Osthole attenuates pulmonary arterial hypertension by the regulation of sphingosine 1-phosphate in rats

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[ABSTRACT] Osthole is observed to have the capacity to treat pulmonary arterial hypertension (PAH) in rats, but molecular mechanisms is still unknown. The present study aims to discover therapeutic targets and explore therapeutic mechanism of osthole against PAH from metabolic perspective. A rat model with PAH was successfully established with MCT, following osthole administration, then untargeted metabolomics assay was performed using UPLC-Q-TOF-MS to identify differential metabolites and associated metabolic pathways, at last mechanistic investigation was done by qRT-PCR, Western blot and ELISA. Differential metabolites characterized in rats with PAH were mostly assigned to sphingolipid metabolism, synthesis of unsaturated fatty acids, glycolysis, nucleotide metabolism, steroid hormone biosynthesis. Furthermore, osthole reversed high level of S1P by modulating metabolic enzyme Sphk1 in rats with PAH. In addition, osthole inhibited the expression of Sphk1 by downregulating microRNA-21, phosphorylation of Akt, phosphorylation of mTOR in vivo and in vitro. These results demonstrated that metabolomics is a promising approach to discover potential drug target for PAH treatment. Importantly, our findings further elucidated therapeutic mechanism of osthole, a natural product, having a role of metabolic regulator to potentially treat PAH by targeting inhibition of Sphk1/S1P via microRNA-21-PI3K/Akt/mTOR signal pathway. Altogether, this discovery paves a critical foundation for enabling osthole to be a candidate compound to treat PAH.

[KEY WORDS] Pulmonary arterial hypertension; Osthole; Metabolomics; Sphk1/S1P; MicroRNA-21- PI3K/Akt/mTOR; Therapeut-ic mechanism

[Introduction] Pulmonary arterial hypertension (PAH) is a severe chronic cardiopulmonary disease with high mortality [1]. Recently, there is increasing evidence to manifest that dysregulated metabolism is supposed to be trigger of PAH development [8], and significant shift in utilization of energy source is considered as pathological hallmarks of PAH [9]. Metabolic disorders in aerobic glycolysis, fatty acid oxidation and tricarboxylic acid cycle play a critical role in pulmonary circulation and right ventricle in rodent models and patients with PAH, medicating cell proliferation, migration and angiogenesis, leading to pulmonary vascular remodeling and right ventricular hypertrophy [4-5]. In addition, many metabolic pathways associated with inflammation and fibrosis are dysregulated during the progression of PAH, including sphingolipid metabolism, carnitine metabolism, heme metabolism, bile acid synthesis, arginine metabolism, fatty acid metabolism [1-8][11]. Collectively, metabolic reprogramming plays critical role in molecular pathogenesis of PAH. Sphingolipids are important biomolecules and play crucial roles in maintaining energy balance, guiding intercellular communication, and regulating membrane dynamics. Moreover, lipids are the primary component of pulmonary surfactant functions to reduce the surface tension at the air/liquid interface in alveoli and prevent lung collapse [10]. Sphingosine 1-phosphate (S1P), as a single-chain lipid produced by phosphorylation of sphingosine and catalyzed by sphingosine kinases1 (Sphk1), is the key mediator in promoting cell proliferation, migration, and angiogenesis [9]. S1P is crucial in PAH development by promoting PASMCS proliferation contributing to pulmonary vascular remodeling and pulmonary arterial pressure increase, and may be a promising small molecular thera-
Osthole is a natural product derived from *Angelica pubescens* Maxim [17-19]. Our previous study preliminarily revealed that osthole has capacity to prevent PAH progression via modulating inflammatory processes and dysregulated metabolism [20-21], but molecular mechanism remains incompletely understood. To this end, in present study, we firstly employed untargeted metabolomics method to identify differential metabolites and associated metabolic pathways with the development of PAH. Then, we figured out to functionally characterize metabolic regulation of osthole upon PAH progression by identifying the key metabolites and associated pathways with therapeutic potential. At last, we aimed at combining the metabolic discovery of PAH development, with functional assessment of osthole, to elucidate therapeutic mechanism of osthole against PAH.

