

Iptakalim influences the proliferation and apoptosis of human pulmonary artery smooth muscle cells

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Abstract. The aim of the present study was to determine the effect of an ATP-sensitive K⁺ (K_{ATP}) channel opener iptakalim (IPT) on the proliferation and apoptosis of human pulmonary artery smooth muscle cells (HPASMCs), and examine the potential value of IPT to hypoxic pulmonary hypertension (HPH) at a cellular level. HPASMCs were divided into the control, ET-1, ET-1+IPT and ET-1+IPT+glibenclamide (GLI) groups. GLI was administered 30 min prior to ET-1 and IPT. The 4 groups were incubated with corresponding reagents for 24 h. Cell viability was evaluated using a CCK-8 assay, cell proliferation by 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay, and cell apoptosis via the expression of apoptosis-related proteins, i.e., Bcl-2-associated X protein (Bax) and B-cell lymphoma 2 (Bcl-2) using western blotting. We incubated HPASMCs with varying concentrations of ET-1 for 24, 48 and 72 h, and found that cell survival rate was increased in a dose-dependent manner (P<0.05) rather than in a time-dependent manner (P>0.05). After co-incubation of HPASMCs with varying concentrations of IPT and ET-1 for 24 h, the cell survival rate was decreased in a dose-dependent manner. The cell survival rate in the IPT+ET-1 group was significantly lower than that in the ET-1 group (P<0.05). The cell viability (P<0.05) and proliferation (P<0.05) in the ET-1 group were higher than those in the control group, and the expression of Bax/Bcl-2 was lower than the control group (P<0.05). The cell viability (P<0.05) and proliferation (P<0.05) in the ET-1+IPT group were lower than those in the ET-1 group, and the expression of Bax/Bcl-2 was higher than that in the ET-1 group (P<0.05). The cell viability (P<0.05) and proliferation (P<0.05) in the ET-1+IPT+GLI group were higher than those in the ET-1+IPT group, and the

expression of Bax/Bcl-2 was lower than that in the ET-1+IPT group (P<0.05). In conclusion, IPT inhibited ET-1-induced HPASMC proliferation and promoted cell apoptosis. Thus, it may play an important role in the treatment of HPH.

Introduction

Hypoxic pulmonary hypertension (HPH) is a severe disease detrimental to human health. The pathophysiology of HPH is contraction and proliferation of human pulmonary artery smooth muscle cells (HPASMCs) as well as small pulmonary arterial remodeling (1). The pathogenesis mechanism of HPH is not well characterized and no effective therapy for HPH is currently available. Although calcium channel antagonists, prostacyclins, endothelin receptor antagonists and phosphodiesterase type 5 (PDE5) inhibitors may treat pulmonary hypertension, these drugs did not inhibit or reverse pulmonary vascular remodeling due to hypoxia (2,3). Therefore, clinical use of these expensive drugs is limited.

Krick *et al* reported that the K⁺ channels on the HPASMC membrane were closely associated with the vasomotor tone (4). The activity of K⁺ channels on the HPASMC membrane decreased in hypoxia, leading to increased intracellular K⁺ concentration, decreased activities of caspases and nuclease, and decreased apoptosis of HPASMCs. On the other hand, decreased activity of K⁺ channels may depolarize the cell membrane, open voltage-dependent Ca²⁺ channels and increase Ca²⁺ influx (5). Furthermore, the release of Ca²⁺ from sarcoplasmic reticulum increased free intracellular Ca²⁺ ([Ca²⁺]_{cyt}) and promoted vascular smooth muscle contraction (2,3). Increase of [Ca²⁺]_{cyt} is a second messenger for many proliferation factors, and may induce the transition from synthesis phase to mitosis phase, promoting cell proliferation and aggravating pulmonary vascular remodeling (6-8).

Currently, ATP-sensitive K⁺ (K_{ATP}) is the only known compensatory open K⁺ channel in ischemia and hypoxia, representing an important compensatory mechanism (9,10). The K_{ATP} channels are a group of widely distributed inward rectifier K⁺ channels, which are heterologous octamers [(SUR/Kir6.x)₄] consisting of the inward rectifier K⁺ channel Kir6.x family and sulfonylurea receptor (SUR) family (11).

