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Isorhynchophylline protects against pulmonary arterial hypertension and suppresses PASMCs proliferation

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ABSTRACT

Increased pulmonary arterial smooth muscle cells (PASMCs) proliferation is a key pathophysiological component of pulmonary vascular remodeling in pulmonary arterial hypertension (PAH). Isorhynchophylline (IRN) is a tetracyclic oxindole alkaloid isolated from the Chinese herbal medicine *Uncaria rhynchophylla*. It has long been used clinically for treatment of cardiovascular and cerebrovascular diseases. However, very little is known about whether IRN can influence the development of PAH. Here we examined the effect of IRN on monocrotaline (MCT) induced PAH in rats. Our data demonstrated that IRN prevented MCT induced PAH in rats, as assessed by right ventricular (RV) pressure, the weight ratio of RV to (left ventricular + septum) and RV hypertrophy. IRN significantly attenuated the percentage of fully muscularized small arterioles, the medial wall thickness, and the expression of smooth muscle α-actin (α-SMA) and proliferating cell nuclear antigen (PCNA). In vitro studies, IRN concentration-dependently inhibited the platelet-derived growth factor (PDGF)-BB-induced proliferation of PASMCs. Fluorescence-activated cell-sorting analysis showed that IRN caused G0/G1 phase cell cycle arrest. IRN-induced growth inhibition was associated with downregulation of Cyclin D1 and CDK6 as well as an increase in p27Kip1 levels in PDGF-BB-stimulated PASMCs. Moreover, IRN negatively modulated PDGF-BB-induced phosphorylation of PDGF-Rβ, ERK1/2, Akt/GSK3β, and signal transducers and activators of transcription 3 (STAT3). These results demonstrate that IRN could inhibit PASMCs proliferation and attenuate pulmonary vascular remodeling after MCT induction. These beneficial effects were at least through the inhibition of PDGF-Rβ phosphorylation and its downstream signaling pathways. Therefore, IRN might be a potential candidate for the treatment of PAH.

1. Introduction

Pulmonary artery hypertension (PAH) is a progressive condition with poor prognosis, which is characterized by a sustained elevation of pulmonary arterial pressure (PAP). This increase in pressure is due to vasoconstriction and remodeling of small pulmonary arteries, causing pulmonary vascular resistance, right ventricular failure and ultimately death [1,2]. Although major advances in the understanding and treatment of the disease have been achieved over the last 20 years, the pathogenesis of PAH remains unclear [3]. Idiopathic PAH, familial PAH, and PAH associated with other conditions such as congenital heart disease and connective tissue disease display similar pathological changes and are considered to share common pathogenic mechanisms. Studies have demonstrated that pulmonary arterial smooth muscle cells (PASMCs) proliferation is a key pathophysiological component of pulmonary vascular remodeling in PAH [4].

Many factors, like inflammation, cytokines, and reactive oxygen species can promote PASMCs proliferation. Recently, plasma platelet-derived growth factor (PDGF)-BB levels were found to be higher in PAH patients than in healthy controls [5]. PDGF has been implicated in the abnormal proliferation of PASMCs, which induces the obliteration of resistant vessels and muscularization of arterioles in association with the pathophysiology of PAH [6,7]. Schermuly has

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demonstrated that inhibiting PDGF signaling pathway can reverse pulmonary vascular remodeling and cor pulmonale in severe experimental pulmonary hypertension regardless of the initiating stimulus [8].

