Int J Clin Exp Med 2016;9(7):12832-12838
www.ijcem.com /ISSN:1940-5901/IJCEM0021363

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 H_2O 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).

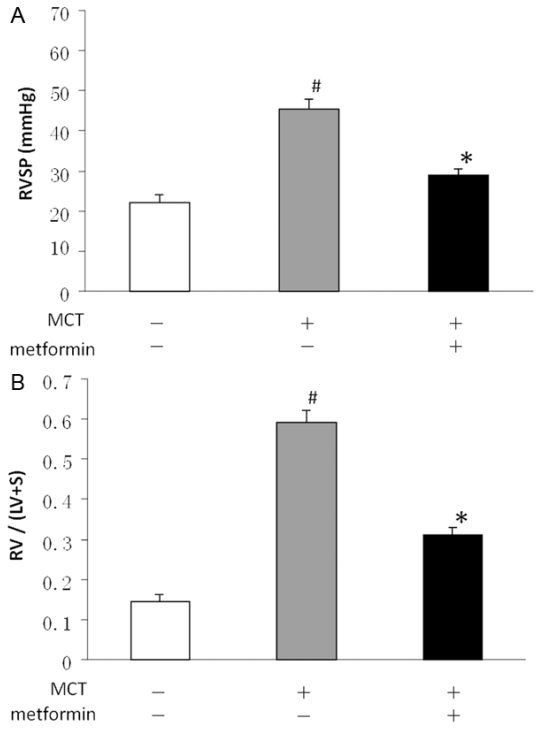

Figure 1. Metformin prevents MCT-induced PAH. A. The RVSP in different groups. After 4-week treatment of MCT, the RVSP was measured by cannulation of the right jugular vein. Metformin treatment significantly decreased the RVSP in MCT-induced model of PAH (MCT + MET). B. The RVH in different groups. The index of the RVH was given as the ratio of right ventricle to left ventricle plus septum weight [RV/(LV + S)]. Metformin treatment significantly prevented the progression of the RVH in MCT-induced model of PAH. #P < 0.05 versus control group; *P < 0.05 versus MCT group.

