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Inhibitory Effect of PPARδ Agonist GW501516 on Proliferation of Hypoxia-induced Pulmonary Arterial Smooth Muscle Cells by Regulating the mTOR Pathway*

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[Abstract] Objective: This study aimed to investigate the effects of the peroxisome proliferator-activated receptor δ (PPARδ) agonist GW501516 on the proliferation of pulmonary artery smooth muscle cells (PASMCs) induced by hypoxia, in order to search for new drugs for the treatment and prevention of pulmonary vascular remodeling. **Methods:** PASMCs were incubated with different concentrations of GW501516 (10, 30, 100 nmol/L) under the hypoxic condition. The proliferation was determined by a CCK-8 assay. The cell cycle progression was analyzed by flow cytometry. The expression of PPARδ, S phase kinase-associated protein 2 (Skp2), and cell cycle-dependent kinase inhibitor p27 was detected by Western blotting. Then PASMCs were treated with 100 nmol/L GW501516, 100 nmol/L mammalian target of rapamycin (mTOR) inhibitor rapamycin and/or 2 μmol/L mTOR activator MHY1485 to explore the molecular mechanisms by which GW501516 reduces the proliferation of PASMCs. **Results:** The presented data demonstrated that hypoxia reduced the expression of PPARδ in an oxygen concentration- and time-dependent manner, and GW501516 decreased the proliferation of PASMCs induced by hypoxia by blocking the progression through the G0/G1 to S phase of the cell cycle. In accordance with these findings, GW501516 downregulated Skp2 and upregulated p27 in hypoxia-exposed PASMCs. Further experiments showed that rapamycin had similar effects as GW501516 in inhibiting cell proliferation, arresting the cell cycle, regulating the expression of Skp2 and p27, and inactivating mTOR in hypoxia-exposed PASMCs. Moreover, MHY1485 reversed all the beneficial effects of GW501516 on hypoxia-stimulated PASMCs. **Conclusion:** GW501516 inhibited the proliferation of PASMCs induced by hypoxia through blocking the mTOR/Skp2/p27 signaling pathway.
Key words: peroxisome proliferator-activated receptor δ; GW501516; hypoxia; pulmonary artery smooth muscle cells; proliferation; mammalian target of rapamycin

Persistent hypoxia can lead to pulmonary vascular remodeling and a progressive increase in pulmonary vascular resistance. The structural changes mainly include thickening of the vessel wall and narrowing of the lumen, which will lead to hypoxic pulmonary hypertension (HPH). HPH is a progressive disease that may cause right ventricular hypertrophy and eventually heart failure, which can result in premature death[1]. A study showed that the inhibition of pulmonary vascular remodeling plays a critical role in the prevention and treatment of HPH[2]. One characteristic of pulmonary vascular remodeling is medial and adventitial hypertrophy due to increased proliferation of pulmonary artery smooth muscle cells (PASMCs) and adventitial accumulation of fibroblasts and myofibroblasts[3]. The abnormal proliferation of PASMCs is critical to the development of HPH[4, 5]. Therefore, exploring the inhibitory mechanism of hyperproliferation in hypoxia-exposed PASMCs may be an efficient preventative and therapeutic strategy for HPH.

The mammalian target of rapamycin (mTOR) is a well-conserved serine/threonine kinase that mainly mediates various physiological and pathological processes, such as cell differentiation, proliferation, apoptosis, hypertrophy, and hyperplasia. Hypoxia can promote the proliferation of PASMCs and pulmonary vascular remodeling by activating the mTOR signaling pathway[6]. Inhibition of mTOR expression by RNA

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interference inhibited the proliferation of PASMCs induced by hypoxia[7]. Moreover, the mTOR inhibitor rapamycin can inhibit PASMCs proliferation induced by platelet-derived growth factor (PDGF)-BB and hypoxia[8, 9]. In the present study, mTOR induced PASMCs proliferation by regulating the S phase kinase-associated protein 2 (Skp2)/ cell cycle-dependent kinase inhibitor p27 (p27) pathway[10, 11].

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated receptors belonging to the nuclear hormone receptor superfamily, which includes three subtypes: PPARα, PPARγ, and PPARδ (also called PPARβ/δ). PPARs have been widely implicated in many pathophysiological processes, including energy homeostasis, cell proliferation and differentiation, glucose metabolism, as well as, fatty acid synthesis and catabolism. More evidence indicates that PPARδ has the effects of improving insulin resistance, and reducing inflammation, oxidative stress, and anti-atherosclerosis[12, 13]. GW501516, also known as GW-1516, cardarine, or endurobol, is a PPARδ-specific agonist developed by GlaxoSmithKline. It can promote fat metabolism, increase the utilization of glucose by skeletal muscles, and improve exercise endurance. Animal experiments have shown that it generates weight loss. Some people buy GW501516 on the black market for weight loss and muscle gain, but its long-term oral safety needs to be further studied[14, 15].

It was found that GW501516 can block cell cycle progression in the G0/G1 phase and inhibit proliferation in oxidized low-density lipoprotein (ox-LDL) induced vascular smooth muscle cells (VSMCs)[16]. Other studies have shown that GW501516 can inhibit the hypertrophy of VSMCs induced by angiotensin Ⅱ by inactivating the PI3K/AKT signaling pathway, while the PI3K/AKT signaling pathway can promote the proliferation of PASMCs under hypoxic conditions[17, 18]. However, the effects of PPARδ on the proliferation of PASMCs induced by hypoxia and its associated mechanisms remain unclear. In this study, it was investigated whether GW501516 suppressed the proliferation of PASMCs induced by hypoxia and elucidated the molecular mechanisms.

