Rutin Reverses Abnormal Autophagy in Hypoxic Pulmonary Arterial Hypertension via Regulation of Mitofusin 1

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Research

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Abstract

Background:

Recently, rutin, a citrus flavonoid occurring in many plants, has been found to be beneficial in preventing hypoxia-induced pulmonary arterial smooth muscle cells (PASMCs) from proliferating via scavenged reactive oxygen species (ROS). However, rutin's underlying mechanism of action against PASMCs in the context of hypoxia is still unclear. Autophagy, the main intracellular degradation and recycling process, exerts a critical adaptive effect on the pathological angiogenesis associated with hypoxic pulmonary arterial hypertension (HPAH) by removing damaged mitochondria and regulating ROS production and cell proliferation. It would be useful to identify the role of rutin and its interaction with autophagy in exerting protective effects against HPAH.

Methods:

We chose 21 SD rats and randomly and equally divided them into three groups of normoxia, hypoxia, and hypoxia + rutin. At the end of the exposure period, we measured the right ventricular systolic pressure (RVSP), the weight of right ventricle (RV), and the ratio of RV weight to left ventricular (LV) weight plus septum (RV/LV+S) of each rat. PASMCs of the three groups of rats were isolated and cultured, and the effect of rutin on autophagy-related protein expression under hypoxia was analyzed using immunofluorescence analysis, transmission electron microscopy, western blot (WB) analysis, and siRNA design and transfection.

Results:

We found RVSP, RV/LV+S, and pulmonary artery wall thickness were reduced by rutin in the pulmonary arterial hypertension (PAH) animal model. WB results showed that rutin regulated expression of autophagy-related proteins. Moreover, rutin downregulated Mitofusin 1 (Mfn1) over-expression induced by hypoxia. But when Mfn1 was silenced, there was little difference in the expressions of beclin-1 (BECN-1), and other marker proteins with or without rutin.

Conclusions:

Rutin suppressed the abnormal autophagy of hypoxia-induced PASMCs via the regulation of the target, Mfn1. This revealed the protective effect of rutin on vascular remodeling caused by hypoxia and demonstrated how rutin could slow down the development of HPAH.

1. Introduction

Pulmonary arterial hypertension (PAH) is a rare and malignant cardiovascular disease (CVD) characterized by continuous increases in mean pulmonary arterial pressure (mPAP) and pulmonary vascular resistance (PVR) that lead to heart failure [1–3]. The average patient's survival time from diagnosis to death is only 2.8 years [3]. Moreover, pulmonary arterial disorders and myocardial lesions

caused by PAH are impossible to cure by conventional therapeutics. Based on this, it is universally acknowledged that identifying an effective treatment for PAH is urgent. Traditional Chinese medicine (TCM) approaches the treatment of various diseases using "multi-target" techniques, which are effective and safe [4]. Hence, TCM has great advantages and developmental prospects [5]. In recent years, studies in TCM has provided many drug candidates that are expected to cure PAH [6].

Rutin (3, 3', 4', 5, 7-pentahydroxyflavone-3-rhamnoglucoside) is a bioactive flavonol glycoside that consists of the flavonol quercetin and disaccharide rutinose [7–11]. Rutin has beneficial antioxidant and anti-inflammatory bioactivities, which have been used to treat a variety of diseases such as obesity, hypertension, and diabetes [12–17]. According to our previous work, rutin reversed the abnormal proliferation of pulmonary artery smooth muscle cells (PASMCs) induced by hypoxia through the reduction of reactive oxygen species (ROS) generated mainly in the mitochondrial respiratory chain [18]. Nonetheless, it remains unclear whether rutin has therapeutic effects on PAH, and what are the underlying mechanisms leading to rutin's mechanism of efficacy.

