Enhanced Ca^{2+}-sensing Receptor Function in Idiopathic Pulmonary Arterial Hypertension

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ABSTRACT

Rationale: A rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in pulmonary arterial smooth muscle cells (PASMC) is an important stimulus for pulmonary vasoconstriction and vascular remodeling. Increased resting $[Ca^{2+}]_{cyt}$ and enhanced Ca^{2+} influx have been implicated in PASMC from patients with idiopathic pulmonary arterial hypertension (IPAH).

Objective: We examined whether the extracellular Ca^{2+}-sensing receptor (CaSR) is involved in the enhanced Ca^{2+} influx and proliferation in IPAH-PASMC and whether blockade of CaSR inhibits experimental pulmonary hypertension.

Methods and Results: In normal PASMC superfused with Ca^{2+}-free solution, addition of 2.2 mM Ca^{2+} to the perfusate had little effect on $[Ca^{2+}]_{cyt}$. In IPAH-PASMC, however, restoration of extracellular Ca^{2+} induced a significant increase in $[Ca^{2+}]_{cyt}$. Extracellular application of spermine also markedly raised $[Ca^{2+}]_{cyt}$ in IPAH-PASMC, but not in normal PASMC. The calcimimetic R568 enhanced, whereas the calcilytic NPS 2143 attenuated, the extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ rise in IPAH-PASMC. Furthermore, the protein expression level of CaSR in IPAH-PASMC was greater than in normal PASMC; knockdown of CaSR in IPAH-PASMC with siRNA attenuated the extracellular Ca^{2+}-mediated $[Ca^{2+}]_{cyt}$ increase and inhibited IPAH-PASMC proliferation. Using animal models of pulmonary hypertension, our data showed that CaSR expression and function were both enhanced in PASMC, whereas intraperitoneal injection of the calcilytic NPS 2143 prevented the development of pulmonary hypertension and right ventricular hypertrophy in rats injected with monocrotaline and mice exposed to hypoxia.

Conclusions: The extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ due to upregulated CaSR is a novel pathogenic mechanism contributing to the augmented Ca^{2+} influx and excessive PASMC proliferation in patients and animals with pulmonary arterial hypertension.

Key Words: Pulmonary artery; smooth muscle cell; proliferation; G protein-coupled receptor

Non-standard Abbreviations and Acronyms

Cytosolic free Ca^{2+} concentration	$[Ca^{2+}]_{cyt}$
Pulmonary arterial smooth muscle cell	PASMC
Idiopathic pulmonary arterial hypertension	IPAH
Ca^{2+}-sensing receptor	CaSR
Voltage-dependent Ca^{2+} channels	VDCC
Receptor-operated Ca^{2+} channels	ROC
Store-operated Ca^{2+} channels	SOC
Diacylglycerol	DAG
Receptor-operated Ca^{2+} entry	ROCE
Inositol 1,4,5-trisphosphate	IP_3
Sarcoplasmic reticulum	SR
Store-operated Ca^{2+} entry	SOCE
Capacitative Ca^{2+} entry	CCE
G protein-coupled receptor	GPCR
Receptor tyrosine kinase	RTK
Chronic thromboembolic pulmonary hypertension	CTEPH
Monocrotaline	MCT
Hypoxia-induced pulmonary hypertension	HPH
Right ventricular systolic pressure	RVSP

INTRODUCTION

Idiopathic pulmonary arterial hypertension (IPAH) is a fatal and progressive disease with unidentified etiological causes. Pulmonary vascular remodeling and sustained pulmonary vasoconstriction are two major causes for the elevated pulmonary vascular resistance and pulmonary arterial pressure in patients with IPAH. A central aspect of pulmonary vascular remodeling is intimal and medial hypertrophy caused by enhanced proliferation and inhibited apoptosis of pulmonary arterial smooth muscle cells (PASMC)[1]. An increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in PASMC is a major trigger for pulmonary vasoconstriction and an important stimulus for PASMC proliferation leading to pulmonary vascular remodeling[2-4].

In PASMC, $[Ca^{2+}]_{cyt}$ can be increased by Ca^{2+} release from the intracellular stores and Ca^{2+} influx through plasmalemmal Ca^{2+} channels[1, 5]. PASMC express various Ca^{2+}-permeable channels including voltage-dependent Ca^{2+} channels (VDCC), receptor-operated Ca^{2+} channels (ROC), and store-operated Ca^{2+} channels (SOC). VDCC are activated by membrane depolarization. ROC channels are mainly opened by vasoconstrictors (e.g., endothelin-1 and serotonin) via an intracellular second messenger, diacylglycerol (DAG), and by growth factors (e.g., epidermal growth factor and platelet-derived growth factor). Activation of ROC by interaction with ligands greatly contributes to the increase in $[Ca^{2+}]_{cyt}$ due to receptor-operated Ca^{2+} entry (ROCE) in PASMC. Upon activation of membrane receptors and subsequent increase in inositol 1,4,5-trisphosphate (IP_3) synthesis, SOC are opened by the depletion of Ca^{2+} from the sarcoplasmic reticulum (SR), an important intracellular store in PASMC, to elicit store-operated Ca^{2+} entry (SOCE) or capacitative Ca^{2+} entry (CCE). SOCE is an important mechanism involved in maintaining a sustained elevation of $[Ca^{2+}]_{cyt}$ and refilling Ca^{2+} into the SR.

We have previously demonstrated that the resting $[Ca^{2+}]_{cyt}$ is increased, while SOCE and ROCE are both enhanced in PASMC isolated from IPAH patients in comparison to PASMC

isolated from normal subjects and patients without pulmonary hypertension[6, 7]. We have also shown that increased Ca^{2+} influx during PASMC proliferation is due largely to the enhancement of SOCE; downregulation and blockade of SOC significantly inhibits PASMC proliferation. These results indicate that SOCE plays an important role in regulating cell proliferation in vascular smooth muscle cells[2-4, 8]. Furthermore, expression and activity of ROC and SOC are both upregulated in PASMC isolated from patients with IPAH and animals with hypoxia-induced pulmonary hypertension[2, 9-11].