**Materials and Methods**

**Chemicals and Reagents**

Compound of osthole (≥ 99%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China); Monocrataline (MCT) (95%) was purchased from Aladdin Co. (Shanghai, China); Sphingosine 1-phosphate (S1P) (≥ 98%) was purchased from Aladdin Co. (Shanghai, China); All the other chemicals were all analytical grade.

**Ethics Statement**

All animal handling and experiments were strictly performed in accordance with the recommendations of the Ethical Committee of Laboratory Animals at Harbin Medical University, Harbin, China and were in compliance with the Chinese national regulations on the use of experimental animals. The experimental protocols were approved by the Center for Laboratory Animals, Harbin Medical University, Harbin, China.

**Animals**

Sparague Dawley rats (SD, male, weight at 200 ± 20 g) were purchased from Experimental Animal Center of Harbin Medical University, which is fully accredited by Institutional Animal Care and Use Committee (IACUC) of Harbin Medical University (Harbin, China) (License No.20160002). We carried out the following animal experiments according to previous study reported [21]. PAH group and osthole group received a single injection of MCT (60 mg·kg⁻¹, i.p.) to induce PAH for 28 days, and control group received equivalent volume of saline injection. Rats of osthole group were orally administrated with 40 mg·kg⁻¹ and 80 mg·kg⁻¹ osthole daily respectively.

**Hemodynamic Evaluation of MCT induced model**

Measurement of hemodynamic parameters was performed using a micro manometer pressure catheter (Model FTH-1912B-8018, Scisense Instruments, London, ON, Canada) which placed in the right jugular vein and advanced into the right ventricle (RV) to monitor RV hemodynamic parameters. The trachea was isolated and connected to a ventilator (Model 28025, Biological Research Apparatus, Comerio, Italy) to assess respiratory function. The right ventricular systolic pressure (RVSP) were recorded (Fig. 1) and analyzed using a data acquisition system (ADV500, Taibei, China). After the animals were deeply anesthetized with an intraperitoneal injection of chloral hydrated (300 mg·kg⁻¹), and the heart and lung were dissected and weighted. The right ventricle free wall (RV) was separated from the left ventricle and septum (LV + S) to determine wet weights and the RV to LV + S weight ratio (RV/LV + S).

**Sample preparation**

Rat blood was obtained from the above-mentioned individuals. The serum was separated immediately by centrifugation at 3500 g for 15 min at 4 °C and stored at −80 °C. Prior to the analysis, serum samples were thawed on ice at 4 °C, then, 120 μL cold methanol was added to 40 μL serum, after vigorous shaking for 1 min, then were centrifuged at 4000 g for 20 min at 4 °C. A pooled QC sample was prepared by mixing equal volumes (20 μL) from each serum samples as they were being aliquoted for analysis.

**UPLC-Q-TOF-MS analysis**

All samples were acquired by the LC-MS system followed machine orders. Firstly, all chromatographic separations were performed using an ultra performance liquid chromatography (UPLC) system (Waters, USA). An ACQUITY UPLC BEH C₁₈ column (100 mm × 2.1 mm, 1.7 μm, Waters, USA) was used for the reversed phase separation. The column oven was maintained at 50 °C. The flow rate was 0.4 mL·min⁻¹ and the mobile phase consisted of solvent A (water modified with 0.1% formic acid) and solvent B (methanol modified with 0.1% formic acid). Gradient elution conditions were set as follows: 0–2 min, 100% phase A; 2–11 min, 0% to 100% B; 11–13 min, 100% B; 13–15 min, 100% A. The injection volume for each sample was 10 μL.

A high-resolution tandem mass spectrometer SYNAPT G2 XS QTOF (Waters, USA) was used to detect metabolites eluted from the column. The Q-TOF was operated in both positive and negative ion mode. The capillary and sampling cone voltages were set at 1 kV and 40 V in positive ion mode and 1.58 kV and 40 V in negative ion mode, respectively. Mass spectrometry data were acquired in centroid mode. The TOF mass range was from 50 to 1200 Da and the scan time was 0.2 s. For the MS/MS detection, all precursors were fragmented using 20–40 eV, and the scan time was 0.2 s. During the acquisition, the LE signal was acquired every 3 s to calibrate the mass accuracy. The stability of the analysis was continuously monitored by analyzing QC samples at intervals of every 10 samples.

