Online Supplementary METHODS

Preparation of human and animal PASMC. Human PASMC were isolated from explanted lung tissues of normal control subjects (2 unsuitable organ donors and 2 COPD patients without pulmonary hypertension) and patients with IPAH (3 patients diagnosed on the basis of NIH IPAH Registry with an averaged mean PAP of 56±5 mmHg).[1, 2] In addition, PASMC were isolated and prepared from endarterectomized pulmonary arterial tissues of patients with chronic thromboembolic pulmonary hypertension (CTEPH, 4 patients with an averaged mean PAP of 39±5 mmHg)[3]. 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 (Invitrogen, Grand Island, NY), 100 U/ml penicillin plus 100 μg/ml streptomycin, 50 μg/ml D-valine (Sigma-Aldrich, St. Louis, MO), and 20 μg/ml endothelial cell growth supplement (BD Biosciences, Franklin Lakes, NJ) at 37ºC. The cells at passages 5 to 8 were used for the experiments. In some experiments, we also used freshly-dissociated PASMC from rats (by a modified protocol based on the methods published previously).[4] In the modified protocol, cell suspensions were plated onto cover slips for a short time (2-3 hrs) before experiments. We also used PASMC from mice by a modified method previously published by Marshall et al.[5].

$[Ca^{2+}]_{cyt}$ measurement. The $[Ca^{2+}]_{cyt}$ measurement was performed as described previously[6]. In brief, cultured PASMC on cover slips were placed in a recording chamber on the stage of an invert fluorescent microscope (Eclipse Ti-E; Nikon, Tokyo, Japan) equipped with an objective lens (S Plan Fluor 20×0.45 ELWD; Nikon), an EM-CCD camera (Evolve; Photometrics, Tucson, AZ), and NIS Elements 3.2 software (Nikon). The cells were loaded with 4 μM fura-2 acetoxymethyl ester (fura-2/AM; Invitrogen/Molecular Probes, Eugene, OR) for 60 min at room temperature (25°C). The HEPES-buffered external solution contained an ionic composition of 137 mM NaCl, 5.9 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 14 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). In the Ca^{2+}-free solution, 2.2 mM $CaCl_2$ was replaced by equimolar $MgCl_2$ and 1 mM EGTA was added to chelate the residual Ca^{2+}. The fura-2-loaded cells in the recording chamber were continuously perfused with HEPES-buffered solution at 32°C for 30 min to wash out extracellular fura-2/AM and allow sufficient time for the intracellular esterase to cleave acetoxymethyl ester from fura-2 so it can stay in the cytosol. The cells were then excited with 340-nm and 380-nm wavelength (D340×v2 and D380×v2 filters, respectively; Chroma Technology, Bellows Falls, VT) by a xenon arc lamp (Lambda LS; Sutter Instrument, Novato, CA) and an optical filter changer (Lambda 10-B; Sutter Instrument). Fura-2 fluorescence (or emission of fura-2) from a region of interest (5×5 μm) of PASMC, as well as background fluorescence, was collected through a dichroic mirror (400DCLP; Chroma Technology) and a wide band emission filter (D510/80m; Chroma Technology). The fluorescence signals emitted from the region of interest in cells were monitored and recorded continuously on a Nikon digital imaging fluorescence microscopy system and recorded in a PC. The ratio (R) of 340-nm and 380-nm fluorescence intensities (F_{340}/F_{380}), recorded every 2 seconds, was then used to calculate $[Ca^{2+}]_{cyt}$ using the following equation: $[Ca^{2+}]_{cyt} = Kd \times (S_{f2}/S_{b2}) \times (R-R_{min})/(R_{max}-R)$, where Sf2 and Sb2 are the emission fluorescence values at 380-nm excitation in the presence of EGTA and triton X-100, respectively; K_d (225 nM) is the dissociation constant of the Ca^{2+}-fura-2 complex; and R_{max} and R_{min} were calculated according to the standard protocol.[7, 8]

