Tropical Journal of Pharmaceutical Research January 2023; 22 (1): 73-79
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v22i1.11

Original Research Article

Pinellia polysaccharide activates PI3K/AKT signaling pathway and regulates the expression of airway smooth muscle-related apoptosis molecules in airway remodeling in rats with cough-variant asthma

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Sent for review: 7 July 2022 *Revised accepted: 19 December 2022*

Abstract

Purpose: *To investigate the effect of Pinellia polysaccharide on airway remodeling in cough-variant asthmatic rats, and the involvement of PI3K/AKT signal route in the process.*
Methods: *Seventy-five Sprague-Dawley (SD) rats were assigned to 5 groups: blank control, model, low-dose Pinellia polysaccharide (100 mg/kg), medium-dose Pinellia polysaccharide (200 mg/kg) and high-dose Pinellia polysaccharide (400 mg/kg) groups, each with 15 rats. Immunoblot assay was employed to measure relative protein concentrations of phosphorylated protein kinase B (p-AKT), phosphorylated phosphoinositide-3-kinase (p-PI3K), MMP-9, B lymphocytes expression of cell tumor-2, B-lymphoma-2 gene-related promoter (Bad), Fas, and Caspase-3.*
Results: *The levels of Wat, Wam, Wai, p-AKT, p-PI3K, MMP-9 and bcl-2 in model group were significantly higher than those in blank control group ($p < 0.05$). However, these factors were significantly up-regulated, relative to blank control levels, but lower than those in the model group ($p < 0.05$). The levels of Bad, Fas and Caspase3 in the model group were significantly lower than the corresponding levels in the blank control group ($p < 0.05$). Moreover, levels of Bad, Fas and Caspase3 differed significantly amongst rats given the 3 doses of Pinellia polysaccharide ($p < 0.05$).*
Conclusion: *Pinellia polysaccharide mitigates cough-variant asthma in rats through stimulation of PI3K/AKT signal route, regulation of expressions of airway smooth muscle-related apoptotic molecules (Bad, Fas and Caspase-3), and slowing down airway remodeling and airway inflammation. Thus, this polysaccharide is a potential agent for the management of cough variant asthma.*

Keywords: *Cough variant asthma, Pinellia polysaccharide, PI3K/AKT, Airway remodeling*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, Web of Science, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Cough-variant asthma is a special type of asthma which results in chronic cough in children. The main clinical manifestation of cough is symptom which is characterized by irritant dry cough, seasonal aggravation and severity during the day and night [1]. Clinical epidemiology

investigations have shown that pediatric cough-variant asthma accounts for 41.95 % of chronic cough in children, and about one-third of patients develop typical asthma in the absence of appropriate and effective treatment [2]. Therefore, early effective diagnosis and targeted preventive treatment for patients with cough-variant asthma are of great significance in preventing further aggravation of the disease and prognostic treatment [3].

The main pathological features of cough-variant asthma are airway remodeling and airway inflammation. Under long-term and sustained airway inflammation, the airway wall structure undergoes changes such as smooth muscle hyperplasia, myofibroblast hyperplasia, collagen fiber deposition and airway basement membrane thickening, resulting in airway remodeling which leads to recurrent asthma [4]. The PI3K/AKT signaling pathway plays an important role in regulating cell survival, proliferation and apoptosis. Studies have shown that the PI3K/AKT signaling pathway plays a crucial role in asthma by promoting airway inflammation and hyperreactivity [5]. Treatment with hormone-based western medicine does not cure cough-variant asthma, and long-term application of these drugs results in adverse side effects, while the clinical effect of TCM is exact [6].

Pinellia ternata is a medicinal plant that grows in the Sichuan Province of China. The important bioactive component of *Pinellia ternata* is a polysaccharide which exerts significant anti-inflammatory, antioxidant and anticancer effects. *Pinellia ternata* has anti-asthma effects, but there are no studies on its effect on airway remodeling in cough-variant asthma [7]. Therefore, the present research was aimed at investigating the influence of *Pinellia* polysaccharide on airway remodeling in cough variant asthmatic rats, and involvement of PI3K/AKT signaling pathway and airway smooth muscle-related apoptotic molecules in the process.