**Data Processing**

Raw data was processed by Progenesis QI (version 2.2, Waters, USA) and further analyzed by MetaX. Principle comp-
ponent analysis (PCA), partial least squares discriminant analysis (PLS-DA) and super partial least squares discriminant analysis (sPLS-DA) were used for multivariate data analysis. The fold change (fold change ≥ 1.2 or ≤ 0.8 and \( P < 0.05 \)) and variable importance in the projection (VIP ≥ 1.0) were used to determine the differential compounds, and presented as heatmap by a more intuitive analysis.

**Cell Culture**

Pulmonary arteries (PAs) were immediately isolated and carefully dissected (without endothelium), and PASMCs were scraped from PAs and cultured as previously described [22]. Cells were maintained at 37 ºC in a humidified incubator with 5% \( \text{CO}_2 \). Cells in hypoxic culture were incubated with a gas mixture containing 92% \( \text{N}_2 \), 5% \( \text{CO}_2 \), 3% \( \text{O}_2 \) for 24 h. Cells were quiesced for 24 h in DMEM without serum before each experiment, then treated with different dose of osthole.

**MTT**

PASMCs were cultured in 96-well plates, and then subjected to growth arrest for 24 h. After the hypoxia and osthole treatments for 24 hours according to the different experiment groups in the incubator, the cells were incubated for 4 h in a medium containing 0.5% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT). The reaction was terminated by adding DMSO to the medium followed by incubation for 10 min at 37 ºC. The absorbance was read at 490 nm in a spectrophotometer to assess cells.

**Enzyme-Linked Immunosorbent Assay**

Concentration of Sphk1 in serum were examined with rat Sphk1 ELISA kit (Oulu Bio) following the manufacturer’s instructions. Optical density (O.D) was determined by microplate reader (Biotek, USA) set at 450 nm. The serum Sphk1 concentration in each sample was calculated by standard curve linear regression equation.

**Western blotting analysis**

Lung tissues and PASMCs were lysed on ice with RIPA (Beyotime, China) including 1% protease inhibitor PMSF. Protein concentrations were determined by the BCA protein assay (BCA, USA). Protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes (Milli-
pore, USA) and blocked with a Tris-buffered saline buffer including 5% skimmed milk, then incubated with the following primary antibodies of Sphk1 (Bioss), Akt (Beyotime), p-Akt (Beyotime), mTOR (Beyotime), p-mTOR (Beyotime), GAPDH (Beyotime), β-actin (Beyotime) for overnight at 4 °C, followed by incubation with secondary antibody (goat anti-rabbit and goat anti-mouse) for 60 min at room temperature, respectively. Immunoreactivity was visualized by fluorography, and immunoblots were scanned with BIO-RAD software.

**qRT-PCR analysis**

Total RNA was extracted from lung tissues and PASMCs using Trizol reagent (Beyotime Biotechnology, Inc.). cDNA was generated using a cDNA synthesis kit (Wanleibio, Inc.). The primers used were as follows: micro RNA-21, forward primer 5'-AGGCTGTAAGCTCTCCCCACT-3' and reverse primer 5'-GCCACACTGCTGCTTTTAGTCCC-3'; GAPDH, forward 5'-ACGCCTCTGGCCGTACC-3' and reverse 5'-TGGTGAAGCTGTAGCCGC-3'. MicroRNA-21 was determined using NCSYB GREEN qPCR Master Mix kit (Life Science, Inc.) and GAPDH was used as a control for normalization. The relative level of microRNA-21 was calculated with the comparative Cq method (2-ΔΔCq). qRT-PCR was performed with SYBR Green I (included in kit) on ABI 7300 (Thermo Fisher Scientific, Inc.). The primers used were as follows: micro RNA-21, forward primer 5′-TGGTGAAGCTGTAGCCGC-3′ and reverse primer 5′-AGGCTGTAAGCTCTCCCCACT-3′ and reverse primer 5′-GCCACACTGCTGCTTTTAGTCCC-3′, GAPDH was used as a control for normalization.

**siRNA design and transfections**

PASMCs were transfected with small interfering RNA (siRNA), which was designed and synthesized by GenePharma (China). PASMCs were cultured till 70% confluence. After growth arrest, 5 μg siRNA and 8 μL X-tremeGene siRNA transfection reagents were separately diluted in serum-free Opti-MEM-1 medium. After gently mixing the siRNA and transfection reagent together, the mixture was incubated at room temperature, and then directly added into the cells for 6 h. Subsequently, the cells were switched to normal culture medium and cultured under normoxic or hypoxic growth conditions for another 24 h.

