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*Original Article*

# Nobiletin protects against monocrotaline-induced pulmonary arterial hypertension in rats by regulating Src/STAT3 signaling pathway

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**Abstract:** Nobiletin, a major polymethoxy flavone, has been demonstrated to exert protective effects on cardivascular diseases. However, the effects of nobiletin on pulmonary arterial hypertension (PAH) have not been studied. Herein in this report, PAH was induced by a single monocrotaline (MCT) injection in rats, and then rats were treated with nobiletin for consecutive three weeks. At the end of three weeks, hemodynamic parameters, right ventricular hypertrophy and pulmonary vascular remodeling were assessed. Moreover, we evaluated anti-proliferative effect of nobiletin in vivo and in vitro. Nobiletin effectively reduced right ventricular systolic pressure (RVSP) and attenuated right ventricular hypertrophy and medial wall thickening in MCT-treated rats. Furthermore, nobiletin inhibited Src/STAT3 activation in the lungs of PAH rats. In rat pulmonary artery smooth muscle cells (PASMCs), nobiletin suppressed PDGF-BB-induced rat PASMCs proliferation and Src/STAT3 activation, accompanied with down-regulation of downstream targets Pim1 and NFATc2. Taken together, our data indicated that nobiletin may exert protective effects on PAH via the inhibition of PASMCs proliferation and Src/STAT3 activation.

**Keywords:** Pulmonary arterial hypertension, nobiletin, PDGF-BB, proliferation, Src/STAT3

## Introduction

Pulmonary arterial hypertension (PAH) is a progressive vascular disorder with high mortality. Pulmonary vascular remodeling is the most typical pathological change, which results in the elevation of pulmonary arterial pressure, right ventricular hypertrophy and dysfunction [1]. Despite recent advances in treatment, the currently available therapeutic strategies are not sufficient to prevent the irreversible progression [2]. Thus, it is necessary to implement effective therapeutic options to inhibit the progression of PAH.

As the key structural characteristic of PAH, pulmonary vascular remodeling is generally described as the thickening of the vascular intima, media, and adventitia [1]. It is widely believed that vascular media thickening resulted from the hyperproliferation of pulmonary artery smooth muscle cells (PASMCs) and resistance to apoptosis [3, 4]. As yet, several signaling pathways have been reported to be closely related to abnormal proliferation and apoptosis of PASMCs, such as JAK/STATs signaling pathway [5], RhoA/ROCKs signaling pathway [6], Notch signaling pathway [7] and Src/STAT3 signaling pathway [8].

Dietary factors play significant roles in the prevention and treatment of various diseases [9]. Nobiletin (also termed 3',4',5,6,7,8-hexamethoxyflavone), is a principal polymethoxylated flavone isolated from peels of citrus fruits. Nobiletin has attracted more and more attention due to its multiple biological activities and therapeutic effects such as anti-inflammatory, anti-oxidant and anti-tumor properties [10]. Nobiletin, which inhibits tumor cell proliferation and induces apoptosis, has been reported to suppress tumor growth, metastasis and invasion in various tumors [11, 12].

These observations prompted us to hypothesize that nobiletin may exert protective effects

on PAH. In the current study, it was investigated whether nobiletin could reduce right ventricular systolic pressure (RVSP) and prevent right ventricular hypertrophy and pulmonary vascular remodeling in monocrotaline (MCT)-induced PAH. We found that administration of nobiletin provided protection for rats against MCT-induced PAH as manifested by the reduced RVSP and the alleviated right ventricular hypertrophy and medial wall thickening. The research on mechanisms revealed that nobiletin regulated Src/STAT3 Signaling, and by which it suppresses PASMCs proliferation. Our data suggested that nobiletin could be a viable strategy for the treatment of PAH.

## Materials and methods

### *Reagents and antibodies*

Rabbit anti-phospho-STAT3 (PY705-STAT3), STAT3, anti-phospho-Src (PY416-Src) and Src antibodies were purchased from Cell Signaling Technology (Danvers, USA). Rabbit anti-PCNA antibody was purchased from Proteintech Group (Wuhan, China). Nobiletin and MCT were purchased from sigma (USA). PDGF-BB was obtained from Peprotech incorporation (Rocky Hill, NJ).

