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Research Paper

Effects of Iptakalim on intracellular free calcium concentration of cultured rabbit pulmonary arterial smooth muscle cells☆

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Abstract

Objective: To explore the effects of Iptakalim on intracellular free calcium concentration and on the proliferation of cultured rabbit pulmonary arterial smooth muscle cells induced by endothelin-1(ET-1) *in vitro*. **Methods:**A cell culture model, [^{3}H]-thymidine([^{3}H]-TdR) incorporation test and confocal microscope were used to observe proliferation and intracellular free calcium concentration($[Ca^{2+}]_i$) of rabbit PASMC induced by ET-1 *in vitro*. **Results:**The value of [^{3}H]-TdR incorporation in ET-1 group was increased 1.468 times higher than that in control group. Iptakalim at the concentration of 10^{-7}mol/L, 10^{-6} mol/L ,10^{-5} mol/L lowered [^{3}H]-TdR incorporation by (19.8 ± 4.6)%, (41.2 ± 9.5)%, (54.7 ± 10.1)%, respectively, compared with the value of the cells treated with ET-1($P < 0.01$); The intracellular fluorescence intensity of PASMC in ET-1 group was increased from 73.70 ± 10.12 to 143.84 ± 28.23, significantly higher than that in control group($P < 0.01$); whereas with Iptakalim,the fluorescence intensity(FI) was only increased from 74.30 ± 10.20 to 86.03 ± 9.82, significantly lower than that in ET-1 group($P < 0.01$). **Conclusion:**Iptakalim inhibited proliferation of PASMC and decreased intracellular free calcium concentration of cultured rabbit PASMC induced by ET-1.

Keywords: smooth muscle cell; endothelin-1; ATP sensitive potassium channels; calcium concentration

INTRODUCTION

Though hypoxic pulmonary hypertension presents various etiology,pulmonary arterial smooth muscle cell (PASMC) proliferation is an important characristical pathological change during the development of hypoxic pulmonary hypertension. However,K^+ channel dysfunction in the plasma membrane plays a key role in PASMC proliferation by modulating cytoplasmic free Ca^{2+} concentration($[Ca^{2+}]$cyt) [1].Hence, ($[Ca^{2+}]$cyt) plays a pivotal and important role in the regulation of pulmonary vasoconstriction and vascular remodeling to hypoxia. As recent evidence suggests, the reduction of the activity of K^+ channels in PASMCs,with the resultant membrane depolarization, may be involved in the development of chronic hypoxic pulmonary hypertension by inducing PASMC proliferation[2]. The purpose of this study was to investigate Iptakalim, an ATP sensitive potassium channel(KATP) opener, which regulates the($[Ca^{2+}]$cyt) and proliferation of PASMC to hypoxia using confocal laser scanning microscopy.

Endothelin-1(ET-1) has also been confirmed as one of the important factors leading to contraction and proliferation of PASMCs as well as the pulmonary arterial remodeling[3-4]. With ET-1 as a tool agent, the proliferation model of PASMCs cultured *in vitro* was

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established in this research, in which ^{3}H-thymidine incorporation and confocal laser scanning microscopy were used respectively to observe:①effect of iptakalim on proliferation of PASMCs; ② cytoplasmic free Ca^{2+} concentration changes of PASMCs; as well as associated mechanisms between KATP channel and PASMCs' proliferation and cytoplasmic free Ca^{2+} concentration.

MATERIALS AND METHODS

Reagents and animals

Pluronic F-127, endothelin-1, propidium iodine, and Me2SO(DMSO) were all purchased from Sigma Co. Fluo-3 AM(Molecular Probes, USA) was dissolved in DMSO and stored at 20~28℃. Dulbecco's Modified Essential Medium(DMEM), fetal bovine serum (FBS), and trypsin were purchased from GIBCO Co. α-Actin antibody was obtained from Boehringer Mannheim. [^{3}H] Thymidine(specific activity, 1 Ci/L) was obtained from Shanghai Institute of Nuclear Research(China). Iptakalim, with a purity of 99.36%, was synthesized and provided by Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences(China). Eight healthy New Zealand rabbits, aged between six weeks and eight weeks, (1.5 ± 0.5)kg, were provided by Animal Experimental Center of Jiangsu Province.