Isorhynchophylline (IRN) is a tetracyclic oxindole alkaloid isolated from the Chinese herbal medicine *Uncaria rhynchophylla*, which has long been used clinically for treatment of cardiovascular and cerebrovascular diseases [9–11]. It is a well-known natural calcium antagonist with antioxidant, anti-inflammatory, anticoagulation and anti-proliferation activities [12–14]. IRN could protect neural and neuroglial cells against amyloid-β-induced neurotoxicity by inhibiting oxidative stress and intracellular calcium overloading [12,15].Yuan has demonstrated that IRN concentration-dependently attenuated LPS-induced production of proinflammatory cytokines [13]. IRN can cause cell cycle arrest and is effective against Angiotension II induced proliferation in rat vascular smooth muscle cells [14]. IRN also participates in the regulation of MAPKs, NF-κB and Akt signal transduction [13,16]. However, the effects of IRN on PAH and the related molecular mechanisms remain unclear. In the present study, we demonstrate that oral administration of IRN prevents PASMCs proliferation and attenuates PAH in MCT induced rats. IRN prevents PASMCs proliferation by reducing Cyclin D1 expression and increasing p27Kip1 accumulation in vitro. The mechanism by which IRN confers its beneficial effects on PASMCs may be the blockade of PDGF-Rβ phosphorylation and its downstream signal transduction.

2. Materials and methods

2.1. Materials

IRN was purchased from Shanghai Huicheng Biotechnology Co. Ltd. Recombinant human PDGF-BB was purchased from R&D Systems Inc (Minneapolis, MN). Antibodies that recognize the total levels and phosphorylation of PDGFR, ERK1/2, Akt, GSK3β and STAT3 were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against proliferating cell nuclear antigen (PCNA), CDK6, P27Kip1 and Cyclin D1 were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Cell Proliferation Reagent WST-1 and BrdU ELISA kits were obtained from Roche Diagnostics (Mannheim, Germany). Monocrotaline, Antibodies against smooth muscle α-actin (α-SMA) and other reagents were purchased from Sigma–Aldrich.

2.2. Monocrotaline (MCT)-induced PAH model

The study was approved by the Ethical Committee of Qilu Hospital of Shandong University. All protocols of the animal experiments were performed under institutional guidelines of animal welfare. Male Wistar rats at age 5 weeks were used for PAH model procedure, as we have described previously [17,18]. Maintain feed or maintain feed containing 0.1% IRN was administered to control or MCT-induced PAH rats. IRN treatment was started immediately after MCT injection.

2.3. Measurements of aortic pressure and RV hemodynamics

At the end of the study protocol, rats were first anesthetized with 1.5% isoflurane. Aortic pressure and RV hemodynamics were measured as we have described previously [17].

2.4. Histological staining

Heart, lung, and other major organs were harvested and weighed. HE and α-SMA immunohistochemical staining was performed to show the condition of pulmonary vascular remodeling. RV was stained with Sirius Red (Sigma) for detection of fibrosis, and HE staining to assist in the evaluation of cardiac myocyte size. The percent volume fibrosis was determined using the method described previously [19,20]. Semiquantification of pulmonary vascular muscularization as described previously [17].

2.5. Cell proliferation, DNA synthesis assay and flow cytometric analysis

Human PASMCs were purchased from ATCC. Cells from passages 3 to 6 were used in all experiments. Cells at 60% confluence were synchronized for 24 h and preincubated with IRN for 1 h, then subsequently treated with PDGF-BB (20 ng/ml) for 48 h. Cell proliferation and DNA synthesis were measured using WST-1 and BrdU incorporation assay kits according to the manufacturer's instructions [21]. Cell viability was evaluated by counting the number of cells that excluded the trypan blue dye. Cell cycle was detected through flow cytometry.

2.6. Western blotting

PASMCs were treated with IRN (25 μM) for 2 h prior to incubation with PDGF-BB (20 ng/ml) for the indicated time. Immunoreactive bands were visualized by blotting with antibodies against α-SMA, PCNA, Cyclin D1, CDK6, P27Kip1, PDGFR, Akt, GSK3β, ERK1/2, STAT3.

2.7. Statistical analysis

The results were expressed as mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. A value of $P < 0.05$ was considered significant.

3. Results

3.1. IRN attenuated the MCT-induced increase of RV pressure

IRN-treated rats grew and developed normally. There were no significant differences in terms of body weight gain (Fig. 1A), lung weight, left ventricular (LV) weight, RV pressure, and the ratio of RV/LV+S weight between IRN and Vehicle-treated rats under control conditions after 3-week's treatment (Fig. 1B and C and Suppl Tables 1 and 2).

