



Original Research

5-HT promotes pulmonary arterial smooth muscle cell proliferation through the TRPC channel

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Abstract: Pulmonary arterial hypertension is caused by an imbalance of pulmonary vasoconstriction and vasodilation. Pulmonary arteriolar remodeling is a primary pathological change and proliferation of pulmonary arterial smooth muscle cells (PASMC) is an important pathological basis for pulmonary arteriolar remodeling. Vasoactive substances, such as 5-HT, may play a role in proliferation of PASMC via unknown mechanisms. *In vitro* experiments with PASMC showed that the TRPC channel inhibitor SKF96365 inhibited the effects 5-HT and DOI on PASMC proliferation and G2M percentage increase, and decreased expression of TRPC1, TRPC6 and calcineurin A/NFATc3 induced by 5-HT and DOI. SKF96365 inhibited binding of NFATc3 and DNA promoted by 5-HT and DOI. Therefore, 5-HT may affect the TRPC channel to promote proliferation of PASMC; upregulate expression of TRPC1, TRPC6, and calcineurin A/NFATc3; and therefore promote NFATc3 nuclear translocation. There may be crosstalk between 5-HT and TRPC, which may contribute to the pathogenesis of pulmonary arterial hypertension and this may be a novel therapeutic target for treating pulmonary arterial hypertension.

Key words: Pulmonary arterial hypertension; 5-HT; TRPC channel; Cell proliferation; NFAT.

Introduction

Pulmonary arterial hypertension (PAH) is caused by an imbalance between pulmonary vasoconstriction and vasodilation (1-5) and pulmonary arteriolar remodeling is a major cause of pathogenesis (4,6-8). Vascular remodeling includes remodeling of the adventitia, tunica media, and tunica intima. At present, it is thought that proliferation of the pulmonary arterioles is dominated by the proliferation of smooth muscle cells to create more muscular arterioles and increase resistance of distal arteries (4,9-11), leading to PAH. Various vasoactive substances such as 5-HT, endothelin, and angiotensin, and growth factors, such as platelet-derived growth factor, epidermal growth factor, and transforming growth factor, can promote pulmonary arterial smooth muscle cell (PASMC) proliferation likely via the TRPC channel, which regulates intracellular calcium to cause vasoconstriction, increased vascular tone, and promote cell proliferation (6,11-20). Calcineurin/NFAT (nuclear factor of activated T cells) signalling pathway is involved in cell proliferation (21). Thus, the mechanism of PASMC proliferation is of interest to elucidate underlying events in PAH.

5-HT (serotonin) is an important neurotransmitter involved in functional regulation of muscle contraction and the cardiovascular system. 5-HT is stored in platelets and is low in plasma (22,23). In the 1990s, appetite suppressants can act on 5-HT system and were found to cause PAH (24). Accordingly, the relationship of 5-HT and PAH was investigated, and studies suggest that

patients with PAH have elevated plasma 5-HT, which may cause pulmonary vasoconstriction and PASMC proliferation (25-29), but the mechanism needs to be further studied. Therefore, we suggest that the 5-HT pathway may interact with TRPC channels and contribute to PAH. To understand any relationship between the 5-HT pathway and the TRPC channel in PAH, we observed the effects of 5-HT on cell proliferation, the TRPC channel and downstream signalling pathways in cultured human PASMC cells.

Materials and Methods

Cell culture

Human PASMCs were purchased from ATCC (ATCC number: PCS-100-023TM, PASMCs, normal human). Cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS). The cells were subcultured to subconfluence in an incubator filled with 5% CO₂ at 37°C before the experiment. PASMCs were randomly divided into 6 groups: control, 5-HT, DOI, 5-HT + ketanserin, 5-HT + SKF96365 and DOI + SKF96365. When cells grew to subconfluence, intervention began. After discarding serum-containing medium and washing with PBS once, serum-free high glucose DMEM was added. These groups were subjected to 24h of growth arrest in FBS-free DMEM. Then, the control group was incubated in DMEM and FBS(0.2%) for 24h. The five other groups were incubated in DMEM plus FBS(0.2%) and one of the following for 24h: 1) 5-HT (10 μmol/L, Tocris), 2)

DOI (1 $\mu\text{mol/L}$, Sigma), 3) 5-HT(10 $\mu\text{mol/L}$, Tocris) + ketanserin(1 $\mu\text{mol/L}$, Sigma), ketanserin was first added, and 5-HT was added 60 min later. 4) 5-HT(10 $\mu\text{mol/L}$, Tocris) + SKF96365(10 $\mu\text{mol/L}$, Tocris), SKF96365 was added first, followed by 5-HT 60 min later. 5) DOI(1 $\mu\text{mol/L}$, Sigma)+ SKF96365(10 $\mu\text{mol/L}$, Tocris) groups, SKF96365 was added first, followed by DOI 60 min later. Then these groups were subjected to the following assay.

