The transient receptor potential vanilloid-3 regulates hypoxia-mediated pulmonary artery smooth muscle cells proliferation via PI3K/AKT signaling pathway

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Abstract

Objectives: Transient receptor potential vanilloid 3 (TRPV3) is a member of the TRP channels family of Ca²⁺-permeant cation channels. In this study, we aim to investigate the role of TRPV3 in pulmonary vascular remodeling and PSMCs proliferation under hypoxia.

Materials and methods: The expression of TRPV3 was evaluated in patients with pulmonary arterial hypertension (PAH) and hypoxic rats, using hematoxylin and eosin (H&E) and immunohistochemistry. In vitro, MTT assay, flow cytometry, Western blotting and immunofluorescence were performed to investigate the effects of TRPV3 on proliferation of PSMCs.

Results: We found that, in vivo, the expression of TRPV3 was increased in patients with PAH and hypoxic rats. Right ventricular hypertrophy measurements and pulmonary pathomorphology data show that the ratio of the heart weight/tibia length (HW/TL), the right ventricle/left ventricle plus septum (RV/LV+S) and the medial width of the pulmonary artery were increased in chronic hypoxic rats. Moreover, the expression of proliferating cell nuclear antigen (PCNA), Cyclin D, Cyclin E and Cyclin A, phospho-CaMKII (p-CaMKII) were induced by hypoxia. In vitro, we revealed that hypoxia promoted PSMCs viability, increased the expression of PCNA, Cyclin D, Cyclin E, Cyclin A p-CaMKII, made more cells from G₀/G₁ phase to G₂/M + S phase, enhanced the microtubule formation, and increased [Ca²⁺]_i, which could be suppressed by Ruthenium Red, an inhibitor of TRPV3, and TRPV3 silencing has similar effects. Furthermore, the up-regulated expression of PCNA, Cyclin D, Cyclin E and Cyclin A, the increased number of cells in G₂/M and S phase, and the enhanced activation and expression of PI3K and AKT proteins induced by hypoxia and in presence of carvacrol (an agonist of TRPV3), was significantly attenuated by incubation of LY 294002, a specific inhibitor for PI3K/AKT.

Conclusions: These findings suggest that TRPV3 is involved in hypoxia-induced pulmonary vascular remodeling and promotes proliferation of PSMCs and the effect is, at least in part, mediated via the PI3K/AKT pathway.

1 | INTRODUCTION

Pulmonary arterial hypertension (PAH) is a complex disease of pulmonary vasculature characterized by an increase in pulmonary vascular resistance leading to right heart failure and eventually death.¹⁻³ The histopathology of PAH is marked by sustained vasoconstriction, thickening of pulmonary artery walls, vascular remodeling.⁴ The pulmonary vascular remodeling in PAH is characterized by pulmonary vascular medial hypertrophy and lumen narrowing, which is mainly caused by excessive proliferation and inadequate apoptosis in pulmonary artery smooth muscle cells (PASMCs). Chronic hypoxia is an important contributor to the development of clinical PAH, a fatal disease consisting of arterial constriction, vasculature remodeling, inflammation ensue and elevated thrombosis. However, the mechanisms of hypoxia-induced PASMCs proliferation remain largely unknown.

AKT, a serine/threonine protein kinase, is activated by a number of growth factors and cytokines in phosphatidylinositol-3 kinase

(PI3K) dependent manner, and it may induce pro-proliferative and anti-apoptotic signaling pathways in a variety of cell types.^{5,6} Most of the malignant diseases are closely bound up with aberrant signaling of PI3K, including hypertension, atherosclerosis, cardiovascular disease and tumour.⁷⁻⁹ In PAH, the PI3K/AKT pathway has been reported to be involved in PASMCs proliferation¹⁰⁻¹² and apoptosis.¹³

The transient receptor potential vanilloid (TRPV) subfamily of TRP channels consists of six structurally similar but functionally unique proteins (TRPV1-V6), which the members of TRPV1-TRPV4 are all heat-activated non-selective cation channels. Transient receptor potential vanilloid 3 (TRPV3) is a Ca^{2+} -permeable cation channel that is abundantly expressed in keratinocytes of the skin, as well as cells of the tongue, palate, testes, cornea, nasal epithelium, distal colon, and inner ear.^{14,15} TRPV3 is currently being investigated for its role in cutaneous physiology, specifically its role in thermal perception, inflammation, irritation and pain, wound healing, maintenance of skin barrier integrity, and hair growth.^{16,17} TRPV3 has been shown to promote the release of pro-inflammatory mediators

