Original Article
Protective role of metformin against chronic experimental pulmonary arterial hypertension

Dong Han, Wei Feng, Cui Zhai, Yanting Zhu, Xinming Xie, Lu Liu, Yang Song, Lan Yang, Manxiang Li

Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Xi’an Jiaotong University, Xi’an 710061, Shaanxi, PR China

Received December 7, 2015; Accepted May 17, 2016; Epub July 15, 2016; Published July 30, 2016

Abstract: Background: Our previous study has indicated that activation of AMPK inhibits the proliferation of rat pulmonary artery smooth muscle cells (PASMCs) in vitro through inhibiting the expression of S phase kinase-associated protein 2 (Skp2), which in turn up-regulates the p27 expression. In the present study, we intended to determine whether similar mechanisms have been involved in rat PAH model. Methods: Rat pulmonary arterial hypertension (PAH) model was established by intraperitoneal injection of monocrotaline (MCT). Metformin was administered to activate AMPK. Parameters including the right ventricle systolic pressure (RVSP), the right ventricular hypertrophy (RVH) and the percentage of medial wall thickness were used to evaluate the development of PAH. Immunoblotting was used to determine the phosphorylation and expression of AMPK, and expression of Skp2 and p27. Results: Metformin significantly decreased the RVSP and inhibited the RVH in MCT-induced rat PAH model, and partially inhibited the pulmonary vascular remodeling. These effects were coupled with the decrease of Skp2 and increase of p27 expressions as well as the activation of AMPK. Conclusions: Metformin benefits PAH by inhibiting proliferation of PASMCs and reducing pulmonary vascular remodeling. The present study suggests that metformin might have potential value in clinical treatment of PAH.

Keywords: Metformin, pulmonary arterial hypertension, AMPK, smooth muscle cell, Skp2, P27

Introduction
Pulmonary artery hypertension (PAH) is a common clinical syndrome, which is characterized by functional and structural changes of pulmonary arterial. These changes usually lead to the increase of pulmonary vascular resistance (PVR) and the development of pulmonary artery pressure, finally the development of right ventricle hypertrophy (RVH), heart failure even death. The pathological mechanisms underlying the development of pulmonary hypertension include persistent pulmonary vasoconstriction, vascular remodeling, and thrombosis in situ [1]. Vascular remodeling is extremely critical among these pathogeneses, which is commonly caused by intima thickness and media hyperplasia, and pulmonary arterial smooth muscle cells (PASMCs) proliferation/migration plays a prominent role in this process [2]. Therefore, it is important to explore the molecular mechanisms responsible for PASMCs proliferation to prevent or to reverse pulmonary vascular remodeling and thus to treat PAH.

AMP-activated protein kinase (AMPK) is a sensor of energy status that maintains cellular energy homeostasis [3]. It arose very early during eukaryotic evolution, and its ancestral role may have been in the response to starvation. Although best known for its effects on metabolism, AMPK has many other functions, including regulation of mitochondrial biogenesis and disposal, autophagy, cell polarity, and cell growth and proliferation [4]. Both tumor cells and viruses establish mechanisms to down-regulate AMPK, allowing them to escape its restraining influences on growth. Recent studies have shown that AMPK phosphorylation is reduced in pulmonary artery endothelial cells (PAEC) of utero pulmonary hypertension (IPH) [5] and in PASMCs of idiopathic PAH (IPAH) [6]. Further studies indicated that enhancing AMPK phosphorylation improves IPH-PAEC angiogenesis [5] and suppresses PASMCs proliferation in IPAH [6]. Metformin has also been shown to inhibit the development of PAH in animal model by suppressing vascular remodeling [7]. Yet, the detailed molecular mechanisms underlying
metformin inhibition of pulmonary vascular remodeling are still unclear.