MTT assay

Cells were seeded in 96-well plates and 20 μL of MTT solution was added to each well followed by incubation for 4 h at 37°C. After aspirating the supernatant, 150 μL DMSO was added per well, followed by shaking for 10 min to fully dissolve the crystals. A microplate reader was used to measure absorbance at 490 nm.

Cell cycle assay

Cells were collected and resuspended in culture medium followed by centrifugation. Pre-chilled 70% ethanol was used to fix the cells which were then centrifuged. Staining was carried out using a cell cycle and apoptosis kit (Biyuntian, China). Flow cytometry was used to measure cell cycle status.

RNA expression measured using RT-PCR

Total RNAs was extracted and reverse transcribed into cDNAs. Expression of TRPC1, TRPC6, calcineurin A and NFATc3 was measured using PCR. Primer sequences are as follows:

TRPC1 Forward 5': GCGCATGTGGCAATCTTTGT, TRPC1 Reverse 5': AAGCATTGCCACCAGCAGTT; TRPC6 Forward 5': TCCTTGTTGCTCCTTGCTGTTG, TRPC6 Reverse 5': GGAGGCTGCGTGTGCTAC; Calcineurin A Forward 5': GCGGAGTACTTTCTG-GAGGG, Calcineurin A Reverse 5': TAAGTTG-GCGTCAGAGGGC; NFATc3 Forward 5': TGCG-GAAGCCACCAGGAGTT, NFATc3 Forward 5': TTGGCGGCTCTTTGGCTCGT; GAPDH Forward 5': AGGTCCACCACTGACACGTT, GAPDH Reverse 5': GCCTCAAGATCATCAGCAAT.

Calculate $\Delta\Delta\text{Ct} = (\text{Ct experiment} - \text{Ct reference}) - (\text{Ct control} - \text{Ct reference})$. Then $2^{-\Delta\Delta\text{Ct}}$ was calculated for analysis of mRNA relative expression. Three samples were used per group, and each sample was repeated three times.

Western blot

Total cellular proteins were extracted and stored at -80°C after denaturation. Proteins were resolved with SDS-PAGE and transferred to nitrocellulose membranes blocked in 5% skim milk powder with slow shaking for 1 h, followed by incubation in primary antibody overnight (TRPC1 antibody 1:200, Alomone; TRPC6 antibody 1:200, Alomone; calcineurin A antibody 1:500, Santa Cruz; NFATc3 antibody 1:100, Santa Cruz; GAPDH monoclonal antibody 1:1000, Epitomics). After TBST washes, membranes were incubated with secondary antibody (HRP-labeled polyclonal antibody, 1:5000, Abcam) at room temperature for 1 h. After washing with TBST, chemiluminescence was used to image the protein in a gel imaging system. Quantity One software was used for image analysis. Three samples were used

per group, and each sample was repeated three times.

EMSA

Nuclear extracts were prepared from the PSMCs treated as described above. Using instructions for non-radioactive EMSA Kit (Pierce, US), PAGE was used to resolve proteins, followed by electrotransfer. After removal of filter paper, membranes were recovered. With the protein-binding face-up, the membrane was placed at 10 cm under a UV lamp and cross-linked for 10 min. Chemiluminescence was used for image display.

Statistics and analysis

Results are expressed as means \pm standard deviation. SPSS 13.0 software was used for statistics. One-way ANOVA was used to compare both groups ($p < 0.05$ was considered statistically significant). Graphs were prepared using GraphPad Prism 5.

Results

SKF96365 inhibits PSMC proliferation and G2M percentage increase induced by 5-HT and DOI

5-HT and 5-HT_{2A} receptor agonist DOI, 5-HT_{2A} receptor inhibitor ketanserin, and TRPC channel inhibitor SKF96365 were evaluated with respect to the proliferation of pulmonary arterial smooth muscle cells using an MTT assay. Compared with controls, 5-HT and DOI groups had increased absorbance values. Ketanserin pre-treatment decreased absorbance values after 5-HT treatment. SKF96365 pretreatment decreased absorbance after either 5-HT or DOI treatment (Figure 1).

Compared with controls, 5-HT and DOI increased cells in the G₂M phase. Compared with the 5-HT group, cells in the G₂M phase decreased after ketanserin intervention, indicating that ketanserin inhibited 5-HT function of promoting G₂M phase increase. Compared with 5-HT and DOI groups, cells in the G₂M phase decreased after SKF96365 intervention, indicating that SKF96365 inhibited G₂M phase increase of PSMC treated with DOI and 5-HT (Figure 2).