FIGURE 1 The expression of TRPV3 in pulmonary vessels from humans, and the Hematoxylin-eosin staining (H&E), right ventricular hypertrophy from hypoxic rats. (A) Immunohistochemical evaluation of TRPV3 protein expression in normal humans and human pulmonary arterial hypertension (PAH). (B) H & E staining showed sections of lung tissues from rats that were exposed to normoxia and hypoxia. (C) The ratio of wall thickness/vessel radius in the pulmonary artery. Boxed regions a, b are shown at a higher magnification in a1, b1. Scale bars, 20 and 50 μm . (D) Changes in the heart weight/tibia length (HW/TL) ratio. (E) Changes in the right ventricle/left ventricle and septum (RV/(LV+S)) ratio. All of the values are denoted as the means $\pm$ SEM. from at least three separate experiments. * $P < .05$ compared to Normoxia. "Nor" means normoxia, "Hyp" means hypoxia

FIGURE 2 The expression of TRPV3 and hypoxia-induced proliferation in vivo. (A) Hypoxia significantly increased the expression of TRPV3 in lung tissues. (B) Immunohistochemical staining of TRPV3 in rat lung tissue. (C, D) Expression levels of cyclin D, cyclin E, PCNA, p-CaMKII and CaMKII were determined using western blot analysis under normoxia and hypoxic rats. All of the values are denoted as the means $\pm$ SEM. Data shown are representative of at least three independent experiments. * $P < .05$ compared to Normoxia; ** $P < .01$ compared to Normoxia. "Nor" means normoxia, "Hyp" means hypoxia

and pruritogens, including ATP, nerve growth factor, chemokines and ILs from cultured keratinocytes.¹⁸⁻²⁰ Moreover, several recent studies have demonstrated that arachidonic acid and other fatty acids, which are produced during inflammation, potentiate TRPV3 function.²¹ In addition, the TRPV3-activator carvacrol has been reported to elevate cytosolic Ca^{2+} concentration and promote proliferation in corneal epithelial cells.²² Previous studies revealed that the proliferation rate in the oral epithelia of TRPV3 knockout mice was less than that of wild-type mice.²³ The TRPV3 agonists could prevent adipogenesis by inhibiting phosphorylation of insulin receptor substrate 1 (IRS-1), downstream PI3K/AKT, and the expression of adipogenic genes peroxisome proliferator-activated receptor gamma (PPAR γ) and CCAAT/enhancer-binding protein alpha (C/EBP α).²⁴ However, the expression and underlying molecular mechanisms of TRPV3 in the PSMCs and pulmonary hypertension have not yet been examined. Therefore, in the current study, we aim at defining TRPV3 expression in lung tissues and whether TRPV3-coupled signaling is involved in the regulation of PSMCs proliferation and to analyse the role of TRPV3 on PI3K/Akt signaling in cultured PSMCs, which may help us better understand the pathophysiological role of TRPV3 in PAH.

2 | MATERIAL AND METHODS

For detailed Material and methods, please see Supporting Information.

3 | RESULTS

3.1 | Morphometric analysis of pulmonary vascular remodeling in human and rats and right ventricular hypertrophy in hypoxic rats

The morphology of pulmonary vessels was examined with TRPV3 staining and hematoxylin-eosin stain to show potential correlations of the morphological changes with remodeling. We found that the expression of TRPV3 in pulmonary vessels was upregulated in PAH humans than the vessels from normal humans (Figure 1A). In addition, the wall thickness of the pulmonary artery was shown by the hematoxylin and eosin stain (H&E). Medial thickening was found in medium-size PAs that were obtained from rats that were exposed to hypoxia for 9 days. There was a significant increase in the Wall Thickness/Vessel Radius ratio compared to that of normoxic rats (Figure 1B,C). More importantly, we observed chronic hypoxia-induced right ventricular hypertrophy, as shown in Figure 1D and E, the ratio of the heart weight/tibia length (HW/TL) and the weights of the right ventricle to the weights of left ventricle plus septum (RV/LV+S) were much higher in the rats that were exposed to chronic hypoxia than in those that were exposed to normoxia.