Opening of ROC and SOC is caused initially by activation of various membrane receptors including G protein-coupled receptors (GPCR) and receptor tyrosine kinases (RTK). The extracellular Ca^{2+}-sensing receptor (CaSR) is a member of GPCR subfamily C (also known as GPRC2A)[12-14] which was originally identified in the parathyroid glands and is activated by its ligands including Ca^{2+}, Gd^{3+}, Mg^{2+}, polyamines, antibiotics and amino acids[12, 15]. The CaSR is involved in multiple cellular processes of the parathyroid glands in response to changes in serum Ca^{2+} concentrations such as proliferation, differentiation, and apoptosis[12, 13, 15]. In addition to parathyroid glands, the CaSR is also expressed in kidney, bone, smooth muscle, endothelium, gastrointestinal tract and brain[13, 16-19]. The physiological and pathological significance of CaSR in the development and progression of pulmonary arterial hypertension, however, remains unknown.

In this study, we examined whether CaSR was involved in the enhanced Ca^{2+} signaling and augmented proliferation in PASMC from patients with IPAH and whether inhibition of CaSR attenuates IPAH-PASMC proliferation and prevents the development of experimental pulmonary hypertension in animal models.

METHODS

Preparation of human and animal PASMC

Human PASMC were isolated from normal control subjects and patients with IPAH and chronic thromboembolic pulmonary hypertension (CTEPH).[8, 20, 21] Approval to use the human lung tissues and cells was granted by the UIC Institutional Review Board. Human PASMC were cultured in Medium 199 supplemented with 10% fetal bovine serum at 37ºC. The cells at passages 5-8 were used for the experiments. In some experiments, we also used freshly-dissociated PASMC from rats[22] and PASMC from mice.[23]

$[Ca^{2+}]_{cyt}$ measurement

$[Ca^{2+}]_{cyt}$ was measured in PASMC using fura-2 and a Nikon digital fluorescent imaging system[24]. Cells were loaded with 4 μM fura-2 acetoxymethyl ester (fura-2/AM) for 60 min at 25°C and $[Ca^{2+}]_{cyt}$ was measured using a ratiometric method at 32°C.

Western blot

Solubilized protein isolated from PASMC, pulmonary arteries and lung tissues was loaded on an 8% acrylamide gel, transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA), and immunoblotted with anti-CaSR monoclonal antibody (MA1-934, 1:200; Thermo Scientific, Rockford, IL). To isolate the fraction of cellular membrane, the cell lysate was centrifuged at 100,000×g and then the pellet was re-suspended to use for western blot analysis. Signals were detected using a Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific). The protein levels were normalized to β-tubulin (sc-9104, 1:200; Santa Cruz Biotechnology) and expressed in arbitrary units.

Transfection of cDNA and small interfering RNA (siRNA)

PASMC were transiently transfected with vector cDNA (2 μg, pcDNA3.1(+)), human CaSR cDNA (2 μg), control siRNA (50 nM, sc-37007; Santa Cruz Biotechnology), or CaSR siRNA (50 nM, s2440; Applied Biosystems, Austin, TX) using an Amaxa Basic Nucleofector kit for primary smooth muscle cells (Lonza). $[Ca^{2+}]_{cyt}$ measurements and western blot using cDNA- and siRNA-transfected cells were preformed 48-72 h after electroporation.

Proliferation assay

Proliferation of PASMC was determined using an automated cell counter (TC10; Bio-Rad Laboratories, Hercules, CA). At 48 h after subculture, PASMC were counted and re-plated (0 h) into 8-well multidishes (Nunclon Δ, 10.5 cm^2 of culture area per well; Thermo Scientific) with 1×10^5 cells/well (0.95×10^4 cells/cm^2).

Preparation of MCT-PH rats and HPH mice

All experiments were approved by the Ethics/Animal Care Committee of University of Illinois at Chicago. For MCT-PH rat experiments, male Sprague-Dawley rats (190-200 g) were treated with single subcutaneous (s.c.) injection of vehicle (dimethyl sulfoxide, DMSO) or 60 mg/kg MCT. For NPS 2143-treated group, rats were intraperitoneally injected (i.p.) with NPS 2143 at a dose of 4.5 mg/kg per day (from day 1 to 10). Fourteen days after injection, rats were anesthetized with ketamine/xylazine and then RVSP was measured using an MPVS Ultra system (Millar Instruments). For HPH mouse experiments, male mice (8-week-old C57BL/6) were exposed to hypoxia (10% O_2) in a ventilated chamber to develop pulmonary hypertension. For

NPS 2143-treated group, mice were injected (i.p.) with NPS 2143 (1.0 mg/kg per day; from day 1 to 22). Four weeks after exposure to normobaric hypoxia, mice were anesthetized with ketamine/xylazine, and then RVSP was measured by right heart catheterization.

Reverse transcription-polymerase chain reaction (RT-PCR)

The extraction of total RNA from rat PASMC and the reverse transcription were performed using TRIzol Reagent (Invitrogen) and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), respectively. For semi-quantitative analysis of mRNA, Platinum PCR SuperMix (Invitrogen) and specific primers for rat CaSR and GAPDH were used. Quantitative real-time PCR analysis was performed based on the SYBR assay (SYBR Green Master Mix; Roche Applied Science, Indianapolis, IN) using gene-specific primers for rat CaSR and GAPDH on a Bio-Rad CFX384 Real-Time System C1000 Thermal Cycler system (Bio-Rad Laboratories, Hercules, CA).

Immunohistochemical and Hematoxylin-Eosin (H&E) staining

Immunohistochemistry and H&E staining were performed using formalin-fixed and paraffin-embedded sections (3 μm) from left lung lobes of rats. The tissue sections were treated with PBS containing 5% normal goat serum and either CaSR (1:100 dilution) or SM α-actin (1:100; EMD Millipore) antibody for 12 h at 4°C. After washing repeatedly in PBS, the sections were covered with PBS containing Alexa Fluor 488-labeled (1:500 dilution) or Cy3-labeled (1:500) secondary antibody for 1 h at room temperature and then rinsed with PBS. Then sections were mounted in VECTASHIELD hard-set mounting medium with DAPI (1.5 μg/ml). To measure the external diameter of pulmonary arteries stained with H&E, the microscopic images were analyzed using ImageScope software (Ver.11).

Drugs

Pharmacological reagents were obtained from Sigma-Aldrich except for KB-R7943, NPS 2143, and R568 (Tocris, Ellisville, MO). All hydrophobic compounds were dissolved in DMSO at the concentration of 10 or 100 mM as a stock solution.

Statistical analysis

Composite data are shown as the mean±S.E. The statistical significance between two groups was determined by Student's *t*-test. The statistical significance among groups was determined by Scheffé's test after one-way analysis of variance. Significant difference is expressed as $^{*}P<0.05$ or $^{**,\,\#\#}P<0.01$.