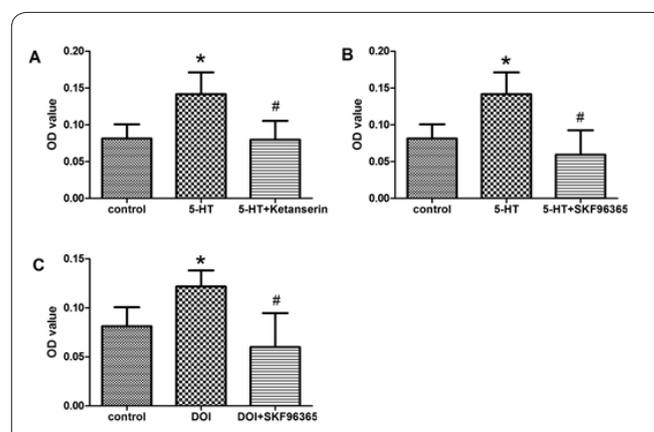


Figure 1. MTT results of each intervention group. A: After ketanserin pre-treatment, 5-HT decreased OD values; B: After SKF96365 pre-treatment, 5-HT decreased OD values; C: After SKF96365 pre-treatment, DOI decreased OD values. Ketanserin: 5-HT_{2A} receptor inhibitor; DOI: 5-HT_{2A} receptor agonist; SKF96365: TRPC channel inhibitor; OD value: absorbance values. * $p < 0.05$, vs control group; # $p < 0.05$, vs. DOI group or 5-HT group, $n = 6$.

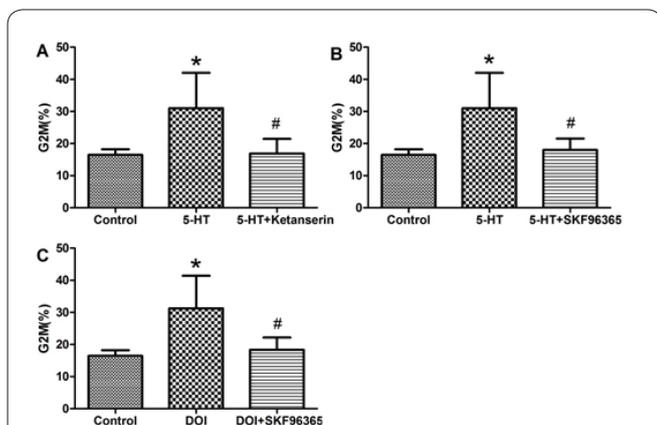


Figure 2. Cell cycle analysis. A: ketanserin inhibited 5-HT-mediated G₂M phase increase of PASM_C; B: SKF96365 inhibited 5-HT-mediated G₂M phase increase of PASM_C; C: SKF96365 inhibited DOI's effects on promoting the G₂M phase increase of PASM_C. Ketanserin: 5-HT_{2A} receptor inhibitor; DOI: 5-HT_{2A} receptor agonist; SKF96365: TRPC channel inhibitor. *p<0.05, vs. Control group; #p<0.05, vs. DOI group or 5-HT group, n = 4.

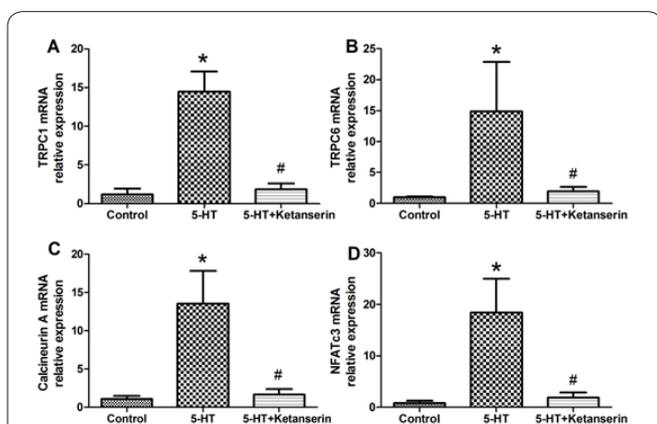


Figure 3. Ketanserin attenuates 5-HT upregulation of TRPC1, TRPC6 mRNA, and calcineurin A/NFATc3 mRNA expression. A: Ketanserin attenuates 5-HT-mediated upregulating TRPC1 mRNA expression; B: Ketanserin attenuates 5-HT-mediated upregulating TRPC6 mRNA expression; C: Ketanserin attenuates 5-HT-mediated upregulating calcineurin A mRNA expression; D: Ketanserin attenuates 5-HT's upregulating NFATc3 mRNA expression. Ketanserin: 5-HT_{2A} receptor inhibitor; DOI: 5-HT_{2A} receptor agonist; SKF96365: TRPC channel inhibitor. *p<0.05, vs. Control; #p<0.05, vs. 5-HT group.