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 percent wall thickness =

$$\frac{2 \times \text{wall thickness} \times 100}{\text{external diameter}}.$$

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 ±

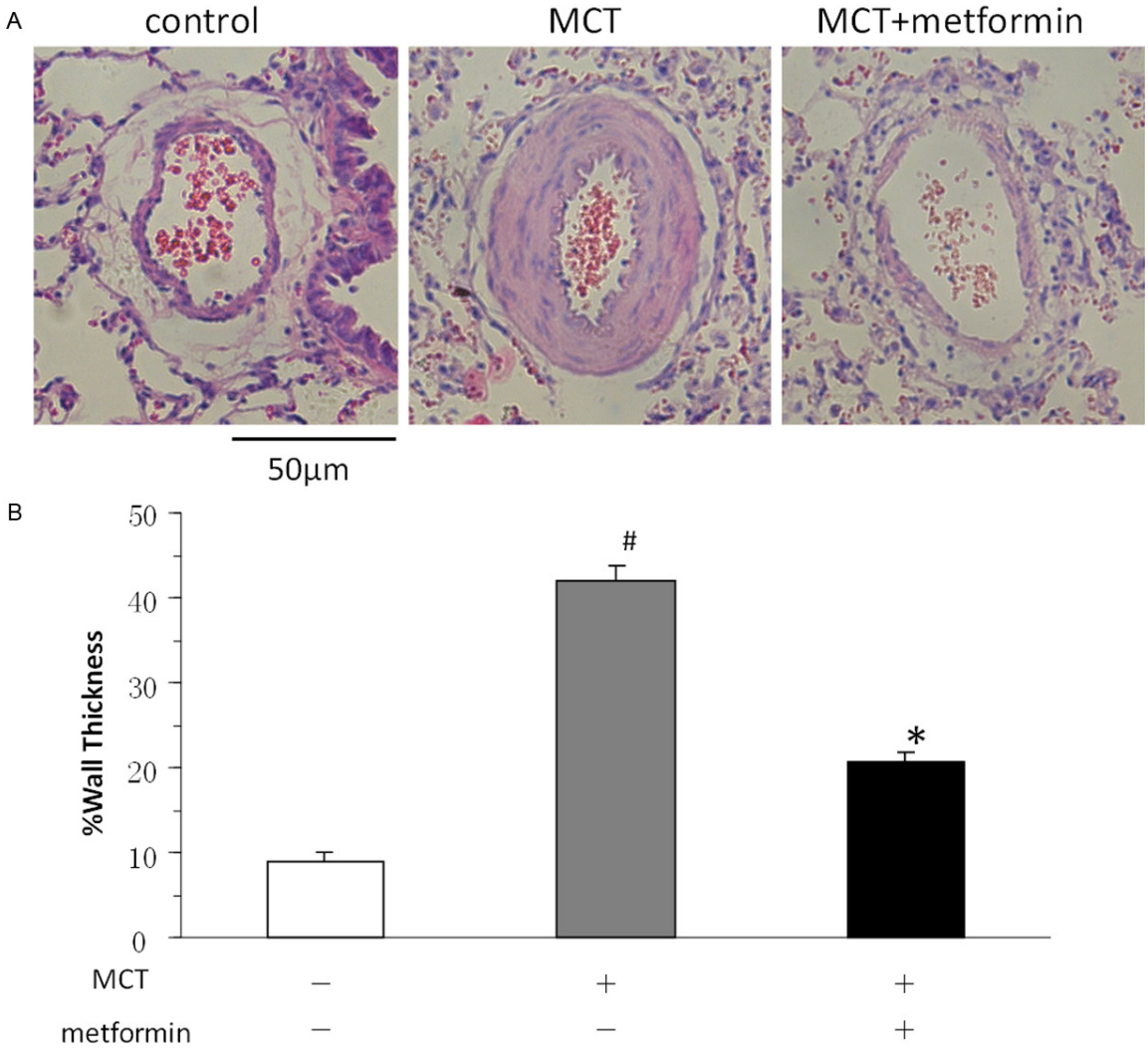

Figure 2. Metformin prevents PAH-associated pulmonary arterial wall remodelling. A. Representative HE-staining photomicrographs of small pulmonary vessels. B. Quantitative analysis of the wall thickness of pulmonary artery. Metformin treatment reduced MCT-induced pulmonary arterial wall thickening. #P < 0.05 versus control group; *P < 0.05 versus MCT group.

1.87)%; P < 0.05]. After giving metformin in MCT-treated rats, this ratio decreased significantly to (32.80 ± 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

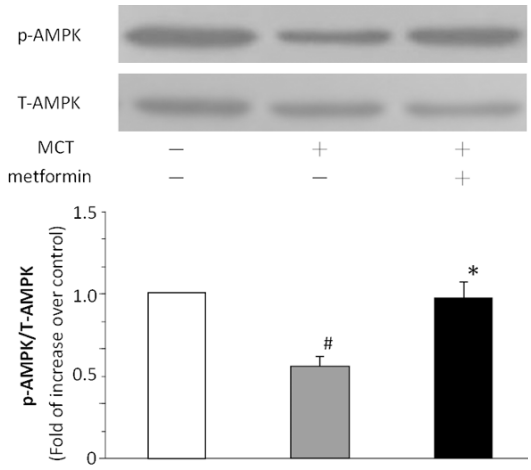

Figure 3. Effects of MCT and metformin on the phosphorylation of AMPK in rat lung tissues. AMPK phosphorylation and expression have been analyzed by Western blot in lung tissues from different groups. A representative blot and quantification of bands are shown (n = 3 each group). #P < 0.05 vs. control, *P < 0.05 vs. MCT group.