Cell preparation and culture

Primary cultures of rabbit PASMCs were incubated in physiological balanced saline containing NaCl 138.0 mmol/L, KCl 5.0 mmol/L, Na_2HPO_4 0.3 mmol/L, KH_2PO_4 0.3 mmol/L, $NaHCO_3$ 4.0 mmol/L, penicillin 10^5 U/L and streptomycin 100 mg/L. A thin layer of adventitia was carefully stripped off with a fine forceps, and endothelium was removed by gently scratching the intimal surface with a surgical blade. The remaining portion of the media was cut into approximately 1 mm squares and placed in culture bottle containing Dulbecco's Modified Essential Medium(DMEM) with 20% fetal bovine serum (FBS). The bottle was placed in a moist tissue culture incubator at 37℃ containing gas with the 95% O_2 and 5% CO_2 mixture. The cells growing from the explants had become relatively confluent within a period of approximately 4 wk. They were trypsinized with a solution of 0.05% trypsin. The resulting suspension of cells was pipetted into a 6 oz culture bottle containing 10 ml of the DMEM for growing large numbers of cultures. The purity of PASMCs in the primary cultures was confirmed by positive staining with smooth muscle α-actin antibody compared with a known positive control of SMCs. Only the third to fifth generations were selected as experimental cells.

Measurement of ^{3}H-TdR incorporation

Well-grown PASMCs were selected, and transferred to 96-well tissue culture plates(10^4 cells each well). Cell were divided into 10 groups,10 wells per group. After cells had been cultured for 24 hours with 10% fetal bovine serum, when cells were made quiescent, the media were replaced with serum-free DMEM for another 24 hours. Then different concentrations of drugs were respectively added into each groups. In ET-1 group, culture solution containing 10^{-7} mol/L of ET-1 was added. The culture solution containing iptakalim at the concentrations of 10^{-7} mol/L, 10^{-6} mol/L and 10^{-5} mol/L were added in EP-1, EP-2 and EP-3 groups respectively. ^{3}H-TdR was added to each well at 18th hour to a final concentration of 18.5 kBq/ml. At the end of the incubation period, the incorporated radioactivity(counts per minute) was measured by fixation and solubilization of cells with a Beckman liquid scintillation counter. Experiments were performed 10 times independently in duplicate.

Measurements of [Ca^{2+}]cyt in pulmonary arterial SMCs

The changes of [Ca^{2+}]cyt were measured using the Fluo-3 fluorescence method under laser scanning confocal microscope. Cells suspended in DMEM containing 10% fetal bovine serum seeded(10^4 cells/well) in 6-well plates on circular 25-mm glass coverslips. After 24 h, cells were loaded with Fluo-3 working solution(Fluo-3 AM 5 mmol/L and Pluronic F-127 0.03% dissolved in standard buffer) at 37℃. Changes in the Fluo-3 fluorescence intensity(FI) indicating fluctuations in[Ca^{2+}]cyt were recorded. After a stable baseline fluorescence intensity was measured, endothelin-1 was added to extracellular medium to yield a concentration of 10 nmol/L, and the fluorescence intensity was recorded for 300 s.

Statistical analysis

All parameters were expressed as *mean* ± *SD*. Analysis of variance was carried out with SPSS 12.0 software.The *q* test was used for paired comparison of means from multiple samples in different groups. $P < 0.05$ was considered as statistical significance.

RESULTS

Effects of iptakalim on proliferation of PASMCs

Effect of ET-1 on ^{3}H-TdR incorporation

Compared with the control group, ^{3}H-TdR incorporation increased by 146.8% in ET-1 group($P < 0.01$).

Effect of iptakalim on ^{3}H-TdR incorporation

When ET-1 was added simultaneously with Iptakalim of 10^{-7}mol/L, 10^{-6} mol/L and 10^{-5} mol/L respectively,

^{3}H-TdR incorporation decreased by 19.8%, 41.2% and 54.7% correspondingly. However, when Iptakalim of 10^{-5} mol/L was added, ^{3}H-TdR incorporation showed no significant difference from that in control group($P>0.05$)(***Tab 1, Fig 1***).

