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Calcilytics enhance sildenafil-induced antiproliferation in idiopathic pulmonary arterial hypertension

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ABSTRACT

Idiopathic pulmonary arterial hypertension (IPAH) is a progressive and fatal disease of the pulmonary artery resulting from currently unidentified etiology. IPAH is pathologically characterized as sustained vasoconstriction and vascular remodeling of the pulmonary artery. Phosphodiesterase type 5 (PDE5) inhibitors have been clinically used in the treatment of IPAH. Recently, we have shown that Ca^{2+}-sensing receptor (CaSR) antagonists, or calcilytics, inhibit excessive cell proliferation of pulmonary arterial smooth muscle cells (PASMCs) from IPAH patients. In this study, the additive or synergistic effect of calcilytics on antiproliferation following PDE5 inhibition was examined in IPAH-PASMCs by MTT assay. Treatment with sildenafil blocked the excessive cell proliferation of IPAH-PASMCs in a concentration-dependent manner with an IC_{50} value of 16.9 μM. However, sildenafil (0.03–100 μM) did not affect the cell growth of PASMCs from normal subjects and patients with chronic thromboembolic pulmonary hypertension (CTEPH). Co-treatment with 0.3 μM NPS2143, a calcilytic, additively enhanced the antiproliferative effect induced by sildenafil (3 or 30 μM) in IPAH-PASMCs. Additionally, the inhibitory effect of calcilytics, NPS2143 or Calhex 231 (1 or 10 μM), on excessive cell proliferation of IPAH-PASMCs was synergistic increased in the presence of 1 μM sildenafil. Similar results were obtained by BrdU incorporation assay. These findings reveal that calcilytics additively/synergistically enhance the antiproliferative activity mediated by PDE5 inhibition, suggesting that a combination therapy of a PDE5 inhibitor with a calcilytic may be useful as a novel therapeutic approach for IPAH.

1. Introduction

Pulmonary arterial hypertension (PAH) is a progressive and fatal disease of the pulmonary artery. Pulmonary vasoconstriction and pulmonary vascular remodeling increase pulmonary arterial pressure. The pulmonary vascular remodeling is mainly due to enhanced cell proliferation and inhibited apoptosis of pulmonary arterial smooth muscle cells (PASMCs) in the media of pulmonary artery. Consequently, an increase of pulmonary arterial resistance leads to right heart failure and eventually death (McLaughlin et al., 2015).

Pulmonary artery abnormalities, including vasoconstriction and vascular remodeling, in PAH patients are mostly triggered by an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) (Morrell et al., 2009). Increased resting $[Ca^{2+}]_{cyt}$ and enhanced Ca^{2+} influx have been reported in PASMCs from PAH patients. PASMCs express several Ca^{2+}-permeable channels including voltage-dependent Ca^{2+} channels (VDCCs), receptor-operated Ca^{2+} (ROC) channels, and store-operated Ca^{2+} (SOC) channels (Fernandez et al., 2015; Firth et al., 2007; Guibert et al., 2007; Yamamura et al., 2011, 2014; Yang et al., 2010). ROC and SOC channels are upregulated in lung tissues and PASMCs from idiopathic PAH (IPAH) patients, compared with PASMCs from normal subjects and normotensive patients. These upregulations contribute to enhanced Ca^{2+} signaling and excessive cell proliferation of PASMCs (Yu et al., 2004; Zhang et al., 2007). These channels are also upregulated in PASMCs during hypoxia (Lin et al., 2004; Smith et al., 2015; Wan et al., 2013; Wang et al., 2006).

Recently, we have found that the extracellular Ca^{2+}-sensing receptor (CaSR), a member of the G-protein-coupled receptor subfamily C (Magno et al., 2011), is upregulated in PASMCs from IPAH patients, compared with those from normal subjects and patients with chronic thromboembolic pulmonary hypertension (CTEPH). The upregulation of CaSRs is involved in the enhanced Ca^{2+} response and subsequent excessive cell proliferation in IPAH-PASMCs. Indeed, the blockade of CaSRs by calcilytics, or CaSR antagonists, attenuates excessive cell proliferation of IPAH-PASMCs, and inhibits the development of pulmonary hypertension in monocrotaline- or hypoxic-induced pulmonary hypertensive animals (Yamamura et al., 2012, 2015).

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Vascular remodeling of PASMCs is thought to be closely related to the dysfunction of prostacyclin, endothelin, and nitric oxide (NO) synthesis pathways. Thus, these signal pathways are considered as screening targets for antiproliferative drugs. Drug therapy for PAH has progressed in recent years with the development of several specific drugs targeting the pathological mechanisms of PAH. In addition to conventional VDCC blockers, three therapeutic options are currently used for the treatment of PAH patients: prostacyclins, endothelin receptor antagonists, and phosphodiesterase 5 (PDE5) inhibitors (Frumkin, 2012; McLaughlin et al., 2015; Seferian and Simonneau, 2013; Yamamura, 2014).

In this study, we examined whether calcilytics (NPS2143 and Calhex 231) enhance the inhibitory effect of a clinically-used PDE5 inhibitor (sildenafil) on excessive cell proliferation of IPAH-PASMCs using MTT and BrdU incorporation assays. We found that the PDE5 inhibition reduced excessive cell proliferation of IPAH-PASMCs. Interestingly, the combination of calcilytic and PDE5 inhibitor acted additively or synergistically to block the excessive cell proliferation of IPAH-PASMCs.