Pulmonary artery endothelial cells may be injured in chronic hypoxia, creating an imbalance of vasoactive substances secreted by endothelial cells, and may affect the

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pulmonary artery (12). The expression of vasoconstrictors increased ET-1, angiotensin II (Ang II) and 5-hydroxytryptamine (5-HT), while the expression of vasodilators decreased prostaglandin I₂ (PGI₂), calcitonin gene-related peptide, adenosine and nitric oxide (NO). These vasoconstrictors act on K_{ATP} channels to promote pulmonary artery smooth muscle contraction and increase pulmonary vascular resistance, thereby promoting HPASMC proliferation and pulmonary vascular remodeling leading to pulmonary hypertension. K_{ATP} channel is an important pathway of pulmonary hypertension. Therefore, K_{ATP} channel opener is a promising novel drug for HPH.

Iptakalim (IPT) is a novel K_{ATP} channel opener and glibenclamide (GLI) is an antagonist to K_{ATP} channel. Wang reported that IPT could increase outward potassium current in rat pulmonary artery smooth muscle cell membrane (13). This could not only prevent rat pulmonary hypertension induced by hypoxia or ET-1, but also reverse pulmonary vascular remodeling and right ventricular hypertrophy in hypoxic rats, thus preventing rat pulmonary hypertension effectively (14). In addition, IPT could open K_{ATP} channels on rabbit pulmonary artery smooth muscle cell membrane, which inhibits Ca²⁺ influx and decrease cytoplasmic Ca²⁺ concentration, thus inhibiting ET-1-induced rabbit pulmonary artery smooth muscle cell contraction and proliferation (15).

Cell proliferation and apoptosis maintain homeostasis in normal cells. In B-cell lymphoma 2 (Bcl-2) gene family, Bcl-2 is the first gene found to be associated with cell proliferation and apoptosis. Bcl-2 proteins are located on inner mitochondrial membrane, endoplasmic reticulum and nuclear membrane. The main biological function of Bcl-2 is to prolong cell life, enhancing cell resistance to various apoptosis-inducing factors. Since the finding by Oltvai *et al* (16) that Bcl-2-associated X protein (Bax) could accelerate apoptosis, the regulation of apoptosis by Bax has been under investigation. Bax formed heterodimers with anti-apoptosis Bcl-2 to inhibit Bcl-2 and induce cell apoptosis. Higher Bax/Bcl-2 ratio promoted cell apoptosis and vice versa (17).

In the present study, a cell proliferation/toxicity detection kit [Cell Counting Kit-8 (CCK-8)] and 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay were used to determine the effect on HPASMC proliferation by ET-1, western blotting to evaluate the expression of Bcl-2 and Bax to detect apoptosis. The potential therapeutic effect of IPT on HPH was clarified on the cellular level.

Materials and methods

Cell culture. HPASMCs were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA). Cells were routinely maintained in the medium containing 2% fetal bovine serum, 1% smooth muscle cell growth supplement (SMCGS) and 1% penicillin/streptomycin, and incubated in a humidified incubator at 37°C with 5% CO₂. The medium was replaced every other day. Cells were passaged every 2 days, and the cells of the 2nd-6th passage were used in the study.

Reagents. ET-1 (Sigma, St. Louis, MO, USA), IPT (Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences), GLI (Sigma), CCK-8 kit (Beyotime Institute of

Biotechnology, Shanghai, China), rabbit anti-Bax polyclonal antibody and rabbit anti-Bcl-2 polyclonal antibody (both from Cell Signaling Technology, Inc., Danvers, MA, USA) were used.

HPASMC viability by CCK-8 assay. Adherent HPASMCs in the logarithmic phase were digested and inoculated in 96-well plates, 100 μl/well in triplicate. Drugs were added after cell adherence for 24 h. CCK-8 solution was prepared in serum-free SMCM medium and 100 μl CCK-8 medium was added into wells instead of medium followed by 2-h incubation. Subsequently, the absorbance values were measured at 450 and 620 nm with a microplate reader (Thermo Fisher, Shanghai, China).