Pharmacological Experiments in Rats with MCT-PH. Adult male Sprague-Dawley rats (190-200 g in body weight; Charles River Laboratories, Wilmington, MA) were randomly divided into two groups: 1) normal control group which was injected subcutaneously of vehicle (DMSO); 2) MCT-PH group which was given a subcutaneous injection of MCT (60 mg/kg, Sigma-Aldrich) to induce pulmonary hypertension. The normal control group was further divided into two subgroups (n=6 for each): one subgroup received the intraperitoneal injection of NPS2143 (Tocris, solubilized into DMSO) at a dose of 4.5 mg/kg per day (from day 1 to day 10) and the other subgroup was injected intraperitoneally with the vehicle (DMSO) (from day 1 to day 10). The MCT-PH group was also further divided into two subgroups (n=6 for each): one subgroup received the intraperitoneal injection of NPS2143 at a dose of 4.5 mg/kg per day (from day 1 to day 10) and the other subgroup was injected intraperitoneally with the vehicle (DMSO) (from day 1 to day 10). Two weeks (14 days) later, the rats were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (26 mg/kg, i.p.) and then right ventricular pressure (RVP) was measured by a pressure-transducer catheter (Millar Instruments: SPF869 and SPF1030) inserted into the right ventricle through the right jugular vein. The hemodynamic data were recorded using an MPVS Ultra® data acquisition system and analyzed with an AD Instruments Lab Chart Pro7.0 software. The protocol used for the rat experiments was approved by the Animal Care Committee of the University of Illinois at Chicago.

Pharmacological Experiments in Mice with HPH. Male C57BL/6N mice (18-22 g in body weight and 6 weeks of age, Charles River Laboratories, Wilmington, MA) were randomly divided into two groups: normoxic control group and hypoxic group which were placed into a normobaric hypoxic chamber (10% O_2) for 4 weeks to establish pulmonary hypertension. The normoxic group was further divided into two subgroups (n=6 for each): one subgroup received the intraperitoneal injection of NPS2143 at a dose of 1 mg/kg per day (from day 1 to day 22) and the other subgroup was injected intraperitoneally with the vehicle (DMSO) (from day 1 to day 22). The hypoxic group was also further divided into two subgroups (n=6 for each): one subgroup received the intraperitoneal injection of NPS2143 at a dose of 1 mg/kg per day (from day 1 to day 22) and the other subgroup was injected intraperitoneally with the vehicle (DMSO) (from day 1 to day 22). Four weeks (28 days) later, the mice were anesthetized with ketamine/xylazine (i.p.) and then right ventricular pressure (RVP) was measured by the right heart catheterization similar to that protocol for rats. The hemodynamic data were recorded using the MPVS Ultra® data acquisition system and analyzed with the AD Instruments Lab Chart Pro7.0 software. The protocol used for the mouse experiments was approved by the Animal Care Committee of the University of Illinois at Chicago.