### *Animal treatment*

Male Sprague-Dawley rats (210 to 250 g body weight) were purchased from Center of Medical Experimental Animals of Hubei Province (Wuhan, China). PAH was induced by MCT (60 mg/kg, i.p.). Rats were randomly assigned into three experimental groups (n = 6): (1) Control group, (2) MCT group, (3) MCT+nobiletin group. Rats that were injected with PBS served as controls. The dose of nobiletin was chosen based on previous studies [13]. Nobiletin (50 mg/kg, once a day) was given by intragastric administration for 3 weeks following MCT injection. All experiments were approved by the Animal Care and Use Committee of Tongji Medical College.

### *Hemodynamic measurements*

Three weeks after MCT injection, rats were anesthetized with sodium pentobarbital (120 mg/kg, i.p.). Through the right jugular vein, a polyethylene catheter was inserted into the right ventricle to detect RVSP as described [3].

### *Right ventricular hypertrophy*

After hemodynamic measurements, the right ventricle (RV) was dissected from the left ventricle (LV) and the septum (S). The wet weights were determined respectively. The weight ratio of RV to (LV+S) was calculated for the index of right ventricular hypertrophy.

### *Morphometric analysis*

Lung tissue sections were stained with hematoxylin and eosin (HE). Small pulmonary arterioles (50-150 μm in diameter) were measured to calculate the percentage of medial wall thickness (%MWT). %MWT = [(medial thickness ×2)/external diameter] ×100%. 8-10 vessels of each rat were measured and average was calculated.

### *Immunohistochemical staining (IHC)*

The Paraffin sections of lung tissue were subjected to immunohistochemistrical staining. Proliferating cell nuclear antigen (PCNA) antibody was used at a dilution of 1:200. The average numbers of PCNA-positive cells in small pulmonary arterioles were calculated to evaluate proliferative activity.

### *Cell culture and treatment*

Primary rat PASMCs were obtained as previously described [3]. Intrapulmonary arteries separated from adult male Sprague-Dawley rats were stripped of adventitia, and endothelium was gently removed with a tweezer. Rat PASMCs were cultured in DMEM/F12 supplemented with 10% FBS (Gibco, USA). Cells were stimulated by PDGF-BB (30 ng/ml) with or without nobiletin for 48 hours.

### *Cell viability assay*

Rat PASMCs were seeded in 96-well culture plates. After adherence, cells were stimulated by PDGF-BB (30 ng/ml) with different concentrations of nobiletin (0, 10, 20, 50 μM) for 48 hours. Finally, CCK-8 (10 μl) (Dojindo, Japan) was added to each well for 2 h at 37°C. A microplate reader (ELX800, BioTek Instruments, USA) was used to determine the absorbance at 450 nm. All experiments were performed in triplicate.