3.2 | The expression of TRPV3 and hypoxia-induced proliferation in vivo

We used western blot and immunohistochemistry stain to determine the expression of TRPV3 in rat lung tissues, the results showed

FIGURE 3 Effect of TRPV3 inhibition on hypoxia induced PSMCs proliferation and cell cycle progression. (A) Cultured PSMCs were exposed to hypoxia for 0 hour as control, 6, 12, 24 hours, respectively, and hypoxia increased protein expression of TRPV3 in a time-dependent manner. (B) The PSMCs were cultured for 24 hours and exposed to normoxia and hypoxia, respectively, and TRPV3 labeled fluorescence images were obtained through immunocytochemistry. The red color denoted TRPV3 stained with cy3, blue color denoted nucleus stained with 4, 6-diamidino-2-phenylindole (DAPI). (C) Ruthenium Red (20 and 40 μ mol L⁻¹) inhibited hypoxia-induced increase in cell viability. (D-F) Treatment with Ruthenium Red reversed the protein expression of PCNA, Cyclin D and Cyclin E induced by 24 hours of hypoxia. (G) Hypoxia increased the cell number in the S plus G₂/M phases compared with normoxia, which was reversed by 20 and 40 μ mol L⁻¹ Ruthenium Red. (H) Cells were fixed and stained with anti- α -tubulin to stain microtubules and DAPI to stain chromosomes and chromatin. (I) The expression of p-CaMKII and CaMKII in PSMCs under hypoxia. (J) Measurement of [Ca²⁺]_i in PSMCs. Fluorescent intensity in [Ca²⁺]_i was recorded by laser scanning confocal microscope in different treatments. Values are denoted as mean $\pm$ SEM from at least three separate experiments. ***P* < .01 compared to Control; #*P* < .05 compared to Hypoxia; ##*P* < .01 compared to Hypoxia. "Con" means control, "Hyp" means hypoxia, "H" means hypoxia, "R20" means 20 μ mol L⁻¹ Ruthenium Red, "R40" means 40 μ mol L⁻¹ Ruthenium Red

that the expression of TRPV3 protein was increased in hypoxic rats compared to normoxic rats (Figure 2A,B). The calcium/calmodulin-dependent kinase II (CaMKII) is known to be activated by intracellular Ca²⁺ concentration.³⁰ Furthermore, PCNA, cyclin proteins, CaMKII and

phospho-CaMKII (p-CaMKII) protein levels were examined by western blot. Cyclin D, Cyclin E and Cyclin A play important roles in both S and G₂/M phases. We found that hypoxia significantly increased the expression of PCNA, Cyclin D, Cyclin E, Cyclin A and enhanced the

FIGURE 3 (Continued)

p-CaMKII protein level (Figure 2C,D; Figure S1A). These results suggest that TRPV3 may be involved in the process of proliferation and affected cell cycle of PAs in hypoxic rats.

3.3 | Effect of inhibitor Ruthenium Red on TRPV3-induced PASCs proliferation and cell cycle progression under hypoxia

We have proved that TRPV3 plays an important role in hypoxia-induced PAH. We endeavored to confirm whether TRPV3 was involved in the proliferation of PASCs. As shown in Figure 3A, the protein level of TRPV3 was increased after 6, 12, 24 hours exposed to hypoxia and among the time course 24 hours hypoxia exposure showed the highest value. Therefore, unless otherwise stated, all of

the subsequent experiments were performed under 24 hours of hypoxia. Furthermore, immunofluorescence analysis for TRPV3 indicated that TRPV3 was upregulated by hypoxia (Figure 3B). To demonstrate the effect of TRPV3 on the PASCs proliferation, we tested the effects of TRPV3 inhibitor Ruthenium Red on cell viability by MTT assay and PCNA protein expression by Western blot. As shown in Figure 3C and D, TRPV3 promoted cell viability, PCNA expression under 24 hours of hypoxia compared to those under normoxia, while the increased effects were inhibited after being treated with 20 and 40 μ mol L⁻¹ Ruthenium Red. To examine whether hypoxia affected the cell cycle progression through TRPV3, the cycle related proteins were measured by western blot, the number of cells in different cell cycle phases was detected by flow cytometry and the organization of microtubules in the mitosis was determined by immunofluorescence