**Statistical analysis**

Statistical analysis was performed using t-test or one-way analysis of variance (ANOVA). All data were presented as mean ± standard errors of the means, P value<0.05 was considered statistically significant.

**Results**

**Therapeutic capacity validation of osthol on PAH**

PAH is characterized by progressive increased pulmonary artery pressure. Right ventricular systolic pressure (RVSP) is the critical index to evaluate PAH [23]. To investigate therapeutic capacity of osthol on PAH, we observed the RVSP and right ventricular hypertrophy index (RVHI). Fig.1 demonstrated the raised RVSP and the increased ratio of right ventricle (RV) weight to left ventricular (LV) weight plus septum (RV/LV + S) obtained from rats induced by MCT, and these changes were both reversed by osthol treatment, and no distinct difference in therapeutic effect between 40 mg·kg⁻¹ and 80 mg·kg⁻¹ osthol, and 40 mg·kg⁻¹ osthol was therefore selected for metabolomics and functional experiments in further study.

**Untargeted metabolomics characterized differential metabolites closely associated with PAH progression**

To identify the key metabolites with therapeutic targets potential, we employed untargeted metabolomics method to identify differential metabolites and associated metabolic pathways with the development of PAH. UPLC-Q-TOF-MS based metabolomics method was developed to comparatively analyze serum metabolomes in both samples collected from healthy control rats (Control group) and the rats with PAH (PAH group).

It has been studied the typical metabolic profile of serum samples, subtle differences can be visualized between PAH group and Control group. Furthermore, pattern recognition analysis was engaged to discover and identify metabolite features whose level changes accounted for the metabolic differences. Obvious group classifications were characterized by PLS-DA score plots between PAH and Control group in negative mode and positive mode (Fig. 2A, B). The variable importance for projection (VIP) exceed 1 were selected based on PLS-DA according to S-por and highlighted with light blue (Fig. 2C, D). In addition, Heatmap illustration clearly demonstrated the metabolic differences attributed to a panel of differential metabolic features (Fig. 2E, F).

To assign metabolic features to the specific compounds, 55 differential metabolites were characterized to mostly distinguish the rats with PAH from healthy control rats, including sphingolipids, free fatty acids, amino acids, nucleotides, bile acids, and steroid hormone(P < 0.05). Glycolytic changes were manifested in this study to increase the levels of fructose 6-phosphate, glucose 6-phosphate and glycolytic intermediate glyceraldehyde-2,3-phosphate in PAH serum compared to healthy control (Fig. 3G). Such finding confirms again metabolic reprogramming is triggered with the increased glycolysis during the development of PAH. Moreover, enhanced levels of sphingosine-1-phosphate (SIP) and dehydro-SIP were observed with the PAH progression in this study (Fig. 3B). In addition, our data illustrated the levels of 20 free fatty acids (FAs) were significantly lower in PAH serum relevant to control levels, including omega-3 PUFA (ALA, ETA, EPA, DHA) (Fig. 3A) and omega-6 PUFA (arachidonic acid, AA; linoleic acid, LA; dihomo-linoleate, DGLA) (Fig. 3C) and omega-9 MUFA (nervonic acid, NA; erucic acid) (Fig. 3A). Furthermore, sterocobilinogen, sterocobilin, esatrene 3-sulfate and estriol (Fig. 3D) were found to be closely associated with PAH. Significantly high level of serum sterocobilinogen (74.92 fold) and sterocobilin (5.17 fold) annotated into Heme metabolism were determined in PAH group (Fig. 3I), indicated increased heme degradation [60]. In addition, Purine metabolism were detected to increase the levels of 20 free fatty acids (FAs) were significantly lower in PAH serum relevant to control levels, including omega-3 PUFA (ALA, ETA, EPA, DHA) (Fig. 3A) and omega-6 PUFA (arachidonic acid, AA; linoleic acid, LA; dihomo-linoleate, DGLA) (Fig. 3C) and omega-9 MUFA (nervonic acid, NA; erucic acid) (Fig. 3A). Furthermore, sterocobilinogen, sterocobilin, esatrene 3-sulfate and estriol (Fig. 3D) were found to be closely associated with PAH. Significantly high level of serum sterocobilinogen (74.92 fold) and sterocobilin (5.17 fold) annotated into Heme metabolism were determined in PAH group (Fig. 3I), indicated increased heme degradation [60]. In addition, Purine metabolism were detected to increase the levels of ITP and Urate-3-ribonucleoside in PAH serum, and Pyrimidine metabolism were disturbed with the higher level of UppppU, dUMP, Thymidine and lower level of Cytidine in PAH serum (Fig. 3H).
Fig. 2  Global metabolomics revealed the distinctively differential metabolites of healthy control rats and PAH rats. (A–B) PLS-DA based group classification of the detected metabolic features of the healthy control rats and PAH rats. (C–D) S-Plot of the PLS-DA model. The variable importance for projection (VIP) exceed 1 were shown in upper-right and low-left quadrants of the PLS-DA corresponding S-plot, and highlighted with blue filled circle. (E–F) Heatmap overview of the detectable metabolic features of the healthy control rats and PAH rats. (A, C, E negative ionization mode; B, D, F Positive ionization mode, n = 8)
In short, our data demonstrated many differential metabolites mostly accounted for the increased glycolysis activity, enhanced sphingolipid metabolites, decreased free fatty acids, dysregulated estrogen, enhanced bile acid biosynthesis, high levels of Heme metabolites, upregulated purine metabolites, abnormal pyrimidine metabolites characterized in serum of the rats with PAH.