Cell viability was calculated as (OD₄₅₀ - OD₆₂₀ in treatment group)/(OD₄₅₀ - OD₆₂₀ in control group) x 100%. Experiments were performed in triplicate independently.

HPASMC proliferation by EdU incorporation assay. EdU incorporation assay was performed following the manufacturer's instructions. Briefly, HPASMCs in the logarithmic phase were digested and inoculated in a 24-well plate with coverslip. Drugs of a pre-determined concentration were added after cell adherence, 50 μmol/l EdU medium were added for 2-h incubation. EdU medium was then discarded, the cells were washed with phosphate-buffered saline (PBS) for 5 min x 2, fixed with 4% paraformaldehyde at room temperature (RT) for 30 min, decolorized in 2 mg/ml glycine for 5 min and washed with PBS for 5 min. Cell permeation was performed by incubating the cells with 0.5% Triton X-100 for 10 min followed by washing. Staining buffer (1X Apollo) was added and incubated in the dark at RT for 30 min. The staining buffer was discarded, permeation reagent (PBS containing 0.5% Triton X-100) was added and incubated for 10 min x 2-3 times. Permeation reagent was discarded, the cells were washed with methanol for 5 min, 1-2 times and then washed with PBS for 5 min. Staining solution (1X Hoechst 33342) was freshly prepared and added to wells for 30-min incubation in the dark at RT. The cells were washed 1-3 times and observed under a fluorescence microscope (EUROIMMUN, Lubeck, Germany). Cell images were taken randomly and the percentage of positive cells was calculated. The assay was performed in triplicate.

Western blotting for Bax and Bcl-2 in HPASMCs to assay apoptosis. Cells were collected for protein extraction and loaded on SDS-PAGE gel for electrophoresis under constant voltage 70 V, and 120 V after protein migration into the separating gel. The proteins were transferred to PVDF membrane (Runwelltac, Shanghai, China) under a constant current 300 mA for 90 min. The membrane was blocked in 5% skimmed milk for 2 h. Primary antibody (Bax, Bcl-2, 1:1,000; β-actin, 1:5,000) was added for incubation overnight in 4°C. The following day, the membrane was washed with TBST for 10 min x 3 and then secondary antibody (horseradish peroxidase (HRP)-labelled goat anti-rabbit antibody, 1:10,000) was added and incubated at RT for 1 h. The membrane was washed with TBST for 10 min x 3 and subjected to color development.

Statistical analysis. SPSS 13.0 software (IBM Corporation, Armonk, NY, USA) was used for statistical analysis.

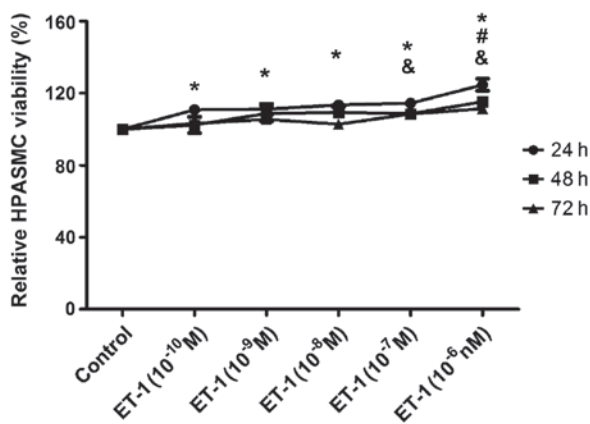


Figure 1. The effect on HPASMC viability by ET-1 (CCK-8 assay). *24-h relative survival, $P < 0.05$, vs. control group. #48-h relative survival, $P < 0.05$, vs. control group. &48-h relative survival, $P < 0.05$, vs. control group. HPASMC, human pulmonary artery smooth muscle cells.

