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Full-length article

5-Hydroxytryptamine-induced proliferation of pulmonary artery smooth muscle cells are extracellular signal-regulated kinase pathway dependent[1]

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Key words

antisense oligodeoxyribonucleotides; serotonin; membrane transport proteins; pulmonary hypertension; vascular smooth muscle; fluxetine; mitogen-activated protein kinases

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Abstract

Aim: To investigate the effect of 5-hydroxytryptamine transporter (5-HTT) inhibitor fluoxetine and antisense oligodeoxynucleotide (ODN) to extracelluar signal-regulated kinases (ERKs) on pulmonary arterial smooth muscle cells (PASMCs) proliferation induced by 5-HT. **Methods:** Liposomal transfection was used to introduce ODNs to ERK1/2 into cultured rat PASMCs and the transfection efficiency was measured by observing the uptake of the fluorecein isothiocynate (FITC)-labeled antisense ODN in PASMCs. The effects of 5-HTT selective inhibitor fluoxetine and ODNs on the proliferation of PASMCs were evaluated by cell number counting and cell cycle analysis, and measured by microculture tetrazolium (MTT) assay and flow cytometry (FCM), respectively. **Results:** Liposomes mediated the transfection of ODNs into PASMCs with high efficiency. MTT assay showed fluoxetine (10 μmol/L, 1 μmol/L, and 100 nmol/L) concentration dependently inhibited the proliferation of PASMCs induced by 5-HT (1 μmol/L) *in vitro*. The proliferation rate of PASMCs by 5-HT was significantly inhibited by pretreatment with ERK1/2 antisense ODN (0.2 μmol/L) from 251%±18% to 86%±5% ($P<0.01$). Flow cytometric analysis of cell cycle distribution showed that the increase of 5-HT induced S phase fraction (SPF) and proliferation index (PI) were significantly inhibited by fluoxetine (1 μmol/L) or antisense ODN with SPF from 36%±4% to 26%±3% and 24%±4%, and PI from 34%±2% to 29%±2% and 24%±2%, respectively. **Conclusion:** 5-HTT mediates the mitogenic effect of 5-HT on PASMCs and the proliferation of PASMCs induced by 5-HT is dependent on ERKs signal pathway.

Introduction

Exposure to acute or chronic hypoxia leads to the development of pulmonary arterial hypertension (PAH). The cardinal features of PAH are persistent vasoconstriction and structural remodeling of the pulmonary vessels[1]. Hyperplasia of pulmonary artery smooth muscle cells (PASMCs) is the main component of pulmonary vascular remodeling which is associated with progressive elevation in pulmonary arterial pressure[2]. However, the exact mechanism of the proliferation of PASMCs was unclear.

Several studies have shown that 5-hydroxytryptamine (5-HT), endothelin-1 (ET), platelet derived growth factors (PDGF), angiotensin II (Ang II), and epidermal growth factor (EGF) participate in the regulation of proliferation in PASMCs[3]. Among these mediators, 5-HT plays an important role in the pathogenesis of PAH. 5-HT exerts potent mitogenic and comitogenic effects on PASMCs, and these effects are associated with cellular internalization of 5-HT mediated by the 5-hydroxytryptamine transporter (5-HTT)[4,5]. Yet, it is not clear if inhibition of 5-HTT may abolish the proliferation of PASMCs to 5-HT.

Furthermore, mitogen activated protein kinases (MAPKs) are a superfamily of serine/threonine protein kinases distributed extensively in cytoplasm. Many stimuli resulting in cell

growth, differentiation, and vascular contraction may activate MAP kinase-dependent signaling pathways.

