Original Article
Pathologic role of peptidyl-prolyl isomerase Pin1 in pulmonary artery remodeling

Caixia Wu1*, Yanping Ha1*, Yuan Zou1*, Xiaomin Liao1, Shuya Zhang2,3, Xiaodian Zhang2,3, Rujia Li1, Jingci Xing1, Wei Jie1,2,3, Junli Guo2,3, Jingquan Li4, Zhihua Shen1

1Department of Pathology & Pathophysiology, School of Basic Medicine Sciences, Guangdong Medical University, Zhanjiang 524023, Guangdong, China; 2Key Laboratory for Tropical Cardiovascular Diseases Research of Hainan Province, The First Affiliated Hospital of Hainan Medical University, Haikou 571199, Hainan, China; 3Key Laboratory of Emergency and Trauma of Ministry of Education, Hainan Medical University, Haikou 571199, Hainan, China; 4Department of Oncology, The First Affiliated Hospital of Hainan Medical University, Haikou 570102, Hainan, China. *Equal contributors.

Received December 21, 2020; Accepted August 6, 2021; Epub October 15, 2021; Published October 30, 2021

Abstract: Peptidyl-prolyl isomerase Pin1 is crucial for cell proliferation, but its role in pulmonary artery remodeling (PAR) is unclear. In the present study, we aimed to evaluate the expression and contribution of Pin1 in PAR. Treatment with Pin1 inhibitor Juglone or Pin1-specific siRNAs ameliorated the expression of Pin1 and proliferating cell nuclear antigen (PCNA) in human pulmonary artery smooth muscle cells (PASMCs) in vitro, and Juglone treatment arrested the cell cycle at the G1 phase. Treatment with transforming growth factor β1 (TGF-β1) also enhanced Pin1 expression and PASMC proliferation. Immunohistochemical staining revealed that Pin1 and PCNA expression levels were increased and positively correlated with each other in PAR samples from humans and monocrotaline-treated Sprague-Dawley rats; these proteins were mainly localized in arteries undergoing remodeling, as well as inflammatory cells, and hyperplastic bronchial epithelial cells. Intraperitoneal injection of Juglone also led to morphologic and hemodynamic changes in PAR rats. Additionally, PAR rats displayed higher serum and lung TGF-β1 levels compared with controls, while administration of Juglone to PAR rats suppressed serum and lung TGF-β1 levels. The findings in this study suggest that TGF-β1 and Pin1 constitute a positive feedback loop, which plays an important role in the pathophysiology of PAR.

Keywords: Pin1, pulmonary artery remodeling, pulmonary artery smooth muscle cells, TGF-β1, cell proliferation

Introduction

Pulmonary artery hypertension (PAH) is a clinical syndrome characterized by a change in the structure and function of the pulmonary vascular bed; this syndrome is caused by a variety of factors. Notably, PAH leads to enhanced pulmonary vascular resistance, and eventually results in right heart failure and possibly death due to progressive pulmonary artery remodeling (PAR) [1, 2]. There have been multiple reports regarding abnormal vascular cells and molecular abnormalities involved in the occurrence and progression of PAH [3-10]; this information has aided in the prevention and treatment of PAH [11]. However, it remains associated with a high clinical mortality rate. Therefore, an improved understanding of the cells and molecules involved in the progression of PAH is necessary for its clinical treatment. Injection of monocrotaline (MCT) into Sprague-Dawley (SD) rats is a common laboratory method to establish PAR model [12], with the main mechanisms relating to endothelial-to-mesenchymal transition [13], endothelial injury, inflammatory response, and the abnormality in pulmonary artery smooth muscle cell (PASMCs) proliferation and apoptosis [14, 15].

PASMCs are an important component of the pulmonary artery. PASMC abnormalities (e.g., hypertrophy, proliferation, and extracellular matrix secretion) are important mechanisms that lead to PAR [14]. PASMCs are heterogeneous cells in which stress responses differ among species and arterial segments [16]. The
proliferation of PASMCs is presumably a key factor involved in PAR [14, 17, 18]. Increasing numbers of factors have been reported to closely associate with the proliferation of PASMCs that eventually causes PAR. These associations provide a theoretical and experimental basis for treatment and prevention of PAR. For example, the widely used phosphodiesterase type 5 inhibitor, sildenafil, the effects of which extend beyond the proliferation of anti-PASMCs, may improve clinical and hemodynamic outcomes in patients with PAH. However, its use was not associated with improvements in mortality or serious side effects [19]. Thus, to fully elucidate the pathways involved in PASMC proliferation, new targeted therapeutic drugs are needed.

Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1) is a member of the parvulin sub-family of peptidyl-prolyl cis/trans isomerases, and is critical in the regulation of mitosis [20]. Through isomerization of specific phosphorylated Ser/Thr-Pro motifs in many proteins that precisely regulate signal transduction, Pin1 extensively mediates cellular biology, including proliferation, metabolism, mobility, cell-cycle progression, survival, and apoptosis [21]. Abnormalities in Pin1 expression have been reported in many types of human cancers [22, 23]. Notably, a report by Lv et al. showed that Pin1 was crucial for vascular smooth muscle cell proliferation and apoptosis [24]. Furthermore, overexpression of Pin1 was reportedly associated with neointima formation [25, 26] and angiotensin II-induced abdominal aortic aneurysm formation [27], indicating that Pin1 has a role in vascular remodeling diseases. Because proliferation of vascular smooth muscle cells is a common pathophysiologic phenomenon in vascular remodeling diseases (e.g., stenosis and PAR), we hypothesized that Pin1 may be crucial in PAR.

In this study, we investigated the role of Pin1 in PAR. Through combined analysis of a rat model of MCT-induced PAR and samples from patients with PAR, we found that Pin1 was upregulated in lung tissues; it was predominantly expressed in PASMCs, proliferating bronchial epithelial cells, and infiltrating inflammatory cells. In vivo administration of the Pin1 inhibitor, Juglone, attenuated MCT-induced PAR and ameliorated hemodynamic dysfunction. Importantly, Pin1-induced PASMC proliferation was associated with changes in transforming growth factor β1 (TGF-β1) levels. Our results provide novel insight concerning Pin1 as a therapeutic target for PAR.

Materials and methods

Human lung tissues and animals

The lung tissues of six patients were acquired from the First Affiliated Hospital of Hainan Medical University. Among these patients, two had undergone lobectomy due to cancer; their adjacent paracancerous tissues were used. The remaining four patients had undergone lung transplantation due to PAR. Pulmonary hypertension is diagnosed when the resting mean pulmonary artery pressure (mPAP) measured by right heart catheterization is ≥25 mmHg [28]. All human lung tissues were paraffin-embedded, sectioned, and subjected to immunohistochemical staining. The demographic data of the patients are shown in Table S1. Adult SD rats (male, weighing 250±30 g) were purchased from the Guangdong Provincial Medical Laboratory Animal Center (Guangzhou, China). Specific pathogen-free-grade rats were fed normal chow and housed in a 12-hour light/dark-cycle animal room with free access to water and food. The use of human tissue samples in this study was approved by the Ethics Council of the Hainan Medical University for Approval of Research Involving Human Subjects (Approval number, HYLL-2018-175). Written informed consent was obtained from all patients prior to participation. All animal procedures were conducted in accordance with protocols approved by the Laboratory Animal Ethical Committee of Guangdong Medical University (Approval number, GDY2016003). The use of human lung tissues and SD rats complied with the tenets of the Declaration of Helsinki.

PAR model establishment

PAR was induced in rats by administration of MCT, as previously described [29, 30]. In this study, 17 SD rats were randomly divided into three groups. Briefly, rats received a single intraperitoneal injection of saline (control group, n = 5) or MCT (#C2401, Sigma-Aldrich, Shanghai, China; 50 mg/kg). Three weeks later, some MCT-treated rats received an intraperito-
neal injection of 3 mg/kg Juglone (5-Hydroxy-1,4-naphthoquinone; #420120, Sigma-Aldrich) dissolved in vehicle (saline and ethanol, 3:2, v/v) at 2-day intervals (group MCT+Juglone, n = 7), while some MCT-treated rats were injected with 5 ml/kg vehicle at 2-day intervals (group MCT, n = 5). After an additional 2 weeks, rats were subjected to the analyses described below. According to a previous report, the LD50 of Juglone was 4.2 mg/kg body weight when administered intravenously to mice [31]. Thus, this study protocol involved Juglone administration at 3 mg/kg every other day to minimize side effects. An illustration of the protocol for establishment of MCT-induced PAR in a rat model is shown in Figure 1.