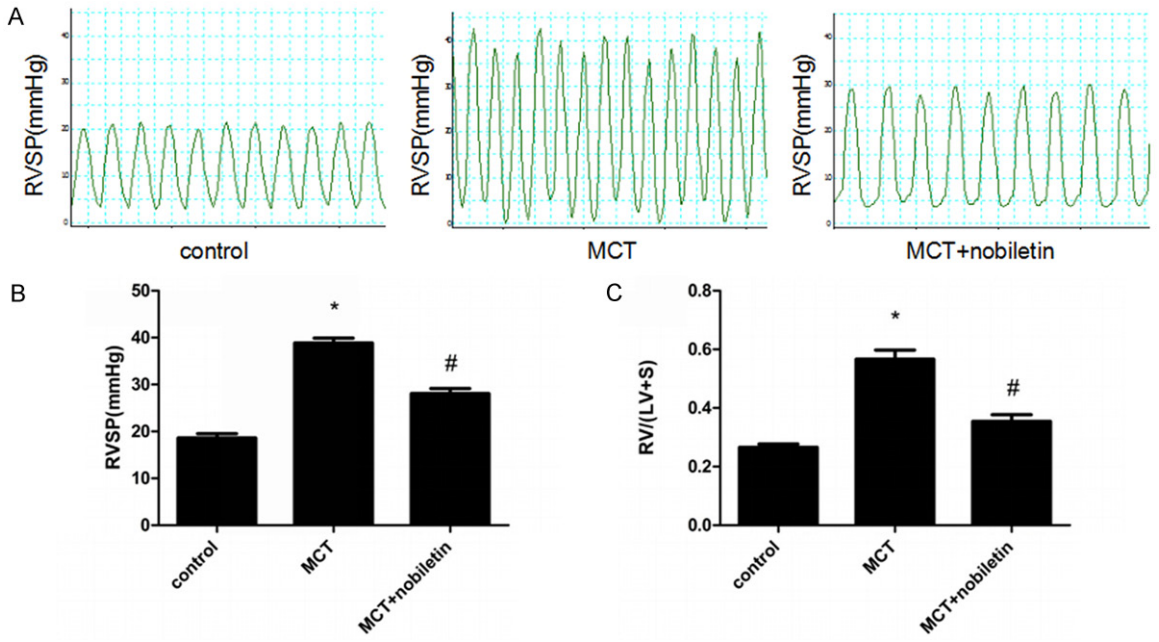


**Figure 1.** Nobiletin reduced right ventricular systolic pressure (RVSP) and prevented right ventricular hypertrophy. A. Demonstrative traces of RVSP. B. Comparison of RVSP among three groups. C. Comparison of RV/(LV+S) among three groups. RV/(LV+S): the weight ratio of the right ventricle (RV) to the left ventricle plus the septum (LV+S). Three groups as follows: the control group; the MCT group; the MCT+nobiletin group. Six mice were included for each study group. *P<0.05, vs. the control group; #P<0.05, vs. the MCT group.

*5-bromo-2-deoxyuridine (BrdU) incorporation assay*

Primary rat PASMCs were stimulated by PDGF-BB (30 ng/ml) with or without nobiletin (50 μM) for 48 hours. BrdU was then added to the culture medium for 4 hours. The cells were fixed with 4% formalin and BrdU was detected with Cy3-labeled anti-BrdU antibody (Sigma, USA). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

*Western blot analysis*

Total proteins were separated by SDS-PAGE and then transferred to PVDF membranes (Millipore, USA). The membranes were incubated with rabbit PY705-STAT3 (1:1000), STAT3 (1:1000), PY416-Src (1:1000) and Src (1:1000) overnight at 4°C, and then incubated with HRP-conjugated secondary antibodies (1:4000) for 1 hour at room temperature. Proteins were detected by ECL (Thermo Fisher Scientific) and GAPDH was used as internal control as described [14].

*Quantitative RT-PCR (qRT-PCR)*

Trizol Reagent (TaKaRa, Dalian, China) was used for total RNA extraction of rat PASMCs. The cDNA obtained by reverse transcription was amplified using the SYBR Premix Ex Taq (TaKaRa). The 2-ΔΔCt method was applied to quantify the relative expression values as described [15]. The qRT-PCR primers used for rat Pim1, NFATc2 and β-actin were listed as follows. Rat Pim1: Forward: 5'-AAGAGATCGTCAAGGGCCAAGTGT-3'; Reverse: 5'-TGC ATC CAC GGA TGG TTC TGG ATT-3'. Rat NFATc2: Forward: 5'-ACATCCGGGTGCCCGTGAAAGT-3'; Reverse: 5'-CTCGGGGCAGTCTGTTGTTGGATG-3'. Rat β-actin: Forward: 5'-CGTAAAGACCTCTATGCCAACA-3'; Reverse: 5'-CGGACTCATCGTACTCCTGCT-3'. Rat β-actin was used as an internal control.

*Statistical analysis*

All results are expressed as mean ± SEM, and statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test. A value of $P<0.05$ was considered to be statistically significant.

## Results

*Nobiletin reduced RVSP and prevented right ventricular hypertrophy in MCT-induced PAH rats*

Nobiletin has been known to exert beneficial effects on various diseases [10, 11, 13].