It has been demonstrated that p27 plays a critical role in regulating cell cycle progression in mammalian cells [8, 9]. p27 as a cyclin-dependent kinases (CDK) inhibitor binds to and inhibits the function of cyclin E/CDK2 complex, and further blocks cell cycle progression from G1 into S phase and suppresses cell proliferation [10]. Recent studies have demonstrated that activation of AMPK reduces the degradation of p27, inhibiting the proliferation of human glioma cell in vitro [11]. Our group has recently demonstrated in vitro that activation of AMPK by metformin inhibits PASMCs proliferation by decrease of S phase kinase-associated protein 2 (Skp2) and increase of p27 [12]. The present study was aimed to investigate whether these mechanisms also work in an in vivo model of PAH and contribute to the inhibition of the development of PAH.

Materials and methods

Materials

Monocrotaline (MCT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Metformin hydrochloride tablets were obtained from Bristol-Myers Squibb (Shanghai, China). Tissue protein extraction buffer was supplied by Wolsen (Beijing, China). Polyclonal antibodies against total-AMPK, phosphor-AMPK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and monoclonal antibodies against p27 and Skp2 were bought from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG was used as the secondary antibody (Sigma). All other chemicals and materials were obtained from local commercial sources.

Animals

Male Sprague-Dawley (SD) rats weighing between 150 and 200 g were used in the present study. All animal care and experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Xi'an Jiaotong University Animal Experiment Centre. All protocols used in this study were approved by the Laboratory Animal Care Committee of Xi'an Jiaotong University. The rats were assigned to the following 3 groups: control group (Con, n = 8), MCT treatment (MCT group, n = 12), and MCT plus metformin treatment (MCT + MET group, n = 8). All the groups were kept in the same room and subjected to the same light/dark cycle.

Generation of PAH models and drug treatment

MCT was dissolved in 1 M HCl. The solutions were then titrated to pH 7.4 with 1 M NaOH with the final concentrations of 10 mg/mL. Metformin hydrochloride tablets were dissolved in 0.9% sodium chloride solution, and the final concentration was 50 mg/mL. The PAH model was induced by subcutaneous injection of MCT (60 mg/kg of body weight) on day 1. Metformin (150 mg/kg of body weight) was given daily by intraperitoneal (IP) injection for 4 weeks until the rats were sacrificed. The normal control rats received an equal volume of vehicle solution. Twenty-eight days after MCT injection, rats were sacrificed, hemodynamic measurement was conducted and tissue samples were collected for morphometric and Western blot analysis.

Measurement of the RVSP and the RVH

On day 28 after MCT injection, all survived rats were anesthetized with 10% chloral hydrate (0.3 mL/kg, intraperitoneal injection). The right ventricle systolic pressure (RVSP) was measured according to Jones JE's protocol [13]. Tracheal cannulation was performed after tracheostomy. The chest was opened via a midline incision, and the animal was ventilated with room air (respiratory rate: 30 breaths per min; tidal volume: 10 mL/kg) using 2-3 cm H₂O positive end expiratory pressure (PEEP). Catheterization of the right ventricle was performed through the right jugular vein with a custom-made silicone catheter, and the right ventricle pressure was measured using a Grass polygraph (Power Lab, Australia). Pulmonary artery pressure was measured as the RVSP, which was assumed to be equal to the pulmonary artery pressure in the presence of a normal pulmonary valve. The right ventricular free wall was dissected from the left ventricle plus septum, and both parts were weighed separately on an analytic scale. The RVH was routinely checked to observe the establishment of PAH in the MCT-injected rats, which was assessed by the ratio of the weight of right ventricular to that of the left ventricle plus septum, RV/(LV + S).
Metformin against PAH

Histological and morphometric analysis

Marginal left lower pulmonary lobes were harvested and fixed with 10% buffered formalin for 4 h, and were then embedded in paraffin wax. Tissue blocks were sectioned to 5 μm in thickness and stained with HE (hematoxylin and eosin). Pulmonary vascular remodeling was assessed by measuring the medial thickness of vessels (diameter 50–250 μm) indexed to terminal bronchioles. Wall thickness was measured using an ocular micrometer by an observer who was blinded to the treatments of the rats. Distorted arteries were not used for the measurement. The percentage of medial wall thickness was calculated by the formula

\[
\text{percent wall thickness} = \frac{2 \times \text{wall thickness}}{\text{external diameter}} \times 100
\]