RESULTS

Extracellular Ca^{2+} induces a significant increase in $[Ca^{2+}]_{cyt}$ in IPAH-PASMC but not in normal PASMC

We first examined and compared the effects of extracellular Ca^{2+} restoration on changes in $[Ca^{2+}]_{cyt}$ in PASMC from normal subjects, IPAH patients and CTEPH patients. In normal PASMC superfused with Ca^{2+}-free solution (plus 1 mM EGTA; for 10 min), restoration of extracellular Ca^{2+} (2.2 mM) into the bath solution had no effect on $[Ca^{2+}]_{cyt}$ (Fig. 1A-C). Even after long exposure (30 and 60 min) of normal PASMC to Ca^{2+}-free solution, there was no change in $[Ca^{2+}]_{cyt}$ after restoration of extracellular Ca^{2+}. In IPAH-PASMC superfused with Ca^{2+}-free solution (for 10 min), however, restoration of extracellular Ca^{2+} resulted in a significant increase in $[Ca^{2+}]_{cyt}$ in 96% of the cells tested. The extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ in IPAH-PASMC was independent of the exposure time (5 to 30 min) to Ca^{2+}-free solution. Interestingly, as shown in Figure 1A-C (IPAH1 vs. IPAH2), the kinetics of the extracellular Ca^{2+}-induced increases in $[Ca^{2+}]_{cyt}$ were different in IPAH-PASMC. Approximately 48% of IPAH-PASMC tested (IPAH1) exhibited a rapid transient increase in $[Ca^{2+}]_{cyt}$, while more than 50% of the cells (IPAH2) had a sustained plateau phase of $[Ca^{2+}]_{cyt}$ increase after the initial transient (Fig. 1A middle panels and C). In PASMC isolated from CTEPH patients superfused with Ca^{2+}-free solution (for 10 min), restoration of extracellular Ca^{2+} had no effect on $[Ca^{2+}]_{cyt}$ (Fig. 1A-C).

The extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase in IPAH-PASMC was dependent on Ca^{2+} concentration (Fig. 1D); the EC_{50} was approximately 1.22 mM (n=57 to 183 cells) which resulted an increase of $[Ca^{2+}]_{cyt}$ by 1,250 nM (Fig. 1D right panel). In contrast, we were unable to detect a significant increase in $[Ca^{2+}]_{cyt}$ in normal PASMC with restoration of 10 mM extracellular Ca^{2+} (Fig. 1D).

Protein expression of CaSR in IPAH-PASMC is greater than in normal PASMC

To investigate the potential mechanism of extracellular Ca^{2+}-mediated increase in $[Ca^{2+}]_{cyt}$, we compared the protein expression level of CaSR in normal and IPAH PASMC. As shown in Figure 2, the protein expression of CaSR in IPAH-PASMC (Fig. 2A and B) and lung tissues from IPAH patients (Fig. 2C and Online Fig. I) was significantly higher than in normal PASMC and lung tissues in both total protein lysates and in the membrane bound fraction (n=5, $P<0.01$). These data indicate that upregulation of CaSR may be involved in the enhanced Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase in IPAH-PASMC.

The extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ rise in IPAH-PASMC is not due simply to Ca^{2+} leakage

To rule out the possibility that the extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase in IPAH-PASMC was caused by membrane leakage to Ca^{2+}, we first compared the resting $[Ca^{2+}]_{cyt}$ in normal and IPAH-PASMC. In cells superfused with 2.2 mM Ca^{2+}-containing solution, the resting $[Ca^{2+}]_{cyt}$ in IPAH-PASMC (n=370 cells) was significantly higher than in normal PASMC (n=96 cells). Removal of extracellular Ca^{2+} negligibly affected the resting $[Ca^{2+}]_{cyt}$ in normal PASMC, but significantly decreased the resting $[Ca^{2+}]_{cyt}$ in IPAH-PASMC (Online Fig. IIA). To examine whether IPAH-PASMC had leaky membranes, we incubated normal and IPAH PASMC with trypan blue (TB). No blue (TB-stained) cells were detected in normal and IPAH PASMC incubated in TB-containing solution, whereas following treatment of the cells with 10 μM ionomycin (for 10 min), a Ca^{2+} ionophore, all normal and IPAH PASMC were TB-stained; there was no difference between normal and IPAH PASMC (Online Fig. IIB). Furthermore, we were unable to detect any fluorescent signals in normal and IPAH PASMC incubated with the membrane-impermeable fura-2 (Online Fig. IIC). All these experiments indicate that the

extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase in IPAH-PASMC was not due to Ca^{2+} leakage through the plasma membrane.

Effects of specific CaSR modulators on the extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase in normal and IPAH PASMC

To further confirm that the upregulated CaSR in IPAH-PASMC is involved in the enhanced extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ rise, we examined whether the polyamine spermine and the calcimimetic R568 induced the same effect on $[Ca^{2+}]_{cyt}$ as did restoration of extracellular Ca^{2+}, and whether the calcilytic NPS 2143 inhibited the extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase. As shown in Figure 3A, extracellular application of 3 mM spermine, a CaSR agonist, induced a slight increase in $[Ca^{2+}]_{cyt}$ (52±24 nM, n=13) in normal PASMC in the presence of 2.2 mM extracellular Ca^{2+}. In IPAH-PASMC, however, extracellular application of spermine caused a huge $[Ca^{2+}]_{cyt}$ increase (1,308±123 nM, n=49; $P<0.01$ vs. normal PASMC) in the presence of 2.2 mM extracellular Ca^{2+}. In IPAH-PASMC, short-time treatment with the positive allosteric modulator of CaSR, R568 (1 μM), significantly enhanced the extracellular Ca^{2+}-mediated increase in $[Ca^{2+}]_{cyt}$ (Fig. 3B), whereas the negative allosteric modulator of CaSR, NPS 2143 (10 μM), significantly inhibited extracellular Ca^{2+}-mediated increase in $[Ca^{2+}]_{cyt}$ (Fig. 3C). Collectively, these results demonstrate that the upregulated CaSR (Fig. 2) is involved in the enhancement of the extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase in PASMC from IPAH patients.

Extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase in IPAH-PASMC is dependent of phospholipase C (PLC) and the inositol-2,4,5-trisphosphate receptor (IP_3R)

To examine the potential signaling pathway involved in the CaSR-mediated increase in $[Ca^{2+}]_{cyt}$ in IPAH-PASMC, we performed pharmacological experiments on the extracellular Ca^{2+}-mediated increase in $[Ca^{2+}]_{cyt}$. As shown in Figure 4, short-time pretreatment of IPAH-PASMC with the inhibitor of phospholipase C (PLC), U73122 (1 μM), or the specific blocker of IP_3R, xestospongin C (3 μM), significantly inhibited the extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ (Online Fig. IIIA and C). The inactive form of U73122, U73343 (1 μM), however, had no effect on the extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ (Online Fig. IIIB). Pretreatment of IPAH-PASMC with the blocker of VDCC, diltizaem (10 μM), or the inhibitor of Na^+/Ca^{2+} exchanger, KB-R7943 (10 μM), however, had no effect on the extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ (Online Fig. IIID and E). These results indicate that activation of PLC and IP_3R is involved in the CaSR-mediated $[Ca^{2+}]_{cyt}$ increase in IPAH-PASMC, while VDCC and the reverse mode of Na^+/Ca^{2+} exchanger are not involved in the Ca2R-mediated Ca^{2+} influx or inward transportation.

Downregulation of CaSR in IPAH-PASMC inhibits extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase and attenuates cell proliferation

To obtain direct evidence for the involvement of CaSR in extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase and cell proliferation, we used siRNA to knockdown CaSR expression and examined whether CaSR was necessary for the extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase in IPAH-PASMC. Treatment of IPAH-PASMC with 50 nM siRNA significantly decreased protein level of CaSR (Fig. 4A) and markedly inhibited the extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ (Fig. 4B). In comparison to normal PASMC, the proliferate rate of IPAH-PASMC, determined by a change in cell number, was much faster (Fig. 4C), while downregulation of

CaSR in IPAH-PASMC by transiently transfecting 50 nM siRNA for CaSR significantly attenuated cell proliferation (Fig. 4D). These experiments provide compelling evidence that CaSR is necessary for the augmented extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase and enhanced cell proliferation in IPAH-PASMC.

Overexpression of CaSR in normal PASMC augments the extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase and enhances cell proliferation

Extracellular application of either 2.2 mM Ca^{2+} or 3 mM spermine failed to induce a significant increase in $[Ca^{2+}]_{cyt}$ in normal PASMC because of a low protein expression level of CaSR (see Figs. 2 and 3). We examined whether CaSR was sufficient to mediate extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase in both human and rat PASMC by transiently transfecting the human CaSR into normal PASMC. As shown in Figure 5, overexpression of CaSR (with 2 μg of the human CaSR cDNA) in normal (human and rat) PASMC significantly augmented the extracellular Ca^{2+}-mediated increase in $[Ca^{2+}]_{cyt}$ (Fig. 5A and B) and enhanced cell proliferation (Fig. 5C) in comparison to normal PASMC transiently transfected with an empty vector. Taken together with the data showed earlier (Fig. 4), these results indicate that *a*) CaSR is sufficient to mediate extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase and cell proliferation in normal PASMC, and *b*) CaSR is necessary for the augmented extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase and enhanced cell proliferation in IPAH-PASMC.

CaSR is functionally upregulated in PASMC from animal models of experimental pulmonary hypertension and blockade of CaSR prevents the development of pulmonary hypertension

The presented *in vitro* experimental data show that the upregulated CaSR and augmented extracellular Ca^{2+}-mediated $[Ca^{2+}]_{cyt}$ increase in PASMC are involved in the enhanced PASMC

proliferation in patients with IPAH. To investigate whether CaSR can be a target for treatment of pulmonary arterial hypertension, we used the rat model of monocrotaline (MCT)-induced pulmonary hypertension (MCT-PH) and the mouse model of hypoxia-induced pulmonary hypertension (HPH) to test the potential therapeutic effect of the calcilytic NPS 2143. We first examined and compared the mRNA and protein expression level of CaSR in PASMC from control and MCT-treated rats. As shown in Figure 6A and B, the mRNA level of CaSR in PASMC isolated from rats (rPASMC) with MCT-PH was much greater than in PASMC isolated from normotensive control rats injected with vehicle. The immunohistochemistry and immunoblotting experimental data indicate that the protein expression level of CaSR in the small pulmonary artery (Fig. 6C) and PASMC (Fig. 6D) of MCT-rats was significantly higher than in the small pulmonary artery and PASMC of control rats. Furthermore, the basal or resting $[Ca^{2+}]_{cyt}$ and the extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ were both enhanced in freshly-dissociated PASMC from MCT-PH rats compared with freshly-dissociated PASMC from normotensive control rats (Fig. 6E). Treatment with NPS 2143 not only decreased the basal $[Ca^{2+}]_{cyt}$, but also inhibited the extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ in PASMC isolated from MCT-PH rats (Fig. 6E). These results imply that upregulation of CaSR and subsequent enhancement of extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase in PASMC contribute to the development of pulmonary hypertension in rats injected with MCT.

To test the *in vivo* therapeutic effect of the CaSR antagonist, we examined and compared the right ventricular systolic pressure (RVSP), the Fulton index [i.e., the ratio of right ventricle/left ventricle+septum, RV/(LV+S)] and muscularization of distal pulmonary arteries in normotensive control (Norm) rats and MCT-injected rats with and without treatment with NPS 2143, a CaSR antagonist. Injection of MCT (60 mg/kg) in rats significantly increased RVSP and caused right ventricular (RV) hypertrophy compared with the normotensive control (Norm) rats

injected with vehicle (DMSO) (Fig. 7A-C). Intraperitoneal injection of NPS 2143 (4.5 mg/kg per day) had little effect on RVSP and RV/(LV+S) ratio in Norm rats, but significantly attenuated the increase in RVSP and the Fulton index in MCT-PH rats (Fig. 7A-C). There were no significant changes in heart rate in Norm rats with (412±17 bpm, n=6) or without (411±21 bpm, n=6) NPS treatment and MCT-injected rats with (414±23 bpm, n=6) or without (413±21 bpm, n=6) NPS treatment. The MCT-induced increases in RVSP and RV hypertrophy were associated with significant pulmonary vascular remodeling; the vascular medial wall thickness of small pulmonary arteries with the outer diameter less than 100 μm was significantly greater in MCT-injected rats than in Norm rats (Fig. 7D and E). Treatment with the CaSR antagonist (NPS 2143) significantly inhibited the muscularization of small pulmonary arteries (Fig. 7D and E). The *in vivo* animal experiments are consistent with the *in vitro* experiments using normal and IPAH PASMC.