Among this family, extracelluar signal-regulated kinases (ERKs) are activated in response to growth and differentiation factors[6–8]. Previous studies have shown that 5-HT induced ERK1/2 activation in rat aortic smooth muscle cells and rabbit isolated renal artery smooth muscle cells[9,10]. However, whether the intracelluar signal pathway of 5-HT in PASMCs is dependent on the ERKs activation is largely unconcerned. Therefore, the present study was designed to observe the effect of fluoxetine, a selective inhibitor of 5-HTT, on the proliferation of PASMCs in response to 5-HT, and then to study the effect of downregulation of ERK1/2, using antisense oligodeoxynucleotides (ODNs), on the proliferation of 5-HT-stimulated PASMCs *in vitro*.

Materials and methods

Drugs and reagents Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from Gibco Co (Grand Island, New York, USA). 5-HT and propidium iodide (PI) were purchased from Sigma Co (St Louis, USA). Lipofectin reagent was from Life Technologies Inc, Ltd (Rockville, USA). Fluoxetine hydrochloride was from Eli Lilly Co (Indianapolis, USA). ODNs were synthesized by Sangon (Shanghai, China).

Pulmonary artery smooth muscle cell culture Lungs were removed from male Wistar rats weighing 250–300 g (supplied by the Animal Center of China Medical University, Grade II, Certificate No LN 03-0009). Under aseptic conditions, proximal pulmonary arteries were isolated. After removing the tissue around the arteries, the pulmonary arteries were cut into small pieces about 1 mm^2 and then seeded into 30-mm Petri dishes and cultured in DMEM (containing 20% FBS, 100 kU/L of penicillin and 100 mg/L of streptomycin, pH 7.2). Then these explants were incubated at the atmosphere of 37 °C and 95% O_2/5% CO_2[11]. When the cells had grown out from the explants and reached the confluence of more than 70%, cells were passaged to T25 flasks. Cells used in experiments were from passages 3 to 9.

Oligodeoxynucleotides On account of GCG package locating in the translation initiation region of rat ERK1/2 mRNA, the sequences of antisense ODN were designed as follows: 5'-GCC GCC GCC GCC GCC AT. This ODN has been used successfully to downregulte ERK1/2 in VSMCs, rat cardiac myocytes and rat cardiac fibroblasts[12]. Sense ODN (5'-AT GGC GGC GGC GGC GGC), random ODN (5'-CGC GCG CTC GCG CAC CC) were used as controls. All bases were protected by phosphorothioation. One batch of antisense ODN was labeled with fluorecein isothiocyanate (FITC) used in fluorescence microscopy experiments.

Microculture tetrazolium (MTT) assay Cells were seeded into 96-well plates at a density of 1×10^4 cells/well. The cells were then incubated in medium containing vehicle (5% FCS DMEM) and 5-HT (1 μmol/L) for 24 h with or without fluoxetine (10 μmol/L, 1 μmol/L, and 100 nmol/L) added 30 min before 5-HT. The effect of different ODNs (0.2 μmol/L) on the proliferation induced by 5-HT was also observed after transfection. The group with the cells incubated in serum-free medium was used as the control. At the end of this period, MTT (5 g/L) was added to each well, and incubation proceeded at 37 °C for 4 h. Thereafter, the medium was removed and the cells were solubilized in 150 μL Me_2SO. Optical density (*OD*) of each well was determined by enzyme-linked ELISA at 490 nm of wavelength. Then the proliferation rates (PR) of each group were calculated.

$$PR=(OD_{experiment}-OD_{control})/OD_{control}$$

Flow cytometry (FCM) PASMCs grown in T25 plates were treated with fluoxetine or antisense ODN (0.2 μmol/L) before 5-HT was added. The cells were harvested by trypsinization, washed twice with PBS, and the pellets were resuspended in 0.5 mL of PBS containing 100 mg/L RNase for incubation at 37 °C for 30 min. Then, 0.5 mL of PI solution (100 mg/L in PBS) was added, and the mixture was incubated in the dark at 4 °C for 30 min. The cells were analyzed with a FACScan flow cytometer. Then S-phase cell fractions (SPF) and proliferation index (PI) of each group were calculated. $SPF=S/(G_0G_1+S+G_2M)$; $PI=(G_2M+S)/(G_0G_1+S+G_2M)$.