Hemodynamic analysis, tissue processing, and morphologic evaluation

At the designated time point, rats were anesthetized with intraperitoneal injection of tribromoethanol (#T48402, Sigma-Aldrich, 250 mg/kg), following hemodynamic evaluation. Using right heart floating catheter technology, mPAP and right ventricular systolic pressure (RVSP) were recorded using a PowerLab system (Shanghai Ranger Apparatus Co., Ltd). Left ventricle blood was collected and the serum was used for TGF-β1 enzyme-linked immunosorbent assay (ELISA) analysis. Anesthetized rats were then sacrificed by CO₂ inhalation; their lungs and hearts were removed. Left lungs were dipped in liquid nitrogen and stored at -80°C for total RNA and protein isolation, while right lungs were used to prepare paraffin-embedded sections. The heart, right ventricle (RV), and left ventricle and septum (LV+S) were weighed; the right ventricular hypertrophy index (RVHI) was calculated. For morphologic evaluation, paraffin-embedded lungs and RVs were sectioned at 4-µm thickness. PAR was comprehensively evaluated through hematoxylin and eosin (H&E) staining and elastic fiber staining, as well as immunohistochemical staining for α-SMA, as described previously [32, 33]. For H&E staining, deparaffinized sections were stained with hematoxylin for 5 min at room temperature, then washed in running water for 5 min, 1% hydrochloric acid-ethanol (v/v) for 30 s and running water for 5 min. Sections were stained with 0.5% eosin solution for 1 min, following washed in running water for 30 min, dehydrated by 100% ethanol and transparentized with xylene. Sealed sections were used for microscopic observation. For elastic fiber staining, deparaffinized sections were stained with elastic fiber staining kit (#MST-8047, MXB Biotechnologies, Fuzhou, China), processed according to the illustration of the kit. For α-SMA immunohistochemical staining, the details are included in the below Immunohistochemical staining section. Pulmonary arteries were classified as non-muscular (0%-25% of the vessel circumference, using α-SMA staining), partially muscular (26%-75% of the circumference), or fully muscular (>75% of the circumference) [32]. The circumference of targeted arteries were measured using Image J software (https://imagej.nih.gov/ij/). For classification of the degree of pulmonary arterial muscularization, each paraffin-embedded rat lung was continuous intermittently sectioned. At least three slices were subjected to α-SMA or elastic fiber staining. Pulmonary arteries with diameter ≥70 μm throughout the section were calculated and mean values were recorded for each rat. RV cardiomyocyte size was measured using ImageJ software based on images of H&E-stained cross-sections. At least one field (original magnification, 40×) was observed for each rat.
Immunohistochemical staining

Immunohistochemistry was performed to determine target protein expression in samples from human and rat lung tissues, using previously described protocols [34, 35]. Briefly, deparaffinized sections were subjected to antigen recovery in heated citrate buffer (10 mmol/l, pH 6.0) for 2 min, then cooled sections were washed with 1×PBS, and incubated with 3% BSA for 20 min at room temperature and with primary antibodies overnight at 4°C, washed with 1×PBS three times, developed with PV9000 kit (#PV-9000, ZSBG-Bio, Beijing, China) and DAB chromogenic Kit (#ZLI-9018, ZSBG-Bio). Finally, sections were counterstained with hematoxylin and subjected to microscopic observation and photographed, the appearance of brown and yellow colors indicated positive results. Antibodies included Pin1 (#3721, Cell Signaling Technology, Danvers, MA, USA; 1:100 dilution), PCNA (#2586, Cell Signaling Technology, 1:100 dilution), TGF-β1 (#21891-1-AP, ProteinTech, Wuhan, China; 1:100 dilution), α-SMA (#sc-53142, Santa Cruz, Dallas, TX, USA; 1:100 dilution) and CD68 (#ZM-0060, ZSGB-BIO, Beijing, China; 1:100 dilution). Non-immune IgG was used as a negative control.

Cell culture and treatment

Human PASMCs (#PCS-100-023, American Type Culture Collection, Manassas, VA, USA) were gifted by Department of Cardiology, the Affiliated Hospital of Guangdong Medical University. Cells were maintained in Dulbecco’s modified Eagle’s medium (#SH30022.01, HyClone, Guangzhou, China) supplemented with 10% fetal bovine serum (HyClone), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂. The medium was changed at 2-day intervals. For drug treatment, human PASMCs were seeded on plates or dishes, followed by addition of Juglone in ethanol (IC50 is 16.47 µmol/l; final concentrations of 0, 0.02, 0.2, 2.0, and 20 µmol/l were used) and TGF-β1 (10 ng/ml; #10021, PeproTech China, Suzhou, China); cells were then cultured for an additional 24 or 48 hours before analysis. For experiments in vitro, PASMCs at passages 5-6 were used. All cells were routinely tested for Mycoplasma and Chlamydia contamination.

In vitro proliferation assays

Proliferation rates were determined using Cell Counting Kit-8 (CCK-8) assays, as described previously [36]. Briefly, human PASMCs were seeded in 96-well plates (3000 cells/well) for 0, 24, or 48 hours after the treatments mentioned above. Subsequently, a 100-µl mixture of CCK-8 reagent (#C0038, Beyotime Institute of Biotechnology, Nanjing, China) and Dulbecco’s modified Eagle’s medium (v/v = 1/10) was added per well. Four hours later, the optical density was measured using a microplate reader (Multiskan MKS, Thermo Fisher Scientific, Waltham, MA, USA) in dual wavelength mode (450/630 nm). Each treatment was repeated in five wells; the data were presented as mean ± standard error of the mean (SEMs) of three independent experiments.

siRNA transfection

Human PASMCs were seeded onto six-well plates. Three pairs of Pin1-specific short-interfering RNA (siRNA) sequences and a pair of negative control siRNA sequences were designed and synthesized by RiboBio (Guangzhou, China). The siRNA target sequences were as follows: siRNA-#1, CATTGGAAGACGCCTCGTT; siRNA-#2, GCAGCAGTGGTGGCAAAAA; and siRNA-#3, CCGGCATCCACATCATCCT. siRNAs (50 nmol/l) were transfected into human PASMCs using the riboFECT™ CP reagent (#C10511-05, RiboBio). Forty-eight hours later, cells were subjected to total RNA isolation, followed by quantitative reverse transcriptase (RT) polymerase chain reaction (PCR) analysis of mRNA levels of Pin1 and PCNA (described below). siRNA transfection efficiency was determined by observing Cy3 fluorescence under a fluorescence microscope (BX73, Olympus, Tokyo, Japan).