1 MATERIALS AND METHODS

1.1 Materials

Collagenase Ⅰ, PPARδ agonist GW501516, and mTOR activator 4,6-dimorpholino-N-(4-nitrophenyl)-1,3,5-triazin-2-amine (MHY1485) were purchased from Sigma-Aldrich (USA). The mTOR inhibitor rapamycin was obtained from Cell Signaling Technology (USA). Cell counting kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies Inc. (Japan). The cell cycle and apoptosis analysis kit was ordered from Beyotime Institute of Biotechnology (China). Antibodies against PPARδ, Skp2, p27, total and phosphorylated forms of mTOR, and GAPDH were purchased from Cell Signaling Technology (USA). Adult male Sprague Dawley rats with a weight of 170–190 g were ordered from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd. (China). All experiments involving animals were performed according to the guidelines of the regulations on experimental animals in Hubei province.

1.2 Cell Culture

Male Sprague-Dawley rats were euthanized by intraperitoneal anesthesia with 50 mg/kg 1% sodium pentobarbital. PASMCs were isolated from the pulmonary arteries of SD rats and digested with 0.2% collagenase Ⅰ[19]. PASMCs were maintained in DMEM/F12 with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic. The purity of PASMCs was determined as the percentage of α-smooth muscle cell actin-stained cells in the total cells, which reached 95% for the experiment. PASMCs from passages 4 to 10 were used in this study. They were grown to 70%–80% confluence and then serum-deprived for 24 h prior to exposure to diverse culture conditions. For the hypoxia group, the cells were kept in a hypoxic incubator at 37ºC with 5% CO_2, 10%–1% O_2, and 85%–94% N_2, whereas the control group was cultured in an incubator at 37ºC with 5% CO_2, 21% O_2, and 74% N_2. The GW501516 + hypoxia, rapamycin + hypoxia, and GW501516 + MHY1485 + hypoxia groups were treated with GW501516, rapamycin (100 nmol/L), and/or MHY1485 (2 μmol/L) prior to exposure to hypoxia[8, 20]. GW501516, rapamycin, and MHY1485 were dissolved in DMSO and stored at –20°C until use.

1.3 Cell Proliferation Assay

Cell proliferation was determined by a CCK 8 assay. PASMCs were inoculated in 96-well plates with 5×10^3 cells per well, and each well contained 100 μL medium. When the cells were grown to 70%–80% confluence, serum-starved for 24 h and then they were exposed to diverse culture conditions. According to the manufacturer's instructions included in the CCK-8 kit, each well was treated with 10 μL CCK-8 solution. At the same time, a blank control well (100 μL medium + 10 μL CCK-8) was set up and incubated in the incubator for the final 3 h. The absorbance (A) of the plate at 450 nm was measured by a spectrophotometer microplate reader. The relative proliferation of cells = $(A_{\text{experiment}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}}) \times 100\%$.

1.4 Cell Cycle Progression Assays

Cell cycle progression was measured by a cell cycle and apoptosis analysis kit. PASMCs were inoculated in a 6-well plate with 1.5×10^5 cells per well, allowed to reach 70%–80% confluence, serum-starved for 24 h, and then exposed to diverse culture conditions. After washing with phosphate-buffered saline (PBS), PASMCs were digested and harvested

by trypsinization and then fixed with 70% ethanol (absolute ethanol + PBS) for 24 h. After centrifugation at 1000×*g* for 5 min, PBS was added to resuspend the cells, staining buffer, RNase and PI were also added, and the cells were incubated at 4ºC for 30 min. Then, cell cycle progression was analyzed by flow cytometry, and Modifit software was used to analyze the experimental data.

1.5 Western Blotting

PASMCs were inoculated in 6-well plates with 1.5×10^5 cells per well. After serum-starved PASMCs were exposed to diverse culture conditions for the indicated time, the cells were lysed in RIPA protein extract buffer for 10 min to extract the total protein, and the protein concentration was measured by a BCA Protein Assay Kit. Approximately 15 μg of protein was separated by SDS-PAGE, blotted onto Immobilon-FL transfer membranes, and then incubated overnight at 4°C with the following primary antibodies: anti-PPARδ (1:1000, cat. No. 74076), anti-Skp2 (1:1000, cat. No. 4313), anti-p27 (1:1000, cat. No. 13715), anti-mTOR (1:1000, cat. No. 2983), anti-phosphorylated (p)-mTOR (1:1000, cat. No. 5536), and anti-GAPDH (1:2000, cat. No. 5174). Secondary antibody was then added for color development. Protein expression was quantified by an Odyssey system (Li-COR, USA).

1.6. Statistical Analysis

All data are represented as the mean ± standard deviation (SD). Data analysis was performed using SPSS 22.0 software (IBM Corp., USA). Multiple group comparisons were assessed by one-way analysis of variance (ANOVA). $P<0.05$ was considered to indicate statistical significance.

2 RESULTS

2.1 Effects of Hypoxia and GW501516 on the Expression of PPARδ in PASMCs

To evaluate the effects of hypoxia and GW501516 on the expression of PPARδ in PASMCs, Western blotting was performed. PASMCs were incubated with different oxygen concentrations (10%, 5%, 1%) for 24 h to explore the effect of oxygen concentration on the expression of PPARδ. Then, the cells were stimulated with 1% oxygen for different time lengths (12, 24, 48 h) to demonstrate the influence of time on the expression of PPARδ. Finally, the PASMCs were incubated with different concentrations of GW501516 (10, 30, 100 nmol/L) for 24 h under normoxic (21% oxygen) or hypoxic (1% oxygen) conditions to determine the effect of GW501516 on the expression of PPARδ. The presented data demonstrated that hypoxia reduced the expression of PPARδ in an oxygen concentration- and time-dependent manner, and GW501516 induced the expression of PPARδ in a concentration-dependent manner both under normoxic (21% oxygen) and hypoxic (1% oxygen) conditions (fig. 1A–1D).