Liposomal transfection Prior to transfection, PASMCs were cultured in serum-free medium for 24 h. ODNs were mixed with antibiotic- and serum-free medium to a concentration of 0.8 μmol/L, then mixed with equal volume of medium containing 80 mg/L of lipofectin and incubated at room temperature for 15 min. ODN/lipofectin mix 20 μL was added to each well of a 96-well plate, 200 μL to each well of a 12-well plate, and 1000 μL to the T25 plates, with equal volume of antibiotic- and serum-free medium. The cells were incubated for a further 6 h with gentle agitation every 2 h. The medium was then replaced with the same volume of liposome-free medium containing the same concentration of ODN and 5% FBS. Cells were incubated for another 24 h before MTT assay and FCM.

Fluorescence microscopy PASMCs were seeded into 12-well plate and transfected using FITC-labeled antisense ODN. After 24 h and 48 h of incubation, the cells were viewed by fluorescence microscopy.

Statistical analysis All the data are presented as mean±SD, and assessed by ANOVA and *t*-test. $P<0.05$ was

considered significant.

Results

Uptake of ODNs by PASMCs Twenty four hours after liposomal transfection, FITC-labeled antisense ODN were observed in both cytoplasm and the nuclei of PASMCs (Figure 1). More than 90% of the cells exhibited fluorescence.

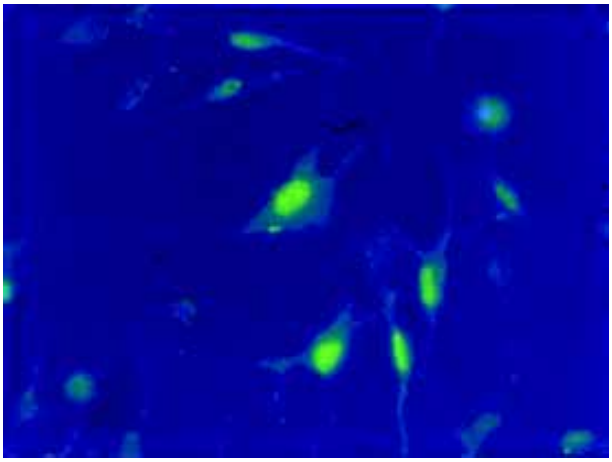

Figure 1. Fluorescence photomicrograph of PASMCs taken 24 h after transfection with the FITC-labeled antisense ODN (× 400). Fluorescence were observed in both cytoplasm and the nuclei of PASMCs.

Effect of fluoxetine and ODNs on the proliferation of PASMCs induced by 5-HT MTT assay showed that 5-HT (1 μmol/L) induced increased proliferation of PASMCs and the PR in response to 5-HT increased from 172%±5% to 217%±4% compared with the vehicle. Pretreatment of the cells with fluoxetine (10 μmol/L, 1 μmol/L, and 100 nmol/L) could produce a concentration-dependent reduction in PR (Figure 2). According to these results, 1 μmol/L was chosen as the concentration of fluoxetine used in following experiments.

Pretreatment of PASMCs with ERK1/2 antisense ODN resulted in a significant inhibition of 5-HT-induced PASMCs proliferation (Figure 3) and this inhibitory effect is rather more potent than fluoxetine. In contrast, sense ODN and random ODN did not have such effect.

Effect of fluoxetine and antisense ODN on cell cycle Flow cytometric analysis of cell cycle distribution showed that the cells treated with 5-HT had larger values of S-phase cell fraction (SPF) and PI than the vehicle. But pretreatment with fluoxetine or antisense ODN decreased these values (Figures 4, 5). These results indicated that 5-HT promoted the PASMCs from the G_0/G_1 phase of the cell cycle into S phase and this effect was inhibited by fluoxetine and antisense ODN.