Tab 1 **Effects of Iptakalim at different concentrations on [^{3}H]-TdR incorporation of PASMCs**

($\bar{x} \pm s, n = 10$)

Group	*n*	[^{3}H]-TdR incorporation(cpm)
Control group	10	167 ± 16
ET-1 group	10	413 ± 60*
ET-1+IPT10^{-7} group	10	330 ± 42△*
ET-1+IPT10^{-6} group	10	241 ± 44△*
ET-1+IPT10^{-5} group	10	183 ± 23△

Compared with control group, *$P<0.01$; Compared with ET-1 group, △ $P<0.01$.

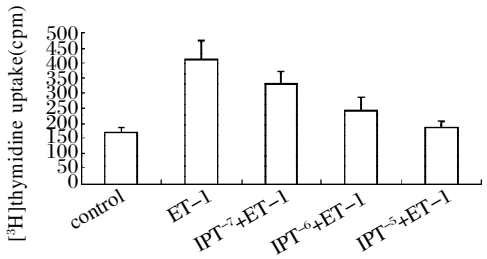
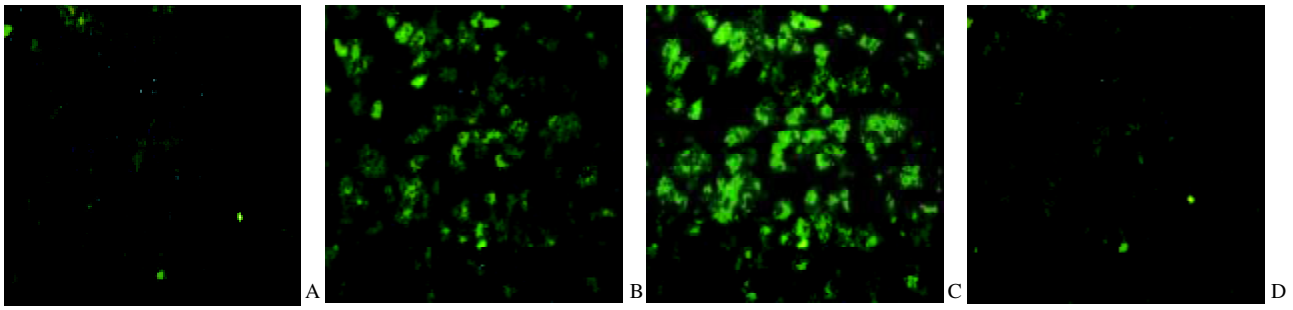


Fig 1 Effects of Iptakalim on ^{3}H-TdR uptake of PASMCs. (Experiments were performed 10 times independently in duplicate).

Effects of Iptakalim on[Ca^{2+}]cyt on PASMCs

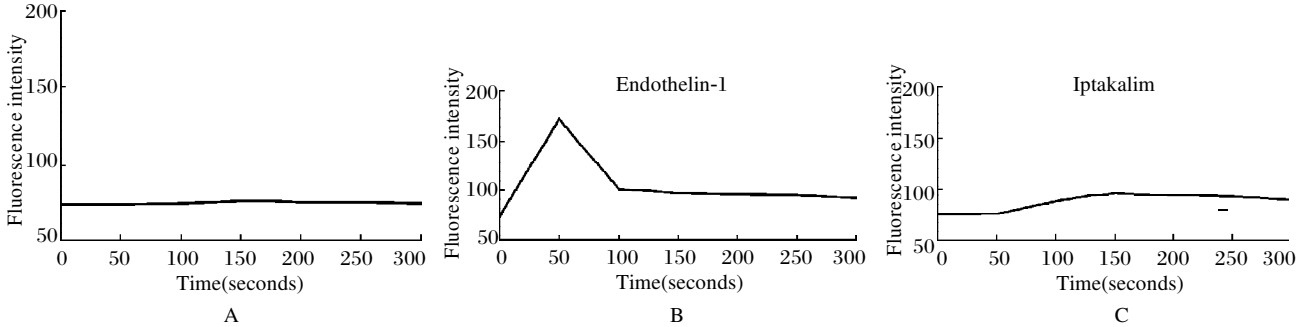
Endothelin-1 at the concentration of 10 nmol/L induced a monophasic increase of Fluo-3 fluorescence intensity(FI); endothelin-1 elicited an initial peak of [Ca^{2+}]cyt, in which fluorescence intensity increased to 143.84 ± 28.23 from 73.70 ± 10.12[(100.72 ± 8.16)% vs. baseline, $n = 6$] at 60s, and then fell to a sustained plateau(***Fig 2***). Pretreatment of PASMCs with iptakalim at the concentration of 10 μ mol/L for 10 min significantly prevented transient increase of[Ca^{2+}]cyt elicited by endothelin-1. The fluorescence intensity in these cells only increased to 86.03 ± 9.82 from 74.30 ± 10.2[(15.78 ± 2.25)% vs. baseline, $n = 6$] at 60 s(***Fig 3***).