2.2 Optimal Oxygen Concentration and Time for Stimulating Proliferation of PASMCs

First, cells were stimulated with different oxygen concentrations (10%, 5%, 1%) for 24 h to determine the optimal concentration of oxygen to induce the proliferation of PASMCs. The proliferation of PASMCs was detected by a CCK-8 assay. Compared to the control group, hypoxia (10%–1% oxygen) stimulated the proliferation of PASMCs in a concentration-dependent manner. Second, PASMCs were cultured under 1% oxygen for different time lengths (12, 24, 48, and 72 h) to determine the appropriate hypoxic stimulation time. Compared to the control group, the proliferation of PASMCs in the hypoxia group was increased significantly from 12 to 24 h. The proliferation rate of hypoxia-stimulated PASMCs decreased after 24 h. Based on the results, inducing the proliferation of PASMCs with hypoxia for 24 h was found to be an effective intervention (fig. 2A and 2B).

2.3 GW501516 Inhibits Proliferation of PASMCs Induced by Hypoxia

To determine the effect of GW501516 on the proliferation of PASMCs induced by hypoxia (1% oxygen), the effects of different concentrations of GW501516 (10, 30, 100 nmol/L) on proliferation under hypoxia-stimulated conditions for 24 h were investigated using a CCK-8 assay. Compared with the control group, exposure to hypoxia for 24 h increased the proliferation of PASMCs, which was inhibited by GW501516 in a concentration-dependent manner (fig. 3).

2.4 GW501516 Induces G1 and S Arrests in PASMCs Cultured Under Hypoxia

The effect of GW501516 (10, 30, 100 nmol/L) on cell cycle progression in PASMCs was examined next. Cell cycle analysis showed that exposure to hypoxia (1% oxygen) for 24 h decreased the number of cells in the G0/G1 phase and increased the number of cells in the G2/M+S phase. Compared with the hypoxia group, treatment with different concentrations of GW501516 increased the number of cells in the G0/G1 phase and decreased the number of cells in the G2/M+S phase. These results indicate that GW501516 arrests cell cycle progression in G0/G1 in PASMCs under hypoxic conditions (fig. 4).

2.5 GW501516 Downregulates Skp2 Levels and Upregulates p27 Levels in Hypoxia-exposed PASMCs

The degradation of cell cycle inhibitory protein p27 is mediated by Skp2[21]. To investigate the inhibitory mechanisms of GW501516 (10, 30, 100 nmol/L) on G0/G1 to S phase arrest, the protein levels of Skp2 and p27 were examined. Hypoxia (1% oxygen) exposure upregulated Skp2 and downregulated p27 in PASMCs, and treatment with GW501516 reversed this

Fig. 1 Effects of hypoxia and GW501516 on the expression of PPARδ in PASMCs

A: the protein levels of PPARδ induced by different oxygen concentrations (10%, 5%, 1%) at 24 h. #*P*<0.05 *vs*. the control group, **P*<0.05 *vs*. cells exposed to 10% oxygen, &*P*<0.05 *vs*. cells exposed to 5% oxygen (*n*=3). B: PPARδ expression in PASMCs after exposure to 1% oxygen for 12, 24 and 48 h. #*P*<0.05 *vs*. the control group, **P*<0.05 *vs*. cells exposed to 1% oxygen for 12 h, &*P*<0.05 *vs*. cells exposed to 1% oxygen for 24 h (*n*=3); C: the protein levels of PPARδ after exposure to different concentrations of GW501516 (10, 30, 100 nmol/L) for 24 h under normoxia (21% oxygen). #*P*<0.05 *vs*. the control group, **P*<0.05 *vs*. cells exposed to 10 nmol/L GW501516, &*P*<0.05 *vs*. cells exposed to 30 nmol/L GW501516 (*n*=3). D: the expression of PPARδ after treatment with different concentrations of GW501516 (10, 30, 100 nmol/L) for 24 h under hypoxia (1% oxygen). #*P*<0.05 *vs*. the hypoxia group, **P*<0.05 *vs*. cells exposed to 10 nmol/L GW501516 + Hypoxia, &*P*<0.05 *vs*. cells exposed to 30 nmol/L GW501516 + Hypoxia (*n*=3). PASMCs: pulmonary artery smooth muscle cells; PPARδ: peroxisome proliferator-activated receptor δ

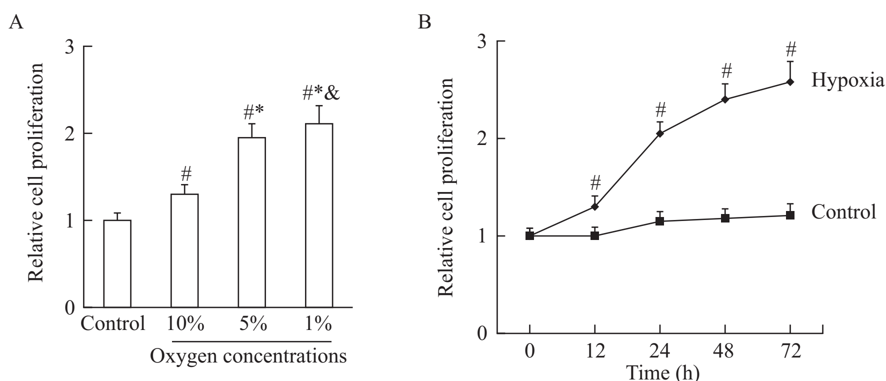

Fig. 2 The optimal oxygen concentration and time that stimulate PASMCs proliferation