Mitochondria, as hallmarks of eukaryotic cells, are significant sites for aerobic respiration and the main sites for synthesizing and producing ROS [19]. Thus, mitochondria play a crucial role in the development of cell biology and multiple diseases. As for mammals, the morphological changes of mitochondria are achieved by fusion and division. The fusion/division of mitochondria depends on Mitofusin 1/2 (Mfn1/2), mitochondrial dynamin-like GTPase (OPA1), and dynamin-related protein (Drp1), which consists of a GTP functional domain and a crimp-crimp domain [20–21]. Under hypoxic conditions, mitochondrial imbalance leads to a large release of ROS that induce morphological changes in the mitochondria, which, in turn, trigger a series of cellular effects, such as the abnormal occurrence of autophagy, apoptosis, and proliferation [22].

Autophagy is a highly conserved evolutionary cellular process, providing a membrane-dependent mechanism for the sequestration, transport, and lysosomal turnover of subcellular components [23–27]. In the absence of nutrients, autophagy can help cells survive, but abnormal autophagy may lead to the occurrence of a variety of human diseases, such as obstructive pulmonary disease and idiopathic pulmonary fibrosis [28–30]. There have been many reports that the abnormal occurrence of autophagy can lead to the pathological angiogenesis of PAH [24–31], and ROS can cause abnormal autophagy under pathological conditions [32]. In this paper, we explored the effects and the molecular mechanism of action of rutin on hypoxic pulmonary artery hypertension (HPAH). Additionally, we also screened for the key protein target that could contribute to rutin's regulation of HPAH.

2. Materials And Methods

2.1 Reagents

Rutin was obtained from the China Biological Products Certification Institute. The antibodies against ATG5 (Boster, BA3525-2, Wuhan, China), ATG7 (Boster, BA3527-2, Wuhan, China), BECN-1 (Santa Cruz

Biotechnology, 11-11427, Texas, U.S.A), and SQSTM1 (Boster, BA2849, Wuhan, China) were used. Additionally, the RFP-eGFP-LC3 plasmid (Beyotime, C3011, Shanghai, China) was used.

2.2 Animal model

Five- to six-week-old adult male SD rats (250 to 300 g) were obtained from the Experimental Animal Center in our university. Twenty-one SD rats were randomly and equally divided into three groups of normoxia, hypoxia, and hypoxia + rutin. All rats were provided with sufficient food and water. The 7 rats in the normoxic group were kept in a normoxic environment with a FiO_2 of 0.21 for 21 days. The other fourteen rats in the hypoxic (7 rats) and rutin (7 rats) groups were housed in a hypoxic environment with a FiO_2 of 0.12 for 21 days [33]. At the beginning of the exposure period, the rats in the rutin group were randomly induced with an intraperitoneal injection of rutin, which was dissolved in 5% cellulose sodium carboxymethyl (CMC) at a dose of $200 \text{ mg kg}^{-1} \cdot \text{d}^{-1}$ for 21 days. The other rats in the normoxic and hypoxic groups were only induced by 5% CMC on the same days.

2.3 RVSP and ventricular weight measurements in rats

At the end of the exposure period, the RVSP of each rat was measured by right heart catheterization. Then, the animals were euthanized by decapitation. The rat hearts were quickly excised, the free wall of the RV was dissected, and each chamber was weighed. The (RV/LV+S) ratio was used as an index of RV hypertrophy. Finally, lung tissue was extracted and retained for subsequent experiments.

2.4 Morphological analysis of the pulmonary artery in rats

The rat lungs were washed with $1 \times$ phosphate-buffered saline (PBS) and stored in the freezer at -80°C . The remaining lung tissue was immersed in 4% paraformaldehyde, and then embedded in paraffin wax and cut into sections. The sections were stained with hematoxylin-eosin (H&E) staining (AR1180, Boster). The lung tissue sections were examined by digital photomicrography and analyzed with Image-Pro Plus 6.0.