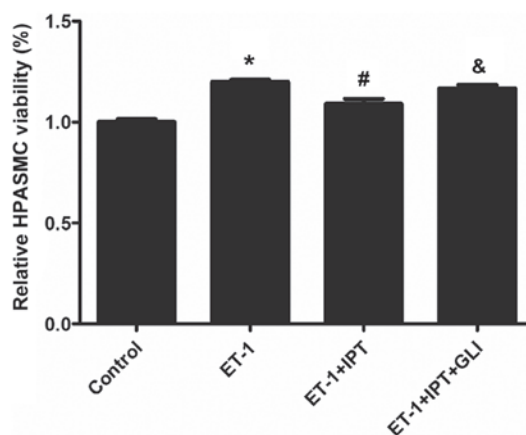


Figure 3. GLI partially reverses the effect on ET-1-induced HPASMC viability by IPT (CCK-8 assay). * $P < 0.05$, vs. control group. # $P < 0.05$ vs. ET-1 group. & $P < 0.05$ vs. ET-1+IPT group. GLI, glibenclamide; HPASMC, human pulmonary artery smooth muscle cells; IPT, iptakalim.

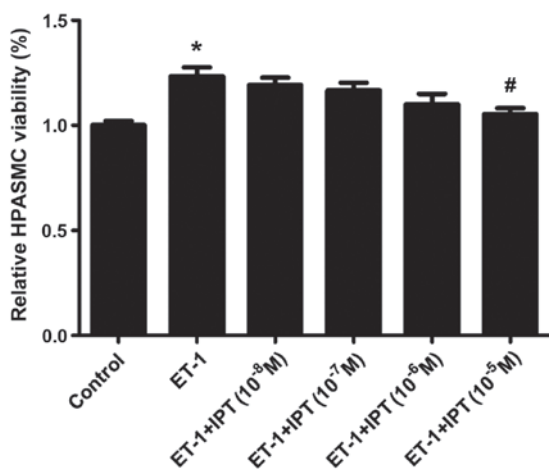


Figure 2. Effect on ET-1-induced HPASMC viability by IPT (CCK-8 assay). * $P < 0.05$, vs. control group. # $P < 0.05$ vs. ET-1 group. HPASMC, human pulmonary artery smooth muscle cells; IPT, iptakalim.

Quantitative data are presented as mean \pm SD. One-way analysis of variance (ANOVA) was used for comparison between multiple groups, and the LSD method was used for comparison between two groups. Independent sample t-test was used in comparison between two groups. $P < 0.05$ was considered of statistical significance.

Results

Effect on HPASMC viability by ET-1. HPASMCs were incubated with ET-1 10⁻¹⁰, 10⁻⁹, 10⁻⁸, 10⁻⁷ and 10⁻⁶ M for 24, 48 and 72 h. As shown in Fig. 1, ET-1 increases HPASMC viability and the effect was enhanced with increasing ET-1 concentration. In addition, with different ET-1 concentrations, the HPASMC viability at 48 and 72 h was similar to that at 24 h and the effect on HPASMCs by ET-1 was not significantly time-dependent. Therefore, we selected ET-1 at 10⁻⁶ M concentration for 24 h for further investigation.

Effect on ET-1-induced HPASMC proliferation by IPT. HPASMCs were incubated with IPT 10⁻⁸, 10⁻⁷, 10⁻⁶ and 10⁻⁵ M,

and ET-1 10⁻⁶ M for 24 h. CCK-8 assay was used to evaluate the change of HPASMC viability after incubation with varying concentrations of IPT. As shown in Fig. 2, IPT inhibited ET-1 induced increasing HPASMC viability and this effect was enhanced with increasing IPT concentration. In comparison with ET-1 alone, IPT (10⁻⁵ M)+ET-1 treatment decreased HPASMC viability significantly ($P < 0.05$).