PASMCs were stimulated with different oxygen concentrations (10%, 5%, 1%) for 24 h and then cultured under 1% oxygen for different durations (12, 24, 48 and 72 h). Cell proliferation was determined using a CCK-8 assay. #*P*<0.05 *vs*. the control group, **P*<0.05 *vs*. cells exposed to 10% oxygen, &*P*<0.05 *vs*. cells exposed to 5% oxygen (*n*=6). PASMCs: pulmonary artery smooth muscle cells; CCK-8: cell counting kit-8

effect in a concentration-dependent manner. These results indicate that GW501516 downregulated Skp2 and upregulated p27 in hypoxia-exposed PASMCs to arrest cell cycle progression in G0/G1 (fig. 5).

2.6 GW501516 Inhibits Hypoxia-induced PASMCs Proliferation by Inhibiting the mTOR/Skp2/p27 Pathway

Studies have shown that mTOR is an important

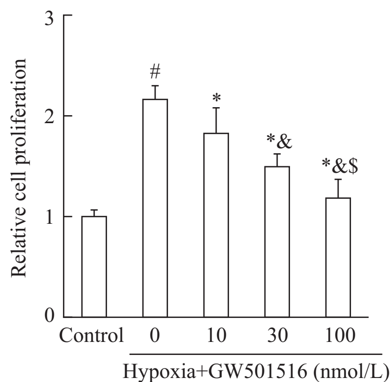

Fig. 3 Effects of GW501516 on the proliferation of hypoxia-exposed PASMCs

PASMCs were exposed to normoxia or hypoxia (1% oxygen) for 24 h in the absence or presence of GW501516 (10, 30, 100 nmol/L). Relative cell proliferation was determined using a CCK-8 assay. #*P*<0.05 *vs*. the control group, **P*<0.05 *vs*. the hypoxia group, &*P*<0.05 *vs*. 10 nmol/L GW501516 + hypxoia group, $*P*<0.05 *vs*. 30 nmol/L GW501516 + hypxoia group (*n*=6). PASMCs: pulmonary artery smooth muscle cells; CCK-8: cell counting kit-8

regulator of both Skp2 and p27 and that mTOR activation promotes Skp2 expression[10, 22]. To explore the molecular mechanisms by which GW501516 reduces the proliferation of PASMCs, PASMCs were treated with 100 nmol/L GW501516, 100 nmol/L rapamycin and 2 μmol/L MHY1485. The results showed that GW501516 blocked the proliferation of hypoxia (1% oxygen)-stimulated PASMCs through G0/G1 arrest, which was associated with an inhibition of the expression of Skp2 and an increase in the expression of p27. Further experiments showed that rapamycin had a similar effect as GW501516 in inhibiting cell proliferation, arresting the cell cycle, regulating the expression of Skp2 and p27, and inactivating mTOR in hypoxia-exposed PASMCs. Moreover, MHY1485 reversed all the beneficial effects of GW501516 on hypoxia-stimulated PASMCs. This study confirms that GW501516 inhibits the proliferation of PASMCs by inactivating the mTOR/Skp2/p27 signaling pathway (fig. 6A–6D).

3 DISCUSSION

In the present study, PPARδ was documented as downregulated after hypoxia stimulation in an oxygen concentration- and time-dependent manner in PAMSCs. This study also demonstrated that GW501516 inhibits the proliferation of PASMCs induced by hypoxia. In accordance with these findings, GW501516 blocked progression through the G0/G1 phase to the S phase of the cell cycle. The GW501516-induced inhibition of the cell cycle was associated with downregulated Skp2 levels and upregulated p27 levels in hypoxia-exposed PASMCs. Further experiments showed that GW501516 treatment significantly reduced the activation of mTOR. Meanwhile, rapamycin had a similar effect as GW501516 in hypoxia-exposed PASMCs. Moreover, MHY1485 reversed all the beneficial effects of GW501516 on PASMCs cultured under hypoxia. Taken together, our study indicates that GW501516 inhibited