EXPERIMENTAL

Animals and reagents

Seventy-five male SD rats aged 1½ - 2 months (weight range, 180 - 200 g) were supplied by the Animal Management Center of Huangjiahu Hospital of Hubei University of Chinese Medicine, Wuhan. Ovalbumin and aluminum hydroxide were products of American Sigma Company. *Pinellia* polysaccharide extract was obtained from National Institute for Drug and Biological Products Testing. Antibodies for p-AKT, p-PI3K, MMP-9, Bcl-2, Bad, Fas, Caspase 3 were purchased from Abcam Biotechnology Co. Ltd (UK). Hematoxylin and eosin (H&E) staining Kit and immunohistochemical sheep anti-rabbit secondary antibody were bought from Shanghai Biyuntian Co. Ltd.

Ethical approval

This study received approval (no. HJH2022003) from the Animal Ethics Authority of Huangjiahu Hospital of Hubei University of Chinese Medicine and also followed NIH guidelines [8].

Establishment of rat model of cough-variant asthma

Seventy-five (75) Sprague Dawley (SD) rats were assigned randomly to blank control, model, and low- *Pinellia ternata* (100 mg/kg), medium-*Pinellia ternata* (200 mg/kg) and high-dose *Pinellia ternata* (400 mg/kg) groups. Each group had 15 rats. From day 1 to day 8, the model group and *Pinellia* polysaccharide intervention group were sensitized *via* intraperitoneal injection of 10 % ovalbumin and aluminum hydroxide suspension (1 mL). On the 15th day, in the excitation stage, 1 % ovalbumin solution in normal saline was given by atomization for 20 min, once a day for 1 week.

Thirty minutes (30 min) before atomizing and inhalation of 1 % ovulin solution in saline, the 3 doses of *Pinellia* polysaccharide were administered orally via gavage, while rats in the model group received equivalent doses of normal saline in place of *Pinellia* polysaccharide.

Sampling and sample preparation

Following drug administration, the rats were fasted for 24 h. After the last atomization, the rats were sacrificed under 10 % chloral hydrate anesthesia. The chest cavity was cut open, and the lung tissue on the right side was excised and preserved in a refrigerator at -80 ℃ for further examination. The left lung was fixed overnight in 4 % paraformaldehyde solution and processed routinely for H&E staining and light microscopy. After cutting, dehydrating, embedding and sectioning, paraffin sections were stained with H&E, and the morphological changes in bronchial texture in lung tissues of newborn rats in the three groups were examined under a light microscope after sealing.

Image analysis of airway remodeling

Image analysis software Image-Pro Plus5.1 was used to conduct analysis of airway remodeling in lung tissue sections. Three complete small and

medium bronchi were randomly selected from each section and enlarged 200 times under the microscope. The bronchial airway smooth muscle area (Wam), inner duct wall area (Wai), total duct wall area (Wat) and basement membrane perimeter (Pbm) were measured. The measured values of Wam, Wai and Wat were normalized with Pbm and presented as Wat/Pbm, Wai/Pbm and Wam/Pbm, respectively.

Western blot assay

Total protein was extracted from lung tissue in each group by preparing a 10 % homogenate in RIPA buffer at 4 ℃. The lysates (10 % homogenate) were centrifuged to obtain supernatants. The protein concentrations of the lysates were determined with BCA method. Thereafter, the proteins were resolved using SDS-polyacrylamide gel electrophoresis, followed by transfer to PVDF membranes which were sealed by incubation with non-fat milk solution. Then, the membranes were incubated overnight at 4 ^{o}C with relevant 1^{o} immunoglobulins, and thereafter with 2^{o} antibodies. The Bio-RAD image laboratory software was used for protein band analysis.

Statistical analysis

The SPSS20.0 was used for statistical analysis. Measurement data are expressed as mean ± standard deviation. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Histomorphological changes in rats

Results from H&E staining showed that in blank control group, there was no obvious infiltration of inflammatory cells in lung tissue. The bronchial mucosa was smooth and complete; there was no obvious thickening of smooth muscle layer or tube wall, and the epithelial cells were orderly arranged. Bronchial structure was clearly disorganized in model rats, with hyperemia and edema in the submucosa and wall of the bronchial tube. Moreover, there was massive infiltration of inflammatory cells around the bronchial wall, obvious thickening of the wall, narrowing of the lumen, hyperplasia and hypertrophic smooth muscle layer, mucosal epithelial degeneration, and exfoliation. Relative to model rats, the pathological changes in lung tissue and bronchus in *Pinellia* polysaccharide intervention groups were mitigated, with low-to-moderate inflammatory cell infiltration, absence of obvious smooth muscle hyperplasia, mild degeneration of mucosal epithelium, and slightly thickened airway wall.