5-HT upregulates TRPC1, TRPC6 channels and calcineurin A, NFATc3 expression through the 5-HT_{2A} receptor

Compared with controls, 5-HT increased TRPC1, TRPC6, and calcineurin A/NFATc3 mRNA expression in PASM_C. Ketanserin reduced this mRNA expression compared with the 5-HT group, indicating that ketanserin attenuated 5-HT upregulated TRPC1, TRPC6, and calcineurin A/NFATc3 mRNA expression (Figure 3). Compared with controls, 5-HT increased PASM_C TRPC1, TRPC6, and calcineurin A/NFATc3 protein expression. Compared with the 5-HT group, TRPC1, TRPC6, and calcineurin A/NFATc3 expression decreased after ketanserin intervention, indicating that ketanserin can down-regulate 5-HT upregulation of TRPC1, TRPC6, and calcineurin A/NFATc3 protein expression (Figure 4).

SKF96365 attenuated upregulation of TRPC1 and TRPC6 channels via 5-HT in PASM_C

Compared with the 5-HT group, TRPC1 and TRPC6 mRNA expression decreased after SKF96365 intervention, indicating that SKF96365 can reduce 5-HT upregulation of TRPC1 and TRPC6 mRNA expression (Figure 5). Compared with the 5-HT group, protein expression of TRPC1 and TRPC6 decreased after SKF96365 intervention, indicating that SKF96365 attenuated upregulation of TRPC1 and TRPC6 protein expression by 5-HT(Figure 6).

SKF96365 can reduce DOI's upregulation of TRPC1, TRPC6 channel expression

Compared with controls, expression of TRPC1 and TRPC6 mRNA in PASM_C increased after 5-HT_{2A} receptor agonist DOI treatment. Compared with DOI intervention group, expression of TRPC1 and TRPC6 mRNA decreased after SKF96365 treatment, indicating that SKF96365 can reduce DOI-mediated upregulation of TRPC1 and TRPC6 mRNA expression (Figure 7).

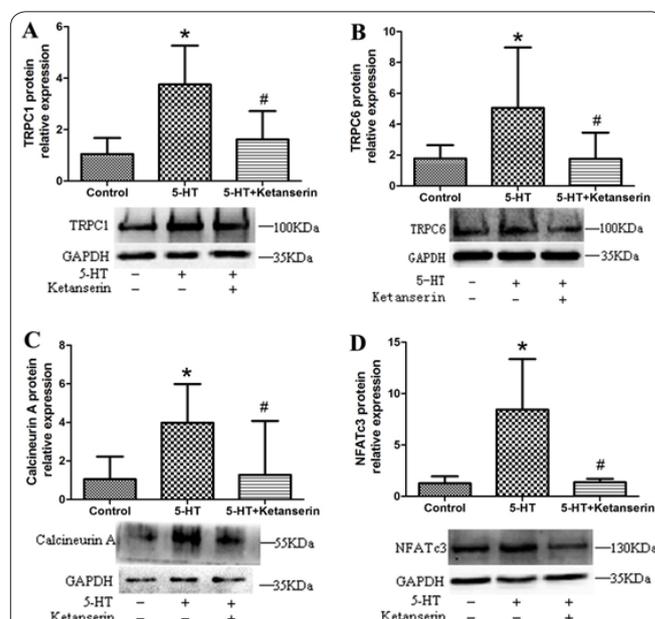


Figure 4. Ketanserin attenuates 5-HT-mediated up-regulation of TRPC1, TRPC6 and calcineurin A/NFATc3 protein expression. A: Ketanserin attenuates 5-HT-mediated up-regulating TRPC1 protein expression; B: Ketanserin attenuates 5-HT-mediated upregulating TRPC6 protein expression; C: Ketanserin attenuates 5-HT-mediated up-regulation of calcineurin A protein expression; D: Ketanserin attenuates 5-HT-mediated up-regulation of NFATc3 Protein. Ketanserin: 5-HT_{2A} receptor inhibitor. *p<0.05, vs. Control; #p<0.05, vs. 5-HT group.

