Journal of Molecular and Cellular Cardiology 65 (2013) 147–155

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

The role of pulmonary vascular contractile protein expression in pulmonary arterial hypertension ☆

Ewa A. Konik, Young Soo Han, Frank V. Brozovich *

Mayo Clinic, Division of Cardiovascular Diseases, 200 First Street SW, Rochester, MN 55905, USA

ARTICLE INFO

Article history:
Received 29 July 2013
Received in revised form 30 September 2013
Accepted 15 October 2013
Available online 23 October 2013

Keywords:
Myosin light chain phosphatase
Myosin phosphatase targeting subunit 1
Smooth muscle myosin
Nonmuscle myosin
Vascular reactivity
Nitric oxide

ABSTRACT

Pulmonary arterial hypertension (PAH) is associated with refractory vasoconstriction and impaired NO-mediated vasodilatation of the pulmonary vasculature. Vascular tone is regulated by light chain (LC) phosphorylation of both nonmuscle (NM) and smooth muscle (SM) myosins, which are determined by the activities of MLC kinase and MLC phosphatase. Further, NO mediated vasodilatation requires the expression of a leucine zipper positive (LZ+) isoform of the myosin targeting subunit (MYPT1) of MLC phosphatase. The objective of this study was to define contractile protein expression in the pulmonary arterial vasculature and vascular reactivity in PAH. In severe PAH, compared to controls, relative LZ+MYPT1 expression was decreased (100 ± 14% vs. 60 ± 6%, $p < 0.05$, $n = 7–8$), and NM myosin expression was increased (15 ± 4% vs. 53 ± 5% of total myosin, $p < 0.05$, $n = 4–6$). These changes in contractile protein expression should alter vascular reactivity; following activation with Ang II, force activation and relaxation were slowed, and sustained force was increased. Further, the sensitivity to ACh-mediated relaxation was reduced. These results demonstrate that changes in the pulmonary arterial SM contractile protein expression may participate in the molecular mechanism producing both the resting vasoconstriction and the decreased sensitivity to NO-mediated vasodilatation associated with PAH.

1. Introduction

In PAH, the pulmonary vasculature is characterized by a resting vasoconstriction and impairment of NO-mediated vasodilatation, and only ~20% of PAH patients respond to conventional vasodilators [1,2]. For untreated patients with PAH, median survival is 2.8 years [3]. Currently, 5-year survival for WHO category I patients is 61%, and the 1-year mortality is ~15% [4]. In addition, pulmonary hypertension contributes to the morbidity and mortality of patients with both pulmonary and cardiovascular diseases [5].

An increase in the proliferation/apoptosis ratio has been suggested to produce PAH. It is hypothesized that widespread endothelial apoptosis results in selection of apoptosis-resistant endothelial precursor cells that proliferate and eventually form plexiform lesions [1,2,6]. In the media, pulmonary artery SM cell apoptosis is suppressed and proliferation is enhanced [3,7,8]. These changes result in the histological findings present in PAH, including intimal hyperplasia, adventitial proliferation and plexiform lesions, which produce an elevation of pulmonary arterial pressure (reviewed in [4,9]). Despite these known factors that contribute to the molecular mechanism that produces PAH, the contribution of dysfunction at the level of SM contractility to the pathogenesis of PAH has not been elucidated.

Vascular tone is regulated by regulatory light chain (LC) phosphorylation of both nonmuscle (NM) and smooth muscle (SM) myosins, which are determined by the activities of MLC kinase and MLC phosphatase [5,10–12]. MLC kinase is regulated by Ca^{2+}-calmodulin, whereas MLC phosphatase activity is regulated by several signaling pathways including those mediated by both NO and Rho kinase [11]. MLC phosphatase is a trimeric enzyme consisting of catalytic, 20 kDa and MYPT1 subunits [10]. Alternative splicing of a 3′ exon produces MYPT1 isoforms that differ by the presence/absence of a LZ (LZ+/LZ−, [13]), and a LZ+MYPT1 isoform is required for NO-mediated activation of MLC phosphatase [14]. We have demonstrated that, in isolation, a decrease in LZ+MYPT1 expression results in a decrease in the sensitivity of SM to NO-mediated relaxation [14]. Thus, changes in LZ+MYPT1 expression contribute to the mechanism that regulates the sensitivity of the vasculature to NO-mediated relaxation [14–16]. Further, LZ+MYPT1 isoform expression decreases in heart failure, pre-eclampsia, and portal hypertension [15–18], and thus, a decrease in LZ+MYPT1 expression contributes to the decrease in sensitivity to NO associated with these diseases.

