ONLINE DATA SUPPLEMENT

Sodium Tanshinone IIA Sulfonate inhibits canonical transient receptor potential expression in pulmonary arterial smooth muscle from pulmonary hypertensive rats

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Online supplemental materials

METHODS

Hemodynamic measurements and lung histochemistry

Right ventricular pressure and right ventricular hypertrophy were measured in the same method as we described previously (31). Briefly, a 23-gauge needle filled with heparinized saline was connected to a pressure transducer, and inserted via the diaphragm into the right ventricle (RV). The right ventricular systolic pressure (RVSP) was then recorded and measured using BIOPAC MP150 (BIOPAC systems, Inc., Santa Barbara, CA). Right ventricular hypertrophy was evaluated as the wet weight ratio of right ventricle (RV) to left ventricle (LV) with septum (S) and RV to body weight. Intrapulmonary vessels were visualized by H&E staining on formalin-fixed and paraffin-embedded lung cross sections at thickness of 5 μm.

Cell proliferation assessment

PASMCs proliferation was assessed using Amersham Cell Proliferation Biotrak ELISA kit (GEhealthcare, Buckinghamshire, UK). Cells were seeded into 96-well plates at 5×10^3 cells/well and cultured for 24 h in SMGM-2 complete medium. After a further 24-hour culture in SMBM containing 0.3% FBS, the cells were subjected to STS (0~25 μM) treatment for 96 h with or without hypoxia (4% O_2) exposure. The labeling reagent containing 5'-bromo-2'-deoxyuridine (BrdU) was added to the cells for 24 h incubation before collection and subsequent ELISA assay for BrdU incorporation according to the manufacturer's instruction.

Cell migration assay

PASMCs treated with 0~25 μM STS for 60 h under normoxic or hypoxic conditions were trypsinized and loaded on the 8 μm polycarbonate membrane of Transwell Permeable Support (24 mm, Corning Inc., Corning, NY) (1×10^5 cells/well) equilibrated with SMBM containing 0.3% FBS. After 24h culture, cells were fixed in 95% freezing-cold ethanol, followed by staining with Brilliant blue R Staining Solution (Sigma) containing 0.5% (w/v) Brilliant Blue R, 45% ethanol and 10% acetic acid for 5min and washing with PBS. Stained cells were observed under inverted microscope. Five photos were taken at fixed positions of the Transwell membrane: top right and left, bottom right and left and the central field. The cells on these photos were counted and taken as total cells. Then the cells on the upper surface of the filter membrane were gently wiped off with a cotton swab. Pictures for cells on the lower surface of membrane were taken at the same fields as indicated above for total cells and these cells were migrated cells. Ratios of migrated cells to the total cells were calculated to represent the migration rate.

Cytotoxicity assessment

Cytotoxicity of serial doses of STS on rat distal PASMCs was analyzed using CytoTox-Glo Cytotoxicity Assay kit (Promega, Madison, WI) according to the manufacturer's instruction. Briefly, the assay sensitivity was determined by running 2-fold serial dilutions of cells from 1 X 10^4 /well to 1.56 X 10^2 /well in 96-well white walled tissue culture plate, and the signal to noise (S:N) was calculated. Based on the determined the assay sensitivity, PASMCs were plated at 5×10^3 cells/well in 96-well

plates, incubated for 24 h with or without STS (0.1~100μM) under normoxic or hypoxic condition, and subjected to cytotoxicity measurements. Cell viability was calculated as percentage ratios of luminescence for viable cells to total per treatment well.

Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

Coverslips with PASMCs loaded with 7.5 μM Fura-2 (Molecular Probes, Inc., Eugene, OR) for 60 min at 37°C under an atmosphere of 5% CO_2-95% air were mounted in a closed polycarbonate chamber clamped in a heated aluminum platform (PH-2, Warner Instrument Corporation, Hamden, CT) on the stage of a Nikon TSE 100 Ellipse inverted microscope (Melville, NY). The chamber was perfused at 0.5-1 ml/min with Krebs Ringer Bicarbonate Solution (KRBS). The KRBS, which contained (in mM) 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 0.57 $MgSO_4$, 1.18 KH_2PO_4, 25 $NaHCO_3$, and 10 glucose, was equilibrated with 16% O_2-5% CO_2 at 38 °C in heated reservoirs and led via stainless steel tubing and a manifold to an in-line heat exchanger (SF-28, Warner Instrument Corporation, Hamden, CT) before it entered the cell chamber. A dual channel heater controller (TC-344B, Warner Instrument Corporation, Hamden, CT) was connected to the heat exchanger to control its temperature. This system maintained temperature at 37°C and oxygen tension at 112 ± 2.0 mmHg at the coverslip. The cells were perfused for 10 min to remove extracellular dye, and ratiometric measurement of Fura-2 fluorescence was performed at 12-30 sec intervals using a collimated light beam from a xenon arc lamp filtered by interference filters at 340 and 380 nm and focused onto PASMCs visualized with a