Western blot analysis

After rats were sacrificed, peripheral lung tissues were dissected and frozen at -70°C. The tissues were homogenized in RIPA protein extraction buffer containing protease inhibitors, and the lung tissue lysates were then separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then, the proteins were transferred onto nitrocellulose membrane in a Bio-Rad Trans-Blot system. After appropriate blocking, the blots were probed with primary antibodies (1:1000 dilution) against total-AMPK, phosphor-AMPK, Skp2, p27 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in 5% powered non-fat dry milk on a rocking platform at 4°C overnight, and were then washed and incubated with 1:10,000 diluted horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. After extensive washing with PBST solution, protein-antibody complexes were visualized using the enhanced chemiluminescence detection system (Amersham Bioscience) and exposed to X-ray film. The optical density of the bands was measured using an Image Lab 4.1 (Bio-Rad).

Statistical analysis

Values are presented as mean ± S.D. Data were analyzed using one-way ANOVA followed by a posthoc Student’s t-test. P < 0.05 was considered to represent significant differences between groups.

Results

Metformin reversed the increase of RVSP and RVH induced by MCT

As shown in Figure 1A, the RVSP measured at day 28 in rats treated with MCT was 45.88 ± 3.45 mmHg, which was significantly higher than that in control group (22.36 ± 2.19 mmHg; P < 0.05). This indicates that MCT successfully induced PAH in rats. After the administration of metformin in MCT-treated mice (MCT + MET), the RVSP at day 28 decreased dramatically to 29.81 ± 1.97 mmHg (P < 0.05 versus MCT group). Similar results were observed in the measurement of the RVH (Figure 1B), another mark for PAH. The ratio of RV/(LV + S) in the MCT rats (58.97 ± 3.62)% was significantly elevated compared with control rats [(15.94 ±
1.87)%; \( P < 0.05 \). After giving metformin in MCT-treated rats, this ratio decreased significantly to \((32.80 \pm 3.05)\)% (MCT + MET versus MCT; \( P < 0.05 \)), suggesting an evident inhibitory effect of metformin on the development of RVH.

*Metformin attenuated MCT-induced pulmonary arterial remodeling*

Figure 2A shows the HE-staining results of the marginal left lower pulmonary lobes. A dramatic increase in pulmonary arterial wall thickness was observed in MCT treated rats, this was accompanied with the increase of PASMC number in medial layer of pulmonary artery, whereas the administration of metformin reduced the wall thickness and PASMC number in pulmonary artery in MCT-treated rats. The quantitative morphometric analysis (Figure 2B) further confirmed that metformin could inhibit the percentage of pulmonary arterial wall thickness [MCT + MET (20.86 ± 1.14)% versus MCT (42.35 ± 2.08)%; \( P < 0.05 \)]. These results suggest that metformin dramatically inhibited the pulmonary arterial remodeling.

*Metformin restores the reduction of AMPK activity in MCT-treated rats*

To address the mechanisms underlying the anti-proliferative effect of metformin, we next...
Metformin against PAH

examined, by Western blot, the phosphorylation of AMPK. As shown in Figure 3, MCT significantly reduced AMPK phosphorylation compared with control rats (0.54 ± 0.08-fold over control, P < 0.05), the administration of metformin in MCT-treated rats restored the phosphorylation level of AMPK (0.96 ± 0.12-fold over control, P < 0.05 versus MCT group).

Metformin decreases Skp2 and increases p27

It has been shown that inhibition of Skp2-mediated p27 degradation underlies AMPK suppression of several types of non-pulmonary smooth muscle cells proliferation [14, 15]. Here, we examined the protein level of Skp2 and p27 in lung lysates. Figure 4A indicates that MCT dramatically increased Skp2 protein level, which rose 2.81-fold compared to control (P < 0.05), while in metformin-treated rats, MCT-induced elevation of Skp2 was significantly reduced to 1.43-fold increase over control (P < 0.05 versus MCT group). Figure 4B shows that MCT significantly reduced p27 protein level, which declined to 0.34-fold compared with control (P < 0.05), while administration of metformin in MCT-treated rats increased p27 protein level to 0.72-fold compared with control rats (P < 0.05 versus MCT group). These results indicate that metformin modulate Skp2 and p27 and contribute to the suppression of PASMCs proliferation.