To further validate the pathogenic role of upregulated CaSR in the development of pulmonary hypertension and the therapeutic effect of CaSR antagonists on experimental pulmonary hypertension, we repeated the experiments mentioned above in the HPH mouse model. As shown in Figure 8, the mRNA and protein expression of CaSR was significantly higher in pulmonary arteries and lung tissues in HPH mice than in normoxic control mice (Fig. 8A and B), while the extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ in freshly-isolated PASMC from HPH mice was significantly enhanced in comparison to cells from normoxic mice (Fig. 8C). These data indicate that CaSR is functionally upregulated in PASMC from HPH mice.

Intraperitoneal injection of NPS 2143 (1 mg/kg per day from day 1 to 10) had little effect on RVSP and RV/(LV+S) ratio in normoxic control mice (Nor), but significantly inhibited the increase in RVSP and RV/(LV+S) ratio in hypoxic mice (Hyp) (Fig. 8D-F). Furthermore,

inhibition of CaSR also significantly inhibited the hypoxia-mediated pulmonary arterial wall thickening (Fig. 8G) and reversed the hypoxia-induced decrease in branch and junction numbers (and total length) of small pulmonary arterial trees (Fig. 8H and I). There were no significant changes in heart rate in normoxc mice with (411±17 bpm, n=6) or without (410±20 bpm, n=6) NPS treatment and hypoxic mice with (412±18 bpm, n=6) or without (413±18 bpm, n=6) NPS treatment.

These data imply that intraperitoneal injection of the CaSR antagonist is an efficient therapeutic approach to inhibit the development and progression of pulmonary vascular remodeling and right ventricular hypertrophy in animal models with experimental pulmonary hypertension induced by injection of monocrotaline and exposure to hypoxia. The observations from this study strongly indicate that increased expression and function of CaSR may play a pathogenic role in the development of pulmonary vascular remodeling and antagonists of CaSR, or calcilytics, may have great therapeutic potential for patients with pulmonary arterial hypertension.

DISCUSSION

In this study, we found that *a*) extracellular application of Ca^{2+} (0.5-10 mM) and spermine (3 mM) induced a large increase in $[Ca^{2+}]_{cyt}$ in IPAH-PASMC but not in normal PASMC; *b*) the protein expression level of CaSR in IPAH-PASMC was greater than in normal PASMC; *c*) downregulation of CaSR in IPAH-PASMC (with siRNA) inhibited the extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ and attenuated cell proliferation, while overexpression of CaSR in normal PASMC augmented the extracellular Ca^{2+}-induced rise in $[Ca^{2+}]_{cyt}$ and enhanced cell proliferation; *d*) the expression and function of CaSR were also increased in PASMC from rats with MCT-PH and mice with HPH, and intraperitoneal injection of a CaSR antagonist (NPS 2143) significantly inhibited pulmonary vascular remodeling and attenuated the development and progression of the experimental pulmonary hypertension. Collectively, the observations from this study indicate that *i*) functionally upregulated CaSR and subsequently augmented extracellular Ca^{2+}-induced rise in $[Ca^{2+}]_{cyt}$ in PASMC contribute to the enhanced Ca^{2+} signaling and excessive cell proliferation in IPAH patients; and *ii*) blockade of the upregulated CaSR with calcilytics may be a novel therapeutic approach for pulmonary arterial hypertension.

$[Ca^{2+}]_{cyt}$ plays an important role in the regulation of contraction, proliferation, and migration of PASMC. An increase in $[Ca^{2+}]_{cyt}$ in PASMC is a major trigger for pulmonary vasoconstriction and an important stimulus for PASMC proliferation and pulmonary vascular remodeling under pathological conditions. Elevation of $[Ca^{2+}]_{cyt}$ in PASMC results from Ca^{2+} release from intracellular stores and Ca^{2+} influx through plasmalemmal Ca^{2+} channels[1, 5]. We previously showed that the resting $[Ca^{2+}]_{cyt}$ was increased, while ROCE and SOCE were enhanced in PASMC from IPAH patients compared to PASMC from normal subjects and patients without pulmonary hypertension[6, 7].

CaSR is a GPCR (belongs to the Family C GPCR) with 1,085 amino acids, which is

present constitutively in a homodimeric configuration formed by covalent and non-covalent linkages[25-27] and is able to form heterodimers with the metabotropic glutamate receptors (mGLuR1 and mGluR5)[27]. One of the hallmarks of CaSR is the cysteine-rich large N-terminal extracellular domain (ECD, approximately 600 amino acids). The region between alanine 116 and proline 136 in the ECD is important for maintaining CaSR in an inactive conformation[28] and is associated with the activating mutations or single nucleotide polymorphism (SNPs) identified in the human CaSR gene. The ligands or activators of CaSR include polyvalent cations (e.g., Ca^{2+}, Mg^{2+}, Gd^{3+}), polypeptides (e.g., amyloid-β peptide), polyamines (e.g., spermine, spermidine, putrescine), aminoglycoside antibiotics (e.g., neomycin, kanamycin), and amino acids (e.g., phenylalanine, tyrosine, tryptophan, glutamate). In addition, there are synthetic CaSR activators, or calcimimetics (e.g., NPS-R-568, NPS-R-467), and CaSR antagonists, or calcilytics (e.g., NPS 2143) that affect CaSR function[12-14, 29-31]. The cysteine-rich domain in the ECD also sensitizes the receptor to redox changes and hypoxia/hyperoxia. In Sprague-Dawley rats, treatment with the CaSR activator or the calcimimetic R-568 attenuates aortic wall thickening induced by uremia.[32] Our data from this study, however, indicate that the extracellular Ca^{2+}-mediated $[Ca^{2+}]_{cyt}$ increase in IPAH-PASMC was potentiated by NPS-R-568, an allosteric agonist of CaSR, and inhibited by NPS 2143, an allosteric antagonist of CaSR. Extracellular application of spermine significantly increased $[Ca^{2+}]_{cyt}$ in IPAH-PASMC superfused in Ca^{2+}-containing solution. These data imply that multiple ligands can activate the upregulated CaSR in IPAH-PASMC leading to cell proliferation, contraction and migration via Ca^{2+} signaling and other signal transduction cascades.