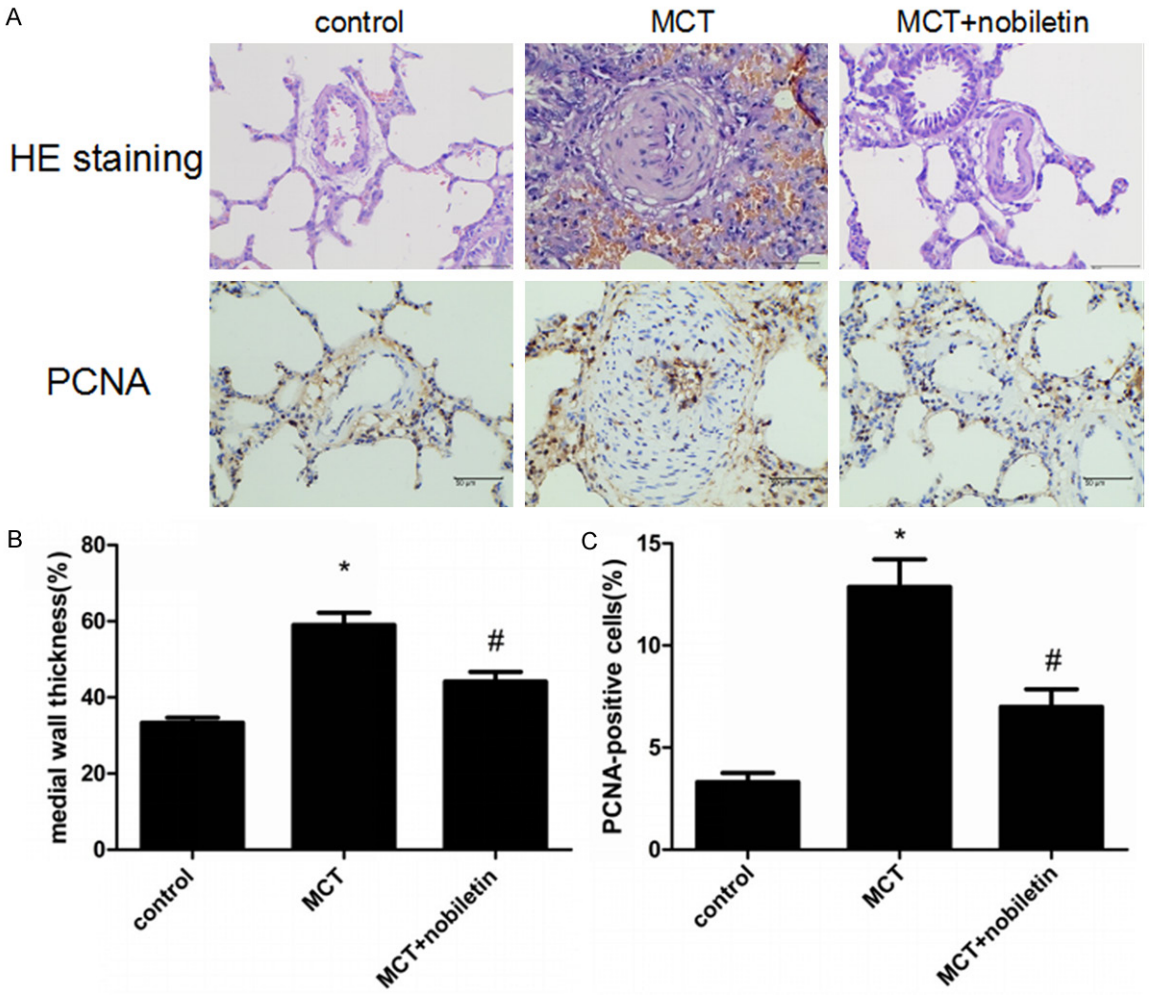


**Figure 2.** Nobiletin ameliorated pulmonary vascular remodeling induced by MCT for three weeks. A. The percentage of medial wall thickness (%MWT) of small pulmonary arterioles was determined by hematoxylin-eosin (HE) staining, and the percentage of PCNA-positive cells in small pulmonary arteries was determined by immunohistochemical staining with anti-PCNA antibody in three groups (n = 6 per group). Magnification: ×400. B. Comparison of %MWT in small pulmonary arterioles among three groups. C. Bar chart of the percentage of PCNA-positive cells in small pulmonary arteries. *P<0.05, vs. the control group; #P<0.05, vs. the MCT group.

However, whether it could be used as an effective approach for MCT-induced PAH has little to be addressed. To investigate effects of nobiletin on MCT-induced PAH, MCT-injected rats were treated with nobiletin for 3 weeks. We firstly detected RVSP which reflected pulmonary arterial pressure. As expected, RVSP was remarkably increased in the rats originated from the MCT group compared with the control group (38.84±1.04 mmHg vs. 18.61±0.91 mmHg; *P*<0.05, **Figure 1A** and **1B**). Surprisingly, administration of nobiletin significantly attenuated MCT-induced increase in RVSP (38.84±1.04 mmHg vs. 28.06±1.09 mmHg; *P*<0.05, **Figure 1A** and **1B**). To evaluate right ventricular hypertrophy, the ratio of RV/(LV+S) was measured. Compared with the control group, an increase in the ratio of RV/(LV+S) was identified in the MCT group (0.57±0.03 vs. 0.27±0.01; *P*<0.05, **Figure 1C**), while nobiletin treatment significantly led to a decrease of RV/(LV+S) ratio in MCT-injected rats (0.57±0.03 vs. 0.35±0.02; *P*<0.05, **Figure 1C**).