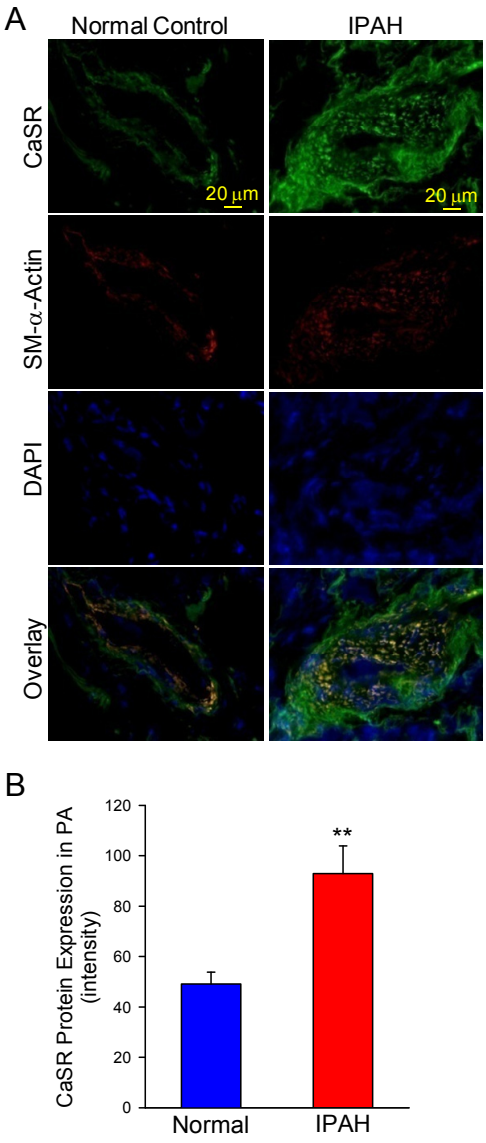
Assessment of Pulmonary Artery Thickness. The left lung lobes (from rats or mice) were fixed in a 3% paraformaldehyde solution and the lobes were dissected in the middle for paraffin embedding. Sectioning at 3 μm was performed for all paraffin-embedded tissue blocks. H&E staining was performed according to common histopathological procedures. Histological analysis was performed in a blinded way: the person who dissected the lobes coded the tissue blocks with numbers and gave to another person to analyze the pulmonary vascular wall thickness. Microscopic images were analyzed using a computerized morphometric system (Aperio Imagescope v11.1.2.752 software) to assess the pulmonary arterial wall thickness. Pulmonary arteries were categorized according to their external diameter: category 1 are the arteries with an

external diameter between 25 and 50 μm; category II are the arteries with an external diameter between 51 and 100 μm; and category III are the arteries with an external diameter greater than 100 μm. Thickness of an artery was determined by the average values obtained in multiple areas of the artery.