Effect on the ET-1-induced increase of HPASMC viability by IPT after blocking K⁺ channel. K⁺ channel blocker GLI was used to block IPT-induced K⁺ channel opening, to determine whether IPT was able to inhibit the ET-1-induced increase of HPASMC viability. Four groups were included in this analysis, i.e., control, ET-1, ET-1+IPT and ET-1+IPT+GLI groups, with ET-1 at 10⁻⁶ M, IPT at 10⁻⁵ M, GLI at 10⁻⁵ M concentration. In the ET-1+IPT+GLI group, GLI was added 30 min prior to ET-1 and IPT, and the incubation time was 24 h. As shown in Fig. 3, after blocking K⁺ channel by GLI, HPASMC viability in the ET-1+IPT+GLI group was significantly higher than that in the ET-1+IPT group ($P < 0.05$). This result demonstrated that GLI partially reversed the effect on ET-1-induced HPASMC viability by IPT, indicating that the inhibition of the ET-1-induced increase of HPASMC viability by IPT may occur through the opening K⁺ channels.

Twenty-four hours after incubation, EdU staining was performed for HPASMCs in all 4 groups. The percentage of EdU-stained cells in total cells was calculated on the basis of 5 fields (20X) randomly selected in each group. As shown in Figs. 4 and 5, the percentage of proliferated cells in the control, ET-1, ET-1+IPT and ET-1+IPT+GLI groups was 3.61 \pm 0.38, 10.35 \pm 0.71, 4.51 \pm 1.92 and 10.50 \pm 0.58%, respectively. After the addition of GLI, the percentage of proliferated cells in the ET-1+IPT+GLI group was significantly higher than that in the ET-1+IPT group ($P < 0.05$).

Effect on ET-1-induced change of apoptotic protein expression in HPASMCs by IPT after blocking K⁺ channel. After incubation with drugs for 24 h, the expression of apoptosis-related Bax and Bcl-2 in HPASMCs was evaluated by western blotting. As shown in Fig. 6, ET-1 downregulated Bax and upregulated

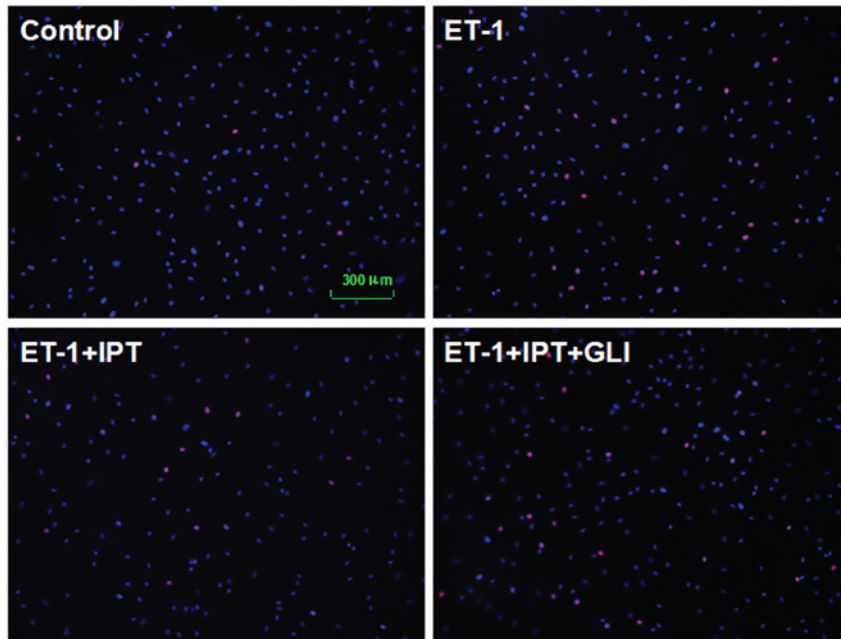


Figure 4. GLI reverses the effect on ET-1-induced HPASMC proliferation by IPT (EdU incorporation assay, n=3). GLI, glibenclamide; HPASMC, human pulmonary artery smooth muscle cells; IPT, iptakalim; EdU, 5-ethynyl-2'-deoxyuridine.