Morphology of airway remodeling

The levels of Wat, Wam and Wai were markedly up-regulated in model rats, relative to blank control rats ($p < 0.05$). However, Wat, Wam and Wai concentrations in the 3 *Pinellia* polysaccharide groups were markedly up-regulated, relative to blank control, but were decreased, when compared to the corresponding levels in model rats. However, levels of Wat, Wam and Wai were comparable in the 3 *Pinellia ternata* polysaccharide groups ($p < 0.05$). These results are shown in Table 1.

Lung tissue protein levels

The levels of p-Akt, p-PI3K and MMP-9 were markedly higher in model rats than in blank control rats. However, p-Akt, p-PI3K and MMP-9 levels in low-dose, medium-dose and high-dose *Pinellia* polysaccharide groups were markedly lower than the corresponding levels in model rats, but were markedly higher than blank control values. In contrast, levels of these parameters were statistically dissimilar in the 3 *Pinellia* polysaccharide groups ($p < 0.05$). These results are shown in Table 2.

Protein expression levels of smooth muscle-related apoptotic factors in rat lung tissues

The level of Bcl-2 was markedly higher in model group than in blank control group, and the levels of Bad, Fas and Caspase3 were significantly lower in model group than in blank control group ($p < 0.05$).

Table 1: Image analysis of airway remodeling in each group (μm, n = 15)

Group	Wat/Pbm	Wai/Pbm	Wam/Pbm
Blank control	34.24±3.52	16.43±2.58	2.63±0.43
Model	65.00±5.34^{a}	40.75±3.26^{a}	8.16±1.42^{a}
Low-dose	54.38±5.04^{ab}	33.92±2.75^{ab}	6.75±1.12^{ab}
Medium-dose	47.47±4.54^{abc}	27.82±2.90^{abc}	4.19±0.95^{abc}
High-dose	40.55±3.70^{abcd}	21.92±2.61^{abcd}	3.25±0.69^{abcd}

$^{a,b,c}P < 0.05$, ^{a}vs blank control; ^{b}vs model; ^{c}vs low *Pinellia* polysaccharide dose, ^{d}vs medium *Pinellia* polysaccharide dose

Table 2: Comparison of protein levels in lung tissues amongst the groups (mean ± SD, n = 15)

Group	p-AKT	p-PI3K	MMP-9
Blank control group	0.18±0.05	0.28±0.04	0.13±0.03
Model group	0.92±0.36[a]	1.25±0.37[a]	0.83±0.35[a]
Lowest dose polysaccharide group	0.59±0.22[ab]	0.84±0.21[ab]	0.59±0.17[ab]
Medium-dose *Pinellia ternata* polysaccharide group	0.36±0.16[abc]	0.58±0.12[abc]	0.39±0.09[abc]
High-dose *Pinellia ternata* polysaccharide group	0.24±0.07[abcd]	0.40±0.06[abcd]	0.21±0.07[abcd]

[a,b,c]$P < 0.05$, [a]vs blank control; [b]vs model; [c]vs low-dose *Pinellia* polysaccharide; [d]$p < 0.05$, vs medium-dose *Pinellia* polysaccharide medium group

Table 3: Protein expression levels of smooth muscle-related apoptotic factors in lung tissues of rats in each group (n=15)

Group	Bad	Fas	Caspase3	bcl-2
Model	0.76±0.29	0.86±0.28	0.71±0.22	0.31±0.12
Low-dose *Pinellia ternata* polysaccharide	0.34±0.11[a]	0.28±0.04[a]	0.33±0.11[a]	0.69±0.37[a]
Medium-dose *Pinellia ternata* polysaccharide	0.45±0.14[ab]	0.37±0.09[ab]	0.41±0.14[ab]	0.54±0.26[ab]
High-dose *Pinellia ternata* polysaccharide	0.52±0.23[abc]	0.47±0.17[abc]	0.55±0.13[abc]	0.44±0.21[abc]
Blank control	0.63±0.21[abcd]	0.63±0.24[abcd]	0.64±0.24[abcd]	0.39±0.15[abcd]