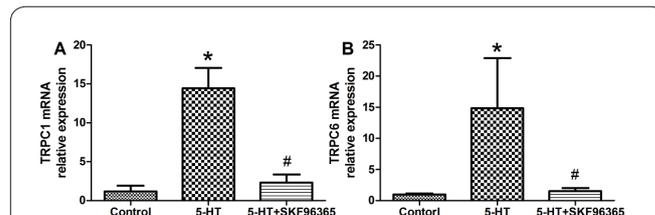
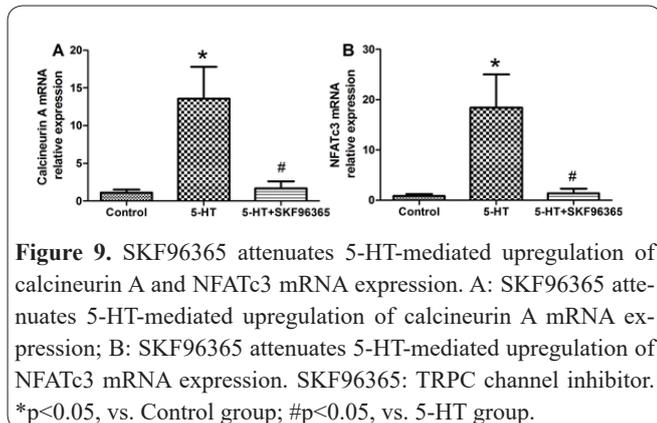
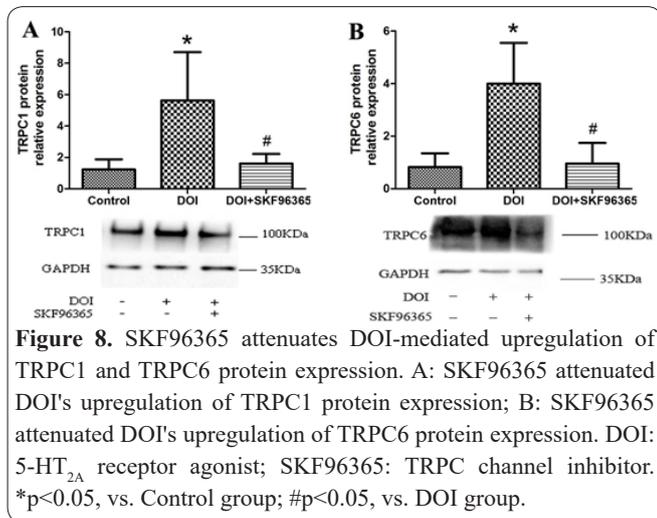
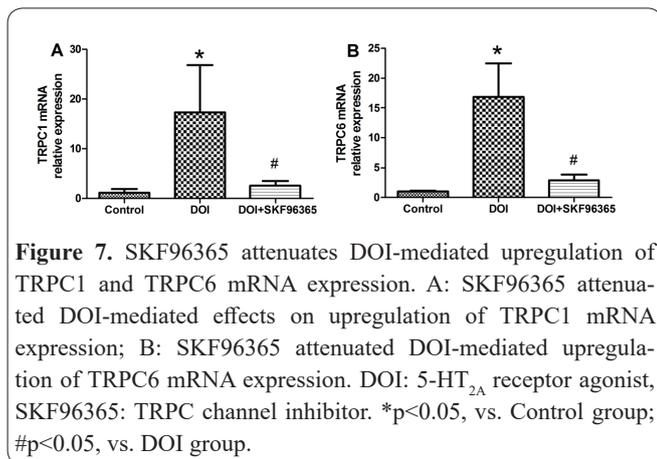
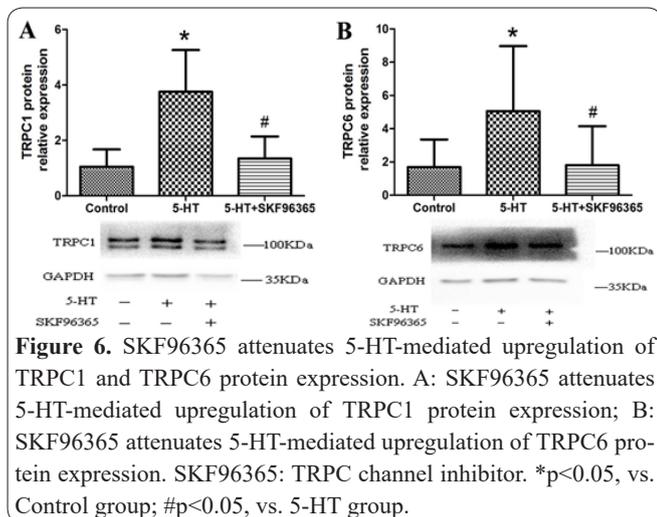


Figure 5. SKF96365 attenuates 5-HT-mediated upregulation of TRPC1 and TRPC6 mRNA expression. A: SKF96365 attenuates 5-HT-mediated upregulation of TRPC1 mRNA expression; B: SKF96365 attenuates 5-HT-mediated upregulation of TRPC6 mRNA expression. SKF96365: TRPC channel inhibitor. *p<0.05, vs. Control group; #p<0.05, vs. 5-HT group.



Compared with controls, DOI treatment increased TRPC1 and TRPC6 protein expression. Compared with the DOI group, SKF96365 reduced TRPC1 and TRPC6

protein expression, indicating that SKF96365 attenuated DOI-upregulated TRPC1 and TRPC6 protein expression (Figure 8).

SKF96365 attenuated 5-HT upregulated calcineurin A/NFATc3 expression

Compared with the 5-HT group, SKF96365 decreased calcineurin A and NFATc3 mRNA expression in PSMC, indicating that SKF96365 attenuated 5-HT upregulated expression of calcineurin A and NFATc3 mRNA (Figure 9). Compared with the 5-HT group, SKF96365 intervention decreased protein expression of calcineurin A and NFATc3 (Figure 10), indicating that SKF96365 attenuated 5-HT upregulated protein expression of calcineurin A and NFATc3.