FIGURE 4 Effect of TRPV3 silencing on hypoxia induced PSMCs proliferation and cell cycle progression. (A) The efficiency and specificity of siRNA directed against TRPV3. (B) Represented the cell viability after the treatment of siRNA against TRPV3 under hypoxic conditions. (C-E) The effect of hypoxia on the PCNA, Cyclin D and Cyclin E expression in PSMCs was decreased by siTRPV3. (F) Hypoxia promoted PSMCs cycle progression and increased the percentage of cells in the S plus G_2/M phase, while the proliferation indices were significantly decreased after treatment with TRPV3 silencing. (G) The mitotic spindle formation in the nucleus of PSMCs was attenuated after the treatment of siRNA against TRPV3. (H) The protein levels of p-CaMKII and CaMKII were measured in PSMCs under hypoxic conditions. (I) The increased $[Ca^{2+}]_i$ was significantly suppressed under hypoxia after treated with siTRPV3. The data are presented as the mean $\pm$ SEM. from at least three separate experiments. * $P < .05$ compared to Control; ** $P < .01$ compared to Control; # $P < .05$ compared to Hypoxia; ## $P < .01$ compared to Hypoxia. "Con" means control, "Hyp" means hypoxia, "H" means hypoxia, "siNC" indicates non-targeted siRNA, "siTRPV3" means siRNA targeted to TRPV3

staining with α -tubulin. The results showed that the treatment of Ruthenium Red inhibited hypoxia-induced upregulation of Cyclin D, Cyclin E and Cyclin A expression (Figure 3E,F; Figure S1B). Hypoxia increased the percentage of cells in the S and G_2/M phase. Ruthenium Red suppressed the cell cycle progression and made more PSMCs arrested at the G_0/G_1 phase (Figure 3G). As shown in Figure 3H, hypoxia significantly enhanced the tubulin polymerization compared with that in normoxia, whereas the microtubule formation was inhibited after treating the cells with Ruthenium Red. Meanwhile, TRPV3 enhanced p-CaMKII protein expression under 24 hours of hypoxia compared with that in normoxia, but was reduced by treatment of Ruthenium Red (Figure 3I). Moreover, from fluorescence intensity taken by laser scanning confocal microscope, after treating PSMCs with Ruthenium

Red, the increased $[Ca^{2+}]_i$ was significantly suppressed under hypoxia (Figure 3J). These results suggested that TRPV3 played a key role on the cell cycle activity and induces PSMCs proliferation, eventually contributing to pulmonary vascular remodeling.

3.4 | Effect of RNA interference on TRPV3-induced PSMCs proliferation and cell cycle progression under hypoxia

To further determine the effects of TRPV3 on cell viability and cycle protein, we applied RNA interference technology to knock down the expression of TRPV3 in PSMCs. To assess the efficiency and specificity of RNA interference, we measured intracellular expression of TRPV3

FIGURE 4 (Continued)

relative to β -actin by western blot. Our results showed that, both in the normoxia group and hypoxia group, the expression of TRPV3 treated with siNC was not different from expression in untreated control cells or cells treated with the Transfection Reagent alone. In contrast, si-TRPV3 reduced TRPV3 protein expression compared to siNC both in normoxia and hypoxia (Figure 4A; Figure S1C). We detected cell viability by MTT assay and PCNA protein expression by Western blot, the results showed that cell viability and PCNA expression were increased by hypoxia but inhibited by TRPV3 silencing (Figure 4B,C). To elucidate whether the TRPV3 participated in hypoxia-mediated cell cycle activity, we detected the cycle related proteins by western blot, the number of cells in different cell cycle phase by flow cytometry and the organization of microtubules in the mitosis by immunofluorescence staining with α -tubulin using TRPV3 silencing. As shown in Figure 4D,E and Figure S1D, hypoxia enhanced the expression of Cyclin D, Cyclin E and Cyclin A but the effect was decreased after the TRPV3 silencing. Furthermore, hypoxia increased the percentage of cells in the S and G₂/M phase, which was inhibited by siTRPV3 and made more PSMCs arrested at the G₀/

G₁ phase (Figure 4F). In addition, our results showed that hypoxia significantly enhanced the tubulin polymerization compared with that in normoxia, however, after treating the cells with TRPV3 silencing, the microtubule formation was inhibited effectively (Figure 4G). Moreover, the increased protein expression of p-CaMKII on hypoxia was significantly suppressed by TRPV3 silencing (Figure 4H). And as shown in Figure 4I, from fluorescence intensity taken by laser scanning confocal microscope, specific siRNA was used to silence TRPV3 gene expression, showing that the increased [Ca²⁺]_i was significantly reduced under hypoxia. These results suggested that TRPV3 has a dominant effect on the cell cycle activity and promotes PSMCs growth and proliferation, eventually contributing to pulmonary vascular medial thickening.