2.5 Cell isolation and culture

PASMCs were prepared from rat pulmonary arterial (PA) tissue. The PA was slit to remove the pulmonary artery endothelial cells (PAECs) and washed with $1 \times$ HEPES buffer. The PASMCs were digested by using 1 mg/mL collagenase II at 37°C for 30 min and were washed once with $1 \times$ phosphate buffered saline (PBS) to remove the collagenase II. Then, PASMCs were cultured in Dulbecco's modified Eagle's medium (DMEM) (SH30022.01B, Hyclone) with 20% fetal bovine serum (FBS) (SV30087.03, Hyclone) and incubated at 37°C . Finally, the cell culture medium was replaced, and the cells were ready to use in subsequent experiments. In vitro PASMCs were treated with $0.1 \mu\text{mol/L}$ rutin and exposed to hypoxia for 24 h by placing the cells in a hypoxia chamber (THERMO) with a water-saturated atmosphere comprising 3% O_2 , 5% CO_2 , and 91% N_2 .

2.6 Immunofluorescence analysis

The PSMCs were plated in 6 well plates and grown on coverslips to 80% confluence. The cells were randomly divided into three groups, including normoxia, hypoxia and hypoxia + rutin. The cells were permeabilized with 0.01% Triton X-100 after fixation. The cells were then incubated with primary antibodies overnight at 4°C. After being washed, cells were incubated with the corresponding Cy3 (A0516, Beyotime) or FITC (A0562, Beyotime) secondary antibodies followed by 4', 6-diamidino-2-phenylindole (DAPI) (C1005, Beyotime) staining. The slices were then examined under a fluorescence microscope (Leica XF 6000).

2.7 Transmission electron microscopy

The PSMCs centrifuged at 3000 × g for 15 min and fixed with 2% glutaraldehyde (electron microscopy grade), and the photographing of autophagic vacuoles was consigned to the Electron Microscopy Center of our university.

2.8 Western blot analysis

The protein samples were extracted from PSMCs and lung tissues of rats as described previously [34]. Protein concentrations were determined by the Bradford assay using bovine serum albumin (BSA) (A8020, Solarbio) as a standard. The protein samples were separated on a 10-12% SDS-PAGE gel, transferred into a nitrocellulose membrane, and blocked in 5% nonfat milk. The membrane was incubated with specific antibodies against ATG 5 (1: 400), ATG7 (1: 400), BECN-1 (1: 400), SQSTM1 (1: 400), Mfn1 (1: 500), and β -actin (1: 1000) followed by reaction with horseradish peroxidase-conjugated secondary antibodies and visualization with enhanced chemiluminescence reagents.

2.9 siRNA design and transfection

Mfn1 and negative-control siRNAs were purchased from GenePharma (China). The siRNA sequences are as follows: siMfn1 (NM_138976.1): sense, 5'-CCAGCUGGUAUACAAUATT-3', antisense,

5'-UAUUCUGUAUACCAGCUGGTT-3'. NC control: sense,

5'-UUCUCCGAACGUGUCACGUTT-3', antisense,

5'-ACGUGACACGUUCGGAGAATT-3'.

The PSMCs were cultured in 6 well plates until 50-70% confluence. After growth arrest, 2 μ g of small interfering RNA (siRNA) and 10 μ L X-tremeGene of siRNA transfection reagents were diluted in serum-free Opti-MEM-1 medium. The siRNA-containing medium was added to a 6 well plate for 24 hours. The knockdown efficiency was determined by western blot (WB) analysis.

2.10 Statistics

The data are presented as the mean $\pm$ standard deviation (SD). The analysis was performed with Student's t-test or one-way analysis (ANOVA) by Dunnett's test, where appropriate. The statistical

significance level was set at $P < 0.05$. Statistical analysis was performed with Statistical Product and Service Solutions version 13.0.