Extracellular Ca^{2+} binding to CaSR is a highly cooperative process. The EC_{50} for extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase in IPAH-PASMC is approximately 1.2 mM (Fig. 1D), while the EC_{50} has been reported to be 1.7 mM in parathyroid cells[33], 1.5 mM in cardiomyocytes[34], and 5.6 mM in bronchial epithelial cells[35]. However, in reconstituted systems

with CaSR clones isolated from parathyroid glands, the EC_{50} for CaSR activation (or the Ca^{2+} sensitivity of CaSR) is 3.0-3.5 mM[12, 13, 15, 36-38]. The lower EC_{50} for Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ rise in IPAH-PASMC and native vascular smooth muscle cells (compared to the EC_{50} for the recombinant CaSR) is presumably due to the high degree of cooperativity in the interaction between the upregulated CaSR (e.g., in IPAH-PASMC) and ligands. CaSR is always exposed to various co-agonists physiologically (e.g., Mg^{2+}, polyamines and amino acids) and other activators pathologically (e.g., antibiotics, heavy metals and amyloid-β peptide). Therefore, the sensitivity of CaSR to extracellular Ca^{2+} is expected to be higher in pathologically conditions than in physiological conditions.

As mentioned earlier, the CaSR senses extracellular Ca^{2+} concentration and transduces it to intracellular space though multiple signal pathways [12-14, 18]. CaSR interacts directly with G proteins ($G_{q\alpha}$ and $G_{11\alpha}$). Activation of CaSR by extracellular Ca^{2+} (or calcimimetics) induces increases in $[Ca^{2+}]_{cyt}$ through phospholipace C (PLC)-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5- trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to the IP_3 receptor (IP_3R) on the SR membrane and induces Ca^{2+} release from the SR to the cytosol. Depletion (or reduction) of Ca^{2+} from the intracellular stores (i.e., SR) via activated IP_3R (or opened Ca^{2+} release channels) then causes SOCE through SOC in the plasma membrane. Furthermore, DAG causes ROCE by activating ROC in the plasma membrane. The IP_3-mediated Ca^{2+} mobilization, the store depletion-mediated SOCE and the DAG-mediated ROCE all contribute to the increase in $[Ca^{2+}]_{cyt}$ upon activation of CaSR in the plasma membrane by extracellular Ca^{2+} and CaSR activators. In IPAH-PASMC, the extracellular Ca^{2+}-mediated $[Ca^{2+}]_{cyt}$ increases were significantly attenuated by the PLC inhibitor (U73122) and the IP_3R blocker (xestospongin C), but not affected by the voltage-dependent Ca^{2+} channel blocker (diltiazem) and the Na^+/Ca^{2+} exchanger inhibitor (KB-R7943) (Online Fig. III). These

data further confirm the important role of the PLC-IP_3 signaling cascade in CaSR-mediated increase $[Ca^{2+}]_{cyt}$ and its proliferative effect on PASMC isolated from patients with IPAH. Most membrane receptors, including many GPCRs, become desensitized with prolonged exposure to agonists. However, CaSR desensitizes very slowly[39], indicating that CaSR signals for long periods of time through the regulation of intracellular signaling cascades and other signal transduction pathways.

In addition to increasing $[Ca^{2+}]_{cyt}$, extracellular Ca^{2+}-mediated activation of CaSR has been linked to several signal transduction cascades. CaSR activates the mitogen-activated protein kinase (MAPK) cascade, e.g. extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-jun N-terminal kinase (JNK), potentially through the interaction with filamin A scaffolding protein and caveolin-1 in cholesterol-rich invaginations in the plasma membrane known as caveolae[16, 17, 40-44]. In IPAH-PASMC, the number of caveoli and the protein expression level of caveolin-1/-2 are both increased, which are associated with increased SOCE[45]. Downregulation of caveolin-1 with siRNA and treatment with methyl-β-cyclodextrin (MβCD) significantly diminish the number of caveoli on the surface membrane of IPAH-PASMC, significantly attenuate SOCE and markedly inhibit cell proliferation[6, 45]. It is unknown, however, whether upregulated CaSR in IPAH-PASMC is exclusively or predominantly localized in caveoli and functionally interacts with upregulated TRPC3/C6 channels[7, 8].

CaSR is widely distributed in the parathyroid glands[15], kidney[46], bone[47], gastrointestinal tract[48], skin[49], brain[50], immune cells[51], and heart[34]. In addition, CaSR is expressed in vascular smooth muscle cells from rat subcutaneous artery[52], aorta[53], pulmonary artery[16, 17], gerbil spiral modiolar artery[54], human renal artery[55], aorta[55, 56], and internal mammary artery[57], as well as in endothelial cells from rat mesenteric artery, porcine coronary artery[58], and human aorta[19]. Activation of CaSR in vascular smooth muscle cells increases $[Ca^{2+}]_{cyt}$ and induces

vasoconstriction[17, 54, 56]; therefore, CaSR is involved in regulating myogenic tone, peripheral vascular resistance[52], and arterial blood pressure[59, 60]. Recent observations demonstrate that the CaSR in vascular smooth muscle cells contributes to regulating cell proliferation and apoptosis through the MEK1/ERK1/2 and PLC signaling pathways[17, 53, 55], and expression of CaSR is involved in the regulation of mouse lung development[61]. Our study indicated that in normal human PASMC, CaSR protein was expressed at a low level and extracellular application of Ca^{2+} (from 0.5 to 10 mM) failed to cause a significant increase in $[Ca^{2+}]_{cyt}$ (Fig. 1D), while extracellular application of spermine caused a small increase in $[Ca^{2+}]_{cyt}$ (Fig. 3A). These observations imply that CaSR is expressed at very low level in normal human PASMC and is not a major contributor to the regulation of pulmonary vascular tone under physiological conditions.

In patients with IPAH and animals with experimental pulmonary hypertension, CaSR is functionally upregulated in PASMC and thus becomes an important GPCR involved in the initiation and progression of pulmonary vascular remodeling and pulmonary hypertension. Knockdown of CaSR with siRNA in PASMC from IPAH patients not only diminished extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$, but also inhibited cell proliferation (Fig. 4). Overexpression of CaSR in PASMC from normal subjects conferred an extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ and enhanced cell proliferation (Fig. 5). These experimental data provide compelling evidence that CaSR is necessary and sufficient for the augmented Ca^{2+} signaling and excessive PASMC proliferation in IPAH patients. In this scenario, Ca^{2+} is an extracellular ligand and an intracellular signaling element involved in the development and progression of sustained pulmonary vasoconstriction and vascular remodeling in patients with IPAH. The pathogenic role of upregulated CaSR in pulmonary hypertension is further confirmed by the therapeutic effect of CaSR antagonists on experimental pulmonary hypertension in rats injected with MCT and in mice exposed to chronic hypoxia.