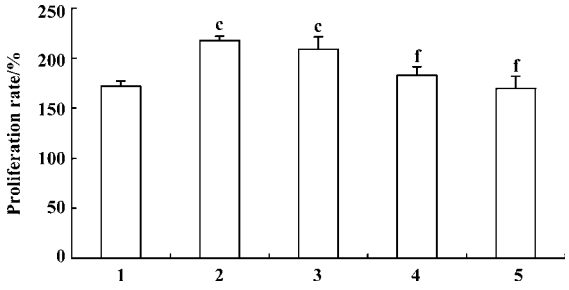

Figure 2. Effect of different concentrations of fluoxetine on the proliferation of PASMCs induced by 5-HT. Experimental groups: 1) vehicle (5% FBS DMEM); 2) 5-HT (1 μmol/L); 3) 5-HT (1 μmol/L) +fluoxetine (100 nmol/L); 4) 5-HT (1 μmol/L)+fluoxetine (1 μmol/L); 5) 5-HT (1 μmol/L)+fluoxetine (10 μmol/L). n=3. ^cP<0.01 *vs* control. ^fP<0.01 *vs* 5-HT (1 μmol/L).

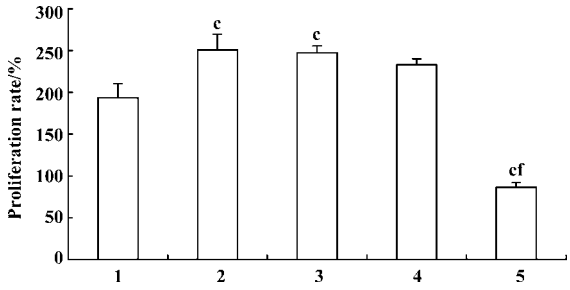

Figure 3. Effect of different ODNs on the proliferation of PASMCs induced by 5-HT. Experiment groups: 1) vehicle (5% FBS DMEM); 2) 5-HT (1 μmol/L); 3) 5-HT (1 μmol/L)+random ODN (0.2 μmol/L); 4) 5-HT (1 μmol/L)+sense ODN (0.2 μmol/L); 5) 5-HT (1 μmol/L) +antisense ODN (0.2 μmol/L). n=3. ^cP<0.01 *vs* control. ^fP<0.01 *vs* 5-HT (1 μmol/L).

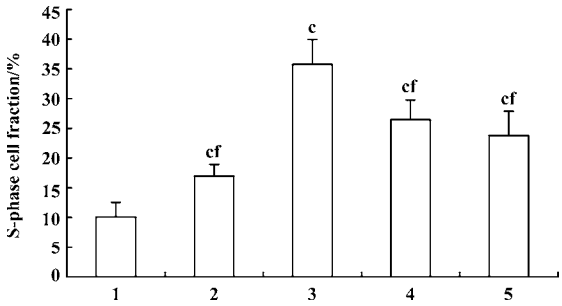

Figure 4. Effect of fluoxetine and antisense ODN on S-phase cell fraction of PASMCs. Experiment groups: 1) control; 2) vehicle (5% FBS DMEM); 3) 5-HT (1 μmol/L); 4) 5-HT (1 μmol/L)+fluoxetine (1 μmol/L); 5) 5-HT (1 μmol/L)+antisense ODN (0.2 μmol/L). n=3. ^cP<0.01 *vs* control. ^fP<0.01 *vs* 5-HT(1 μmol/L).

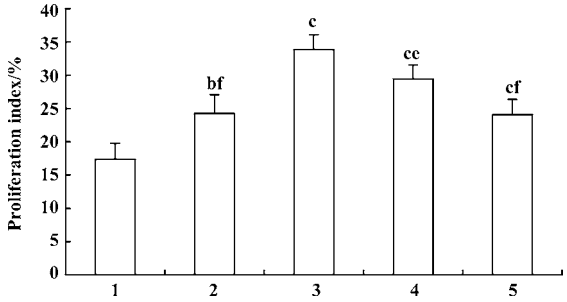

Figure 5. Effect of fluoxetine and antisense ODN on proliferation index of PASMCs. Experiment groups: 1) control; 2) vehicle (5% FBS DMEM); 3) 5-HT (1 μmol/L); 4) 5-HT (1 μmol/L)+fluoxetine (1 μmol/L); 5) 5-HT (1 μmol/L)+antisense ODN (0.2 μmol/L). n=3. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control. $^{e}P<0.05$, $^{f}P<0.01$ *vs* 5-HT (1 μmol/L).