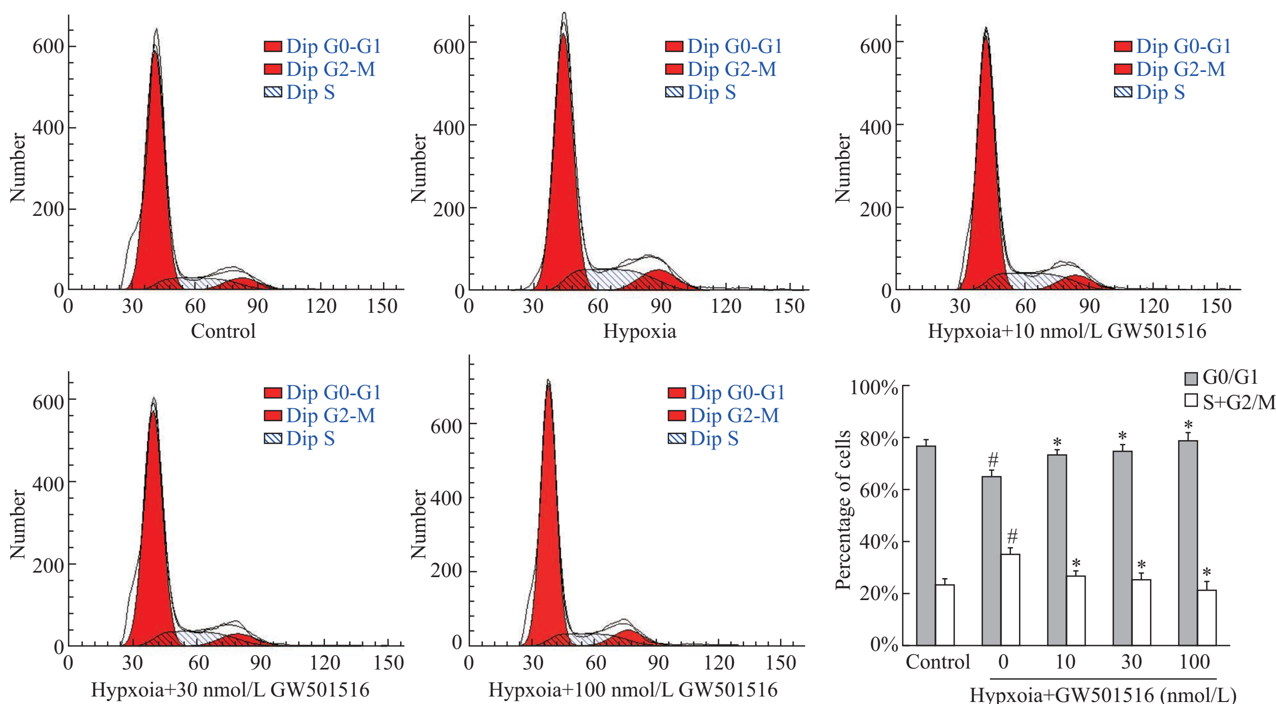

Fig. 4 Effects of GW501516 on cell cycle progression in hypoxia-exposed PASMCs

PASMCs were exposed to normoxia or hypoxia (1% oxygen) for 24 h in the absence or presence of GW501516 (10, 30, 100 nmol/L). Quantification of cells in the G0/G1 and S + G2/M phases was performed by flow cytometry. #*P*<0.05 *vs*. control, **P*<0.05 *vs*. hypoxia group (*n*=3). PASMCs: pulmonary artery smooth muscle cells

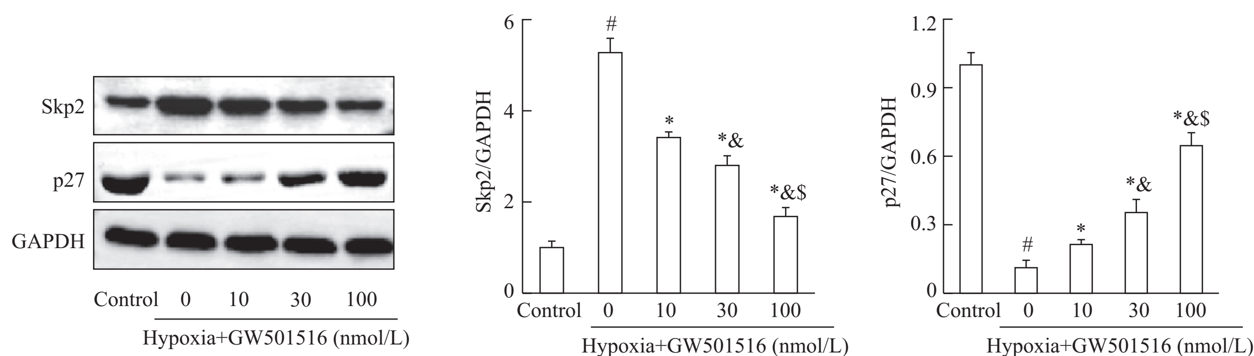

Fig. 5 Effects of GW501516 on the protein expression of Skp2 and p27 in hypoxia-stimulated PASMCs

PASMCs were grown with GW501516 (10, 30, 100 nmol/L) and exposed to hypoxia (1% oxygen) for 24 h. The protein expression levels of Skp2 and p27 were measured by Western blotting. #P<0.05 *vs*. the control group, *P<0.05 *vs*. the hypoxia group, &P<0.05 *vs*. 10 nmol/L GW501516 + hypxoia group, $$P$<0.05 *vs*. 30 nmol/L GW501516 + hypxoia group (n=3). PASMCs: pulmonary artery smooth muscle cells; Skp2: S phase kinase-associated protein 2

the proliferation of PASMCs induced by hypoxia by blocking the mTOR/Skp2/p27 signaling pathway.