3. Results

3.1 Rutin attenuated the development of HPAH in the rat model

To observe the effect of rutin on the development of PAH, the rats received intragastric administration of rutin during the preparation of HPAH rats. Then, we characterized the PH parameters in detail, mainly including the indirect index of cardiac function (RVSP and RV/LV + S ratio) and vascular remodeling. As shown in Fig. 1 (A, B), hypoxia significantly elevated the RVSP and RV/LV + S ratio, while this impact was reversed by rutin. In addition, H&E staining showed that the ratio of the intimal-to-medial area was significantly increased in HPAH models, and rutin reversed the wall thickening induced by hypoxia (Fig. 1C). Taken together, these results indicate that rutin prevented the pathological development of pulmonary arterial hypertension.

3.2 Rutin affected the expression of BECN-1 in lung tissue and PSMCs

According to reports, hypoxia-induced autophagy is a characteristic of HPAH [25]. Therefore, we investigated whether rutin protected against HPAH through inhibiting the autophagy of PSMCs in rats. We observed the changes of the autophagy-related protein BECN-1 with cell immunofluorescence, WB, and immunohistochemical experiments. As shown in Fig. 2 (A), the immunofluorescence staining displayed that hypoxia led to up-expression of BECN-1, but rutin reversed over-expression of BECN-1. Furthermore, we found the autophagy occurred in the smooth muscle cell layer under hypoxic conditions, and rutin could reverse abnormal autophagy. WB results indicated the expression of BECN-1 in hypoxic lung tissue and PSMCs was significantly up-regulated, however, the expression of BECN-1 was down-regulated when the rats and PSMCs under hypoxia were pre-treated with rutin, shown in Fig. 2 (B, C). At the same time, immunohistochemical analysis displayed the expression of BECN-1 in lung tissues, and we discovered that rutin could decrease the expression of BECN-1 under hypoxic conditions (Fig. 2D). Thus, our results show that rutin could induce a decrease in BECN-1 expression under hypoxia.

3.3 Rutin reduced occurrence of autophagy-induced chronic hypoxia

To investigate molecular mechanism of rutin affecting autophagy of PSMCs, we used an electron microscope to analyze the autophagy morphology. There were immature autophagy vacuoles (AVi) and degradative autophagic vacuoles (AVd) in PSMCs under hypoxia, which are usually generated during the development of autophagy. But we observed few AVi and AVd in the PSMCs pretreated with rutin under hypoxia (Fig. 3A). Then, we performed the plasmid eGFP-mRFP-LC3 fluorescence assay. Compared with the normoxic group, the autolysosomes (yellow dots) and autophagosomes (red dots) in the hypoxia group were significantly increased, however; rutin decreased the numbers of autolysosomes and autophagosomes (Fig. 3B). WB was used to detect the expression of autophagy-related proteins, such as sequestosome 1 (SQSTM1) in lung tissues and PSMCs. As shown in Fig. 3 (C, D), rutin significantly

down-regulated the over-expression of ATG5 and ATG7, and up-regulated low-expression of SQSTM1 induced by hypoxia both in the rats and PSMCs. These findings suggest that rutin could inactivate the autophagy process in PSMCs under hypoxic conditions.

3.4 Mfn1 played a key role in the process of rutin suppressing PSMCs abnormal autophagy

It has been reported that Mfn1 influences the mitochondrial dynamics involved in hypoxia-induced proliferation in PSMCs [35]. Therefore, we explored the changes in Mfn1 expression in rat lung tissue and PSMCs induced by hypoxia. We found that Mfn1 expression was up-regulated under hypoxic conditions, while rutin suppressed Mfn1 over-expression induced by hypoxia (Fig. 4). Then, we used silencing Mfn1 with siRNA transfection to verify our findings, and the transfection efficiency is shown in Fig. 5A. The WB experiment was used to observe the effects of rutin on the expression levels of the proteins ATG5, ATG7, BECN-1, and SQSTM1 after silencing Mfn1 with siRNA. The expression of ATG5, ATG7, and BECN-1 in the hypoxic group (silenced Mfn1 with siRNA) were down-regulated and SQSTM1 was up-regulated. A similar effect was obtained in the hypoxic group treated by rutin. More importantly, the hypoxic group with siMfn1 and rutin did not show further inhibition of hypoxia-induced abnormal expression of autophagy-related proteins (Fig. 5B,C). These results demonstrate that Mfn1 played a prominent role in rutin regulating autophagy in PSMCs under hypoxia.