*Nobiletin alleviated pulmonary vascular remodeling induced by MCT*

Pulmonary vascular remodeling is the key characteristic of PAH, which leads to the irreversible progression of PAH [1, 2]. To evaluate effects of nobiletin on pulmonary vascular remodeling, we determined medial wall thickness of small

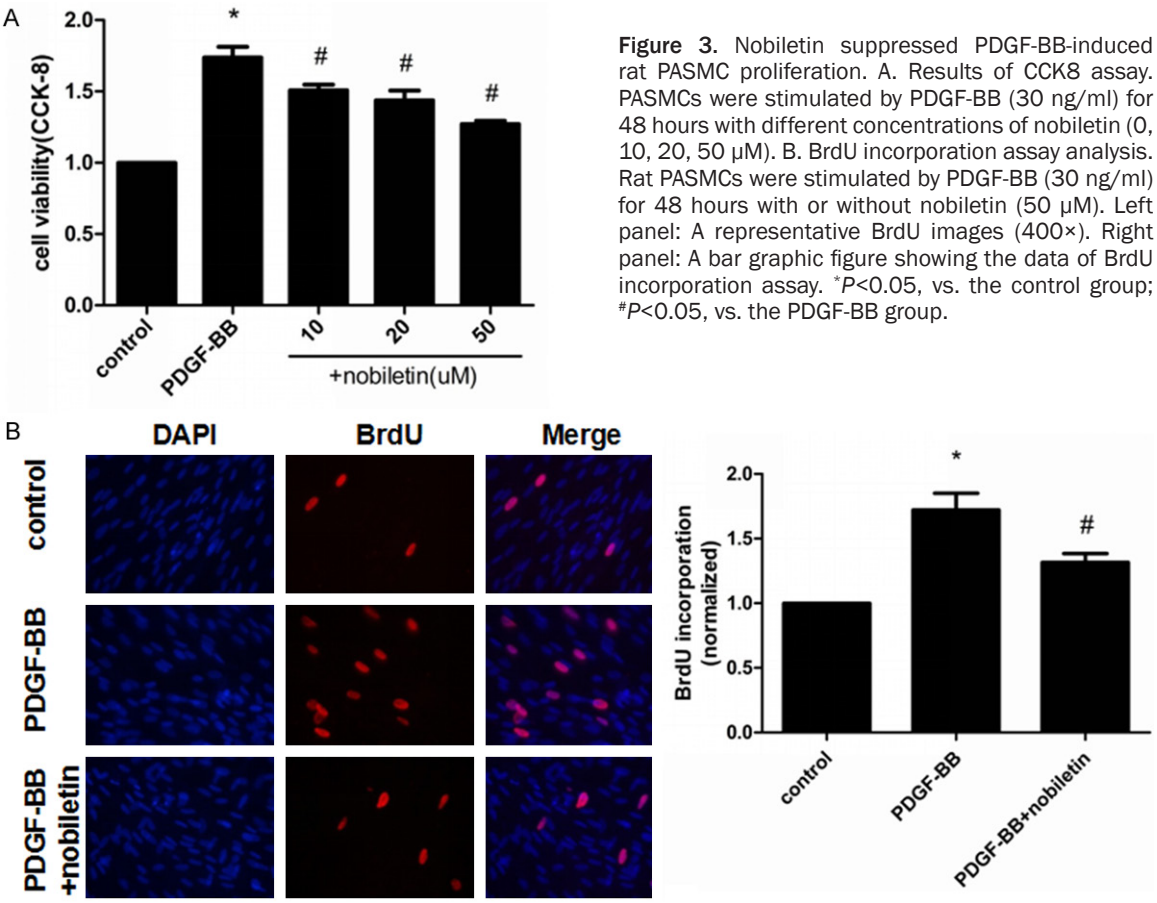


**Figure 3.** Nobiletin suppressed PDGF-BB-induced rat PASMC proliferation. A. Results of CCK8 assay. PASMCs were stimulated by PDGF-BB (30 ng/ml) for 48 hours with different concentrations of nobiletin (0, 10, 20, 50 μM). B. BrdU incorporation assay analysis. Rat PASMCs were stimulated by PDGF-BB (30 ng/ml) for 48 hours with or without nobiletin (50 μM). Left panel: A representative BrdU images (400×). Right panel: A bar graphic figure showing the data of BrdU incorporation assay. $^{*}P<0.05$, vs. the control group; $^{\#}P<0.05$, vs. the PDGF-BB group.