ELISA

Rat serum was subjected to ELISA analysis of TGF-β1 levels, in accordance with the manufacturer’s instructions (#MB100B, R&D Systems, Minneapolis, MN, USA).

Cell-cycle distribution analysis

Human PASMCs were seeded onto six-well plates in the following groups (as described above): MOCK, Juglone (2.0 µmol/l), and
Vehicle. Forty-eight hours later, harvested cells (>10^6 cells/sample) were treated with RNase and propidium iodide for 30 min at room temperature in the dark. The cells were then subjected to flow cytometric analysis of the cell-cycle distributions using a FACS Canto II (BD Bioscience, San Jose, CA, USA). Each treatment was repeated in three wells.

Immunofluorescence staining

Cells grown on coverslips were subjected to indirect immunofluorescence to identify the expression and location of Pin1, as described previously [37]. Antigenic epitopes were identified with FITC-labeled IgGs (#SA00003-8, ProteinTech) and nuclei were counterstained with DAPI (#D9542, Sigma-Aldrich). Images were captured using a laser scanning confocal microscope (TCS SP5 II; Leica Microsystems, Wetzlar, Germany).

RNA isolation and quantitative RT-PCR

Total RNA from lung tissues and human PASMCs was extracted with TRIzol reagent (#15596026, Thermo Fisher Scientific, Guangzhou, China). Total RNA (500 ng) was used to generate cDNA by means of an RT kit (#K1622, Thermo Fisher Scientific) with an oligo(dT18) primer. Quantitative PCR was conducted using a LightCycler480 II instrument (Roche China, Guangzhou, China), as described previously [34, 35]. Primers were synthesized by Sangon Biotech (Shanghai, China) as follows: human Pin1 forward, 5'-CGGGAGGAGGAGGAGTTCGGAGAGGAGTGAGT3'; human Pin1 reverse, 5'-TCAGTGCGGAGGATGATGT-3'; human PCNA forward, 5'-CTGACAAA-TGCTTGCTGACC-3'; human PCNA reverse, 5'-CTAGCTGGTTTGCGGCTTCAG-3'; human β-actin forward, 5'-TGACGTGGAGATCCGCAAAG-3'; human β-actin reverse, 5'-CTGGAAGGTGGACAGCGAGG-3'; rat Pin1 forward, 5'-GTCAGGAGAGGACTTTGAAT-3'; rat Pin1 reverse, 5'-ATTCTGTGCGTAGGATGATATGG-3'; rat PCNA forward, 5'-GGGTGAATTTTCTGCGAGT-3'; rat PCNA reverse, 5'-CAGTGGAGTGGCTTTTGTGA-3'; rat β-actin forward, 5'-CCCATCATATGGGAGTTAGC-3'; and rat β-actin reverse, 5'-TTTAATGTCACGCACGATTTC-3'. For PCR analyses, the expression level of β-actin served as a loading control. The 2^(-ΔΔCq) method was used to determine the expression levels of target genes.

Western blotting

Rat lung tissues and human PASMCs were homogenized with RIPA buffer (#P0013B, Beyotime Institute of Biotechnology) plus protein inhibitors. Protein homogenates were separated by 15% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with primary antibodies against Pin1 (#3721, Cell Signaling Technology, 1:1000), PCNA (#2586, Cell Signaling Technology, 1:1000), and β-actin (#sc-47778, Santa Cruz, 1:1000) in Tris-buffered saline with Tween plus 3% skim milk overnight at 4°C. Membranes were then washed twice in Tris-buffered saline with Tween and incubated with horseradish peroxidase-coupled secondary antibodies (#SA0001-2, ProteinTech, 1:3000,) for 2 hours at room temperature. Bands were visualized using enhanced chemiluminescence reagents (Thermo Fisher Scientific) and analyzed with a Tanon-4200 system (Tanon, Shanghai, China).

Statistical analysis

Statistical analyses were conducted using GraphPad Prism (Version 7.0, GraphPad Software, La Jolla, CA, USA). Data were expressed as mean ± SEM. Differences between two groups were compared using unpaired t-tests. Differences among multiple groups were compared using analysis of variance, followed by Tukey's multiple comparisons test. Pearson correlation analysis was used to analyze the correlation of Pin1 with PCNA in human and rat lungs. P<0.05 was considered significant.