**Dysregulated metabolic pathways mostly affected by PAH progression**

Capturing biological significance underlying the dysregulated metabolic pathways will assist in better understanding of metabolic regulatory mechanisms of PAH progression, thus we further home the above differential metabolites to their metabolic pathways in this section. The identified differential metabolites were annotated to 19 metabolic pathways by MetaX involving Sphingolipid metabolism, Glycolysis, Fatty acid metabolism, Amino acid metabolism, Heme metabolism, Purine metabolism, Pyrimidine metabolism, etc. The mostly affected metabolic pathways with PAH progression are schematically illustrated in Fig. 4.

**Differential S1P was characterized as a molecular target of Osthole to treat PAH**

Based on differential metabolites and associated dysregulated metabolic pathways characterized with PAH progression by metabolomics assay, we further explored the therapeutic target and intervention mechanism of a natural product YAO Li, et al. / Chin J Nat Med, 2020, 18(4): 308–320

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**Graphs**

- **A** Unsatuated fatty acid metabolism
- **B** Sphingolipid metabolism
- **C** Linoleic acid metabolism
- **D** Steroid hormone metabolism
- **E** α-Lenolenic acid metabolism
- **F** Arachidonic acid metabolism
- **G** Glucose metabolism

- **Graph Details:**
  - ALA, ETA, EPA
  - Docosadienoic acid
  - Erucic acid, Nervonic acid
  - γ-Linolenic acid
  - Dihomo-γ-Linolenic acid
  - Docosahexaenoic acid
  - Dihydro-S1P
  - Dihydroxy-S1P
  - Dihydroxy-S1P
  - 17α-Hydroxypregnenolone
  - 17α, 21-Dihydroxypregnenolone
  - Estrone 3-sulfate
  - Estriol
  - Estradiol-17β 3-sulfate
  - 13(S)-HpOTrE
  - 17-Hydroxylinolenic acid
  - 9(S)-HOTrE
  - Volicitin
  - 17α-Hydroxypregnenolone
  - 17α, 21-Dihydroxy-pregnadiolone
  - Estriol 3-sulfate
  - Estradiol 17β 3-sulfate
Osthole to treat PAH in rats. The developed metabolomics method was applied to compare serum metabolomes among healthy control rats (Control group), the rats with PAH (PAH group) and the PAH rats with osthole treatment (Os group). The result manifested that dysregulated metabolomes with PAH progression were markedly recovered by osthole (Figs. 5A, B). Simultaneously, Heatmap overview of interactive metabolomes clearly demonstrated metabolic modifications in PAH and restoration by osthole treatment, as the alterations of differential metabolites well identified the therapeutic effect of osthole upon PAH (Figs. 5C, D). Of differential metabolites, the high level of S1P in the rats with PAH was substantially restored by osthole treatment to the serum level in healthy control rats for the first time (Fig. 6B), indicated that S1P might be the therapeutic target of osthole.