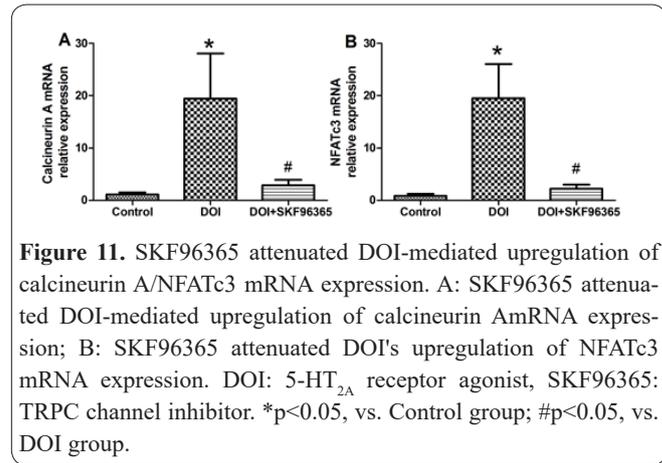
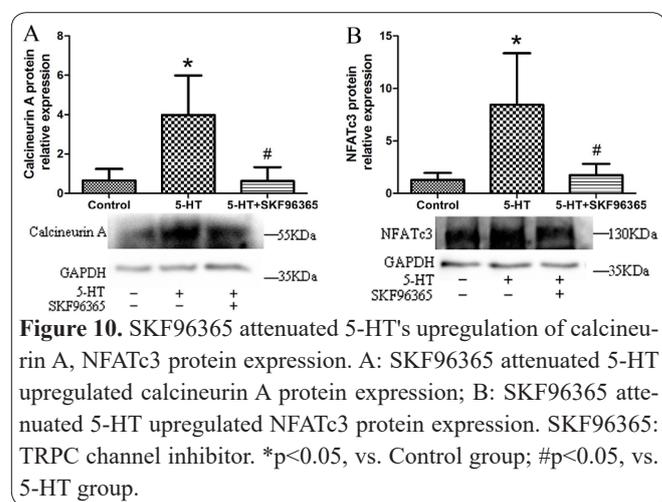
SKF96365 attenuates DOI upregulated calcineurin A/NFATc3 expression

Compared with controls, DOI increased expression of calcineurin A/NFATc3 mRNA. Compared with the DOI group, SKF96365 decreased calcineurin A/NFATc3 mRNA expression, indicating that SKF96365 attenuated DOI-mediated upregulation of calcineurin A/NFATc3 mRNA expression (Figure 11).

Compared with controls, DOI treatment increased protein expression of calcineurin A/NFATc3. Compared with the DOI group, SKF96365 treatment decreased calcineurin A/NFATc3 protein expression (Figure 12), indicating that SKF96365 attenuates DOI-induced calcineurin A/NFATc3 protein expression.

NFATc3 nuclear translocation

Dephosphorylated NFAT translocated from the



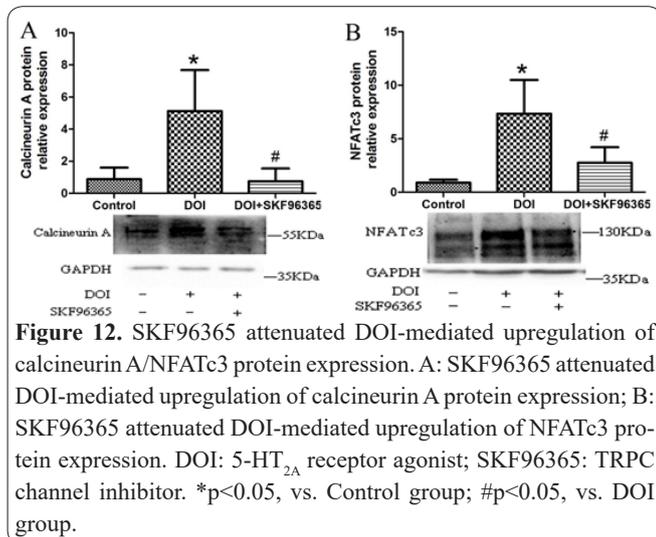


Figure 12. SKF96365 attenuated DOI-mediated upregulation of calcineurin A/NFATc3 protein expression. A: SKF96365 attenuated DOI-mediated upregulation of calcineurin A protein expression; B: SKF96365 attenuated DOI-mediated upregulation of NFATc3 protein expression. DOI: 5-HT_{2A} receptor agonist; SKF96365: TRPC channel inhibitor. **p*<0.05, vs. Control group; #*p*<0.05, vs. DOI group.