Results

Pin1 was involved in PASMC proliferation in vitro

To investigate the role of Pin1 in human PASMC proliferation, we used a CCK-8 assay to test the effects of inhibition of Pin1 by Juglone on human PASMC viability. As shown in Figure 2A, Juglone suppressed viability of PASMCs in vitro in a concentration-dependent manner. At both protein and mRNA levels, we observed that Juglone inhibited Pin1 expression in PASMCs (Figure 2B and 2C), which paralleled the attenuated expression of PCNA, a marker for cell proliferation (Figure 2C). Analysis of the cell-
cycle distribution of PASMCs showed that administration of Juglone led to an increased G1 phase proportion and a decreased S phase proportion (Figure 2D). Finally, using Pin1-specific siRNA, we found that interference with Pin1 in PASMCs led to downregulation of PCNA (Figure 2E); the siRNA knockdown results were consistent with the CCK-8 assay results. These findings suggested that Pin1 was involved in human PASMC proliferation in vitro.

**Pin1 and PCNA expression in human pulmonary arteries**

To explore the expression patterns of Pin1 and PCNA in human lung tissues from patients with PAR, samples were collected from four patients (two with idiopathic pulmonary fibrosis, one with chronic obstructive pulmonary disease, and one with PAH) and two controls (Table S1). We performed H&E staining to assess pulmonary artery morphology and immunohistochemical staining to detect Pin1 and PCNA protein expression patterns. As shown in Figure 3A, patients with PAR underwent substantial pulmonary artery remodeling. Moreover, both Pin1 and PCNA proteins were present in human lung tissues, but the intensity of Pin1 protein staining in the arteries was weaker than the intensity of PCNA staining. The proportions of Pin1- and PCNA-positive cells were significantly lower in the control group than in the PAR group (Figure 3B). Notably, Pin1 protein expression levels were positively correlated with PCNA protein expression levels in human pulmonary arteries (Figure 3C).

**Expression levels and correlations of Pin1 and PCNA in PAR rats**

To further investigate the expression patterns of Pin1 and PCNA in arteries affected by PAR,
Figure 3. PAR morphology, Pin1 expression, and PCNA protein expression in human pulmonary arteries. A. Lung samples from two controls and four patients with PAR were subjected to H&E staining. Pin1 and PCNA protein
expression patterns were assessed by immunohistochemistry. Blue arrows indicate Pin1-positive staining and red arrows indicate PCNA-positive staining. Scale bar = 200 μm or 50 μm. B. Percentages of Pin1- and PCNA-positive cells in each group. Values were determined as the number of Pin1- or PCNA-positive cells/the number of nuclei × 100%; mean values were obtained from at least five representative arteries for each section. *p<0.05; #p<0.05. C. Pearson correlation analysis was used to evaluate the relationship between Pin1 expression and PCNA expression in human pulmonary arteries.

Figure 4. Representative images of pulmonary arterial remodeling (PAR) morphology in rats. H&E staining, elastic fiber staining, and immunohistochemical staining for α-SMA were used to assess PAR morphology in control, MCT-treated, and MCT+Juglone-treated rats. Scale bar = 200 μm.

we established a rat PAR model by MCT injection, in accordance with existing protocols [29, 30]. Both MCT- and MCT+Juglone-treated rats exhibited fatigue and reduced activity. The results of H&E, elastic fiber, and α-SMA staining analyses indicated that PAR had been successfully established in rats that received MCT injections (Figure 4, left and middle panels). We then used multiple methods to test the expression levels of Pin1 and PCNA in whole lung tissues. Notably, both mRNA and protein levels of Pin1 and PCNA were significantly higher in MCT-treated rats than in normal controls, while MCT+Juglone-treated rats exhibited reduced expression levels (Figure 5A and 5C). For the original bands for Pin1 protein expression, please reference to Figure S1. Morphologic changes were also ameliorated in MCT+Juglone-treated rats (Figure 4, right panels). At both mRNA and protein levels, Pin1 expression was positively correlated with PCNA expression in PAR rat lung tissues (Figure 5B and 5D). Furthermore, both Pin1 and PCNA proteins showed higher expression levels in PAR pulmonary arteries than in control pulmonary arteries. Importantly, the intimal endothelium, medial smooth muscle, and adventitial fibroblasts in pulmonary arteries expressed Pin1. Juglone treatment partially reduced this Pin1 staining intensity (Figure S2). Immunohistochemical staining analysis also revealed massive macrophage infiltration in lung tissues from MCT-
Pin1 in pulmonary artery remodeling

Figure 5. Pin1 and PCNA expression in rat lung tissues. mRNA and protein levels of Pin1 and PCNA were tested in whole lung tissues from control (n = 5), MCT-treated (n = 7), and MCT+Juglone-treated (n = 5) rats by real-time quantitative PCR and western blotting, respectively (A, C), *P<0.05; #P<0.05; **P<0.01; ##P<0.01. Pearson correlation analysis was used to evaluate the relationships between Pin1 and PCNA at both mRNA and protein levels in PAR rats (B, D).