Osthole restored dysregulated S1P in the rats with PAH by regulating Sphk1

What mechanisms underlie the downregulation of S1P by osthole? To address these questions, we studied the expression of sphingosine kinase 1 (Sphk1), the key enzyme of S1P synthesized in PAH rats [14]. To determine whether the changes of serum S1P level resulting from the activity alternation of Sphk1, ELISA and western blot were used to measure the expression of Sphk1 in serum and lung among Control group, PAH group and Os group. The analytical results showed that the expression of Sphk1 in serum and lung were significantly increased in PAH group while compared with Control group (Figs 6A, C, D). Furthermore, osthole was evidenced to significantly restore the expression of Sphk1 in PAH group to the normal levels in Control group. So we argued that the upregulation of Sphk1 promoted the biosynthesis of S1P, and osthole inhibited Sphk1 expression to reduce S1P production in the rats with PAH, it might be regarded as a small-molecule inhibitor of Sphk1 to block the biosynthesis of S1P. This discovery is supposed to account for molecular mechanism of osthole to treat PAH in rats.

Osthole significantly inhibited the expression of Sphk1 through microRNA 21- PI3K/Akt/mTOR signal pathway in vivo and vitro

How osthole affects the enzyme activity of Sphk1 in PAH? What signal pathways are involved in osthole induced suppression of Sphk1/S1P? microRNAs (miRs) have been identified as critical regulators in regulating the pathological process of PAH [24]. Previous reports suggested the regulatory interaction of Sphk1 and miR-21 in chronic kidney disease, but it is incompletely unclear in PAH [25]. To explore
these questions, we first detected the levels of miR-21 by qRT-PCR and western blot. qRT-PCR assay confirmed that the expressional level of miR-21 was significantly upregulated in PAH rats (Fig. 6E). MTT assay was used to assess the suitable dosage of osthole on hypoxia induced PASMCs proliferation, $10^{-7}$ was therefore selected for functional experiments in our study (Fig. 7A). Then to assess the efficiency and specificity of the siRNA, the intracellular miR-21 was measured. Expression of miR-21 treated with non-targeted control siRNA (NC) was not different from that in untreated control cells (BLK) (Fig. 7C).

Subsequently, we examined miR-21 and Sphk1 expression under hypoxic conditions in PASMCs, revealing that hypoxia upregulated miR-21 expression at transcriptional levels, and increased the expression of Sphk1 (Figs 7B, F, I, K). We then examined whether miR-21 knockdown prevented hypoxia-induced Sphk1 expression, our results indicated that si miR-21 inhibited hypoxia-induced Sphk1 upregulation (Fig. 7F). These results indicated that miR-21 regulated Sphk1 expression in PAH rats and in PASMCs. Furthermore, the upregulation of miR-21 and Sphk1 were considerably reversed by osthole treatment in vivo and in vitro (Figs. 6C, 6D, 6E, 7B, 7F).

It is known that PI3K/Akt signal pathway involves in osthole induced pulmonary arteries relaxation [20], but it is unknown whether PI3K/Akt/mTOR is crucial for miR-21 mediated Sphk1 expression in PAH. To address this issue, we assessed the phosphorylation level of Akt and mTOR by western blot. We identified the phosphorylation occurred to Akt and mTOR, and their levels were obviously increased in PAH in vivo (Figs. 6F, G). Moreover, miR-21 knockdown inhibited hypoxia-induced increase in phosphorylation of Akt and mTOR in PASMCs (Figs. 7D, E). In addition, treatment of LY294002 (PI3K inhibitor, 1 μmol·L$^{-1}$) to PASMCs significantly lowered the expressional levels of p-Akt/Akt and p-mTOR/mTOR (Figs. 7G, H). These results indicated that miR-21...
activated PI3K/Akt/mTOR signal pathway in PAH rats and in PASMCs. Likewise, the upregulation of phosphorylation of Akt and mTOR were accordingly restored by osthole treatment in vivo and in vitro (Figs. 6F, 6G, 7D, 7E). Next, we detected whether blocking PI3K/Akt/mTOR signal pathway prevented hypoxia-induced Sphk1 increase, our results suggested that blocking PI3K/Akt/mTOR signal pathway with LY294002 (PI3K inhibitor, 1 μmol·L⁻¹) and Rapamycin (mTOR inhibitor, 100 nmol·L⁻¹) both significantly inhibited hypoxia-induced Sphk1 upregulation (Figs. 7I, K). These results indicated that PI3K/Akt/mTOR signal pathway modulated Sphk1 levels in PAH rats and in PASMCs.