MCT injection caused a significant increase of RV pressure in both Vehicle and IRN-treated rats, whereas MCT caused a significantly greater increase of RV systolic pressure in Vehicle-treated rats (Fig. 1B and Suppl Table 2), indicating attenuated PAH in IRN-treated rats.

3.2. IRN alleviated the MCT-induced increase of RV hypertrophy and fibrosis

IRN had no significant effect on LV or RV hypertrophy in rats under control conditions. Consistent with the significantly greater increase of RV systolic pressure in Vehicle-treated rats after MCT, Vehicle-treated rats had significantly greater increases of RV weight and the ratio of RV/LV+S weight in response to MCT (Fig. 1C and D, Suppl Table 1), indicating that IRN alleviated MCT-induced RV hypertrophy. Histological analysis indicated that MCT caused a significantly increase of RV fibrosis and cardiac myocyte cross sectional area (Fig. 1E and F, Suppl Fig. 1).

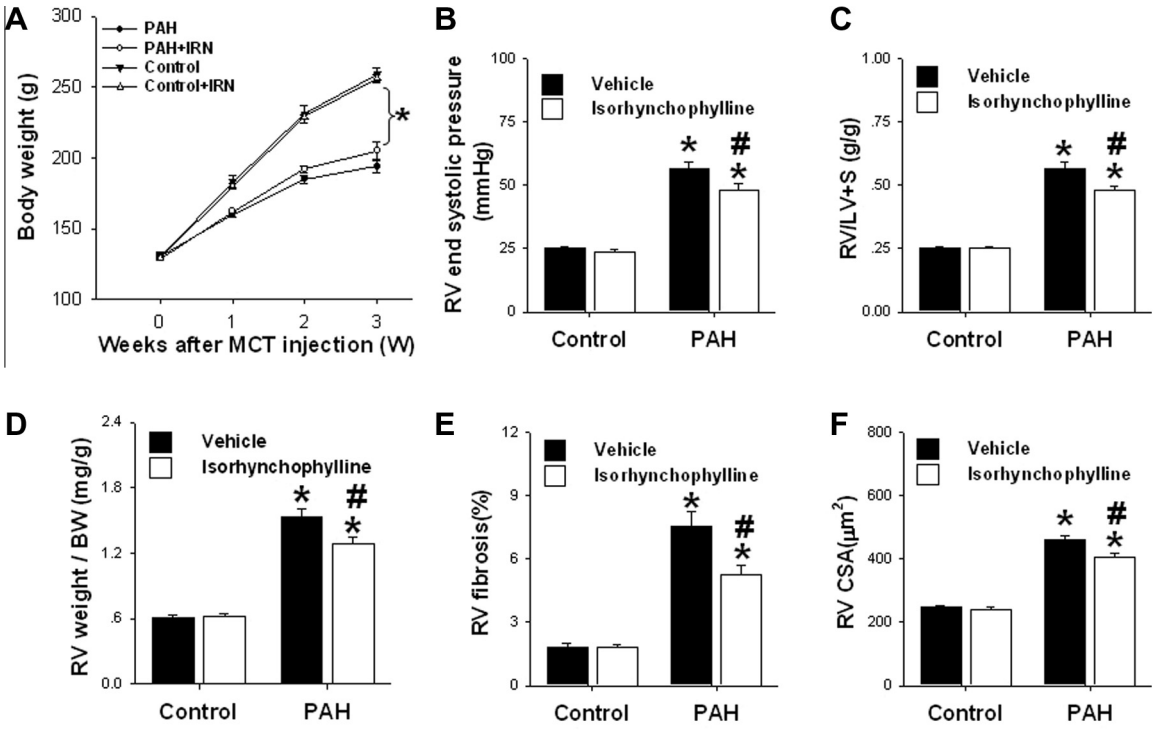

Fig. 1. IRN had no significant effect on growth but attenuated MCT-induced increase of RV pressure. IRN had no effect on growth under both control conditions and after MCT injection respectively (A). IRN had no effect on RV pressure under control conditions but alleviated the MCT-induced increase of RV pressure (B). IRN attenuated the MCT-induced increase of the RV hypertrophy as indicated by the ratio of RV to LV+S weight and the ratio of RV to body weight (C,D), the RV fibrosis (E), and RV cardiac myocyte hypertrophy as indicated by an increase of the cross-sectional area of cardiac myocytes in rats (F). $^{*}P < 0.05$ vs. sham control; $^{\#}P < 0.05$ vs. corresponding vehicle rats.

3.3. IRN attenuated the MCT-induced pulmonary vascular remodeling and PASMCs proliferation

To determine the effect of IRN on pulmonary vascular remodeling, we determined the percentage of non-muscularized (NM), partially muscularized (PM), and fully muscularized small arterioles (FM) in rats with and without MCT injection. MCT caused increasing of fully muscularized small arterioles in both Vehicle rats and IRN rats, but these increases were significantly lower in IRN rats (Fig. 2A). Meanwhile, MCT also caused decreases of NM small arterioles in both vehicle and IRN rats, but these decreases were significantly lower in IRN rats. In addition, IRN treated significantly alleviated MCT-induced medial wall thickness (Fig. 2B) of small arteries (50–200 μm). Together, these data indicate that IRN significantly attenuated MCT-induced pulmonary vascular remodeling in rats.