Juglone ameliorated hemodynamic responses in PAR rats

Proliferation and hypertrophy of PASMCs mainly contribute to the remodeling of medium to large pulmonary arteries, but not distal alveolar arterioles [16]; thus, we analyzed the degree of muscularization within medium to large pulmonary arteries, particularly those with diameter ≥70 μm. As shown in Figure 6A, MCT treatment considerably enhanced the proportion of arteries with full muscularization, but reduced the proportion of arteries with partial muscularization, while Juglone treatment reduced the proportion of arteries with full muscularization induced by MCT. These trends were consistent with changes in % medial thickness (% MT, ratio of wall thickness to vessel lumen) of remodeled pulmonary arteries (Figure 6B). Further assessment of the extent of remodeling in PAR rats revealed that the RVSP, mPAP, RV/LV+S, and cardiomyocyte size were significantly greater in PAR rats than in controls. However, Juglone treatment effectively ameliorated the hemodynamic responses in PAR rats (Figure 6C-G), indicating that inhibition of Pin1 contributes to improved respiratory and cardiac function in PAR rats. In this work, wheat germ agglutinin (WGA) staining was also tried for measuring the size of cardiomyocytes but produced unsatisfactory results (data not shown).

TGF-β1 contributes to Pin1-mediated PASMC proliferation

To elucidate pathways involved in Pin1-mediated PASMC proliferation, we focused on TGF-β1 because of its critical contribution to treated rats (Figure S3). Some infiltrating macrophages and hyperplastic bronchial epithelial cells exhibited obvious Pin1 and PCNA protein expression in both human and rat PAR samples. Juglone treatment suppressed this expression pattern in the rat model (Figure S4).
Pin1 in pulmonary artery remodeling

We found that treatment with exogenous recombinant TGF-β1 effectively induced Pin1 expression in human PASMCs (Figure 7A). Additionally, treatment with exogenous recombinant TGF-β1 triggered PASMC proliferation in vitro (Figure 7B). Furthermore, TGF-β1 protein expression levels were suppressed in the serum of MCT+Juglone-treated rats, compared to MCT-treated rats (Figure 7C). Immunohistochemical staining revealed TGF-β1 protein in the arterial walls of MCT-treated rats (Figure 7D). Finally, TGF-β1 protein was localized in inflammatory cells and hyperplastic bronchial epithelial cells in PAR samples of rats (Figure S5). Overall, our results implied that TGF-β1 and Pin1 constitute a positive feedback loop in lungs with PAR.

Figure 6. Rat pulmonary artery muscularization and hemodynamics. (A) Degree of muscularization in medium to large pulmonary arteries with diameter ≥70 μm was assessed based on α-SMA and elastic fiber staining. The % MT (B), RVSP (C), mPAP (D), right ventricular hypertrophy index (RVHI, defined as RV/LV+S; %) (E), and cardiomyocyte size (F) were examined in rats (n = 5-7) at designated time points. *P<0.05; **P<0.01; ***P<0.001. (G) Representative images of cardiomyocytes. Scale bar = 100 μm.
Discussion

We found that Pin1 was required for human PASMC proliferation in vitro. Moreover, Pin1 was upregulated in the lung tissues of patients with PAR, particularly in pulmonary arterial walls, inflammatory cells, and hyperplastic bronchial epithelial cells. This elevated Pin1 expression was positively correlated with PCNA expression. These preliminary results suggest that Pin1 may participate in the pathophysiology of PAR. Through intraperitoneal injection of MCT, we successfully established a rat model of PAR. Using that model, we found that Pin1 and PCNA were increased in whole rat lungs with PAR, including in remodeled pulmonary arteries, hyperplastic bronchial epithelial cells, and infiltrating inflammatory cells. Notably, Pin1 expression was positively correlated with PCNA expression in PAR rats. The results of animal experiments were consistent with the outcome in specimens from patients with PAR. To further explore the role of Pin1 in PAR, we used Juglone, a standard Pin1 inhibitor, to eliminate the effects of Pin1 on MCT-treated rats. The results showed that Juglone-mediated inhibition of Pin1 effectively attenuated PAR, leading to improved hemodynamics. On the basis of our findings, targeted inhibition of Pin1 might be beneficial for treatment of PAR.