3.5 | TRPV3 activated PI3K/AKT signal pathway in hypoxic PSMCs

Because PI3K/AKT signal pathway promoted cell survival and proliferation in PSMCs under hypoxia,³¹ we further determined whether

FIGURE 5 The expression of PI3K and AKT in PSMCs under hypoxia. (A) The phosphorylation and total PI3K and AKT protein levels were reduced after administrated to Ruthenium Red. (B) The inhibited effect of phosphorylation and total PI3K and AKT proteins expression was measured after treatment with siTRPV3. The data are presented as the mean $\pm$ SEM, from at least three independent experiments. ** $P < .01$ compared to Control; # $P < .05$ compared to Hypoxia; ## $P < .01$ compared to Hypoxia. "Con" means control, "Hyp" means hypoxia, "H" means hypoxia, "R20" means $20 \mu\text{mol L}^{-1}$ Ruthenium Red, "R40" means $40 \mu\text{mol L}^{-1}$ Ruthenium Red, "siNC" indicates non-targeted siRNA, "siTRPV3" means siRNA targeted to TRPV3

PI3K/AKT pathway is involved in TRPV3-induced PSMCs proliferation. As shown in Figure 5A, hypoxia not only significantly increased PI3K and AKT phosphorylation but also enhanced the total PI3K and AKT protein levels at 24 hours of hypoxia exposure compared to that of normoxia, and the effects were obviously reversed by the TRPV3 inhibitor. As Ruthenium Red is a chemical blockage of TRPV3, we used RNA interference technology to knock down the expression of TRPV3. In Figure 5B, we can see the same conclusion that siTRPV3 inhibited the phosphorylated and total protein levels of PI3K and AKT pathway under hypoxia. The ratio of the phosphorylated PI3K and AKT to the total PI3K and AKT did not change, suggesting that the increase in PI3K and AKT phosphorylation are due to the increase in protein expression.

3.6 | TRPV3 promoted PSMCs proliferation via PI3K/AKT pathway

To elucidate whether the PI3K/AKT pathway participates in TRPV3 mediated proliferation, we used carvacrol, a TRPV3 agonist and blocked PI3K/AKT with inhibitor LY294002 under hypoxia. MTT assay was conducted to determine the cell viability in PSMCs. As shown in Figure 6A, carvacrol ($60 \mu\text{mol L}^{-1}$) augmented the result of cell viability caused by hypoxia in PSMCs, which was inhibited by PI3K/AKT inhibitor LY294002 ($20 \mu\text{mol L}^{-1}$). Meanwhile, the upregulation of PCNA expression under hypoxia was augmented by carvacrol. In contrast, LY294002 suppressed the protein expression of

FIGURE 6 Effect of PI3K/AKT pathway on carvacrol induced proliferation of PSMCs under hypoxia. (A) Carvacrol ($60 \mu\text{mol L}^{-1}$), a TRPV3 agonist, increased cell viability in PSMCs under the presence of 24 hours hypoxia exposure, which was attenuated by PI3K/AKT inhibitor LY 294002 ($20 \mu\text{mol L}^{-1}$). (B-D) The protein levels of PCNA, Cyclin D and Cyclin E were measured in the absence or presence of $60 \mu\text{mol L}^{-1}$ carvacrol under hypoxia in PSMCs, and the enhanced expression were suppressed after incubated with LY 294002. (E) Hypoxia promoted the cell number in the S plus G_2/M phases compared with normoxia, which was augmented by carvacrol under hypoxia. However, the effect was reversed by using LY 294002 in PSMCs. (F, G) Hypoxia increased the expression of PI3K and AKT in PSMCs under hypoxia alone or in the presence of carvacrol, whereas LY 294002 decreased the effects. All of the values are denoted as means $\pm$ SEM. Data shown are representative of at least three independent experiments. ** $P < .01$ compared to Control; # $P < .05$ compared to Hypoxia; ## $P < .01$ compared to Hypoxia; \$ $P < .05$ compared to H + Car. "Con" means control, "Hyp" means hypoxia, "H" means hypoxia, "Car" means carvacrol, "C" means carvacrol, "LY" means LY 294002