To assess PASMCs proliferation, lung sections were stained with the anti-α-SMA antibody. MCT caused increases of proliferation of vessel smooth muscle cells, but these increases were significantly reduced in the IRN rats (Fig. 2C). Meanwhile, Western blot demonstrated that IRN significantly diminished MCT-induced increase in the protein expression of α-SMA and PCNA in pulmonary arteries (Fig. 2D). Together, these data indicate that IRN significantly attenuated MCT-induced PASMCs proliferation in rats.

3.4. IRN suppressed PDGF-BB-stimulated PASMCs proliferation

To further confirm the in vivo results, WST-1 cell proliferation assay was performed to evaluate the in vitro effect of IRN on PASMCs proliferation. As expected, PASMCs proliferation was significantly stimulated with PDGF-BB (20 ng/ml) for 48 h compared with untreated controls. IRN inhibited PDGF-BB induced PASMCs proliferation in a concentration-dependent manner (Suppl Fig. 2A). The inhibitory effect of IRN on DNA synthesis was illustrated by BrdU incorporation assay. Supplemental Fig. 2B showed that PDGF-BB treatment increased DNA synthesis in PASMCs, while IRN concentration-dependently inhibited DNA synthesis. Trypan blue exclusion showed that IRN did not induce cell necrosis of PASMCs up to 48 h (Suppl Fig. 2C).

3.5. IRN arrested PDGF-BB-stimulated cell cycle in the G0/G1 phase

The effects of IRN, PDGF-BB, or the combination on the percentage of cell population distribution were monitored by flow cytometry after 24 h treatment. As revealed in Fig. 3A, PDGF-BB alone increased the S phase cell population ratio ($P < 0.05$). 25 μM IRN decreased the numbers of S phase cells and increased the fraction of G0/G1 phase cells in PDGF-BB treated PASMCs, indicating that IRN prevented PDGF-BB caused cell cycle entry/progression in the G0/G1 phase.

3.6. IRN inhibited Cyclin D1, CDK6 expression and p27Kip1 degradation

Cyclins, cyclin dependent kinases (CDKs) and CDK inhibitors play major roles in cell cycle regulations. The expression of cyclin-CDK complexes and p27Kip1 were analyzed to characterize IRN caused cell cycle arrest. As shown in Fig. 3B, the expression of Cyclin D1 and CDK6 was induced by PDGF-BB, while IRN treatment significantly decreased expression of these molecules. Western blotting analysis showed that p27Kip1 was constitutively expressed in serum-starved quiescent PASMCs and was downregulated following PDGF-BB stimulation. In contrast, pretreatment with IRN partly restored p27Kip1 expression (Fig. 3B and C).