Prostacyclin (PGI2) is an endogenous PPARδ agonist, and PGI2 and its analogs can promote pulmonary vasodilation and inhibit the proliferation of PASMCs and are used for the treatment of pulmonary hypertension[23]. However, little is known about the effect and mechanism of PPARδ on hypoxic pulmonary vascular remodeling. Hypoxia can promote the proliferation of the pulmonary arterial wall in a variety of cell types; in particular, the proliferation of PASMCs plays a key role in hypoxic pulmonary vascular remodeling, while inhibiting the proliferation of PASMCs can inhibit hypoxic pulmonary vascular remodeling and relieve HPH[24–26]. In the present study, PPARδ was documented as downregulated in an oxygen concentration- and time-dependent manner in PAMSCs, and GW501516 induced the expression of PPARδ in a concentration-dependent manner both under normoxic (21% oxygen) and hypoxic (1% oxygen) conditions. Therefore, the authors speculated that the proliferation of PASMCs induced by hypoxia might be related to PPARδ downregulation. For this reason, the effects of GW501516 on the proliferation of PASMCs induced by hypoxia were investigated. In the present study, PASMCs proliferated significantly after 24 h of stimulation with 1% oxygen, while GW501516 inhibited hypoxia-induced PASMC proliferation in a dose-dependent manner. The current study demonstrated that GW501516 can inhibit the proliferation of VSMCs induced by ox-LDL, while inhibition of the expression of PPARδ by RNA interference or the PPARδ inhibitor GSK0660 can reverse the inhibitory effect of GW501516 on the proliferation of VSMCs[16]. DL-propargylglycine can promote aortic vascular remodeling and VSMCs proliferation by inhibiting the expression of PPARδ, while GW501516 can protect vascular remodeling and proliferation of VSMCs[26]. A stent coated with the PPARδ agonist GW0742 can inhibit intimal hyperplasia, restenosis after stenting and proliferation of VSMCs[27]. Cell proliferation is related to the cell cycle. Under normal physiological conditions, PASMCs stay in the G0 phase of the cell cycle, and pathological stimuli induce PASMCs to enter and progress through the various stages of the cell cycle, which causes the abnormal proliferation of PASMCs and leads to pulmonary vascular remodeling[28]. Our results showed that GW501516 arrested cell cycle progression in G0/G1. Cell cycle progression is tightly regulated through CDKs and cyclin, which can form specific CDK-cyclin complexes to promote cell cycle progression. In addition, p27 is a cyclin-dependent kinase (CDK) inhibitor that forms heterotrimeric complexes with cyclin D-CDK4 and cyclin E-CDK2 to inhibit their function, which further arrests the cell cycle in the G0/G1 phase and suppresses cell proliferation[29, 30]. Skp2, an F-box component of Skp1-Cullin-F-box protein (SCF)-type ubiquitin ligase, is implicated in the ubiquitination and proteasomal degradation of p27[32]. The inhibition of Skp2 with siRNA inhibited the degradation of p27 and blocked the proliferation of PASMCs induced by PDGF[10]. The present study demonstrated that hypoxia exposure upregulated Skp2 and downregulated p27 in PASMCs, and treatment with GW501516 reversed these effects.

The mTOR signaling pathway is essential for multiple cellular processes, such as cell proliferation, differentiation, and apoptosis. Studies have shown that the activation of mTOR is critical in the hypoxia- and PDGF-BB-induced proliferation of PASMCs[7, 8, 32]. Hypoxia induced the activation of mTOR, and treatment with rapamycin reversed hypoxia-induced PASMC proliferation through the mTOR/Skp2/p27 signaling pathway[11]. The mTOR signaling pathway induces Skp2-mediated degradation of p27. A study in PASMCs found that siRNA-mediated knockdown of Skp2 can upregulate the expression of p27, while rapamycin reversed the phosphorylation of mTOR, Skp2 upregulation and p27 downregulation in PDGF-