2. Materials and methods

2.1. Cell culture

Cell lines of PASMCs (passages 5–10) from normal subjects (Lonza, Walkersville, USA), IPAH patients (Yu et al., 2004), and CTEPH patients (Ogawa et al., 2009) were cultured in Medium 199 supplemented with 10% fetal bovine serum, 100 U/ml penicillin plus 100 μg/ml streptomycin (Invitrogen/GIBCO, Grand Island, USA), 50 μg/ml D-valine (Sigma-Aldrich, St. Louis, USA), and 20 μg/ml endothelial cell growth supplement (BD Biosciences, Franklin Lakes, USA) at 37 °C.

2.2. MTT assay

PASMCs were subcultured in 96-well plates ($\sim 1 \times 10^4$ cells per well) and incubated at 37 °C for 6 h before assay. Then PASMCs were exposure to the culture medium including vehicle or drug (s) for 48 or 72 h. In the combination therapy, two different classes of drugs were applied into the medium at the same time. Cellular viability of PASMCs was evaluated using Cell Counting Kit-8 (Dojin, Kumamoto, Japan) based on MTT (3-(4,5-dimethyl-2-thiazolyl)−2,5-diphenyl-2*H*-tetrazolium bromide) assay. The result was quantified colorimetrically as the absorbance at 450 nm (A_{450}) using Benchmark Plus Microplate Reader and Microplate Manager (ver. 5.2; Bio-Rad Laboratories, Hercules, USA).

2.3. BrdU incorporation assay

Cell preparation and drug application were performed in the same way as MTT assay. Cell proliferation of PASMCs was evaluated using Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche Diagnostics, Mannheim, Germany) based on BrdU (bromodeoxyuridine) incorporation assay. The colorimetrical quantification was measured using the absorbance at 370 nm (A_{370}) by a method similar to the MTT assay.

2.4. Drugs

The chemical structures of sildenafil (1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*-pyrazolo[4,3-*d*]pyrimidin-5-yl)-4-ethoxyphenyl]sulfonyl]-4-methylpiperazine), NPS2143 (2-chloro-6-[(2*R*)-2-hydroxy-3-[(2-methyl-1-naphthalen-2-ylpropan-2-yl)amino]propoxy]benzonitrile) (Tocris Bioscience, Ellisville, USA), and Calhex 231 (4-chloro-*N*-[(1*S*,2*S*)-2-[[(1*R*)-1-(1-naphthalenyl)ethyl]amino]cyclohexyl]benzamide) (Sigma-Aldrich) are shown in Fig. 1. All hydrophobic compounds were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 or 100 mM as a stock solution. The assay medium contained 0.1% DMSO throughout the experiments, regardless of the presence/absence of drug(s). It was confirmed that up to 0.1% DMSO did not affect these responses.

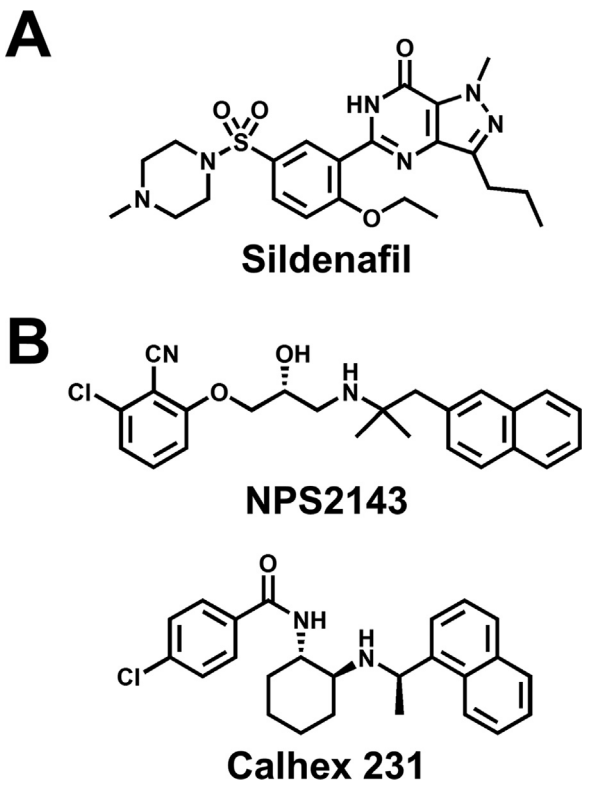

Fig. 1. *Chemical structure of PDE5 inhibitor and calcilytics (CaSR antagonists).* The chemical structures of a PDE5 inhibitor, sildenafil (*A*), and calcilytics, or negative allosteric modulators of CaSR, NPS2143 and Calhex 231 (*B*).

2.5. Statistical analysis

Pooled data are shown as the mean ± S.E.M. The statistical significance of differences between two groups was determined by Student's *t*-test. The statistical significance of differences among groups was determined by Scheffé's test following one-way analysis of variance (ANOVA). Significant differences are expressed in the figure as $^{*}P < 0.05$ or $^{**, \#\#}P < 0.01$. The data of the relationship between drug concentrations and cell viability (Fig. 3B) were fitted using the following equation: MTT $(A_{450}) = A_1 - (A_1 - A_2)/\{1 + (K_d/[\text{drug}])^n\})$, where K_d is the apparent dissociation constant of drug (IC_{50}), [drug] is the concentration of drug, n is the Hill coefficient, A_1 is the control value before the application of drug, and A_2 is the component resistance to the drug.

3. Results

3.1. Excessive cell proliferation in PASMCs from IPAH patients

At first, the proliferation rates of PASMCs from normal subjects and patients with IPAH and CTEPH were analyzed by quantitative colorimetric assay based on the MTT test for cellular viability (Fig. 2), as reported previously (Yamamura et al., 2015). In normal-PASMCs, the cell number was gradually increased until 72 h after subculture ($A_{450} = 0.58 \pm 0.02$ at 48 h and 0.85 ± 0.04 at 72 h, $P < 0.01$ vs. 0.47 ± 0.01 at 0 h, $n = 14$). The proliferation rate in