3.7. Molecular mechanisms involved in IRN inhibition of PASMCs proliferation

PDGFR signaling pathway is important in pulmonary vascular remodeling. To further investigate the mechanisms by which IRN inhibits PASMCs proliferation, we examined the expression of

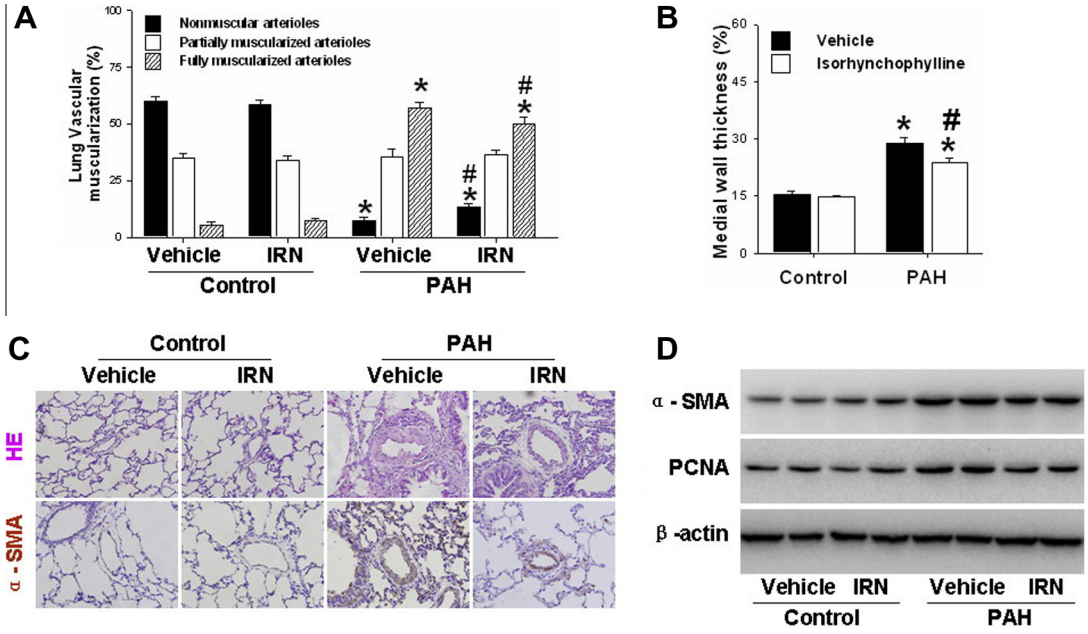

Fig. 2. IRN attenuated MCT-induced pulmonary vascular remodeling and PASMCs proliferation. Distribution of nonmuscular, partially muscular, and fully muscularized small arterioles in control rats and MCT-induced PAH rats. IRN significantly attenuated MCT-induced pulmonary vascular muscularization (A). IRN significantly attenuated MCT-induced medial wall thickness of small arteries (B). HE and α-SMA staining showed that IRN significantly suppressed pulmonary vascular remodeling (C). IRN significantly attenuated MCT-induced increase of the α-SMA and PCNA (D). $^{*}P < 0.05$ vs. sham control; $^{\#}P < 0.05$ vs. corresponding vehicle rats.