PCNA (Figure 6B). To understand whether TRPV3 inhibition affected the cycle progression, we detected the expression of Cyclin D, Cyclin E and Cyclin A by Western blot and the number of cells in the different cell cycle phases by flow cytometry. Our results showed that carvacrol augmented the hypoxia-induced increase in Cyclin D, Cyclin E and Cyclin A expression. LY294002 inhibited carvacrol-induced augmentation effect (Figure 6C,D; Figure S1E). Furthermore, hypoxia increased the percentage of cells in the S plus G_2/M phase, which was augmented by carvacrol. In contrast, LY294002 suppressed the cell cycle progression and made more PSMCs arrested at the G_0/G_1 phase (Figure 6E). As shown in Figure 6F and G, carvacrol significantly enhanced the PI3K and AKT protein levels under hypoxia compared with that in hypoxia alone, whereas the PI3K and AKT protein levels were suppressed after administration of LY294002 to the PSMCs. These results suggested that PI3K/AKT pathway may be involved in TRPV3-induced proliferation of PSMCs under hypoxia.

4 | DISCUSSION

In this study, the expression property and biological function of the TRPV3 protein were detected in PSMCs, and we demonstrated for the first time that the activation and expression of TRPV3 protein both promote proliferation of PSMCs and improve cell cycle progression under hypoxic condition, which is, at least in part, mediated via the PI3K/AKT pathway. These data support the hypothesis that TRPV3-PI3K/AKT axis plays an important role in pulmonary vascular remodeling in response to hypoxic stress.

Studies have indicated that some other TRP channels are expressed in lung tissues and PSMCs, and these channels were widely involved in pulmonary vasoconstriction, differentiation, migration, proliferation and pulmonary vascular remodeling, such as TRPC1, TRPC6, TRPM7, TRPV1, TRPV4.³²⁻³⁶ Accumulating evidence suggests that carvacrol activated TRPV3-like cation currents in cerebral artery endothelial cells,³⁷ and the activation of TRPV3 channel could

FIGURE 6 (Continued)

promoted the proliferation process of non-small cell lung cancer,³⁸ and the activation of TRPV3 decreased proliferation and preserved the pluripotency of Embryonic stem cells (ESCs).³⁹ However, the functional orientation, expression and the underlying mechanisms that whether TRPV3 activation participates in hypoxia-mediated proliferation in PAH is still unclear. Consistent with this notion, in our experiment, we found that TRPV3 is overexpressed in the pulmonary vessels of human PAH lungs and hypoxic rats. Furthermore, chronic hypoxia exposure resulted in significantly increased HW/TL and RV/LV + S and media thickening of the pulmonary artery, which indicated the role of TRPV3. To further validate the potential role of TRPV3 in the development of PAH, we used TRPV channel blocker Ruthenium Red and siRNA to inhibit and knockdown TRPV3 expression in PSMCs, respectively. The result was confirmed by MTT assay, which could reflect cell proliferation indirectly. After treating PSMCs with Ruthenium Red and siTRPV3 the increased effect of TRPV3 on cell viability was significantly decreased. Meanwhile, we measured the expression of PCNA, which was an essential substance in the DNA synthesis phase of eukaryotic cells and an important index for evaluating the status of cell proliferation.⁴⁰ We found that the PCNA expression was up-regulated on hypoxic condition, but the effect was

reduced in the presence of Ruthenium Red and siTRPV3. We further observed the TRPV3 on cell cycle progression.

The cell cycle plays a central role in cell proliferation,^{41,42} whereas how the cycle responds to the TRPV3 remains unclear. The cell cycle is subdivided into four phases: DNA replication occurs during S phase, and chromosome segregation occurs during M phase. The S and M phases are separated by G₁ (before DNA replication) and G₂ (before mitosis). Our findings demonstrate that hypoxia enhanced the proportion of cells in the S and G₂/M phases and promotes polymerization of mammalian α-tubulin into microtubules in the mitosis phase, while the effect was inhibited after treated with Ruthenium Red and siTRPV3. Cyclin D is required for the G₁/S transition, Cyclin E and Cyclin A are essential for progression through the S and G₂/M phases.⁴³⁻⁴⁵ Our results showed that blockade of the TRPV3 with Ruthenium Red decreased the expression of Cyclin D, Cyclin E, Cyclin A and TRPV3 silencing has the similar effect. Thus, our data provide a new molecular mechanism that hypoxia-induced cell cycle progression is mediated by the TRPV3 pathway.