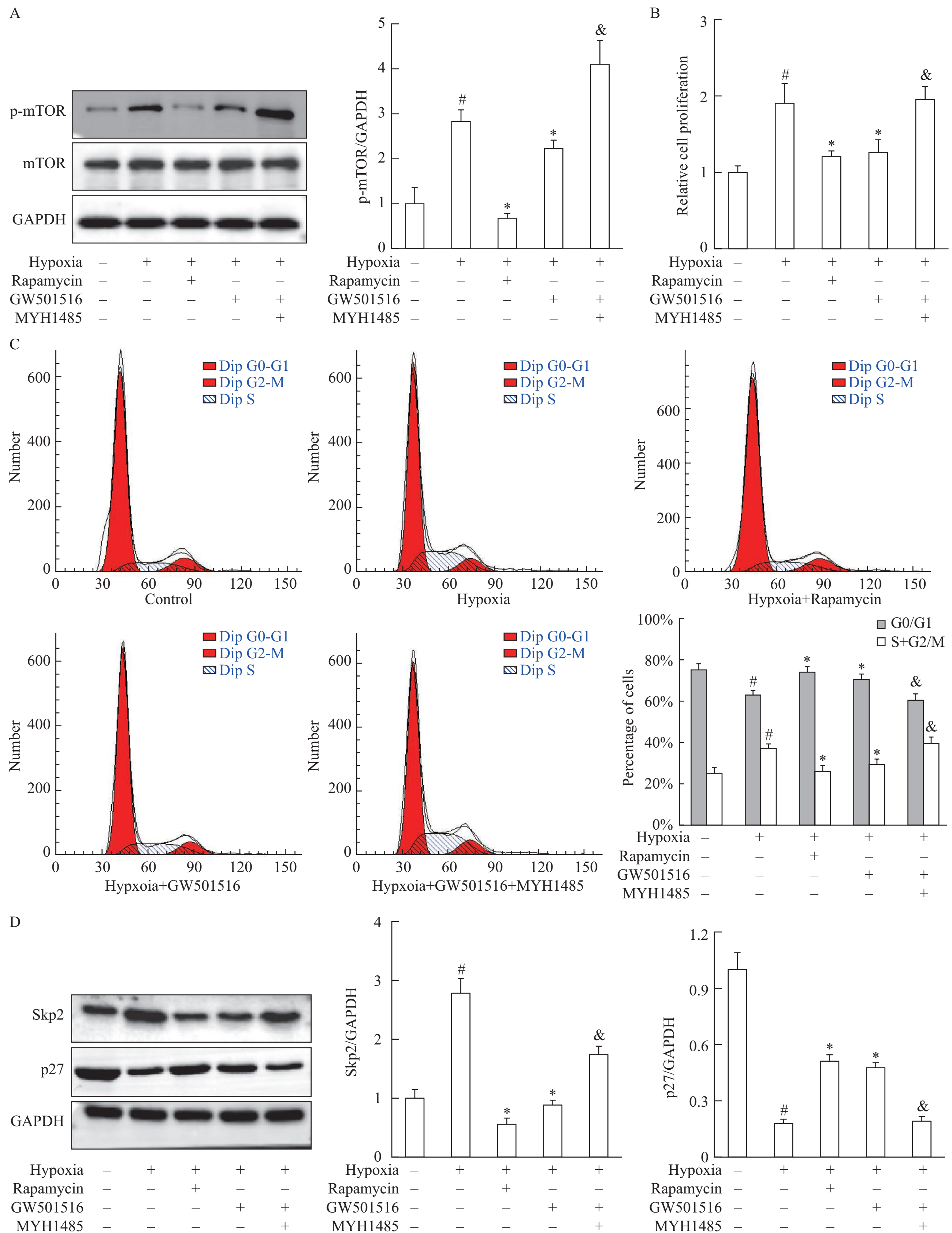

Fig. 6 GW501516 inhibits hypoxia-induced PASMC proliferation by inactivating mTOR/Skp2/p27

PASMCs were treated with GW501516 (100 nmol/L), rapamycin (100 nmol/L), and/or MHY1485 (2 μmol/L) prior to exposure to hypoxia (1% oxygen). A: The protein levels of total and phosphorylated mTOR were measured by Western blotting (*n*=3). B: Cell proliferation was determined by a CCK-8 assay kit (*n*=6). C: Cell cycle progression was analyzed by flow cytometry (*n*=3). D: The protein levels of Skp2 and p27 were analyzed by Western blotting (*n*=3). $^{\#}P$<0.05 *vs*. control group, $^{*}P$<0.05 *vs*. hypoxia group, $^{\&}P$<0.05 *vs*. hypoxia + GW501516 group. PASMCs: pulmonary artery smooth muscle cells; CCK-8: cell counting kit-8; Skp2: S phase kinase-associated protein 2; mTOR: mammalian target of rapamycin

stimulated PASMCs[10]. In this study, it was documented that GW501516 can decrease the phosphorylation of mTOR in hypoxia-exposed PAMSCs. Further experiments showed that rapamycin had similar effects as GW501516 in inhibiting cell proliferation, arresting the cell cycle, regulating the expression of Skp2 and p27, and inactivating mTOR in hypoxia-exposed PASMCs. Moreover, MHY1485 reversed all the beneficial effects of GW501516 on hypoxia-stimulated PASMCs. These data provide strong support for the hypothesis that GW501516 blocks the proliferation of PASMCs induced by hypoxia by arresting cells in the G0/G1 phase due to the downregulation of Skp2 and the upregulation of p27 by mTOR inhibition.

In summary, our study demonstrated that GW501516 inhibits the proliferation of PASMCs induced by hypoxia by blocking the cell cycle at the G0/G1 phase to the S phase by inhibiting the mTOR/Skp2/p27 signaling pathway. The current results may have a potential impact on therapeutic applications in hypoxic pulmonary vascular remodeling diseases.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

REFERENCES

1 Chai T, Qiu C, Xian Z, *et al*. A narrative review of research advances in hypoxic pulmonary hypertension. Ann Transl Med, 2022,10(4):230

2 Li C, Zhu H, Zhang S, *et al*. Astragaloside IV ameliorates pulmonary vascular remodeling in hypoxia-induced pulmonary hypertension by restraining the T follicular helper cell response and expanding T follicular regulatory cell response. Phytomedicine, 2022,102:154171

3 Rajagopal S, Yu YA. The Pathobiology of Pulmonary Arterial Hypertension. Cardiol Clin, 2022,40(1):1-12

4 Hu HH, Zhang RF, Dong LL, *et al*. Overexpression of ACE2 prevents hypoxia-induced pulmonary hypertension in rats by inhibiting proliferation and immigration of PASMCs. Eur Rev Med Pharmacol Sci, 2020,24(7):3968-3980

5 Liu Y, Nie X, Zhu J, *et al*. NDUFA4L2 in smooth muscle promotes vascular remodeling in hypoxic pulmonary arterial hypertension. J Cell Mol Med, 2021,25(2):1221-1237

6 Wang AP, Yang F, Tian Y, *et al*. Pulmonary Artery Smooth Muscle Cell Senescence Promotes the Proliferation of PASMCs by Paracrine IL-6 in Hypoxia-Induced Pulmonary Hypertension. Front Physiol, 2021,12:656139

7 Liu X, Wang G, You Z, *et al*. Inhibition of hypoxia induced proliferation of pulmonary arterial smooth muscle cells by a mTOR siRNA-loaded cyclodextrin nanovector. Biomaterials, 2014,35:4401-4416

8 Zha LH, Zhou J, Tan Y, *et al*. NLRC3 inhibits PDGF-induced PASMCs proliferation via PI3K-mTOR pathway. J Cell Physiol, 2020,235(12):9557-9567

9 Krymskaya VP, Snow J, Cesarone G, *et al*. mTOR is required for pulmonary arterial vascular smooth muscle cell proliferation under chronic hypoxia. FASEB J, 2011,25(6):1922-1933