4. Discussion

In this study, we provided the first evidence of the protective molecular mechanisms of rutin in PAH and PSMCs under hypoxia in an animal model. The novel findings in this paper consist of two points. Firstly, rutin alleviated hypoxia-induced pulmonary vascular remodeling and prevented the development of pulmonary hypertension. Secondly, rutin reduced the occurrence of hypoxia-induced autophagy in PSMCs. Mfn1 played a crucial role in this process, which indicates that rutin inhibited PSMCs autophagy by regulating the expression of Mfn1.

PAH is a malignant cardiovascular disease with poor prognosis and a high fatality rate [36–37]. Recently, PAH treatment has consisted of systemic vasodilatation drugs, which lack specificity to pulmonary circulation. Moreover, these drugs have strong side effects, undermining the treatment effect and causing great harm to the human body. Seeking safe and effective treatments, researchers have turned to TCM. Rutin has many pharmacological activities, such as strengthening and regulating the permeability of blood vessel walls [38]. Based on this, we hypothesized that rutin could have a certain inhibitory effect on the progress of HPAH. Through establishing an HPAH rat model, we observed the effects of rutin on right ventricular function in HPAH rats. We found that rutin reduced the increased RVSP and RV/LV + S induced by hypoxia, which proved that rutin improved the right ventricular function in the setting of HPAH. Moreover, H&E staining results indicated that rutin could also delay pulmonary vascular remodeling. These results suggest that rutin had a preventive effect on HPAH. However, the specific mechanism involved in the process of HPAH prevented by rutin remains unclear.

Autophagy is a vital process in maintaining cellular homeostasis and survival. Many reports have shown that autophagy could affect the progression of PAH [31]. Based on this, we explored the effects of rutin on autophagy in rats with HPAH and PASMCs. Initially, using immunofluorescence and immunohistochemistry experiments, we found the application of rutin could effectively reduce the production of autophagy, which occurs in the smooth muscle cell layer under hypoxic conditions. Next, we employed WB analysis to detect the change in the autophagy marker protein expressions, such as ATG5, ATG7, BECN-1, and SQSTM1 [24]. The results showed the over-expression of ATG5, ATG7, and BECN-1 as well as the down-expression of SQSTM1 (under hypoxic conditions) were effectively reversed by rutin. In addition, immunofluorescence, electron microscope, and eGFPmRFP-LC3 plasmid transfection experiments were administered to observe immature autophagosomes and degraded autophagosomes in PASMCs. We discovered autolysosomes and autophagosomes returned to their normal levels in PASMCs pre-treated with rutin under hypoxia. All these results suggest that rutin inhibited the abnormal autophagy of PASMCs induced by hypoxia, which further verified that rutin attenuated the remodeling of pulmonary arteries by suppressing the occurrence of autophagy in PASMCs.

