_{20} [23]. However the most important target in terms of vascular smooth muscle contraction is the 130 kDa myosin-binding subunit of MLCP, MYPT-1. Phosphorylation of MYPT-1 inhibits MLCP, which prevents dephosphorylation of MLC_{20} and hence increases the sensitivity of the contractile apparatus to Ca^{2+}. RhoA activation can be opposed either by inhibition of GEF activity by guanine nucleotide dissociation inhibitors (GDIs); [24,25] or by inhibition of translocation of GTP-bound RhoA to the membrane by GTPase-activating protein dephosphorylation or protein kinase A (PKA)/protein kinase G (PKG) phosphorylation [26]. In pathological states of hyperconstriction, MLC_{20} may be phosphorylated at Thr^{18} in addition to Ser^{19} [27–29]. This is termed 'diphosphorylation' and in a swine model of coronary artery vasospasm, the diphosphorylation was prevented by ROK inhibition [30]. It therefore is possible that ROK contributes to hyperconstriction not only *via* inhibition of MLCP but also by direct phosphorylation of MLC_{20}.

2.1. RhoA/ROK and cellular proliferation

Smooth muscle cells demonstrate both contractile and proliferative phenotypes, and somewhat paradoxically, it appears that RhoA/ROK is important for maintaining both. The contractile phenotype is defined by the expression of SMC-specific proteins such as SMC α-actin (SMA), SM22 and calponin. Expression of these is increased by serum response factor (SRF) and its cofactors (*e.g.* myocardin, myocardin-related transcription factors (MRTFs)) which translocate to the nucleus and bind to CArgA domains in the promoter regions of these genes [31]. This process depends on actin polymerisation, which is in turn enhanced by RhoA/ROK [32].

On the other hand, inhibiting the RhoA/ROK pathway attenuates vascular SMC proliferation in response to a variety of stimuli [33]. Studies carried out mainly in fibroblasts show that signalling *via* RhoA/ROK is important in ensuring progression of cells through the G_1 phase of the cell cycle in response to mitogens. This requires the activation of cyclin-dependent kinases (CDKs) which act to phosphorylate proteins of the retinoblastoma family, leading to the transcription of proteins such as cyclin A which are necessary for the subsequent S phase of the cell cycle. CDKs are activated by the binding of cyclins including cyclin D1 and