The roles of TRP channels in PAH progression may involve changes in intracellular Ca²⁺. CaMKII, as a general integrator of Ca²⁺ signaling,

is activated upon binding to Ca^{2+} /calmodulin (CaM), which undergoes autophosphorylation.^{30,46} It has been reported that CaMKII has been involved in lung diseases, as many are the factors that modulate the intracellular Ca^{2+} concentration.⁴⁷⁻⁴⁹ Our results suggest that hypoxia increased the phosphorylation of CaMKII, which was reversed by Ruthenium Red and siTRPV3. Moreover, TRPV3 is a member of the TRP family of Ca^{2+} -permeant channels and we found that blocking or knockdown TRPV3 remarkably decreased $[\text{Ca}^{2+}]_i$ of PSMCs by laser of scanning confocal microscope.

To further determine whether PI3K/AKT pathway is involved in TRPV3-induced proliferation, we measured the activation of PI3K/AKT in hypoxic PSMCs. As stated previously, the activation of TRPV3 suppressed adipogenesis via restraining downstream of PI3K/AKT axis and the expression of PPAR γ , an adipogenic gene.²⁴ PI3K/AKT is an intracellular signaling pathway and AKT is a downstream target of the PI3K pathway and plays a key role in cell growth and proliferation.^{50,51} There has been accumulating evidence indicating that the activation of PI3K/AKT pathway contributed to pulmonary vascular medial thicken, migration, proliferation and apoptosis of PSMCs.⁵²⁻⁵⁴ Consistent with those reports, our findings demonstrated that hypoxia activated and promoted the expression of PI3K/AKT signaling pathway, whereas the phosphorylation and total protein levels of PI3K and AKT were abolished by Ruthenium Red and siTRPV3. In addition, the cell viability was increased by 24 hours of hypoxia exposure and in presence of carvacrol but reversed by LY294002. Meanwhile, we measured the expression of PCNA, Cyclin D, Cyclin E, and cell cycle progression. Our results prove that carvacrol augmented the hypoxia induced increase in PCNA, Cyclin D, Cyclin E expression and the percentage of cells in the S plus G₂/M phase, which were decreased by administration of LY294002. Furthermore, carvacrol significantly enhanced the phosphorylation and total protein expression of PI3K and AKT under hypoxia compared to the hypoxia alone, while the effects were reduced after treated with LY294002 at PSMCs. As previously mentioned, the increase in PI3K and AKT phosphorylation are due to the increase in protein expression, one possible explanation for this phenomenon is that there may be some factors and transcription factors in the middle for bridge, which regulate the transcription and translation level of PI3K and AKT. Further investigation, which is also important in revealing the TRPV3 and PI3K/AKT complexes interaction, should be addressed in future studies.

In conclusion, our results show that the expression of TRPV3 is apparently increased under hypoxia, TRPV3 inhibition and silencing decrease the effect of proliferation induced by hypoxia in PSMCs. We elucidated a novel mechanism that the role of TRPV3 channel in hypoxic pulmonary vascular remodeling and its effect on cell viability, cell cycle progression in the process of PSMCs proliferation, which is, at least in part, mediated via the PI3K/AKT pathway. Furthermore, we can have hypothesis that upregulated TRPV3 channels in PSMCs may make patients more sensitive to carvacrol and some other aromatic compounds, and more likely to develop pulmonary vascular wall thickening, and ultimately, pulmonary hypertension. These results indicate that developing pharmacological interventions specially aimed

at decreasing the gene expression of TRPV3 or inhibiting the function of TRPV3 may greatly help in the development of new therapeutic strategies for PAH.

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AUTHOR CONTRIBUTIONS

H.S. conceived and designed the experiments; Q.Z. performed the experiments; Q.Z. and Y.C. analysed the data; J.R. and B.F. contributed reagents/materials tools; Q.Z. wrote the paper; P.W. and P.S. conducted the Western blot; Q.L., C.S. and M.E. conducted other experiments.

CONFLICTS OF INTEREST

The authors declared no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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