Discussion

The novel finding of the present study was the identification of the anti-diabetic drug metformin as an effective therapeutic agent in well-established model of severe PAH. Metformin normalized haemodynamic parameters and RV hypertrophy. We further showed that metformin had an efficient anti-remodeling effect on pulmonary vasculature by inhibiting pulmonary artery cell proliferation. The present study provides direct evidence that MCT stimulates PASMCs proliferation by increasing Skp2 and decreasing p27, and activation of AMPK reversed MCT-induced elevation of Skp2 and reduction of p27, therefore suppressed PA-
SMCs proliferation. Our study indicates that enhancing the activity of AMPK might have potential value in the treatment of PAH by modulation of vascular remodeling.

The cell cycle is controlled by CDK and CDK inhibitors and has been a key therapeutic target in vascular proliferation-associated diseases. p27 as a CDK inhibitor binds to and inhibits the function of cyclin E/CDK2 complex, and further blocks cell cycle progression from G1 into S phase and suppresses cell proliferation [10]. Nabel et al. has reported that p27 is one of the potent inhibitors of vascular smooth muscle cell growth in vitro and in vivo [16, 17]. Fouty et al. showed that p27 modulated PASMCs proliferation during mitogenic stimulation, and over-expression of p27 decreased PASMCs proliferation [18]. Consistent with this notion, we have found that MCT reduced the level of p27 protein in rat lungs. Metformin reversed the MCT-induced reduction of p27 at protein level.

Studies have shown that activation of AMPK signaling pathway benefits a variety of types of malignant tumors and atherosclerosis by inhibiting the proliferation of tumor cells and artery vascular smooth cells [6, 19]. Here, we confirmed that activation of AMPK also suppressed the proliferation of pulmonary artery smooth muscle cells in vivo. Inhibition of pro-proliferation signaling cascades, such as PI3K/Akt signaling, underlies the effect of activation of AMPK inhibiting cell proliferation [20]. Activation of mTOR by PI3K/Akt is associated with proteins synthesis required for cell proliferation [21], while AMPK inhibits mTOR activity by activation of tuberous sclerosis (TSC) and further suppresses cells proliferation [22]. The present study found that metformin reversed MCT induced p27 reduction; this was accompanied with the activation of AMPK and increase of Skp2 protein level. It has been shown that mTOR promotes cell cycle progression by proteasome-dependent p27 degradation which is associated with the increase of one subunit of ubiquitin protein ligase complex SCFs (SKP1-cullin-F-box), Skp2. Skp2 is a F-box protein acts as the substrate recognition factor [23, 24]. It is known that the level of p27 protein is correlated with its expression and phosphorylation [14, 25]. AMPK inhibits p27 degradation by suppressing mTOR-dependent Skp2 expression and by increasing phosphorylation of p27 at site of threonine 197 which makes it resistant to degradation [25].

Metformin is an in vitro synthetic AMPK agonist which has been commonly used in clinic to treat type 2 diabetes with wide clinical experience and safety record [26]. Results of the present and previous studies indicate that activation of AMPK by metformin suppresses PASMCs proliferation, inhibits pulmonary vascular remodeling, suggesting that metformin has potential value in the treatment of pulmonary artery hypertension. Yet, this needs to be verified in clinical trials.

Acknowledgements

This work was supported by Chinese National Science Foundation (No. 81070045 and No. 81330002).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Manxiang Li, Department of Respiratory Medicine, The First Affiliated Hospital of Xi’an Jiaotong University, No. 277, Yanta West Road, Xi’an 710061, Shaanxi, P. R. China. Tel: +86-029-85323850; Fax: +86-029-85323850; E-mail: manxiangli@hotmail.com

References