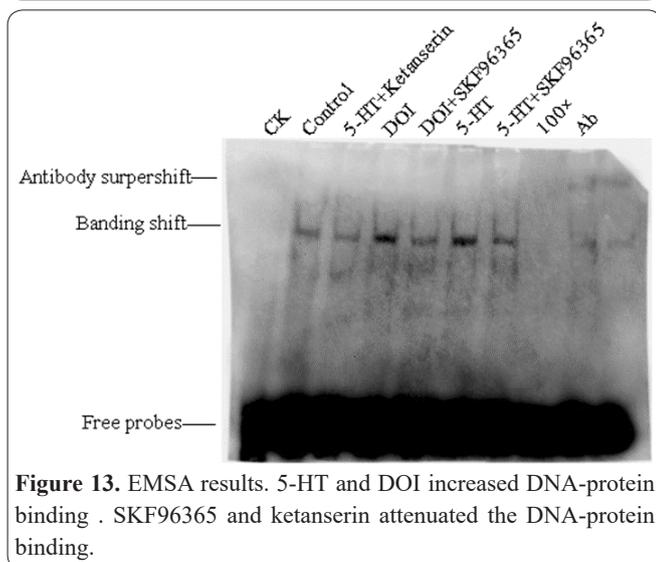


Figure 13. EMSA results. 5-HT and DOI increased DNA-protein binding. SKF96365 and ketanserin attenuated the DNA-protein binding.

cytoplasm to the nucleus in order to promote cell proliferation. To evaluate this, EMSA was used to study NFAT nuclear translocation. NFAT enters the nucleus to bind with genetic motifs to promote transcription and protein synthesis. The complex of NFAT and DNA slows during electrophoresis and can be measured with EMSA. Figure 13 shows that DNA-protein binding increased in the 5-HT and DOI intervention groups compared to controls. DNA-protein binding decreased after SKF96365 intervention compared to the 5-HT group. Compared with the DOI group, DNA-protein binding decreased after SKF96365 intervention. DNA-protein binding decreased after ketanserin intervention compared to 5-HT. A supershift showed a binding band, indicating the presence of NFATc3 protein in the mixture. Thus, DOI and 5-HT promote NFATc3 translocation into the nucleus. Ketanserin, a 5-HT_{2A} receptor antagonist, attenuated the effect of 5-HT on nuclear translocation of NFATc3. Inhibition of DOI and 5-HT by SKF96365, a TRPC channel inhibitor, also attenuated the nuclear translocation of NFATc3.

Discussion

Pulmonary arterial smooth muscle cell proliferation is key to pathological changes in PAH (4, 11). Various vasoactive substances and growth factors such as 5-HT, endothelin, platelet-derived growth factor, epidermal

growth factor, and transforming growth factors promote smooth muscle cell proliferation. It is known that 5-HT is stored in platelets and its circulating level is normally low (20, 22, 23). In the late 1990s, it was found that appetite suppressants enhanced the development of PAH, and these were identified as 5-HT receptor agonists which bring about increases in local and systemic levels of 5-HT (24). Studies have confirmed that PAH patients have increased 5-HT levels which promote endothelial and smooth muscle cell proliferation (25, 30). Precursors of 5-HT and its metabolites do not promote cell proliferation (31, 32). It has been reported that 5-HT acts through receptors and transporters (33, 34). Moreover, studies have revealed that 5-HT increases intracellular calcium and promotes proliferation of PSMC (20, 33, 35, 36). In the current study, MTT assay and cell cycle analysis were used to assess cell proliferation and changes in cell cycle. The results showed that 5-HT and DOI, a 5-HT and 5-HT_{2A} receptor agonist, promoted proliferation of PSMC and increased percentage of cells in the G2M phase. In addition, ketanserin, a 5-HT_{2A} receptor antagonist, inhibited 5-HT-induced cell proliferation and increase in percentage of cells in G2M phase. The TRPC channel inhibitor, SKF96365 inhibited 5-HT- and DOI-induced PSMC proliferation, and blocked the increase in percentage of cells in G2M phase. Therefore, it can be proposed that 5-HT stimulates proliferation of PSMC and percentage of cells in G2M phase via the TRPC channel and the 5-HT_{2A} receptor pathway.

The effect of 5-HT on TRPC channels and downstream calcineurin A/NFATc3 signaling was also studied. The TRPC channel belongs to the TRP channel superfamily associated with the cardiovascular system. There are seven subtypes of TRPC channel i.e. TRPC1–TRPC7 (37, 38). Numerous studies have demonstrated that PSMC expresses mainly TRPC1 and TRPC6, although TRPC3 and TRPC4 are sometimes expressed, but never TRPC5 and TRPC7 (39). The TRPC channel is a component of the non-voltage-dependent calcium channel or store-operated calcium channel (SOCC) (39 - 43). When SOCC opens, store-operated Ca²⁺ entry (SOCE) induces elevation in intracellular Ca²⁺ which promotes proliferation of PSMC. Diverse vasoactive substances, growth factors, and other stimuli may upregulate TRPC channel expression in PSMC and promote cell proliferation (39 - 44). The opening the TRPC channel increases intracellular calcium, thereby activating downstream, the calcineurin/NFAT signaling pathway. Calcineurin comprises calcineurin A and calcineurin B. The catalytic subunits are activated by binding of calcium to the regulatory subunits, and to the calcium/calmodulin complexes (45). Calcineurin is involved in many physiological and pathological processes such as cell proliferation, cell hypertrophy, and apoptosis (46). The NFAT family consists of five members designated as NFATc1–NFATc5. Common features of the NFAT family include an N-terminal NFAT homology domain (NHR) and a C-terminal Rel homology domain. The NHR is involved in regulatory functions: it is the binding site for calponin, and it is involved in nuclear localization and nuclear export. It is also linked to some serine/threonine kinase phosphorylation sites. Rel homodomain mediates DNA binding (47). In-