However, the question remains as to what molecular mechanism was responsible for the regulation of autophagy by rutin in PASMCs. In our previous work, we discovered that rutin, as an antioxidant, exhibited significant ROS scavenging properties, with ROS mainly generated by mitochondria under hypoxic conditions [18]. Meanwhile, the balance of mitochondrial fusion and fission normalizes mitochondrial networks when the loss of homeostasis would lead to mitochondrial dysfunction. This has long been linked to the pathogenesis of neurodegenerative diseases, diabetes, myopathies, and many other human diseases. Recently, mitochondrial fusion and fission have been linked to both mitophagy (mitochondrial autophagy) and global autophagy in mouse embryonic fibroblasts, human fibroblasts, and cardiac cells. One group reviewed fusion was brief and triggers fission. Following a fission event, the daughter mitochondrion may either maintain intact membrane potential, or depolarize. In the case mitochondrial depolarization is transient and $\Delta\psi_m$ resumed, fusion capacity was restored. However, if $\Delta\psi_m$ depolarization is sustained, elimination by autophagy occurs [10]. And other observations showed Mfn1 may affect occurrence of autophagy both mitophagy (mitochondrial autophagy) and global autophagy, that is to sequester portions of cytosol or damaged and surplus organelles into autophagosomes and degrade them in autolysosomes, some signaling pathways, such as NF κ B and TP53 involved in these process [11–14]. However, it is still unclear whether the balance of mitochondrial fusion and fission induces autophagy, and what their combined effects are on apoptosis or proliferation. Our previous work showed rutin reduced the production of ROS to reverse the abnormal proliferation induced by hypoxia [15], so it will be of interest to explore whether Mfn1 is a key target protein in the process of rutin regulation of abnormal autophagy. Based on this, in our current study, we used WB to observe that up-expression of the altered autophagy markers under hypoxia were inhibited by rutin, which could be regulated by siMfn1 to further to verify our hypothesis. Thus, these findings suggest that rutin could decrease the anomalous expression of Mfn1 induced by hypoxia, which alleviated the abnormal autophagy in PASMCs under hypoxia.

In the present study, we focused on exploring rutin's bioactivity, and its mechanism of action in the setting of HPAH. Because of its chemical structure, rutin solubility in water is poor leading to low bioavailability in vivo. Today, we have little knowledge about the potential use of this compound in patients with HPAH. We face the problem of how to improve rutin solubility in water, decrease its dose administration to humans and increase its possible applications in clinical treatment. Thus, our next work will explore rutin's pharmacodynamics (how rutin is metabolized and how to increase its bioavailability in vivo) so that rutin can be used as a treatment in a clinical setting.

5. Conclusions

In conclusion, our study elucidates the protective role of rutin and how it slowed progression of HPAH in rats. The underlying mechanism contributing to this finding was that rutin attenuated the abnormal autophagy of PASMCs under hypoxia and HPAH in rats. We discovered the mitochondria-Mfn1-ROS pathway involved in this process, especially Mfn1 regulated by rutin as a target protein, played a key role in rutin's suppression of the remodeling of pulmonary arteries that ultimately slowed the development of HPAH in rats. Our study will help to deepen our understanding of HPAH treatment and guide us to significant insights for the development of new PAH treatment strategies.

Abbreviations

CVDs: cardiovascular diseases; DAPI: 4',6-diamidino-2-phenylindole; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; H&E staining: Hematoxylin-eosin staining; Hyp: Hypoxia; HPAH: hypoxic Pulmonary arterial hypertension; LV: Left ventricular; mPAP: mean pulmonary arterial pressure; NC, Non-targeted control siRNA; NOR: normoxia; PA: Pulmonary arterial; PAEC: pulmonary artery endothelial cell; PAH: Pulmonary arterial hypertension; PASMC: Pulmonary artery smooth muscle cell; PBS: Phosphate buffer saline; RV/LV+S: The ratio of RV weight to (LV) weight plus septum; PVR: pulmonary vascular resistance; ROS: Reactive oxygen species; Ru: *Rutin*; RV: Right ventricle; RVPS: Right ventricular systolic pressure; siRNA: Small interfering RNA; TCM: Traditional Chinese medicine.

Declarations

Ethics approval and consent to participate

All experimental procedures in animals which were approved by the Institutional Animal Care and Use Committee were carried out in accordance with guidelines for the Care and Use of Laboratory Animals, and it was conducted in compliance with the NIH guide. The study protocol in the Ethics of Animals was approved by the Committee Experiments of the Harbin Medical University (Permit Number: 2010-0006). All surgeries were carried out under sodium pentobarbital anesthesia, and all efforts were made to minimize pain.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Attenuation HPAH of rat models treated by rutin. (A) The measurement and statistical results of RVSP. (B) The statistical results of RV/LV+S. (C) The morphology of pulmonary arteries was identified using hematoxylin-eosin (H&E) staining. "Nor" means normoxia; "Hyp" means hypoxia; "Hyp + Rutin" means rutin treats hypoxia. Values are denoted as means $\pm$ SD from three or more independent experiments. * $P < 0.05$, ** $P < 0.01$ versus normoxia; # $P < 0.05$, ## $P < 0.01$, versus hypoxia; The arrows show the smooth muscle layer of pulmonary artery.