Discussion

Results from the present study demonstrated that 5-HTT played a key role in the mitogenic effect of 5-HT on PASMCs. Fluoxetine, a highly selective inhibitor of 5-HTT inhibited the proliferation of PASMCs induced by 5-HT *in vitro*. Meanwhile, antisense ODN to ERK1/2 inhibited 5-HT-induced proliferation of PASMCs. These findings suggest that 5-HT-induced proliferation of PASMCs is 5-HTT and ERK pathway dependent.

In response to hypoxia, 5-HT is released from pulmonary neuroendocrine cells and neuroepithelial bodies distributed throughout the airways. An increase in 5-HT may contribute to secondary pulmonary artery hypertension[13]. The proliferation of PASMCs induced by 5-HT is an important component of pulmonary arterial remodeling. RT-PCR analyses of PASMCs indicated the presence of 5-$HT_{1B/1D}$, 5-HT_2 receptors and 5-HTT mRNA[14,15]. The present results show that the proliferation of PASMCs induced by 5-HT is inhibited by fluoxetine in a manner that is concentration-dependent. In contrast, the 5-$HT_{1B/1D}$ receptor antagonist GR127935, the 5-HT_{2A} receptor antagonist ketanserin, or the 5-$HT_{2B/2C}$ receptor antagonist SB206553 had no this effect[16]. It has been recently reported that exposure of PASMCs to hypoxia increased 5-HTT expression and activity, and this effect was associated with potentiation of the mitogenic action of 5-HT[5,17]. Some scholars found that mice deficient in 5-HTT or treated with selective inhibitors of 5-HTT developed less PAH than controls when exposed to hypoxia[18,19]. These evidences suggest that 5-HTT in PASMCs may be a key determinant of pulmonary arterial remodeling and the development of PAH. Therefore, our result means that 5-HTT is one of the important mechanisms of PAH and this may provide a novel therapeutic target for PAH.

The uptake of FITC-labeled antisense ODN by PASMCs with liposomes proved the high efficiency of transfection. In the present study, the antisense ODN to ERK1/2 delivered by lipofectin resulted in an effective suppression of the proliferative response to 5-HT in PASMCs. Meanwhile, the effect of antisense ODN stronger than fluoxetine suggests that antisense ODN to ERK1/2 could inhibit the proliferation of PASMCs not only induced by 5-HT, but also by the serum[20]. The present study proves that 5-HT induced the proliferation of PASMCs and is dependent on the activation of ERK1/2. Therefore, it is reasonable to consider that 5-HT induced activation of ERKs through 5-HTT in PASMCs. Previous studies showed that 5-HT induced the Tyr phosphorylation of GTPase-activating protein (GAP) and the effect was mediated by 5-HTT, which was the upstream of the ERK pathway, not by 5-HT receptors[21]. The mechanism of 5-HT signaling for PASMCs through 5-HTT has also been shown to involve the production of reactive oxygen species (ROS) such as superoxide and H_2O_2 via the activation of NAD(P)H oxidase, and the activation of the ERK pathway occurs secondary to ROS formation[22,23]. Therefore, the activation of ERKs induced by 5-HT is mediated by 5-HTT.

The present study showed that fluoxetine concentration-dependently inhibited 5-HT-induced proliferation of PASMCs *in vitro*, and demonstrated that antisense ODN to ERK1/2 significantly inhibited mitogenesis of PASMCs. Therefore, we concluded that 5-HT-induced mitogenesis of PASMCs was mediated by 5-HTT, in which the signal transduction for 5-HT was dependent on ERKs signal pathway.

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