pulmonary arterioles via HE staining. Compared with the control group, the percentage of medial wall thickness (%MWT) remarkably was increased in response to MCT (59.06%±3.19% vs. 33.35%±1.36%; $P<0.05$, **Figure 2A** and **2B**), while nobiletin markedly prevented MCT-induced medial wall thickening (59.06%±3.19% vs. 44.19%±2.48%; $P<0.05$, **Figure 2A** and **2B**). It was noted that pulmonary vascular cells proliferation contributes to pulmonary vascular remodeling. Therefore, we determined proliferating cell nuclear antigen (PCNA)-positive cells in vascular walls of small pulmonary arterioles by immunohistochemical staining. The percentage of PCNA-positive cells in the MCT group was markedly increased compared with the control group, whereas nobiletin markedly inhibited pulmonary vascular cells proliferation in vivo (**Figure 2A** and **2C**).

*Nobiletin suppressed PDGF-BB-induced rat PASMCs proliferation*

Previous studies have shown that abnormal PASMCs proliferation was a major factor contributing to pulmonary vascular remodeling [16]. The above results indicated that administration of nobiletin alleviated pulmonary vascular remodeling, and thereby improved MCT-induced PAH. To further demonstrate anti-proliferative effect of nobiletin, primary rat PASMCs were obtained from intrapulmonary arteries, and then subjected to PDGF-BB stimulation as described. We adopted CCK-8 and BrdU incorporation assay to detect the proliferation of PASMCs. As expected, PDGF-BB remarkably promoted rat PASMCs proliferation. Interestingly, nobiletin significantly suppressed PDGF-BB-induced rat PASMCs proliferation (**Figure 3A** and **3B**). These findings implied that nobiletin may prevent pulmonary vascular remodeling through suppression of PASMCs proliferation.

*Nobiletin suppressed Src/STAT3 activation and its target genes expression in PAH model and rat PASMCs*

To further investigate the mechanisms underlying protective effects of nobiletin on PAH, the

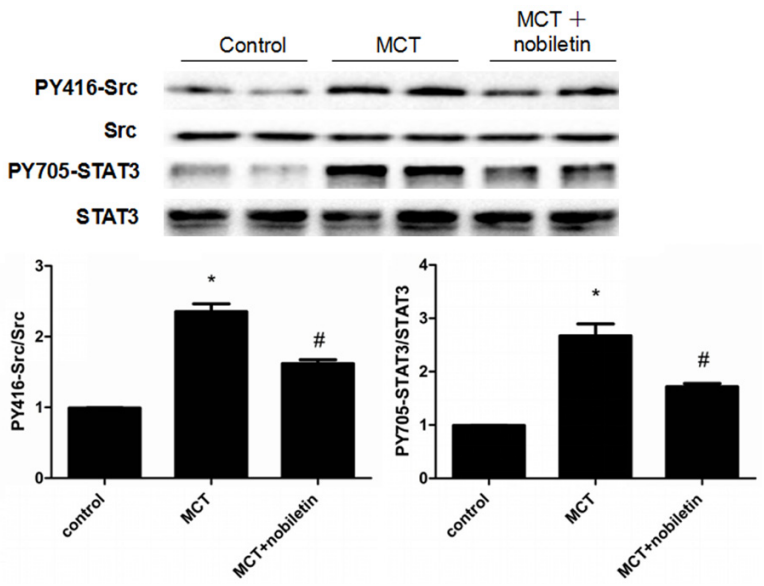


**Figure 4.** Nobiletin inhibited Src/STAT3 activation in the lungs of MCT-treated rats. Western blot analysis of Src/STAT3 activation in the lung homogenates. Up panel: A representative figure of Western blot. Six rats were included for each study group. Down panel: Bar graphic figures for Western blot analysis showing the data of Src and STAT3 phosphorylation. $^{*}P<0.05$, vs. the control group; $^{\#}P<0.05$, vs. the MCT group.

phosphorylation levels of Src and STAT3 in lungs were assessed by Western blot. In the MCT group, the levels of phosphorylated Src and STAT3 in the lungs of rats were markedly increased compared with the control group, whereas nobiletin reversed MCT-induced Src/STAT3 activation (**Figure 4**). Accordingly, a significant increase in the levels of phosphorylated Src and STAT3 was observed in rat PASMCs after PDGF-BB stimulation (**Figure 5A** and **5B**), while nobiletin significantly inhibited PDGF-BB-induced Src (**Figure 5A**) and STAT3 (**Figure 5B**) activation. Meanwhile, the expression of STAT3-targeted genes Pim1 and NFATc2 were detected by qRT-PCR. As expected, both Pim1 (**Figure 5C**) and NFATc2 (**Figure 5D**) were also significantly decreased by nobiletin in rat PASMCs.