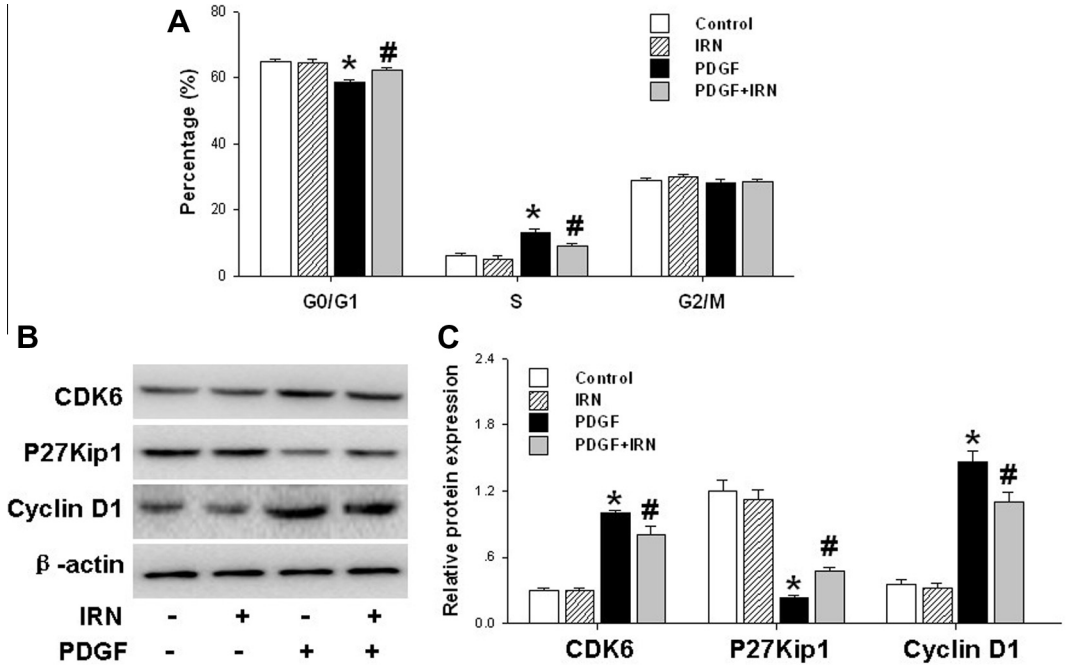

Fig. 3. IRN prevented cell cycle progression in PASMCs induced by PDGF-BB. PASMCs were grown in IRN (25 μM) with or without PDGF-BB (20 ng/ml) for 24 h, quantification of PASMCs in the G0/G1, S, and G2/M phases was determined by flow cytometric evaluation (A). Cell cycle related proteins were measured with western blot analysis, β-actin detection served as a loading control (B and C). $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. PDGF alone.

PDGFRβ phosphorylation induced by PDGF-BB. As shown in Fig. 4, PDGF-BB induced a rapid and sustained phosphorylation of PDGF-Rβ. Pre-treatment with IRN significantly inhibited PDGF-Rβ phosphorylation. The inhibitory effect was not due to the reduction of total protein levels (Fig. 4A).

Akt/GSK3β signal transduction pathways are reported to critically involve in PASMCs proliferation [29]. In this study, we found that PDGF-BB induced phosphorylation of Akt and GSK3β, while IRN inhibited PDGF-induced activation of the Akt/GSK3β pathways. The observed inhibitory effects of IRN on PDGF-induced Akt/GSK3β activation were not due to decreases in total protein levels (Suppl Fig. 3).

ERK1/2 and STAT3 are downstream components of PDGFR, which are important in the regulation of cell growth and differentiation [30]. Next, we examined the effects of IRN on PDGF-BB-induced activation of ERK1/2 and STAT3. Accordingly, PASMCs