creased intracellular calcium promotes calcineurin activation, thereby dephosphorylating NFAT which is then translocated from the cytoplasm to the nucleus. Within the nucleus, NFAT binds to motifs, promotes cell proliferation and inhibits apoptosis, thereby regulating gene transcription and protein expression. Due to the binding site of NFAT to the *TRPC* gene, it can be combined with TRPC to upregulate expression of the TRPC channel, thereby forming a positive feedback pathway for promoting proliferation (48, 49). In the present study, it was found that 5-HT and DOI upregulated expression of TRPC1 and TRPC6, and also upregulated the expression of downstream signaling pathway calcineurin A/NFATc3 in PASM_C *in vitro*. Ketanserin, a 5-HT_{2A} receptor antagonist, decreased the expressions of TRPC1, TRPC6, and the downstream signaling pathway calcineurin A/NFATc3. Moreover, TRPC1, TRPC6, and calcineurin A/NFATc3 which were upregulated by 5-HT and DOI, were decreased after treatment with the TRPC channel blocker SKF96365. Thus, it can be speculated that, in addition to its effect on the expression of TRPC channel and the downstream calcineurin A/NFATc3 signaling pathway through the 5-HT_{2A} receptor, 5-HT may directly activate the TRPC channel and upregulate expression of the TRPC channel and downstream calcineurin A/NFATc3 signaling pathway via a positive feedback mechanism. At present, very few studies have focused on the relationship between 5-HT and TRPC channels. In a study of POMC neurons, it was found that TRPC channels are involved in mCPP-mediated neuronal activation of the 5-HT_{2C} receptor agonist (50). In a study on cardiomyocytes, it was reported that the TRPC1 channel participates in the activation of calcineurin/NFAT by 5-HT (51). Vasoactive substances such as ET-1, angiotensin-2 or growth factor can increase intracellular calcium and promote calcineurin/NFAT activation (47, 52), but information about the effects of 5-HT on calcineurin/NFAT is limited.

In the present study, EMSA results showed that 5-HT and DOI increased binding of NFATc3 to DNA. Ketanserin attenuated this binding, and SKF96365 attenuated 5-HT and DOI-induced binding of NFATc3 and DNA. These results indicate that the TRPC channel may be involved in 5-HT-mediated promotion of NFATc3 and DNA binding. It has been reported that in PAH, pulmonary arterial NFATc2 expression is elevated and translocated to the nucleus. The NFATc2 is expressed mainly in the cytoplasm of pulmonary arteries in normal and secondary PAH patients (53). Studies have revealed that TRPC channels participate in activation and translocation of NFAT, and overexpression of TRPC channels in cells leads to redistribution of NFAT from the cytoplasm to nucleus (49, 54).

In summary, this study found that SKF96365, a TRPC channel inhibitor, attenuated the effects of 5-HT and DOI on PASM_C cell proliferation and increase in percentage of cells in G2M phase. It also attenuated the upregulation of TRPC1, TRPC6 and calcineurin A/NFATc3 by 5-HT and DOI, and attenuated NFATc3 and DNA binding which were enhanced by 5-HT and DOI. Therefore, 5-HT may promote proliferation of PASM_C, upregulate the expressions of TRPC1, TRPC6 and calcineurin A/NFATc3, and promote nuclear translocation of NFATc3 by affecting TRPC channels. It is likely that

5-HT and TRPC channels operate through a common pathway in promoting PASM_C proliferation and PAH pathogenesis.

The samples used in this study were small. Thus, more work is required to validate these preliminary data. Among available drugs for treating PAH, calcium antagonists are effective only in few patients (9). Other drugs such as endothelin receptor antagonists, are expensive. Therefore, 5-HT and TRPC channels are worth considering as a novel therapeutic targets for PAH.

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Conflict of Interest

There is no conflict of interest to be declared by the authors.

Authors' contribution

All work was done by the authors named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Tian Hongyan; Liu Ya, Fan Fenling collected and analysed the data; Han Junli did the research work and wrote the text and all authors have read and approved the text prior to publication.

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