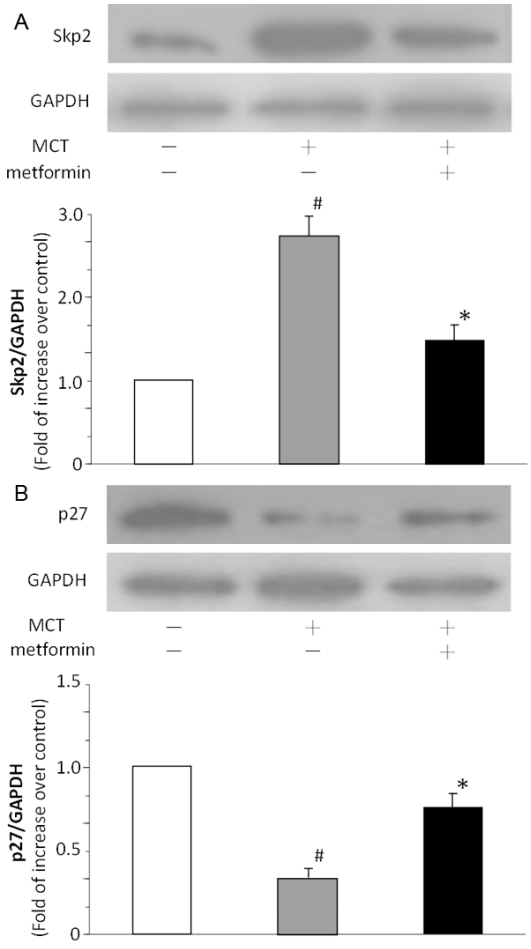

Figure 4. Effects of MCT and metformin on the expression of Skp2 and p27 protein in rat pulmonary tissues. The expressions of Skp2 and p27 have been analyzed by Western blot in lung tissues from different groups. GAPDH served as loading control. A representative blot and quantification of bands are shown (n = 3 each group). #P < 0.05 vs. control, *P < 0.05 vs. MCT group.

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 overexpression 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 Affiiated 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

[1] Miura Y, Fukumoto Y, Sugimura K, Oikawa M, Nakano M, Tatebe S, Miyamichi S, Satoh K and Shimokawa H. Identification of new prognostic factors of pulmonary hypertension. Circ J 2010; 74: 1965-1971.

[2] Firth AL, Yao W, Remillard CV, Ogawa A and Yuan JX. Upregulation of Oct-4 isoforms in pulmonary artery smooth muscle cells from patients with pulmonary arterial hypertension. Am J Physiol Lung Cell Mol Physiol 2010; 298: L548-557.

[3] Han D, Li SJ, Zhu YT, Liu L and Li MX. LKB1/AMPK/mTOR signaling pathway in non-small-cell lung cancer. Asian Pac J Cancer Prev 2013; 14: 4033-4039.

[4] Hardie DG. AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. Genes Dev 2011; 25: 1895-1908.

[5] Teng RJ, Du J, Afolayan AJ, Eis A, Shi Y and Konduri GG. AMP kinase activation improves angiogenesis in pulmonary artery endothelial cells with in utero pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 2013; 304: L29-42.

[6] Goncharov DA, Kudryashova TV, Ziai H, Ihida-Stansbury K, DeLisser H, Krymskaya VP, Tuder RM, Kawut SM and Goncharova EA. Mammalian target of rapamycin complex 2 (mTORC2) coordinates pulmonary artery smooth muscle cell metabolism, proliferation, and survival in pulmonary arterial hypertension. Circulation 2014; 129: 864-874.

[7] Agard C, Rolli-Derkinderen M, Dumas-de-La-Roque E, Rio M, Sagan C, Savineau JP, Loirand G and Pacaud P. Protective role of the antidiabetic drug metformin against chronic experimental pulmonary hypertension. Br J Pharmacol 2009; 158: 1285-1294.

[8] Chu IM, Hengst L and Slingerland JM. The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. Nat Rev Cancer 2008; 8: 253-267.

[9] Larrea MD, Wander SA and Slingerland JM. p27 as Jekyll and Hyde: regulation of cell cycle and cell motility. Cell Cycle 2009; 8: 3455-3461.

[10] Ammit AJ and Panettieri RA Jr. Invited review: the circle of life: cell cycle regulation in airway smooth muscle. J Appl Physiol (1985) 2001; 91: 1431-1437.

[11] Jiang YS, Lei JA, Feng F, Liang QM and Wang FR. Probucol suppresses human glioma cell proliferation in vitro via ROS production and LKB1-AMPK activation. Acta Pharmacol Sin 2014; 35: 1556-1565.