Many studies have proposed that Pin1 is required for cell proliferation, predominantly in the context of oncologic diseases [21, 22, 40]. Our current findings suggested that Pin1 and PCNA expression levels were greater in inflammatory cells and hyperplastic bronchial epithelial cells than in pulmonary arteries undergoing remodeling. Therefore, high expression of Pin1 may mediate PASMC, bronchial epithelium, and inflammatory response, thereby contributing to PAR pathogenesis. In this report, we focused on the effects of Pin1 on PASMC proliferation. In vitro experiments showed that Pin1 was
indeed required for PASMC proliferation, as inhibition of Pin1 by Juglone or by Pin1-specific siRNAs led to attenuated human PASMC viability and reduced PCNA levels. These in vitro results were consistent with the findings concerning Pin1 and PCNA expression in PAR in vivo. Notably, this Pin1 suppression was accompanied by substantial cell-cycle arrest at the G1 phase. Juglone is a classical Pin1 inhibitor that enhances the deterioration of peptidyl-prolyl cis/trans isomerase activity and transcription potential [41, 42]. Fila et al. reported that Juglone affected the cell-cycle progression of fibroblasts at several levels, including through tubulin aggregation and spindle assembly inhibition, but not mitotic arrest [43]. However, we found that Juglone disturbed mitosis in human PASMCs by arresting the cell cycle at the G1 phase. We also observed that the Pin1 protein was present in the cytoplasm before Juglone treatment, whereas it translocated into the nucleus following treatment. In some cancer cells, Pin1 protein has also been reported to exhibit cytoplasmic and nuclear distributions [44, 45]. In a zebrafish model, Pin1 mRNA and protein distribution patterns exhibited variation among developmental stages [46]. The biologic implications of differences in the cellular distribution of Pin1 have not been elucidated.

The mechanism mediating Pin1 expression is unclear. Because TGF-β1 signaling is a critical factor in PAR [47, 48], we focused on the role of TGF-β1. When human PASMCs were treated with exogenous TGF-β1, Pin1 expression increased; this was accompanied by enhanced cell viability. Because Juglone-mediated Pin1 inhibition led to attenuated PAR in MCT-treated rats, the current results suggested that TGF-β1-induced PASMC proliferation is at least partly mediated by Pin1. However, we also observed a reduction in the serum level of TGF-β1 in MCT+Juglone-treated rats. Thus, the inhibition of Pin1 may weaken TGF-β1 expression and secretion. TGF-β has three isoforms (TGF-β1, TGF-β2, and TGF-β3); this multifunctional set of peptides controls proliferation, differentiation, and other cellular functions. Many cell types, including immune cells and vascular wall cells, synthesize TGF-β; nearly all of these cells have specific receptors for TGF-β. A prior report indicated that TGF-β2 and TGF-β3, but not TGF-β1, played roles in PAR [49]. However, there is increasing evidence that TGF-β1 is upregulated in animal pulmonary arteries undergoing remodeling, as well as in cultured PASMCs [50-53]. In this study, we found that TGF-β1 was upregulated in lung tissues from PAR rats. TGF-β1 has biologic effects on pulmonary arteries, particularly with respect to PASMC proliferation, apoptosis, and migration; secretion of collagens and extracellular matrix proteins; and fibroblast transdifferentiation. These effects eventually lead to the onset of PAR.

A potential feedback loop may exist between Pin1 and TGF-β1 in rats with MCT-induced PAR, as evidenced by the reduction of TGF-β1 following inhibition of Pin1, as well as the enhancement of Pin1 expression following treatment with exogenous TGF-β1. Previous studies suggested that Pin1 could negatively influence TGF-β1 signaling [54, 55]. However, other studies have demonstrated contrasting findings [56-58]. Shen et al. reported that Pin1 was important for regulation of TGF-β1 production in eosinophils, an important process during airway remodeling [56]. In samples from patients with liver fibrosis, Pin1-mediated TGF-β1 expression was also observed [57]. However, TGF-β1 may regulate Pin1 expression, as Pin1 is considered a downstream effector of TGF-β1 signaling [59]. In this study, we also detected TGF-β1 and Pin1 expression in airway tissue undergoing remodeling. Thus, the interaction between Pin1 and TGF-β1 merits close attention. Notably, the TGF-β1/Pin1 positive feedback loop has been proposed to play a role in cardiac fibrosis [60]. Our current results imply the existence of a positive feedback loop between Pin1 and TGF-β1 in PAR rats; these findings may facilitate understanding of PAR pathogenesis.

Intraperitoneal injection of MCT is an effective method for establishment of PAR in a rat model. In addition to interference with pulmonary vascular endothelial cell function, the inflammatory response is considered another major mechanism in the context of MCT-induced PAR [15]. We also observed abundant inflammatory cells in lung tissues from rats with MCT-induced PAR. Importantly, many inflammatory cells (e.g., macrophages) express the Pin1 protein. Thus, Juglone-mediated inhibition of Pin1 may weaken the inflammatory response in MCT-induced PAR; further research is needed regarding this point.
There were some limitations in our study. First, the mechanism of Pin1 involvement in PAR was not clearly defined. For example, the contributions of changes in the inflammatory response, airway epithelial lesions, and pulmonary vascular endothelial cells in PAR after Pin1 inhibition were not investigated. Second, because Pin1 plays a critical role in regulating the stability of many proteins and in maintaining E3 ligase activity, more experiments should have been performed to identify key molecules potentially involved in PASMC proliferation. Third, Juglone exhibits low solubility and has potential side effects; novel Pin1 inhibitors are needed to overcome these problems. Finally, a tissue-specific Pin1 gene knockout mouse may have been a more powerful model for this research, and should be considered for future investigations.