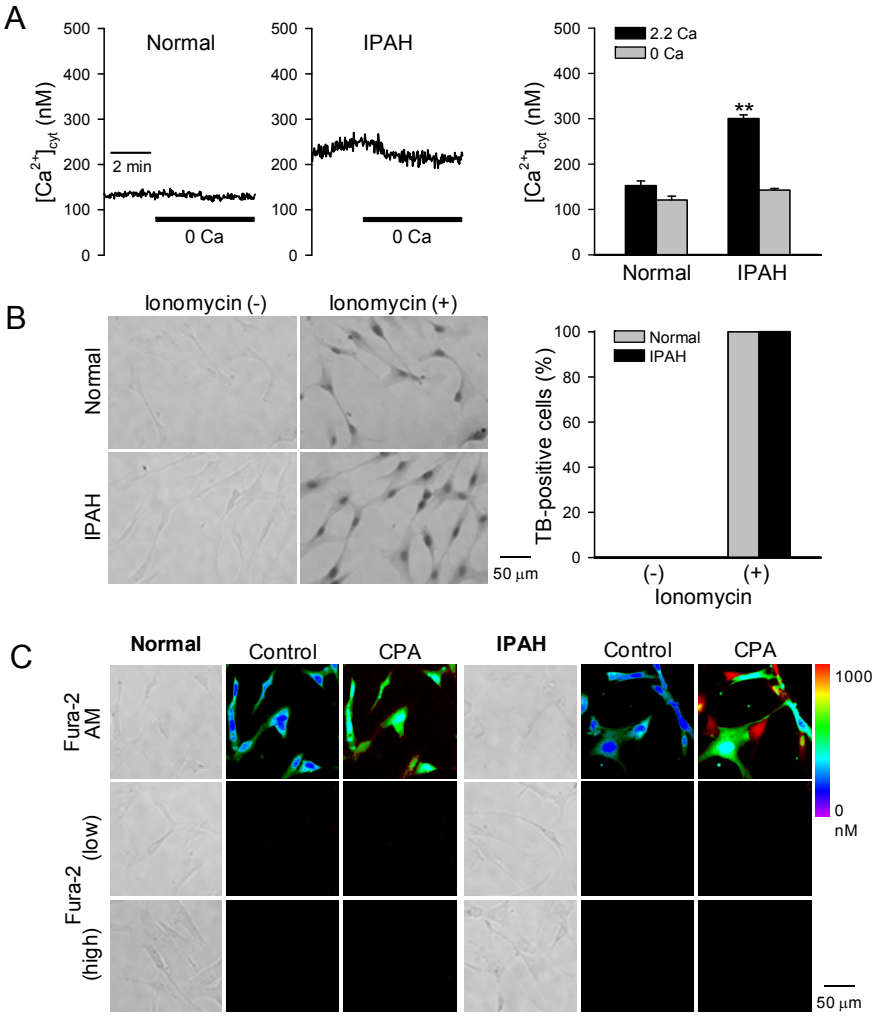
Angiography in Rats and Mice. Rats and mice were anesthetized to dissect out the whole lungs. A PE10 (BD) tube was inserted into the main pulmonary artery and superfused with warm PBS solution (36-38°C) via a syringe pump (Syringepump.com NE-300) at a rate of 0.25 ml/min for 4 min. Then MICROFIL® Silicone Rubber Injection Compounds (Flow-Tech, Inc., Carver, MA), a liquid silicon polymer, was infused into the main pulmonary artery at 0.25 ml/min for 4 min using the syringe infusion pump. The MICROFIL-infused lung tissues were stored at 4°C for 12 hrs before the lungs were sequentially bathed in ethanol at gradually increased concentrations. The tissues were then placed in methyl-salicylate and photographed with a digital camera (MD600E, Amscope, USA) through a dissecting microscope (WILD M651, Leica, Switzerland). The ImageJ software was used to evaluate the number of arterial branches, the number of arterial junctions and the total length of arterial segments in a given area (usually a square mm area, i.e., 1 mm^2). We selected the areas (in 1 mm^2 unit) of the lung vascular images with Photoshop CS software, and made binary images using ImageJ software. The pictures were then skeletonized for analyzing the vessel branches, junctions and total length. These data were normalized by the area selected in each lung.

Immunofluorescence Staining of Human and Rat Lung Sections. Immunohistochemistry for CaSR and SM-α-actin was performed on formalin-fixed, paraffin-embedded (human, rat and mouse) lung tissue sections. Briefly, sections were first deparaffinized in xylene, rehydrated in graded ethanol solutions, and rinsed in deionized water. Then, sections were incubated (for 30 min) in a Tris-EDTA Buffer (10 mM Tris Base,1 mM EDTA solution, with 0.05% Tween20; pH 9.0) for heat-induced epitope retrieval. After washing in PBS, the sections were blocked by incubating with 5% bovine serum albumin (Sigma) for 1 hr. The slides were rinsed in PBS and incubated first with a CaSR polyclonal antibody (rabbit anti-mouse IgG, Abcam) at a dilution of 1:50 for 1 day at 4°C. Then the slides were incubated with a monkey anti-rabbit IgG antibody conjugated with Alexa488 (Invitrogen) as the secondary antibody for 1 hr and a monoclonal antibody against the SM-α-actin clone 1A4 (Cy3 conjugate) (Purified Mouse Immunoglobulin) at a dilution of 1:200. After washing in PBS, sections were mounted in Vectashield hard-set mounting medium with DAPI (Vector Laboratories) and viewed under an Axiovert 200M fluorescence microscope. The expression of CaSR was quantified by grey value using the of Image J software.