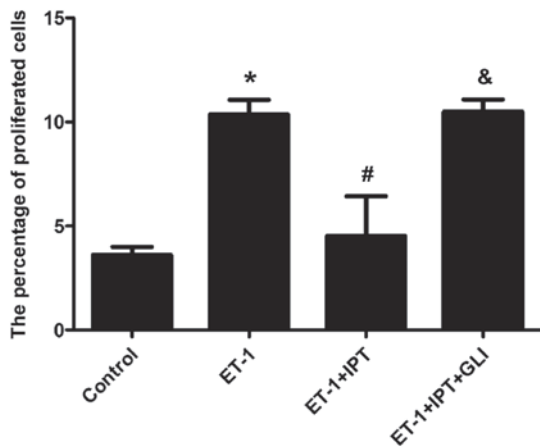


Figure 5. GLI reverses the effect on ET-1 induced HPASMC proliferation by IPT (n=3). *P<0.05 vs. control group. #P<0.05 vs. ET-1 group. &P<0.05 vs. ET-1+IPT group. GLI, glibenclamide; HPASMC, human pulmonary artery smooth muscle cells; IPT, iptakalim.

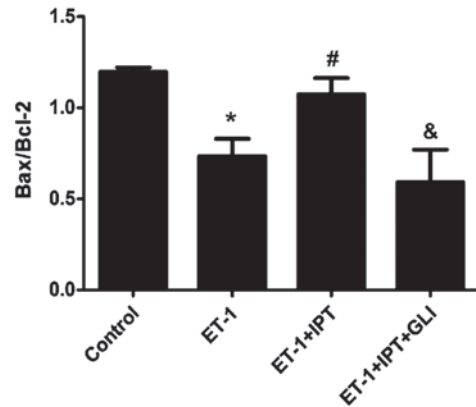
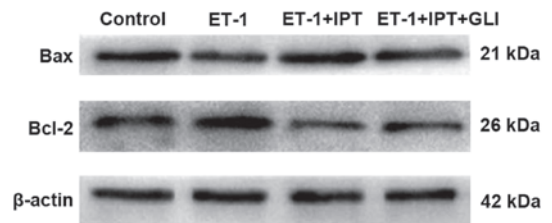


Figure 6. GLI reverses the effect on ET-1 induced change of apoptotic protein expression in HPASMCs by IPT (western blot, n=3). *P<0.05 vs. control group. #P<0.05 vs. ET-1 group. &P<0.05 vs. ET-1+IPT group. GLI, glibenclamide; HPASMC, human pulmonary artery smooth muscle cells; IPT, iptakalim.

Bcl-2, led to lower Bax/Bcl-2 ratio (P<0.05) vs. control group. IPT upregulated Bax and downregulated Bcl-2, leading to higher Bax/Bcl-2 ratio (p<0.05) vs. ET-1 group. GLI could block K⁺ channels, downregulated Bax and upregulated Bcl-2, leading to lower Bax/Bcl-2 ratio (P<0.05) vs. ET-1+IPT group.

Discussion

Pulmonary hypertension is a disease detrimental to human health, its incidence is only second to hypertension and coronary heart disease in cardiovascular diseases (18-20). The pathophysiological characteristics are predominantly pulmonary vasoconstriction and pulmonary vascular remodeling. Pulmonary hypertension potentially causes right-sided heart failure and death (21). In China, the incidence of

pulmonary hypertension due to hypoxia and respiratory diseases is on the increase, and the reasons include: i) smoking, chronic bronchitis and chronic obstructive pulmonary disease (COPD) due to cigarette smoking, common pulmonary diseases that lead to HPH; ii) the incidence of respiratory disorder during sleep is increasing due to an increase in obese population, with pulmonary hypertension due to higher pulmonary vascular

resistance; and iii) high altitude pulmonary hypertension, a common chronic hypoxic pulmonary disease in China as compared to other countries (22). Therefore, the investigation of pathogenesis mechanism underlying HPH and development of new therapeutic drugs are of important clinical significance.