10 Song Y, Wu Y, Su X, *et al*. Activation of AMPK inhibits PDGF-induced pulmonary arterial smooth muscle cells proliferation and its potential mechanisms. Pharmacol Res, 2016,107:117-124

11 Ke R, Liu L, Zhu Y, *et al*. Knockdown of AMPKα2 Promotes Pulmonary Arterial Smooth Muscle Cells Proliferation via mTOR/Skp2/p27(Kip1) Signaling Pathway. Int J Mol Sci, 2016,17(6):844

12 Degueurce G, D'Errico I, Pich C, *et al*. Identification of a novel PPARbeta/delta/miR-21-3p axis in UV-induced skin inflammation. EMBO Mol Med, 2016,8(8):919-936

13 Cheang WS, Wong WT, Zhao L, *et al*. PPARδ Is Required for Exercise to Attenuate Endoplasmic Reticulum Stress and Endothelial Dysfunction in Diabetic Mice. Diabetes, 2017,66(2):519-528

14 Kintz P, Ameline A, Gheddar L, *et al*. Testing for G W501516 (cardarine) in human hair using LC/MS-MS and confirmation by LC/HRMS. Drug Test Anal, 2020,12(7):980-986

15 Weihrauch M, Handschin C. Pharmacological targeting of exercise adaptations in skeletal muscle: Benefits and pitfalls. Biochem Pharmacol, 2018,147:211-220

16 Hwang JS, Ham SA, Yoo T, *et al*. Sirtuin 1 Mediates the Actions of Peroxisome Proliferator-Activated Receptor δ on the Oxidized Low-Density Lipoprotein–Triggered Migration and Proliferation of Vascular Smooth Muscle Cells. Mol Pharmacol, 2016,90(5):522-529

17 Kang ES, Hwang JS, Lee WJ, *et al*. Ligand-activated PPARdelta inhibits angiotensin II-stimulated hypertrophy of vascular smooth muscle cells by targeting ROS . PLoS One, 2019,14(1):e0210482

18 Zhang S, Wang J, Qi X, *et al*. Plasminogen activator Inhibitor-2 inhibits pulmonary arterial smooth muscle cell proliferation in pulmonary arterial hypertension via PI3K/Akt and ERK signaling. Exp Cell Res, 2021,398(1):112392

19 Chen C, Tang Y, Deng W, *et al*. Salidroside blocks the proliferation of pulmonary artery smooth muscle cells induced by platelet derived growth factor BB. Mol Med Rep, 2014,10(2):917-922

20 Gui D, Cui Z, Zhang L, *et al*. Salidroside attenuates hypoxia-induced pulmonary arterial smooth muscle cell proliferation and apoptosis resistance by upregulating autophagy through the AMPK-mTOR- ULK1 pathway. BMC Pulm Med, 2017,17(1):191

21 Jeong K, Murphy JM, Ahn EE, *et al*. FAK in the nucleus prevents VSMC proliferation by promoting p27 and p21 expression via Skp2 degradation. Cardiovasc Res, 2022,118(4):1150-1163

22 Liu L, Pan Y, Song Y, *et al*. Activation of AMPK α2 inhibits airway smooth muscle cells proliferation. Eur J Pharmacol, 2016,791:235-243

23 Parikh V, Bhardwaj A, Nair A. Pharmacotherapy for pulmonary arterial hypertension. J Thorac Dis, 2019,11(Suppl 14):S1767-S1781

24 Deng H, Tian X, Sun H, *et al*. Calpain-1 mediates vascular remodelling and fibrosis via HIF-1α in hypoxia-induced pulmonary hypertension. J Cell Mol Med, 2022,26(10):2819-2830
25 Nan X, Yang Z, Su S, *et al*. The Mechanism of Volatile Oil of Rhodiola tangutica against Hypoxia-Induced Pulmonary Hypertension in Rats Based on RAS Pathway. Biomed Res Int, 2022,2022:9650650
26 Tian D, Teng X, Jin S, *et al*. Endogenous hydrogen sulfide improves vascular remodeling through PPARdelta/SOCS3 signaling. J Adv Res, 2020,27:115-125
27 Hytönen J, Leppänen O, Braesen JH, *et al*. Activation of Peroxisome Proliferator-Activated Receptor-delta as Novel Therapeutic Strategy to Prevent In-Stent Restenosis and Stent Thrombosis. Arterioscler Thromb Vasc Biol, 2016,36(8):1534-1548
28 Fouty BW, Grimison B, Fagan KA, *et al*. p27(Kip1) is important in modulating pulmonary artery smooth musclecell proliferation. Am J Respir Cell Mol Biol, 2001,25(5):652-658
29 Chen MJ, Cheng AC, Lee MF, *et al*. Simvastatin induces G(1) arrest by up-regulating GSK3β and down-regulating CDK4/cyclin D1 and CDK2/cyclin E1 in human primary colorectal cancer cells. J Cell Physiol, 2018,233(6):4618-4625
30 Luan L, Li N, Zhang K, *et al*. Diversin upregulates the proliferative ability of colorectal cancer by inducing cell cycle proteins. Exp Mol Pathol, 2023, 129:104850
31 Fujita T, Liu W, Doihara H, *et al*. Regulation of Skp2-p27 axis by the Cdh1/anaphase-promoting complex pathway. Am J Pathol, 2008,173(1):217-228
32 Zhang H, Gong Y, Wang Z, *et al*. Apelin inhibits the proliferation and migration of rat PASMCs via the activation of PI3K/Akt/mTOR signal and the inhibition of autophagy under hypoxia. J Cell Mol Med, 2014,18(3):542-553

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