## Discussion

The pathogenesis of PAH is not fully elucidated. However, aberrant proliferation of PASMCs, chronic inflammation, thrombosis and sustained pulmonary vasoconstriction are considered to be implicated in the pathogenesis of PAH [17]. Nobiletin, a polymethoxylated flavonoid, has been reported to exert protective effects on some cardiovascular diseases due to its anti-oxidant, anti-inflammatory and anti-proliferative properties [10, 13]. Nobiletin decreased neointimal hyperplasia in balloon-injured rat carotid arteries by regulating ROS/NF-κB pathway and inflammation [18] and ameliorated inflammation and apoptosis in STZ-induced diabetic cardiomyopathy [19, 20]. In addition, nobiletin exhibited antiplatelet activity and prevented arterial thrombosis [21]. However, the effect of nobiletin on PAH still remains unclear. In this study, we provided the evidences that nobiletin could effectively prevent the progression of MCT-induced PAH in rats, suggesting that nobiletin may be a potential treatment for the prevention of PAH.

MCT-induced PAH model has been extensively applied in experiments for several years. A single injection of MCT can cause significant hemodynamic and morphometric changes such as the elevation of RVSP and right ventricular hypertrophy [22]. Consistent with previous findings, hemodynamic and morphometric alterations in response to MCT were identified in our study. More importantly, we found that Administration of nobiletin for three weeks not only significantly reduced the elevation in RVSP, but also attenuated right ventricular hypertrophy in MCT-injected rats. These results indicated that nobiletin can lead to hemodynamic improvement and attenuation of right ventricular hypertrophy in the PAH rat model.

Pulmonary vascular remodeling, which results mainly from excessive PASMCs proliferation, is considered as the pivotal structural characteristics of PAH [23, 24]. Our findings showed that medial wall thickness in pulmonary vessels of MCT-treated rats was significantly increased. Nevertheless, nobiletin efficiently prevented this change, indicating that nobiletin exerts a beneficial effect on PAH by preventing pulmonary vascular remodeling.

In addition, the percentage of PCNA positive cells in pulmonary vessels of PAH rats were