Herein, these results suggested miR-21 upregulation stimulated PI3K/Akt/mTOR signal pathway thus to increase Sphk1 in PAH rats and PASMCs, and miR-21-PI3K/Akt/mTOR signal pathway involved in osthole mediated downregulation of Sphk1/S1P.

Discussion

We demonstrated for the first time that osthole exerted therapeutic effect on PAH by targeting inhibition of S1P metabolism via regulating miR-21-PI3K/Akt/mTOR/Sphk1 signal pathway. The novel findings of this study are two fold. Firstly, we discovered a novel therapeutic target of osthole in therapy for PAH from metabolic perspective, inhibition of S1P metabolism by osthole prevented the increase in RVSP and RV/LV + S in PAH rats, and osthole was a potential molecular inhibitor of S1P. Secondly, we revealed novel metabolomic modulation mechanism of osthole against PAH, and osthole inhibited the elevated level of S1P produced by Sphk1 via miR-21-PI3K/Akt/mTOR signal pathway in vivo and in vitro.
Given metabolic reprogramming might trigger PAH development, we sought to discover and identify the dysregulated metabolomes underlying the PAH pathological progression by metabolomics method, and also verify the key metabolic targets of osthole intervention. Our data revealed substantially dysregulated metabolomes occurred in PAH involving glycolysis, sphingolipid metabolism, amino acid metabolism, nucleotide metabolism, heme metabolism, unsaturated fatty acids metabolism. To reveal biological significance underlying these dysregulated metabolic pathways, we found that warburg shift from mitochondrial oxidative phosphorylation to glycolysis is a dominant metabolism feature in PAH, thus to adapt to short bouts of cellular stress and resist apoptosis result in initiating proliferation and inflammatory. We speculated that increase of sphingolipids and decrease of omega-3 and omega-9 PUFA might initiate the inflammation
Fig. 7  Osthole dramatically inhibited the expression of Sphk1 via miR-21-PI3K/Akt/mTOR signal pathway in PASMCs. MTT was used to assess the suitable dosage of osthole on PASMCs proliferation induced by hypoxia (n = 6) (A) miR-21 was significantly elevated under hypoxia compared with that grown in normoxia, and obviously reversed by osthole treatment (n = 5). The data represented as mean ± SEM (p < 0.05, ##p < 0.01, ###p < 0.001 vs Normoxia; *p < 0.05, **p < 0.01, ***p < 0.001 vs Hypoxia; Nor represented Normoxia; Hyp represented Hypoxia; BLK represented blank control, NC represented negative control). Treatment of LY294002 to PASMCs significantly decreased the expression of p-Akt/Akt (G), p-mTOR/mTOR (H) and Sphk1 (J) compared to hypoxia group, and the regulatory role of osthole was dramatically restrained by Rapamycin (n = 5). The data represented as mean ± SEM (p < 0.05, ##p < 0.01, ###p < 0.001 vs Normoxia; *p < 0.05, **p < 0.01, ***p < 0.001 vs Hypoxia; Nor represented Normoxia; Hyp represented Hypoxia; BLK represented blank control, NC represented negative control).

process in response to increase the pulmonary pressure

Markedly low levels of circulating Estriol, 17-β estradiol, and higher level of estrone 3-sulfate confirmed that hormone might involve in controlling, adjusting, and promoting right ventricular hypertrophy. Increased steroclubinogen, sterocobin and bile acids indicated intravascular hemolysis.
which efficiently distributed oxygen throughout the body in PAH \[4, 8, 10\]. In addition, we found a direct evidence as disturbed pyrimidine metabolites in the serum of rats with PAH, which is a novel discovery for PAH pathogenesis. We found some differential metabolomes were highly associated with PAH progression for the first time that largely distinguished our work from previous metabolomics studies of PAH. Lipid metabolites were identified as the mostly metabolic phenotypes of PAH progression in our metabolomics study.