A:Fluo-3 basal fluorescence. B:Fluo-3 fluorescence 5 s after addition of endothelin-1(10 nmol/L). C:Fluo-3 fluorescence 60 s after addition of endothelin-1(10 nmol/L). D:Fluo-3 fluorescence 600 s after addition of endothelin-1(10 nmol/L)

Fluo-3 is used as intracellular calcium indicator. An increase in Fluo-3 fluorescence indicates the increase in intracellular calcium.

Fig 2 [Ca^{2+}]cyt of PASMCs before and after addition of endothelin-1 to PASMCs. (under inverted fluorescence microscope, × 400)



A:Control(6 cells). B:Endothelin-1 induced a monophasic increase in[Ca^{2+}] cyt of pulmonary arterial SMCs(6 cells). C:Pretreatment of pulmonary arterial SMCs with Iptakalim significantly prevented[Ca^{2+}]cyt rise induced by endothelin-1(6 cells).

Changes in the Fluo-3 fluorescence intensity(FI) indicating fluctuations in[Ca^{2+}]cyt were recorded. After stable baseline fluorescence intensity was measured, endothelin-1 was added to extracellular medium to yield a concentration of 10 nmol/L, and the fluorescence intensity was recorded for 300 s.

Fig 3 The effects of iptakalim on changes of[Ca^{2+}] cyt in pulmonary arterial SMCs. (Changes in[Ca^{2+}] cyt were estimated by fluorescence measurement using Ca^{2+} indicator Fluo-3)

DISCUSSION

Excess of ET-1 can impair pulmonary artery endothelium, initiate and exacerbate the development of hypertension through its vasoconstrictive and promitogenic properties[5-6].The binding of excessive ET-1 to ETA receptors stimulated both the calcium channels, which were coupled with the ETA receptors and phospholipase C, to cause an influx of calcium and an increment in the intercellular free calcium concentration through G protein[7].A rise in intracellular calcium was harmful to impair endothelium and vascular smooth muscle in pulmonary arterioles. Recent studies have indicated that increased intracellular calcium was also a determinant to promote cell proliferation as a signal transform factor[8].

The constriction and proliferation of pulmonary arteries are an import pathogenic factor in chronic hypoxic pulmonary hypertension. Therefore, to control the constriction and proliferation of PASMCs is of important significance in the prevention and treatment of hypoxic pulmonary hypertension. The increase in the intracellular free calcium activates not only the actinmyosin apparatus resulting in contraction, but also genes required for cells to enter a proliferative phase[9].The cytoplasmic ionized Ca^{2+} acts as a signal transduction element, which is critical in cellular proliferation .A rise in$[Ca^{2+}]$cyt increases nuclear Ca^{2+} concentration propelling the quiescent cells into cell cycle and mitosis. Furthermore, $[Ca^{2+}]$cyt increases the activity of protein kinse C, enhances the expression of cancer genes of c-myc,c-fos, growth factors and so on, thus promoting the proliferation of PASMCs[10].

Through successful establishment of the proliferation model of rabbit PASMCs using exogenous ET-1, this research confirmed that Iptakalim could completely inhibit the increase of ^{3}H-TdR incorporation in rabbit PASMCs induced by ET-1, which suggested that Iptakalim should suppress DNA synthesis in PASMCs, thereby inhibiting the proliferation of PASMCs.

Potassium channels may play a significant role in regulating intracellular calcium concentration of pulmonary artery smooth muscle cells[11].Hypoxic agents, which can cause pulmonary hypertension, inhibit potassium currents in pulmonary artery smooth muscle cells causing membrane depolarization, activate the voltage-dependent Ca^{2+} channels resulting in increasing Ca^{2+} influx, which can promote intracellular calcium concentration increase and vasoconstriction[12]. Iptakalim-ATP sensitive potassium channel opener[13], can promote membrane hyperpolarization. This effect can lower the opening probability of voltage-dependent Ca^{2+}channels, restrain Ca^{2+} release from intracellular sources through inhibiting inositol trisphosphate formation, decrease the sensitivity of intracellular contractile elements to Ca^{2+}, and accelerate the clearance of intracellular calcium via the Na^{2+} /Ca^{2+} exchanger[14].As a result, there was a decrease in intracellular Ca^{2+} and vasodilatation.