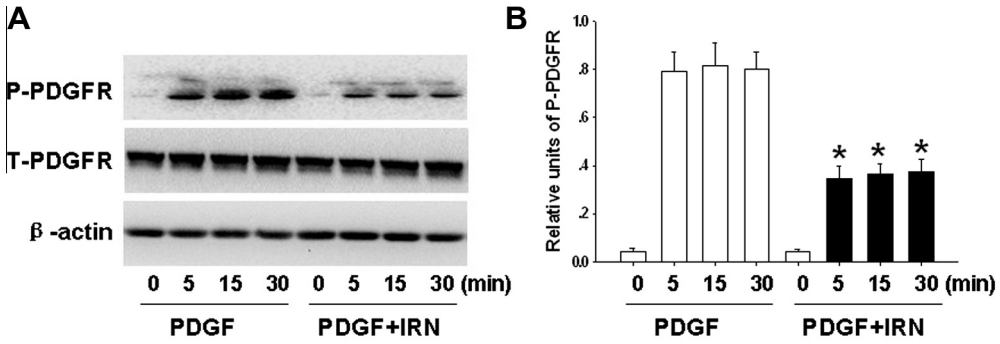

Fig. 4. Inhibitory effects of IRN on PDGF-Rβ activation in PDGF-BB-stimulated PASMCs. Serum-starved PASMCs were stimulated with PDGF-BB(20 ng/ml) for an indicated time with or without IRN (25 μM). The total and phosphorylated protein levels of PDGF-Rβ were determined with western blot analysis (A). The ratio of P-PDGFR/PDGFR was determined with western blot analysis (B). $^{*}P < 0.05$ vs. PDGF alone.

stimulated with PDGF-BB produced strong ERK1/2 and STAT3 activation, as demonstrated by the phosphorylation of ERK1/2 and STAT3. Pre-treatment with IRN significantly alleviated ERK1/2 and STAT3 phosphorylation (Suppl Fig. 3). These results indicated that IRN could inhibit PDGF-Rβ phosphorylation and its downstream components, such as Akt/GSK3β, ERK1/2 and STAT3 phosphorylation in the same pattern.

4. Discussion

Here we characterized the biological effect of IRN on PASMCs in vitro and in vivo models. The results from our current study demonstrated that there were PASMCs proliferation after MCT-injection, and IRN administration prevented increase of RV pressure, the ratio of RV/LV+S, muscularization of pulmonary arterioles and PASMCs proliferation in rat model of MCT-induced PAH. We provide evidence that IRN inhibited PDGF-BB-induced DNA synthesis in a concentration-dependent manner. IRN downregulated Cyclin D1 and stabilized p27Kip1, subsequently, prevented the proliferation of PASMCs in vitro. The ability of IRN to reduce PASMCs proliferation was mediated by inhibition of PDGF-Rβ phosphorylation and its downstream Akt/GSK3β, ERK1/2 and STAT3 signaling pathways.

In the past decade, the pharmacological interventions for the treatment of PAH have increased dramatically. However, these therapies are mainly focus on vasoconstriction and endothelia dysfunction. Little proof is shown that these drugs have any direct effect or have the capacity to reverse the proliferation changes in PAH. IRN, the major and bioactive components extracted from the Chinese herb medicine *U. rhynchophylla*, has been extensively used by Chinese practitioners for treatment of cardiovascular and cerebrovascular diseases, such as hypertension, arrhythmia, vascular dementia, and sedation [10,11]. A growing body of evidence demonstrates that IRN has anti-inflammatory, anti-oxidative stress and anti-proliferation effect. Zhang et al. found that IRN was effective in blocking Angiotension II induced proliferation via inducing cell cycle arrest in rat vascular smooth muscle cells [14]. So, it is postulated that IRN's pharmacological action might also inhibit the development of the PAH.

In this study, we found that IRN not only alleviated MCT-induced RV hypertension, decreased muscularization of pulmonary arterioles, but also decreased the expression of α-SMA and PCNA in pulmonary arterioles. Excessive PASMCs proliferation in the pulmonary arterial walls plays a pivotal role in the development of PAH [4]. PDGF was found to be implicated in abnormal PASMCs proliferation [6]. Our in vitro study showed IRN inhibited PASMCs proliferation and PDGF-BB-induced DNA synthesis in a concentration-dependent manner.