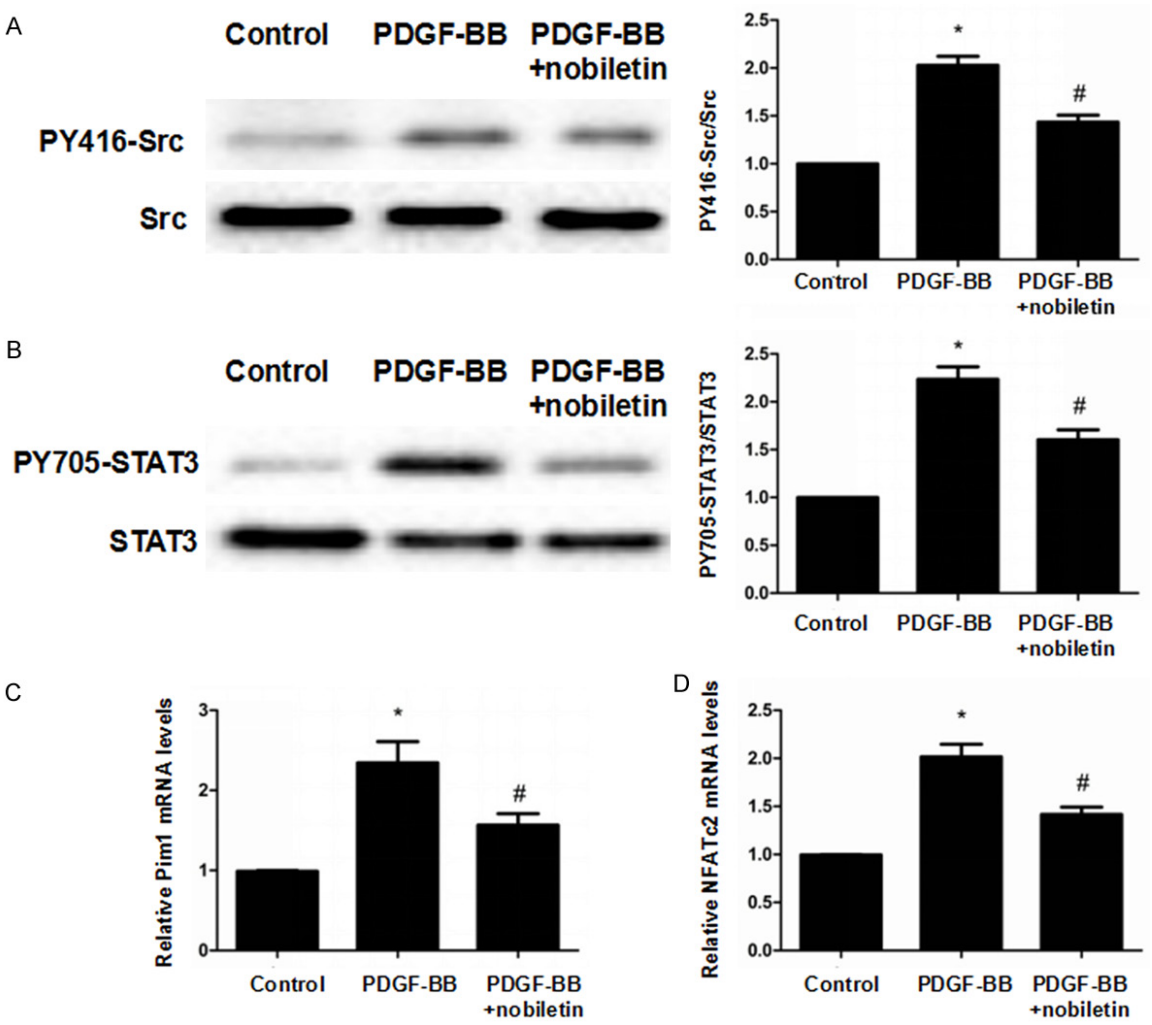


**Figure 5.** Nobiletin inhibited PDGF-BB-induced Src/STAT3 activation and STAT3-targeted genes expression in rat PASMCs. Rat PASMCs were stimulated by PDGF-BB (30 ng/ml) with or without nobiletin (50 μM) for 48 hours. A. The expression of PY416-Src and Src was measured by western blot. B. The expression of PY705-STAT3 and STAT3 was measured by western blot. C, D. Real-time PCR results for analysis of Pim1 and NFATc2 expression. $^{*}P<0.05$, vs. the control group; $^{\#}P<0.05$, vs. the PDGF-BB group.

found to be increased, while the change was effectively prevented by nobiletin. These findings signified that nobiletin suppressed pulmonary vascular cells proliferation. Previous studies have reported that nobiletin suppressed vascular smooth muscle cells proliferation [18, 25]. In our study, we demonstrated that nobiletin also suppressed PDGF-BB-induced PASMCs proliferation. Based on above results, we concluded that nobiletin prevented pulmonary vascular remodeling, at least in part, through inhibition of PASMCs proliferation.

Accumulating evidences suggest that the Src-dependent activation of STAT3 contributes to pulmonary vascular remodeling and the progress of PAH [8]. STAT3 could be phosphorylated and translocated to the nucleus in response to PDGF, IL-6, endothelin-1 and angiotensin II stimulation, all of which were increased and led to the procession of PAH [26, 27]. STAT3 activation triggered the oncogene provirus integration site for Moloney murine leukemia virus (Pim1) expression, and then promoted the expression and activation of nuclear factor of activated T-cells (NFATc2) [28]. In PAH, NFATc2 activation promoted PASMCs proliferation and resulted in apoptosis inhibition [29]. Therefore, STAT3/Pim1/NFATc2 signaling pathway plays significant roles in the pathogen-

esis of PAH. In line with previous findings, the current study showed that Src and STAT3 phosphorylation were significantly increased in the lungs of MCT-treated rats. However, nobiletin significantly suppressed the phosphorylation of Src and STAT3 in the lungs of PAH rats. Besides, we further demonstrated that nobiletin inhibited PDGF-BB-induced Src/STAT3 activation in cultured rat PASMCs, accompanied with decreased expression of both Pim1 and NFATc2. Taken together, these results suggested that nobiletin ameliorated MCT-induced PAH in rats through inhibition of Src/STAT3 axis.

As mentioned above, our study showed that nobiletin can prevent the progression of MCT-induced PAH in rats, suggesting nobiletin may be a potential treatment for prevention of PAH. However, additional research is needed to determine whether nobiletin can reverse the progression of PAH in established PAH models and access the potential clinical value of this agent.

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## Disclosure of conflict of interest

None.

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