[a,b,c,d]$P < 0.05$, [a]vs blank control; [b]vs model; [c]vs low-dose *Pinellia* polysaccharide, [d]vs medium-dose *Pinellia* polysaccharide

The levels of Bcl-2 in the 3 *Pinellia* polysaccharide groups were markedly up-regulated, relative to that in blank control rats, but were low, relative to model values. The levels of Bad, Fas and Caspase-3 were markedly down-regulated in the 3 *Pinellia* polysaccharide dose groups, when compared with blank control, but they were higher than the corresponding levels in model group ($p < 0.05$). There were statistically significant differences in levels of bcl-2, Bad, Fas and Caspase-3 amongst the 3 *Pinellia* polysaccharide dose groups ($p < 0.05$; Table 3).

DISCUSSION

Bronchial asthma is shared by a variety of cells and cellular elements involved in the formation of airway hyperresponsiveness. It is a reversible, obstructive respiratory disease characterized by recurrent cough, wheezing and shortness of breath and other clinical symptoms. Severe cases may result in status asthmaticus, difficulty in breathing, deterioration of lung function, and increased risk of pulmonary encephalopathy. Bronchial asthma seriously affects the growth and development of patients and their daily lives [9].

Cough-variant asthma is a special variant of asthma in which patients present no obvious symptoms or signs such as wheezing and shortness of breath: the major clinical presentation is cough. Clinical studies have revealed that about one-third of the patients are prone to developing typical bronchial asthma which seriously affects physical and mental health and daily life [10]. In recent years, the number of patients with cough-variant asthma has increased, which brings great pressure to the society and the affected families. At present, no radical cure has been found for cough-variant asthma. Glucocorticoids are often used to alleviate the clinical symptoms in patients, but studies have shown that some patients treated with glucocorticoids had no significant reductions in clinical symptoms [11]. Therefore, it is of great clinical significance to seek effective treatment for cough-variant asthma so as to improve their short- and long-term prognosis.

Pinellia ternata is one of the traditional Chinese medicines in China. Pharmacological studies have shown that *Pinellia ternata* polysaccharide, the main bioactive component of *Pinellia ternata*, has a wide range of pharmacological activities, including anti-tumor, anti-fibrosis and anti-inflammatory effects, and it has been widely used in a variety of chronic lung diseases [12].

Airway remodeling and airway inflammation are characteristics of recurrent cough-variant asthma in patients. The two main pathological changes in airway remodeling are due to the deposition of collagen, airway epithelium goblet cells and hyperplasia, increases in airway smooth muscle thickness, and thickening of basement membrane and extracellular matrix. Increases in new blood vessels are caused by epithelial thickening which is generally considered to be the result of repeated inflammatory damage and repair of the airway, and it is the major morphological and functional change in structural cells of the airway wall [13,14]. Airway remodeling is usually irreversible, and it often predisposes to pathological features such as

airway hyperresponsiveness, airway stenosis and airflow limitation, as well as repeated attacks and gradual exacerbation of asthma symptoms [15].

In this study, the model group rats were sensitized and repeatedly stimulated with OVA and alumina for 15 days. Results from H&E staining revealed that the lung tissues of blank control group rats had no obvious infiltration of inflammatory cells; the bronchial mucosa was smooth and complete, with no obvious thickening of smooth muscle layer and tube wall. Moreover, there was ordered arrangement of epithelial cells. The bronchial structure in model rats was obviously disordered, with hyperemia and edema in the submucosa and wall of the bronchial tube, infiltration of a large population of inflammatory cells around the bronchial wall, thickening of the wall, narrowing of the lumen, hyperplasia and hypertrophic smooth muscle layer, mucosal epithelial degeneration, and exfoliation. Thus, airway remodeling model of cough-variant asthma was successfully established in the rats.