The results demonstrated that Iptakalim could effectively inhibit constriction and proliferation of PASMCs, the main pathological changes of hypoxic pulmonary hypertension. So Iptakalim, the ATP sensitive potassium channel opener, displayed a promising future in clinical treatment of chronic hypoxic pulmonary hypertension[15].

References

[1] Mandegar M, Yuan JX. Role of K^+ channels in pulmonary hypertension. *Vasc Pharmacol* 2002,38;25-33.

[2] Platoshyn O, Golovina VA, Bailey CL, Limsuwan A, Krick S, Juhaszova M,et al. Sustained membrane depolarization and pulmonary artery smooth muscle cell proliferation. *Am J Physiol Cell Physiol* 2000;279:C1540-9.

[3] Li P, Oparil S, Sun JZ, Thompson JA, Chen YF. Fibroblast growth factor mediates hypoxia-induced endothelin-a receptor expression in lung artery smooth muscle cells. *J Appl Physiol* 2003;95:643-51.

[4] Davie N,Haleen SJ,Upton PD, Polak JM, Yacoub MH, Morrell NW, et al. ET(A) and ET(B) receptors modulate the proliferation of human pulmonary artery smooth muscle cells. *Am J Respir Crit Care Med* 2002 ;165:398-405.

[5] Freitas CF,Faro R,Dragosavac D,Clozel M,De Nucci G,Antunes E. Role of endothelin-1 and thromboxane A2 in the pulmonary hypertension induced by heparin-protamine interaction in anesthetized dogs. *J Cardiovasc Pharmacol* 2004:43:106-12.

[6] Kim H, Yung GL, Marsh JJ, Konopka RG, Pedersen CA, Chilea PG,et al. Endothlin mediates pulmonary vascular remodeling in a canine PH model of chronic embolic pulmonary hypertension. *Eur Respir J* 2000;15:640-8.

[7] Chew DK, Orshal JM, Khalil RA. Elastase induced suppression of endothelin-mediated Ca^{2+} entry mechanisms of vascular contraction. *Hypertension* 2003; 42:818-24.

[8] Li SJ, Sum NL. Regulation of intracellular Ca^{2+} and calcineurin by NO/PKG in proliferation of vascular smooth muscle cells. *Acta Pharmacol Sin* 2005; 26:323-8.

[9] Platoshyn O,Golovina VA,Bailey CL, Limsuwan A, Krick S, Juhaszova M, et al.Sustained membrane depolarization and pulmonary artery smooth muscle cell proliferation. *Am J Physiol Cell Physiol* 2000;279: C1540-9.

[10] Sweeney M,Yu Y,Platoshyn O, Zhang S, McDaniel SS, Yuan JX. Inhibition of endogenous TRP1 decreases capacitative Ca^{2+} entry and attenuates pulmonary artery smooth muscle cell proliferation. *Am J Physiol Lung Cell Mol Physiol* 2002 ; 283:L144-55.

[11] Cui Y,Tran S,Tinker A, Clapp LH.The molecular composition of k (ATP) channels in human pulmonary artery smooth muscle cells and their modulation by growth. *Am J Respir Cell Mol Biol* 2002; 26:135-43.

[12] Olschewski A, Hong Z, Nelson DP, Weir EK.Graded response of k^+ current, membrane potential, and$[Ca^{2+}]$i to hypoxia in pulmonary arterial smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2002; 283:L1143-50.

[13] Wang H, Pharmacological characteristics of the novel antihypertensive Drug iptakalim hydrochloride and its molecular mechanisms. *Drug Dev Res* 2003;58: 65-8.

[14] ZOU Wei, HE Guo-wei, YIM Anthony PC. Effects of Potassium Channel Opener KRN_(4884) on EDHF-mediated Relaxation in Porcine Coronary Micro-arteries. *Acta Nanjing medicinalis universitatis* 2004;24:383-6.

[15] Cole WC,Clement-Chomienne O.ATP-sensitive k^+ channels of vascular smooth muscle cells. *J Cardiovase Electrophysiol* 2003;14: 94-103.