Recent evidence suggests that NM myosin also participates in the force maintenance phase of SM contraction and hence to the regulation of vascular tone. Compared with SM myosin, the kinetics of NM myosin are slow [19,20]. Consequently, NM myosin spends a majority of its kinetic cycle attached to actin to support force [19], and changes in NM myosin expression have been demonstrated to influence vascular tone [21].

* Corresponding author at: 200 First Street SW, Rochester, MN 55905, USA. Tel.: +1 507 774 3727; fax: +1 507 538 6418.
E-mail address: brozovich.frank@mayo.edu (F.V. Brozovich).

0022-2828/$ – see front matter © 2013 The Authors. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.yjmcc.2013.10.009

This study was designed to determine the expression of contractile proteins in pulmonary arterial smooth muscle and pulmonary vascular reactivity in PAH. Our data suggest that MYPT1 and NM myosin expression is altered in PAH, and that these changes contribute to the molecular mechanism that produces PAH.

2. Material and methods

2.1. MCT rat model of PAH and pulmonary artery preparation

The Institutional Animal Care and Use Committee of the Mayo Clinic approved all experimental protocols and animal care, and the study conformed to the guidelines of the National Institutes of Health.

We used the rat model of monocrotaline (MCT)-induced PAH, in which a single MCT injection leads to severe PAH within 4 wks [22]. The pathological changes occurring in the lung vasculature as well PAH progression in this model have been well characterized [23,24]. Briefly, PAH was induced in male Sprague–Dawley rats weighing 250 g by a single i.p. injection of MCT (60 mg/kg). Control rats were injected with normal saline. The rats were sacrificed at 2 and 4 wks after injection. Prior to sacrifice, RV pressure was assessed using a 22G catheter connected to a pressure transducer and inserted into RV from abdominal cavity via the diaphragm. Subsequently, the heart was exposed by a midline thoracotomy and the heart and lungs were harvested. Tissue was then placed in Ca^{2+}-free Tyrode's solution (in mM: 135 NaCl, 4 KCl, 1 $MgCl_2$, 0.33 Na_2HPO_4, 0.03 EDTA, 10 glucose, 10 HEPES; pH 7.4) on ice. The main, right and left pulmonary arteries, and their lobar branches were isolated (with an intact endothelium) and cleaned of connective tissue. PA tissue was processed immediately as appropriate for studies described below. The RV was separated from the LV and the interventicular septum (IVS). The RV wt and RV to (LV + IVS) ratio (Fulton index) was used to assess RV hypertrophy. The left lung was placed in the neutral buffered 10% formalin and submitted to the Mayo Clinic Pathology Department for further processing.

2.2. Echocardiography

Echocardiography was performed at baseline, 2-wk, and 4-wk time points using the Vevo 770 ultrasound system (VisualSonics), as previously described [23]. Briefly, transthoracic 2D, M-mode, and pulsed wave (PW) Doppler were obtained with a 17 MHz broadband scan head. M-mode and 2D modalities were applied to measure RV free-wall thickness during end diastole using the right parasternal short-axis view. Main pulmonary artery diameter was measured at the level of pulmonary outflow tract during midsystole using superior angulation of the parasternal short-axis view. PW Doppler was used to obtain pulmonary artery flow velocity time integral (VTI) and to measure the pulmonary artery acceleration time (PAAT). For these measurements, the sample volume was centrally positioned within the main pulmonary artery, just distal to the pulmonary valve with orientation of the beam parallel to flow.

2.3. Pathology

The lung tissue preserved in the neutral buffered 10% formalin was processed by the Mayo Clinic Pathology Department. The tissue was embedded in paraffin, cut in 5 μm slices (thickness), and stained with either haematoxylin and eosin (H&E) or Van Gieson. Morphometric analysis of pulmonary vasculature was performed using ImageJ.