The pathological changes in lung tissue and bronchus in *Pinellia* polysaccharide intervention group were mitigated, relative to model rats, with low-to-moderate inflammatory cell infiltration, absence of smooth muscle hyperplasia, mild degeneration of mucosal epithelium, and slightly thickened airway wall. There were markedly higher Wat, Wam and Wai levels in model rats than in blank control rats, while concentrations of Wat, Wam and Wai were up-regulated in rats given the 3 *Pinellia* polysaccharide doses, relative to blank control rats, but they were low, when compared with model rat values. The 3 *Pinellia ternata* polysaccharide dose groups had statistically marked variations in Wat, Wam and Wai levels. These results suggest that *Pinellia* polysaccharide inhibited the pathological changes in airway remodeling, and also suppressed airway remodeling in rats with cough-variant asthma.

Pinellia ternata is one of the traditional Chinese medicines in China. It produces the effects of eliminating *ruffian* and *dispersive knot*, reducing reflux, stopping vomiting, eliminating *dampness*, and reducing phlegm. Pharmacological studies have shown that polysaccharide extracted from *Pinellia ternata* exerted significant anti-inflammatory and antioxidant effects which make it suitable for use for scavenging free radicals and as an anti-inflammatory agent. *Pinellia* polysaccharide also stabilizes the ratio of Th1/Th2 cells in the body and relieves asthma [16].

The PI3K/AKT signaling pathway is a signal transduction channel with a wide range of functions in humans, and it is important in regulation of cell proliferation, differentiation, migration and apoptosis [17]. It is known that AKT is the direct target gene of PI3K and the downstream effector of PI3K. Phosphorylation of AKT is an indicator of PIK3 activity, and phosphorylation of AKT regulates downstream proteins, thereby regulating basic cell functions [18]. A member of matrix metalloproteinases, MMP-9 is a downstream target of PI3K/AKT signaling pathway, and one of the major proteases involved in asthma; it plays an important role in airway remodeling by degrading and reshaping dynamic balance in extracellular matrix [19]. Clinical studies have shown that the PI3K/AKT signal route is closely associated with the pathogenesis of human asthma. Indeed, the PI3K/AKT signal route is crucial in pathogenesis of bronchial asthma via its influence on inflammatory cells and secretion of airway mucus, as well as regulation of the proliferation, differentiation, migration and apoptosis of airway smooth muscle cells [20].

This investigation demonstrated markedly higher levels of p-Akt, p-PI3K and MMP-9 in model rats than in blank control rats, and their levels in the low-, medium-and high-dose *Pinellia* polysaccharide groups were markedly higher than blank control values, but lower than the corresponding levels in model group. There were marked variations in levels of p-Akt, p-PI3K and MMP-9 amongst the 3 *Pinellia* polysaccharide dose groups. These results suggest that PI3K/AKT signaling pathway plays an important role in suppression of airway remodeling in this disease.

Previous studies reported that reduced airway smooth muscle cell apoptosis is one of the possible mechanisms of airway remodeling in patients with cough-variant asthma [21]. Other studies have found that abnormal expression of Fas pathway (exogenous apoptotic pathway) is an important factor in inhibition of apoptosis of airway smooth muscle cells. The most representative pro-apoptotic molecules are Bad, Fas and Caspase3, while Bcl-2 is an anti-apoptotic molecule. All of these factors are closely related to the apoptosis of airway smooth muscle cells. The phosphorylation of Bad by AKT blocks the pro-apoptotic function of Bad [22,23].

The results obtained in this study showed that the expressions of pro-apoptotic factors i.e. Bad, Fas and Caspase-3 were markedly up-regulated in *Pinellia* polysaccharide-fed rats, relative to model rats, while the anti-apoptotic molecule Bcl-

2 in *Pinellia* polysaccharide group was reduced, relative to model rats. Cough-variant asthma rats exhibited lower expressions of molecules that promote apoptosis, thereby inhibiting high expressions of apoptotic molecules. *Pinellia* polysaccharide promoted apoptosis by up-regulating Bad, Fas and Caspase-3, and it reduced the inhibition of apoptosis by decreasing the expression of Bcl-2. Thus, *Pinellia* polysaccharide increased apoptosis of smooth muscle cells and slowed down airway remodeling.

CONCLUSION

Pinellia polysaccharide slows down airway remodeling and reduces airway inflammation in rats with cough-variant asthma by regulating the expressions of the apoptotic molecules, viz, Bad, Fas and Caspase-3, in airway smooth muscles through activation of PI3K/AKT signaling pathway. Therefore, *Pinellia* polysaccharide has a potential for use in the treatment of asthma.

DECLARATIONS

Acknowledgements

None provided.

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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