[12] Wu Y, Liu L, Zhang Y, Wang G, Han D, Ke R, Li S, Feng W and Li M. Activation of AMPK inhibits pulmonary arterial smooth muscle cells proliferation. Exp Lung Res 2014; 40: 251-258.

[13] Jones JE, Mendes L, Rudd MA, Russo G, Loscalzo J and Zhang YY. Serial noninvasive assessment of progressive pulmonary hypertension in a rat model. Am J Physiol Heart Circ Physiol 2002; 283: H364-371.

[14] Hsu JD, Kao SH, Ou TT, Chen YJ, Li YJ and Wang CJ. Gallic acid induces G2/M phase arrest of breast cancer cell MCF-7 through stabilization of p27(Kip1) attributed to disruption of p27(Kip1)/Skp2 complex. J Agric Food Chem 2011; 59: 1996-2003.

[15] Ferri N. AMP-activated protein kinase and the control of smooth muscle cell hyperproliferation in vascular disease. Vascul Pharmacol 2012; 56: 9-13.

[16] Akyurek LM, Boehm M, Olive M, Zhou AX, San H and Nabel EG. Deficiency of cyclin-dependent kinase inhibitors p21Cip1 and p27Kip1 accelerates atherogenesis in apolipoprotein E-deficient mice. Biochem Biophys Res Commun 2010; 396: 359-363.

[17] Tanner FC, Boehm M, Akyurek LM, San H, Yang ZY, Tashiro J, Nabel GJ and Nabel EG. Differential effects of the cyclin-dependent kinase inhibitors p27(Kip1), p21(Cip1), and p16(Ink4) on vascular smooth muscle cell proliferation. Circulation 2000; 101: 2022-2025.

[18] Fouty BW, Grimison B, Fagan KA, Le Cras TD, Harral JW, Hoedt-Miller M, Sclafani RA and Rodman DM. p27(Kip1) is important in modulating pulmonary artery smooth muscle cell proliferation. Am J Respir Cell Mol Biol 2001; 25: 652-658.

[19] Ben Sahra I, Le Marchand-Brustel Y, Tanti JF and Bost F. Metformin in cancer therapy: a new perspective for an old antidiabetic drug? Mol Cancer Ther 2010; 9: 1092-1099.

[20] Memmott RM and Dennis PA. Akt-dependent and -independent mechanisms of mTOR regulation in cancer. Cell Signal 2009; 21: 656-664.

[21] Motoshima H, Goldstein BJ, Igata M and Araki E. AMPK and cell proliferation--AMPK as a therapeutic target for atherosclerosis and cancer. J Physiol 2006; 574: 63-71.

[22] Inoki K, Ouyang H, Zhu T, Lindvall C, Wang Y, Zhang X, Yang Q, Bennett C, Harada Y, Stankunas K, Wang CY, He X, MacDougald OA, You M, Williams BO and Guan KL. TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. Cell 2006; 126: 955-968.

[23] Shapira M, Kakiashvili E, Rosenberg T and Hershko DD. The mTOR inhibitor rapamycin down-regulates the expression of the ubiquitin ligase subunit Skp2 in breast cancer cells. Breast Cancer Res 2006; 8: R46.

[24] Bond M and Wu YJ. Proliferation unleashed: the role of Skp2 in vascular smooth muscle cell proliferation. Front Biosci (Landmark Ed) 2011; 16: 1517-1535.

[25] Short JD, Dere R, Houston KD, Cai SL, Kim J, Bergeron JM, Shen J, Liang J, Bedford MT, Mills GB and Walker CL. AMPK-mediated phosphorylation of murine p27 at T197 promotes binding of 14-3-3 proteins and increases p27 stability. Mol Carcinog 2010; 49: 429-439.

[26] Liu SC, Tu YK, Chien MN and Chien KL. Effect of antidiabetic agents added to metformin on glycaemic control, hypoglycaemia and weight change in patients with type 2 diabetes: a network meta-analysis. Diabetes Obes Metab 2012; 14: 810-820.