Online Supplementary FIGURES and FIGURE LEGENDS

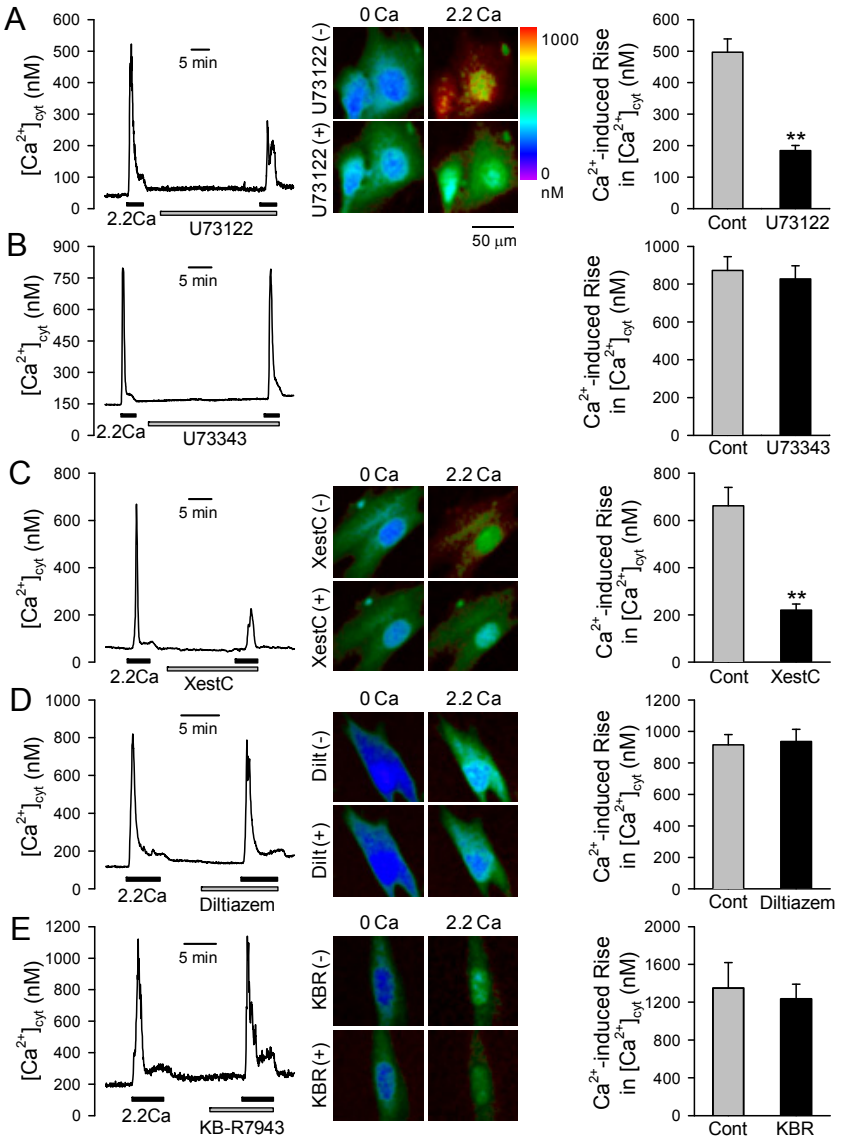


Online Figure I. Upregulated CaSR in pulmonary arteries from IPAH patients.
A. Immunohistochemistry data showing CaSR expression level (green fluorescence intensity) in pulmonary arteries of normal subjects and IPAH patients. The lung tissue sections were stained with antibodies against CaSR (green) and smooth muscle α-actin (SM-α-Actin, red) and with DAPI (blue). The overlay images are shown in the bottom. B. Summarized data (means±SE, n=3 for each group) showing CaSR expression level (green fluorescence intensity) in pulmonary arteries of normal subjects (Norm) and IPAH patients. $^{**}P<0.01$ vs. Normal.



Online Figure II. Extracellular Ca^{2+}-induced increase $[Ca^{2+}]_{cyt}$ in IPAH-PASMCs is not due to Ca^{2+} leakage.