The pathogenesis mechanism of HPH is not well established. Currently, the main mechanism underlying higher pulmonary vascular resistance due to chronic hypoxia is pulmonary vascular proliferation and remodeling (23). On the one hand, hypoxia induces the production of reactive oxygen species in mitochondria and inhibits the activity of voltage-gated K^+ channels on the HPASMC membrane leading to depolarization of the cell membrane and influx of extracellular Ca^{2+} , which may cause vascular smooth muscle cell contraction. On the other hand, hypoxia may cause dysfunction in pulmonary artery endothelial cells, leading to the decreased production of vasodilator factors (NO and prostacyclin) and overexpression of vasoconstrictive and pro-proliferation factors (thromboxane A₂ and ET-1). An imbalance between vasodilators and vasoconstrictors may cause increased small pulmonary arterial tone. The proliferation of endothelial cells, smooth muscle cells and fibroblasts may lead to small pulmonary artery remodeling and increased synthesis of extracellular matrix, such as collagen, elastin and fibronectin (24).

ET-1 is a vasoconstrictor synthesized and secreted by pulmonary vascular endothelial cells. Excess ET-1 may cause pulmonary vascular smooth muscle cell contraction and proliferation (25,26). ET-1 binds to the endothelin-A (ET_A) receptor, inhibit G protein (Gi), adenylate cyclase or phospholipase C (PLC), and K_{ATP} channels (27), leading to a decreased outflow of intracellular K^+ , cell membrane depolarization, voltage-dependent Ca^{2+} channel opening, increased influx of extracellular Ca^{2+} , release of Ca^{2+} in sarcoplasmic reticulum, which would increase free intracellular Ca^{2+} , promoting the expression of the genes related to cell proliferation (c-Myc and c-Fos), and inducing cell proliferation (28).

In the present study, we stimulated HPASMCs with ET-1 to develop the model of HPASMC proliferation. We found that IPT inhibited ET-1 and induced HPASMC proliferation to promote HPASMC apoptosis. To verify whether the inhibition of ET-1-induced HPASMC proliferation by IPT was mediated through K_{ATP} channels, we blocked K_{ATP} channel with K_{ATP} channel blocker GLI and evaluated the effect on IPT. As shown in Figs. 4 and 5, in ET-1+IPT+GLI group, GLI could block IPT, thus IPT could not inhibit ET-1-induced HPASMC proliferation. This result confirms that IPT could inhibit ET-1 induced HPASMC proliferation through opening K_{ATP} channels.

It was reported that mitochondria was sensitive to hypoxia, mitochondrial dysfunction was closely associated with cell apoptosis and played important roles in the pathogenesis of HPH. Hypoxia could inhibit the release of cytochrome *c* (cyt-*c*) from mitochondria into cytoplasm, thus inhibiting the mitochondrial apoptotic pathway, leading to HPASMC proliferation and pulmonary hypertension (29-32). The release of mitochondrial cyt-*c* and other pro-apoptotic components was regulated by Bcl-2 family, upstream of the mitochondrial apoptosis pathway. Bcl-2 family proteins were classified into anti-apoptosis proteins (Bcl-2, Bcl-xL and Bcl-W) and pro-apoptosis proteins (Bax, Bcl-Xs and Bak). Among others, Bcl-2 and Bax were similar in structure and antagonistic to

each other (33). Bcl-2 and Bax played important regulatory roles in the mitochondrial apoptosis pathway. The present findings demonstrate that ET-1 downregulated Bax and upregulated Bcl-2 in HPASMCs to inhibit cell apoptosis while IPT reversed the effect of ET-1, indicating that IPT regulated the proliferation and apoptosis of HPASMCs by increasing the Bax/Bcl-2 ratio. K_{ATP} channel inhibitor GLI could partially reverse the effect of IPT, indicating that IPT could increase Bax/Bcl-2 ratio through opening the K_{ATP} channel, and inhibited ET-1-induced HPASMC proliferation.

In conclusion, IPT inhibited ET-1-induced HPASMC proliferation and promoted apoptosis through opening K_{ATP} channels, therefore, it may be a promising novel drug for HPH.

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