Figure 2

Effects of rutin on the autophagy protein BECN-1. (A) PSMCs were administrated by Immunofluorescence staining. The cells were exposed under Nor, Hyp and induced by rutin (0.1 $\mu\text{mol/L}$) before exposing under Hyp for 24 h $n = 6$, DAPI (blue), BECN-1 (red), the arrows indicate red fluorescence of BECN-1. (B) and (C) BECN-1 expression were detected by western blotting in lung tissues and PSMCs from Nor, sustained Hyp-treated and Hyp induced by rutin of rats. β -actin served as the standard, $n = 6$. (D) Immunohistochemically analysis of BECN-1 in lung tissues. All panels are at 20 $\times$ magnification except for the zoomed ones. Scale bars = 100 μm (upper). * $P < 0.05$, ** $P < 0.01$, versus normoxia; # $P < 0.05$, ## $P < 0.01$, versus hypoxia; Nor, Normoxia; Hyp, hypoxia; PSMCs, pulmonary arterial smooth muscle cell.

Figure 3

Reducing autophagy in chronic hypoxia induced by rutin. (A) PASCs were observed by transmission electron microscopy. Scan bars = 500 nm; n = 4; the arrows indicate immature autophagic vacuoles (AVi, white arrows) and degradative autophagic vacuoles (AVd, white arrows). (B) PASCs were transfected with eGFP-mRFP-LC3 plasmid and exposed under Nor, Hyp and Hyp + rutin for 24 h. Yellow and red dots refer to autolysosomes and autophagosomes, respectively. Scale bars = 50 mm; n = 3. (C) and (D) ATG5,

ATG7, BECN-1, and SQSTM1 expression was detected by western blotting in lung tissues and PSMCs from NOR, sustained HYP-treated and Hyp lungs induced by rutin of rats. β -actin served as the standard; $n = 6$; * $P < 0.05$, ** $P < 0.01$, versus normoxia; # $P < 0.05$, ## $P < 0.01$, versus hypoxia; Nor, Normoxia; Hyp, hypoxia; PSMCs, pulmonary arterial smooth muscle cells.

Figure 4

The expression of Mfn1 reduced by rutin in PSMCs. (A) Western blotting of Mfn1 expression was obtained from PSMCs, including Nor, sustained Hyp-treated, Hyp-PSMCs induced by rutin of rats. (B) Western blotting of Mfn1 expression was obtained from lung tissues, including Nor, sustained Hyp-treated, Hyp-PSMCs induced by rutin of PSMCs. β -actin served as the standard; $n = 6$; * $P < 0.05$, ** $P < 0.01$, versus normoxia; # $P < 0.05$, ## $P < 0.01$, versus hypoxia; Nor, Normoxia; Hyp, hypoxia.

Figure 5

Rutin regulation of autophagy of PSMCs by Mfn1. (A) Interference efficiency of Mfn1. (**P < 0.01 versus NC group, n = 4). (B) and (C) Western blotting of ATG5, ATG7, BECN-1, and SQSTM1 expression in PSMCs was obtained from five groups, including Nor, sustained Hyp-treated, Hyp-PSMCs induced by siRNA or Hyp-PSMCs induced by rutin, Hyp-PSMCs induced by siRNA and Hyp-PSMCs induced by rutin. β-actin served as the standard; n = 6; * P < 0.05, ** P < 0.01, versus normoxia; # P < 0.05, ## P < 0.01, versus hypoxia; Nor, Normoxia; Hyp, hypoxia; siRNA, Small interfering RNA; PSMCs, pulmonary arterial smooth muscle cells.