The gain-of-function (or activating) mutations or single-nucleotide polymorphisms (SNP) in the human CaSR gene causes autosomal dominant hypocalcemia[62] and Bartter's syndrome type V[12, 63]. CaSR antagonists (calcilytics), i.e. negative allosteric modulators that indirectly stimulate parathyroid hormone secretion through a decrease in CaSR activity, are potential drug candidates for the treatment of osteoporosis and other bone metabolism diseases[64, 65]. Using two well-established animal models of pulmonary hypertension (MCT-injected rats and chronic hypoxic mice), we found that increased RVSP and pulmonary vascular medial hypertrophy, as well as right ventricular hypertrophy [determined by the Fulton index, RV/(LV+S)] were associated with upregulated CaSR expression and enhanced extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ rise (and basal $[Ca^{2+}]_{cyt}$) in PASMC (Figs. 6-8). Intraperitoneal injection of the CaSR antagonist, NPS 2143 (4.5 mg/kg once a day), had little effect on the pulmonary hemodynamics and the Fulton index in control rats or normoxic mice, but significantly decreased RVSP, RV/(LV+S) ratio and small pulmonary vascular wall thickening in rats with MCT-PH (Fig. 7) and mice with HPH (Fig. 8). These results strongly suggest that CaSR is involved in the development of experimental pulmonary hypertension and a potential target for developing therapeutic approach for pulmonary arterial hypertension.

In conclusion, upregulated expression of CaSR in PASMC and augmented function of CaSR through intracellular Ca^{2+} signaling (and other signal transduction cascades) are new pathogenic mechanisms involved in the initiation and progression of pulmonary vascular remodeling in patients with pulmonary arterial hypertension. Pharmacological blockade of CaSR in the pulmonary vasculature by synthetic calcilytics and downregulation of CaSR by siRNA (and/or specific microRNA) may be a novel therapeutic approach for IPAH patients who do not respond to the conventional drug therapy.

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DISCLOSURES

None.

FOOTNOTE

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FIGURE LEGENDS

Figure 1. Enhancement of extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ in PASMC from IPAH patients

A-C. Representative records (A), pseudo-color images (B), and summarized data (means±SE, C) showing extracellular Ca^{2+}-mediated changes in $[Ca^{2+}]_{cyt}$ in PASMC from normal subjects (n=45 cells), IPAH patients (n=160 cells), and CTEPH patients (n=27 cells). Two kinetically different responses of $[Ca^{2+}]_{cyt}$ to extracellular Ca^{2+} in PASMC from two IPAH patients are shown in the middle panels. D. Representative traces of $[Ca^{2+}]_{cyt}$ changes in response to extracellular application of 0.1, 0.5, 1.1, 2.2, and 10 mM Ca^{2+} (left panels) and the dose-response curves (right panel) in normal PASMC (upper panel) and IPAH-PASMC (lower panel). The EC_{50} for extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase in IPAH-PASMC is 1.22 mM.

Figure 2. Upregulation of CaSR in PASMC from IPAH patients

A. Western blot analysis on CaSR in total proteins (upper panels) and membrane proteins (lower panels) isolated from normal PASMC (Nor) and IPAH-PASMC (IPAH). B. Summarized data (means±SE, lower panel) showing CaSR protein levels that were normalized to the β-tubulin level. $^{**}P<0.01$ vs. normal PASMC. C. Western blot analysis on CaSR in total and membrane proteins isolated from lung tissues of normal subjects and IPAH patients.

Figure 3. Effects of CaSR modulators on $[Ca^{2+}]_{cyt}$ in IPAH-PASMC

A. Representative traces (left panels) and summarized data (means±SE, right panel) showing the effect of spermine (3 mM), a CaSR agonist, on $[Ca^{2+}]_{cyt}$ in the absence (0Ca) and presence (2.2Ca) of extracellular Ca^{2+} (2.2 mM) in normal (n=30) and IPAH PASMC (n=52). $^{**}P<0.01$ vs.

normal PASMC (spermine/0Ca); $^{\#\#}P<0.01$ vs. normal PASMC (spermine/2.2Ca). B and C. Representative records of $[Ca^{2+}]_{cyt}$ changes (left panels) and summarized data (means means±SE, right panels) of the stimulatory effect of R568 (1 μM, n= 63, B), a positive allosteric modulator of CaSR, and the inhibitory effect of NPS-2143 (10 μM, n=120, C), a negative allosteric modulator of CaSR, on the 0.5-mM extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increases in IPAH-PASMC. $^{**}P<0.01$ vs. Control IPAH-PASMC (without treatment with vehicle, R568 or NPS 2143).

Figure 4. Downregulation of CaSR with siRNA attenuates extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increases in IPAH-PASMC and inhibits IPAH-PASMC proliferation

A. Western blot analysis on CaSR in IPAH-PASMC treated with a control (or scrambled) siRNA (Control) and siRNA for CaSR (at 25 and 50 nM, n=3 for each group). B. Representative records of $[Ca^{2+}]_{cyt}$ changes (left panels) and summarized data (means±SE, right panel) showing the extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increases in IPAH-PASMC transfected with control siRNA (n=57) and CaSR-siRNA (n=57). $^{**}P<0.01$ vs. control-siRNA. C. Summarized data (means±SE) showing the total cell numbers of normal PASMC (open circles) and IPAH-PASMC (solid circles) after cultured in growth media for 8, 24, 48, 72, and 96 hrs. The growth curves for normal and IPAH PASMC are significantly different ($P<0.001$, n=3 experiments). D. Summarized data (means±SE, n=3 experiments) showing inhibitory effects of CaSR-siRNA on the cell proliferation in IPAH-PASMCs. $^{**}P<0.01$ vs. control-siRNA.