A. Representative traces showing change in resting $[Ca^{2+}]_{cyt}$ before and during application of Ca^{2+}-free solution in normal and IPAH PASMC. Summarized data (right panel, n=96-370 cells) showing the resting $[Ca^{2+}]_{cyt}$ in normal and IPAH PASMC superfused with 2.2 mM Ca^{2+}-containing solution (black bars) or Ca^{2+}-free solution (grey bars). $^{**}P<0.001$ vs. normal PASMC. B. Representative images (left panels) of trypan (TB) blue staining (0.4%, 1 min) before (-) and during (+) treatment with 10 μM ionomycin for 10 min in normal (upper panels) and IPAH (lower panels) PASMC. Summarized data (right panel) showing no correlation between the extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ and the Ca^{2+} leakage through the plasma membrane. C. Representative images showing $[Ca^{2+}]_{cyt}$ (or Fura-2 fluorescence intensity) in normal (left panels) and IPAH (right panels) PASMC in the absence and presence of 10 μM CPA, an inhibitor of Ca^{2+}-ATPase in the SR. The cells were loaded with the membrane-permeable fura-2/AM (4 μM, upper panels) or the membrane-impermeable fura-2 (4 and 40 μM, middle and lower panels). Data were obtained from 21 to 64 cells.



Online Figure III. Inhibition of PLC and IP_3R attenuates extracellular Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ increases in IPAH-PASMC

Representative records of $[Ca^{2+}]_{cyt}$ changes (left panels), pseudo images (middle panels) and summarized data (means±SE, right panels) showing extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_{cyt}$ before and during treatment with 1 μM U73122 (a PLC inhibitor; n=57, A), 1 μM U73343 (an inactive form of U73122; n=45, B), 3 μM xestospongin C (an IP_3R blocker; n=34, C), 10 μM diltiazem (a VDCC blocker; n=46, D), and KB-R7943 (an Na^+/Ca^{2+} exchanger inhibitor; n=22, E) in IPAH-PASMC. ***P*<0.01 vs. control.

Online Supplementary REFERENCES

1. Yuan JX, Aldinger AM, Juhaszova M, Wang J, Conte JV, Jr., Gaine SP, Orens JB, Rubin LJ. Dysfunctional voltage-gated k+ channels in pulmonary artery smooth muscle cells of patients with primary pulmonary hypertension. *Circulation*. 1998;98:1400-1406
2. Yu Y, Fantozzi I, Remillard CV, Landsberg JW, Kunichika N, Platoshyn O, Tigno DD, Thistlethwaite PA, Rubin LJ, Yuan JX. Enhanced expression of transient receptor potential channels in idiopathic pulmonary arterial hypertension. *Proc Natl Acad Sci U S A*. 2004;101:13861-13866
3. Ogawa A, Firth AL, Yao W, Madani MM, Kerr KM, Auger WR, Jamieson SW, Thistlethwaite PA, Yuan JX. Inhibition of mtor attenuates store-operated ca^{2+} entry in cells from endarterectomized tissues of patients with chronic thromboembolic pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol*. 2009;297:L666-676
4. Yuan XJ, Goldman WF, Tod ML, Rubin LJ, Blaustein MP. Ionic currents in rat pulmonary and mesenteric arterial myocytes in primary culture and subculture. *Am J Physiol*. 1993;264:L107-115
5. Marshall C MA, Verhoeven AJ, Marshall BE. Pulmonary artery nadph-oxidase is activated in hypoxic pulmonary vasoconstriction. *Am. J. Respir. Cell Mol Biol.* 1996;15:633-644
6. Yamamura A, Yamamura H, Zeifman A, Yuan JX-J. Activity of ca^{2+}-activated cl^- channels contributes to regulating receptor- and store-operated ca^{2+} entry in human pulmonary artery smooth muscle cells. *Pulm Circ*. 2011;1:269-279
7. Grynkiewicz G, Poenie M, Tsien RY. A new generation of ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem*. 1985;260:3440-3450
8. 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:L144-155

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