2.4. Mechanical studies

Mechanical studies were conducted following protocols established in our laboratory as described previously [25]. Briefly, isolated main pulmonary artery and the right and left pulmonary arteries were cut in 4 rings of ~2 mm (length). Rings isolated from the same animal were used for both mechanical and molecular studies. For the mechanical studies, rings were transferred to a vessel chamber containing continuously oxygenated physiological saline solution (PSS, in mM: 140 NaCl, 3.7 KCl, 2.5 $CaCl_2$, 0.81 $MgSO_4$, 1.19 KH_2PO_4, 0.03 EDTA, 5.5 glucose, 25 HEPES; pH 7.4), mounted on a DMT (Mulvany) 4-channel myograph system and stretched to Lo (the length for maximal force, which was determined in control preparations). Following the stretch to Lo, the rings were allowed to equilibrate for 30 min, and then the passive force was recorded. The rings were stimulated to contract with 80 mM KCl depolarization (in mM: 64.5 NaCl, 80 KCl, 2.5 $CaCl_2$, 0.81 $MgSO_4$, 1.19 KH_2PO_4, 0.03 EDTA, 5.5 glucose, 25 HEPES; pH 7.4) or 1 μM Ang II. Active force is defined as the increase in force above the resting passive force, and the force traces are the active component of force. To determine the sensitivity to ACh-mediated relaxation, the SM was depolarized (80 mM KCl) and after force reached a steady state, the dose response relationship for ACh-mediated relaxation was determined by sequential addition of a higher [ACh].

2.5. Determination of NM and SM expression

NM and SM myosin expression was determined using 2D SDS-PAGE as described [21,25]. We have previously demonstrated that this technique resolves the nonphosphorylated and phosphorylated SM myosin light chain (SM LC) and NM myosin light chain (NM LC) as four distinct spots [21]. Briefly, rings from the main PA, the right and left PAs and their lobar branches were manually homogenized in 2D gel extraction buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% 3–5.6 immobilized pH gradient (IPG) buffer and EDTA-free Protease Inhibitor and PhosStop Phosphatase Inhibitor (Roche, Indianapolis, Ind., USA)). The homogenates were cleared of lipids and extraneous salts using the 2D gel clean up kit (GE Healthcare). The acidic halves of 13-cm IPG DryStrip gels (pH 3–5.6 NL) were rehydrated in the presence of suitable amounts of sample in rehydration buffer solution (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% pH 3.5–5 IPG buffer, 0.002% bromophenol blue and 12 μM/ml Destreak Reagent) for at least 10 h in the 'face-down' mode on the Ettan IPG rehydration tray and then resolved by isoelectric focusing in the 'face-up' mode on an Ettan IPGphor III (GE Healthcare). Following isoelectric focusing, the gel strips were equilibrated in 6 M urea, 50 mM Tris–HCl, pH 6.4, 30% glycerol, 2% (w/v) SDS and 0.002% bromophenol blue, first containing 130 mM DTT for 15 min and then containing 135 mM iodoacetamide for 15 min before undergoing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation by molecular weight using the Bis-Tris buffering system with 12% gels (29:1). Subsequently, resolved 2D SDS-PAGE gels were silver stained. Gels were scanned using a Personal Densitometer SI, and the spots were quantified using ImageQuant TL software. The two spots closest to the anode (spots 1 & 2) represent the phosphorylated and nonphosphorylated NM LC and the two spots nearest the cathode (spots 3 & 4) represent the phosphorylated and nonphosphorylated SM LC. The expression of NM myosin is calculated as $[(1+2)/(1+2+3+4)] \times 100\%$], while LC phosphorylation for SM myosin is $(3/(3+4)) \times 100\%$ and LC phosphorylation for NM myosin is $(1/(1+2)) \times 100\%$ [21].

2.6. Immunoblotting

Western blots were used to determine protein expression in pulmonary arteries of normal versus rats with PAH as previously described [21,25]. Briefly, pulmonary arterial rings were homogenized in an SDS sample buffer. The total extracted protein was resolved by SDS-PAGE using the Bis-Tris buffering system with 8% gels (29:1). The actin band on Coomassie stained gels was used to normalize protein loading among samples. After SDS-PAGE separation, proteins were transferred onto a Hybond™ (GE Healthcare) membrane. MYPT1, LZ+MYPT1, SM myosin, NMIIA, NMIIB, and actin were visualized