Figure 5. Overexpression of CaSR in normal PASMC enhances extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increase and promotes cell proliferation

A and B. Representative records of $[Ca^{2+}]_{cyt}$ changes (left panels) and summarized data (means±SE, right panels) extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increases in human (A) and rat (B) PASMC transfected with an empty vector (Vector, n=12) and the human CaSR cDNA (CaSR, n=8). C. Summarized data (means±SE) showing the total numbers of normal human PASMC transfected with the vector (solid circles) and CaSR gene (grey circles) after incubation in growth media for 24, 48, 72, 96, and 120 hrs. The growth curves for vector control PASMC and IPAH PASMC are significantly different ($P<0.01$, n=3 experiments). **$P<0.01$ vs. vector. D. Western blot analysis on CaSR in human PASMC transfected with an empty vector (Vector) or CaSR.

Figure 6. Upregulation of CaSR in PASMC from rats with MCT-induced pulmonary hypertension

A and B. Regular (A) and real-timer (B) RT-PCR analyses on CaSR in PASMC isolated from normal rats (Norm, n=6) and rats with MCT-induced pulmonary hypertension (MCT, n=5). **$P<0.01$ vs. Norm. C. Immunohistochemistry data showing CaSR expression level (green fluorescence intensity) in normal and MCT-treated rats. D. Western blot analysis on CaSR in PASMC isolated from normal and MCT-PH rats. *$P<0.05$, **$P<0.01$ vs. Norm. E. Summarized data (means±SE) showing the basal $[Ca^{2+}]_{cyt}$ (left panel, n=12) and the amplitude of extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increases (right panel, n=11) in PASMC freshly isolated from normal rats and MCT-injected rats that are treated with vehicle (-NPS) or 10 μM NPS 2143 (+NPS), a synthetic calcilytic. **$P<0.01$ vs. Norm (-/+NPS).

Figure 7. Blockade of CaSR by NPS 2143 inhibits the development of pulmonary vascular remodeling and pulmonary hypertension in rats injected with MCT

A and B. Representative record of right ventricular pressure (RVP, A) and summarized data (means±SE) showing the peak value of right ventricular systolic pressure (RVSP, B) in normal control rats (Norm, n=6) and MCT-injected rats (MCT, n=6) that are treated with vehicle (-NPS) or NPS 2143 (+NPS, 4.5 mg/kg once a day). C. Averaged Fulton index [RV/(LV+S) ratio, means±SE] showing that RV hypertrophy is significantly inhibited in MCT-rats treated with NPS. **P*<0.05 vs. MCT along. D and E. Representative H&E images of small pulmonary arteries (D) and summarized data of the medial thickness of pulmonary arteries with a diameter (Ø) less than 50 μm, between 50 and 100 μm and greater than 100 μm (E) in normal control rats (Norm, n=6) and MCT-injected rats (MCT, n=6) that are treated with vehicle or NPS 2143. **P*<0.05 vs. MCT-treated rats (red bars).

Figure 8. Upregulation of CaSR in PASMC from HPH mice and blockade of CaSR by NPS 2143 inhibits the development of HPH in mice

A and B. Real-time RT-PCR (A) and Western blot (B) analyses on CaSR in PA and lung tissues isolated from normoxic (Nor) and hypoxic (Hyp) mice. C. Representative record (left panels) and summarized data (means±SE, right panel) showing the extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ in PASMC isolated from normoxic and hypoxic mice. ***P*<0.01, ****P*<0.001 vs. Nor. D and E. Representative record of right ventricular pressure (RVP, D) and summarized data (means±SE) showing the peak value of right ventricular systolic pressure (RVSP, E) in normoxic (Nor, n=6) and hypoxic (Hyp, n=6) mice that are treated with vehicle or NPS 2143 (+NPS, 1 mg/kg once a day). F. Averaged Fulton index [RV/(LV+S) ratio, means±SE] in normoxic and hypoxic mice treated with or without NPS 2143. **P*<0.05 vs. Hyp along. G. Representative H&E images of small pulmonary arteries showing that the medial thickness is significantly increased in Hyp mice and the hypoxia-induced medial hypertrophy is inhibited by NPS 2143. H.

Representative angiography of the whole lung (upper panels) and enlarged area of the whole lung (lower panels, indicated by the box in the upper panels) in normoxic and hypoxic mice treated with vehicle or NPS (+NPS). The vertical bars denote 2 mm (upper panels) and 0.5 mm (lower panels). I. Summarized data (means±SE) showing the number of branches, the number of junctions, and the total length of vascular segments per square millimeter (mm^2). $^{**}P<0.01$ vs. other bars.

Novelty and Significance

What is known?

- Pulmonary vascular remodeling and sustained pulmonary vasoconstriction contribute to the development of idiopathic pulmonary arterial hypertension (IPAH).
- Increased cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) stimulates pulmonary arterial smooth muscle (PASMC) proliferation leading to pulmonary vascular remodeling.
- Ca^{2+}-sensing receptor (CaSR) is a G protein coupled receptor important for multiple cellular processes of the parathyroid glands such as proliferation, differentiation, and apoptosis.

What new information does this article contribute?

- CaSR is functionally upregulated in IPAH-PASMC contributing to enhanced Ca^{2+} signaling and excessive cell proliferation in IPAH patients.
- Blockade of the CaSR with an antagonist inhibits the development of pulmonary hypertension in animal models.
- Targeting the CaSR may be a novel therapeutic approach for IPAH patients.

Idiopathic pulmonary arterial hypertension (IPAH) is a rare, progressive and fatal disease that predominantly affects women. The pathogenic mechanisms involved in the pulmonary vascular abnormalities (e.g., arterial remodeling and sustained vasoconstriction) in IPAH patients remain unclear. An increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in pulmonary arterial smooth muscle cells (PASMC) is a major trigger for pulmonary vasoconstriction and an important stimulus for PASMC migration and proliferation (which subsequently cause pulmonary vascular

wall thickening leading to the increase in pulmonary vascular resistance). In this study, we report that a unique G protein-coupled receptor (GPCR), Ca^{2+}-sensing receptor (CaSR), is significantly upregulated in PASMC isolated from patients with IPAH and animals with experimental pulmonary hypertension. The upregulated CaSR is necessary for the enhanced extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ and the augmented cell proliferation in IPAH-PASMC. Pharmacological blockade of CaSR with a calcilytic, NPS 2143, markedly inhibits the extracellular Ca^{2+}-induced rise in $[Ca^{2+}]_{cyt}$ and attenuates the development of experimental pulmonary hypertension in animal models. These data indicate that functionally upregulated CaSR in PASMC may play an important role in causing sustained pulmonary vasoconstriction and excessive pulmonary vascular remodeling in IPAH patients. Targeting CaSR in PASMC may help develop novel therapeutic approach for pulmonary hypertension.