Taken together, our findings indicated that Pin1 and PCNA were strongly expressed in PAR samples from both humans and rats; moreover, their expression levels were positively correlated. Pin1 was required for PASMC proliferation, whereas Juglone-mediated inhibition of Pin1 ameliorated PAR in rats and improved their cardiac function. Notably, we found that TGF-β1 contributed to Pin1 expression; MCT+Juglone-treated rats produced a lower TGF-β1 level than rats treated with MCT alone. Our findings constitute novel evidence concerning Pin1-induced PAR. The Pin1/TGF-β1 feedback loop in lung tissues offers a target for PAR treatment.

Acknowledgements

The authors thank Ryan Chastain-Gross, Ph.D., from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript. This work was supported by grants from the Key Research and Development Projects of Hainan Province (ZDYF2018102), the YangFan Plan of Guangdong Province (4YF16007G), and the CAMS Innovation Fund for Medical Sciences (2019-I2M-5-023). The funders had no role in the design of the study, the collection, analysis, and interpretation of data, or in writing the manuscript.

Disclosure of conflict of interest

None.

Address correspondence to: Zihhua Shen, Department of Pathology & Pathophysiology, School of Basic Medicine Sciences, Guangdong Medical University, No. 2, Eastern Wenming Road, Zhanjiang 524023, Guangdong, China. Tel: +86-759-2388587; E-mail: szh75@126.com; Jingquan Li, Department of Oncology, The First Affiliated Hospital of Hainan Medical University, No. 31, Longhua Road, Haikou 570102, Hainan, China. Tel: +86-898-66773409; Fax: +89-898-66773791; E-mail: jingquanli2012@163.com

References

Pin1 in pulmonary artery remodeling


[32] Zhu P, Huang L, Ge X, Yan F, Wu R and Ao Q. Transdifferentiation of pulmonary arteriolar endothelial cells into smooth muscle-like cells regulated by myocardin involved in hypoxia-in-


Pin1 in pulmonary artery remodeling


Table S1. Clinical parameters of patients who underwent lobectomy or lung transplantation

<table>
<thead>
<tr>
<th>Number</th>
<th>Patient identity</th>
<th>Final diagnosis</th>
<th>mPAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>Male in his 60 s</td>
<td>Mediastinal squamous cell carcinoma</td>
<td>28 mmHg</td>
</tr>
<tr>
<td>Case 2</td>
<td>Male in his 60 s</td>
<td>Lung adenocarcinoma</td>
<td>34 mmHg</td>
</tr>
<tr>
<td>Case 3</td>
<td>Male in his 60 s</td>
<td>COPD</td>
<td>36 mmHg</td>
</tr>
<tr>
<td>Case 4</td>
<td>Male in his 50 s</td>
<td>IPF</td>
<td>42 mmHg</td>
</tr>
<tr>
<td>Case 5</td>
<td>Male in his 50 s</td>
<td>IPF</td>
<td>51 mmHg</td>
</tr>
<tr>
<td>Case 6</td>
<td>Male in his 60 s</td>
<td>PAH</td>
<td>66 mmHg</td>
</tr>
</tbody>
</table>

mPAP, Mean pulmonary artery pressure; COPD, Chronic obstructive pulmonary disease; IPF, Idiopathic pulmonary fibrosis; PAH, Pulmonary arterial hypertension.

Figure S1. Original bands for western blotting. Bands in red frame were cropped and shown in main text.

Figure S2. Representative images of Pin1 and PCNA protein expression in pulmonary arteries of rat lungs. Pin1 and PCNA proteins were assessed by immunohistochemical staining. Antigens were developed with DAB kit and produced brown color. Arrows indicate the positive expression of Pin1 (blue) and PCNA (red).
Figure S3. Detection of macrophage in rat lungs using immunohistochemical staining of CD68. A. CD68 expression in lung tissues detected by immunohistochemical staining. Antigens for CD68 were developed with DAB kit and produced brown color. Scale bar = 100 μm or 50 μm. B. Quantitative analysis of CD68+ cells. *P<0.05, **P<0.01.
Figure S4. Representative images of Pin1 and PCNA protein expression in bronchial and interstitial of human and rat lungs. Pin1 and PCNA proteins were assessed by immunohistochemical staining. Antigens for Pin1 and PCNA were developed with DAB kit and produced brown color. Scale bar = 100 μm or 200 μm.
Figure S5. Detection of TGF-β1 protein in bronchial epithelial cells and inflammatory cells infiltrated in rat lungs through immunohistochemical staining. Antigens were developed with DAB kit and produced brown color. Scale bar = 100 μm.