Cell proliferation is primarily controlled by regulation of the cell cycle. As is expected, flow cytometry analysis showed that IRN led to G1 phase arrest, exhibiting remarkably decreased proportion of cells in S phase and significant accumulation of cells in G0/G1 phase. Cell cycle progression is critically regulated by sequential activation of cyclins and cyclin-dependent kinases (Cdks). Cyclin D1 is a key protein that regulates cell cycle G1 phase. It is a more sensitive indicator than other cyclins. CDK6 is Cyclin D1 binding partners, and activated Cyclin D1/CDK6 complex could induce the expression of target genes essential for S phase entry [22]. p27Kip1, one of the CDK inhibitors can inhibit a wide spectrum of cyclin/CDK complexes to arrest cell growth [23]. In this study, IRN treatment significantly decreased Cyclin D1/CDK6 and restored p27Kip1 expression, consistent with the inhibitory effect of IRN on PASMCs proliferation. These results suggest that the anti-proliferative activity of IRN is accomplished by cycle arrest.

PDGF is synthesized by many different cell types, and has the ability to induce the proliferation of smooth muscle cells (SMCs) and fibroblasts. Perros has demonstrated that PDGF and PDGFR mRNA levels were higher in PAH patients than in healthy controls [5]. Schermuly has found that inhibiting PDGF signaling pathway can reverse pulmonary vascular remodeling and cor pulmonale [8]. Our results demonstrated that IRN inhibited the phosphorylation of PDGF-Rβ, which suggest that IRN's antiproliferation effect on PASMCs is considered to be at least partly due to the inhibition of the kinase activity of PDGFR.

It is known that binding of PDGF-BB to PDGF receptor leads to phosphorylation of PDGF-Rβ, then leads to activation of PI3K/AKT, Jak/STAT, ERK1/2 signaling pathways [24]. In the present study, PDGF-BB activated PDGF-Rβ and enhanced the phosphorylation of Akt/GSK3β, while IRN pre-treatment reversed these changes. GSK3β is constitutively active in unstimulated cells, and is inactivated during stimulation. PI3K/AKT induced GSK3β phosphorylation inhibits its activity. GSK3β has inhibitory role in Wnt, c-Myc, endothelin and VEGF signaling pathway [25]. It can directly regulate expression of Cyclin D1, independent of β-catenin [26]. In many types of cancer and vascular remodeling models, GSK3β is phosphorylated. In a recent study, Sklepkiewicz found that GSK3β was upregulated in PAH lung homogenates, and PDGFR inhibitor, lmatinib, attenuated PDGF-BB induced GSK3β phosphorylation in PASMCs [27]. IRN, as an inhibitor of PDGFR in this study, was hypothesized to suppress PASMCs proliferation by Akt/GSK3β pathway. Consistent with previous studies, PDGF-BB induced rapid activation of Akt/GSK3β [7]. Pretreatment with IRN attenuated phosphorylation of Akt and its substrate GSK3β.

ERK1/2 and STAT3 signaling pathways have been reported to participate in PDGF-BB-induced biological effects in VSMCs [28]. STAT3 can directly regulate Cyclin D1 transcription [29]. Paulin found that inhibition of the inappropriate activation of STAT3

could decrease PASMCs proliferation through NFATc2 inhibition [30]. Levels of ERK1/2 and STAT3 phosphorylation were increased by PDGF-BB in this study and IRN attenuated PDGF-BB-stimulated phosphorylation of these proteins. Therefore, these findings indicate that Akt/GSK3β likely synergizes with ERK1/2 and STAT3 signaling pathways to regulate PASMCs proliferation.

Taken together, the present study presents evidences that IRN can inhibit PASMCs proliferation and attenuate PAH in MCT induced rats. The antiproliferation activity of IRN is mediated by decreasing Cyclin D1 expression and increasing p27Kip1 accumulation via blockade of PDGF-Rβ phosphorylation, and its downstream Akt/GSK3β, ERK1/2 and STAT3 signal transduction. Our observation may explain in part mechanistic basis for the treatment of PAH. However, the safety and efficacy of IRN in human PAH should be evaluated in future.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.044.

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