Based on the above identify differential metabolites with therapeutic targets potential, we then functionally characterized metabolic regulation of osthole upon PAH to hunt for therapeutic target. Importantly, SIP was confirmed as the therapeutic target of osthole. SIP is a bioactive lipid produced by sphingolipids with proproliferation, antiapoptosis, proinflammatory signaling properties \[23-30\], and involved in the increase in RVSP and RVH, evidenced as a future metabolic therapeutic target for PAH treatment \[14\]. SIP is synthesized by Sphk1, Sphk1/SIP signaling is crucial for promoting PASMCs proliferation contributing to pulmonary vascular contraction/remodeling and pulmonary pressure increase, and intervention of Sphk1/SIP might therefore prevent and treat PAH \[4, 8, 10, 14\]. Consistent with previous findings, we confirmed again Sphk1/SIP was significantly increased in the rats with PAH, then we were first to find that osthole can inhibit the biosynthesis of SIP in the serum of PAH rats by decreasing the expression level of Sphk1 in serum and lung tissues. Herein, we argue that osthole is a novel small-molecule inhibitor of Sphk1/SIP, which can be used to efficiently treat PAH by alleviating pulmonary pressure and right ventricular hypertrophy.

Whether osthole could directly inhibit Sphk1? which intracellular signal pathway involved in osthole downregulated Sphk1? Subsequent experiments will focus on the mechanisms that clarified the potential inhibition action of osthole on Sphk1/SIP. Recently, microRNAs have been identified as potential therapeutic targets for several diseases and as critical regulators in regulating the pathological process of PAH, and participating in cellular differentiation, proliferation, metabolism and apoptosis \[24\]. Recent studies highlight the regulation of miR-21 on Sphk1 in chronic kidney disease, but whether miR-21 as a critical regulator in modulating of Sphk1 in the pathogenesis of PAH is still incompletely unclear \[25-33\]. This interaction is supported by our evidence showing that siRNA of miR-21 ablated the Sphk1 upregulation in PASMCs under hypoxia \[34\].

Sphk1 activation is highly associated with Akt phosphorylation. Akt is a serine/threonine kinase and PI3K/Akt signaling pathway has been widely reported to be associated with the development and transformation of PAH, we hypothesized that PI3K/Akt/mTOR signaling pathway might contribute to miR-21 regulating Sphk1 in PAH \[35-38\]. Next, we determined PI3K/Akt/mTOR signal pathway participated in the role of miR-21 regulation in vitro, evidenced by the decreased level of Akt phosphorylation and mTOR phosphorylation after siRNA miR-21 in PASMCs under hypoxia, then ascertained the activation of Sphk1 was correlated with stimulation of Akt/mTOR, the blockade induced by the PI3K inhibitor LY294002 was markedly associated with a decrease in Akt phosphorylation, mTOR phosphorylation and Sphk1 activity, the blockade induced by the mTOR inhibitor rapamycin was markedly associated with a decrease in both mTOR phosphorylation and Sphk1 activity. Collectively, we verified that miR-21 stimulated the activity of PI3K/Akt/mTOR signal pathway resulting in enhanced Sphk1 expression in PAH rats and in PASMCs under hypoxia condition \[37-39\]. Importantly, we found that osthole could reverse MCT induced PAH via inhibition of SIP synthesis through regulating miR-21-PI3K/Akt/mTOR-Sphk1 signal pathway.

In short, our findings revealed a novel mechanism that osthole exerts therapeutic effect on PAH by targeting inhibition of Sphk1/SIP via regulating miR-21-PI3K/Akt/mTOR signal pathway.

**Conclusion**

In the present study, we employed metabolomics to firstly characterize metabolic disorders in PAH rats, and then deciphered the modulatory effects of osthole on the dysregulated metabolic pathways during PAH treatment. We are first to find that SIP biosynthesis is mostly affected by osthole during PAH treatment. Furthermore, we confirmed that osthole exerts therapeutic effect on PAH in rats by the inhibition of SIP through miR-21-PI3K/Akt/mTOR/Sphk1 signal pathway. In summary, our study provides a novel insight into therapeutic mechanism of osthole upon PAH, and osthole was verified again as a valuable lead compound for future drug discovery to treat PAH.

**References**


[8] Zhao YD, L Chu, K Lin, et al. A biochemical approach to understand the pathogenesis of advanced pulmonary arterial hypertension: metabolomic profiles of arginine, sphingosine-1-