20x fluorescence objective (Super Fluor 20, Nikon, Torrance, CA). Light emitted from the cells at 510 nm was returned through the objective and detected by a cooled CCD imaging camera. An electronic shutter (Vincent Associates, Rochester, NY) was used to minimize photobleaching. Protocols were executed and data collected on-line with InCyte software (Intracellular Imaging Inc, Cincinnati, OH). $[Ca^{2+}]_i$ was estimated by linear regression from a calibration curve created by measuring Fura-2 fluorescence in calibration solutions with $[Ca^{2+}]$ ranging from 0 to 1350 nM.

As we previously described (27), to assess SOCE, we perfused PASMCs for at least 10 min with Ca^{2+}-free KRBS containing 5 μM nifedipine to prevent calcium entry through L-type voltage-dependent Ca^{2+} channels (VDCC) and 10 μM cyclopiazonic acid (CPA) to deplete SR calcium stores. Next, we measured $[Ca^{2+}]_i$ at 30 sec intervals before and after restoration of extracellular $[Ca^{2+}]$ to 2.5 mM, and SOCE was evaluated from the increase in $[Ca^{2+}]_i$ caused by restoration of extracellular $[Ca^{2+}]_i$ in the continued presence of CPA and nifedipine. In these experiments, KRBS perfusate also contained 1 mM ethyleneglycol-bis (beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) to chelate any residual Ca^{2+}. Additionally, we monitored Fura-2 fluorescence excited at 360 nm at 30 sec intervals before and after addition of $MnCl_2$ (200 μM) to the cell perfusate, and SOCE was evaluated from the rate at which Fura-2 fluorescence was quenched by Mn^{2+}, which enters the cell as a Ca^{2+} surrogate and reduces Fura-2 fluorescence upon binding to the dye. Fluorescence excited at 360 nm is the same for Ca^{2+}-bound and Ca^{2+}-free Fura-2; therefore, changes in fluorescence can be assumed to be caused by

Mn^{2+} alone. In the experiment evaluating the effect of membrane poteintial change on Mn^{2+} quenching, NaCl was replaced with equimolar KCl in the perfusate with final concentration of KCl at 122.7 mM.

Small interference RNA (siRNA) treatment

The targeted sequence and treatment of siRNA specific for TRPC1 (siT1) or TRPC6 (siT6) were described previously (33). Briefly, PASMCs at about 50~60% confluence were transfected with 25 nM siGENOME SMARTpool (Dharmacon, Lafayette, CO) of non-targeting control siRNA (siNT), siT1 or siT6 using GeneSilencer (Genlantis, San Diego, CA) as carrier for 6h in serum-free SMBM. Serum was then added to the culture to a final concentration of 0.3%, and cells were subjected to hypoxia (4% O_2) or normoxia exposure for 60 h before proliferation and migration assay.

Real-time quantitative polymerase chain reaction

Total RNA was extracted using TRIzol method for de-endothelialized distal PA, or using RNeasy kit (Qiagen, Valencia, CA) for PASMCs, as previously described (27, 29, 31). DNA contamination in RNA preparations was removed by on-column DNase digestion using RNeasy column and RNase-free DNase (Qiagen).

Reverse transcription was performed using TaKaRa RT kit (Takara, Dalian, China) with reaction mixture containing 1 μg total RNA in a 20 μl volume. cDNA of rat TRPC1, TRPC6 or 18s were amplified by real-time quantitative PCR (qPCR) using Scofast^{TM} EvaGreen SuperMix (Bio-Rad, Carlsbad, CA) in a CFX96^{TM} real-time system (Bio-Rad). The PCR protocol consisted of initial enzyme activation

at 95°C for 3 min, followed by 40 cycles at 95°C for 5 s and at 60°C for 15 s. (31). Primer sequences in the measurements were 5'- AGCCTCTTGACAAACGAGGA-3' (sense) and 5'-ACCTGACATCTGTCCGAACC-3' (antisense) for TRPC1; 5'-TACTGGTGTGCTCCTTGCAG-3' (sense) and 5'-GAGCTTGGTGCCTTCAAATC-3' (antisense) for TRPC6; and 5'-GCAATTATTCCCCATGAACG-3' (sense) and 5'-GGCCTCACTAAACCATCCAA-3' (antisense) for 18s. Relative concentration of each transcript was calculated using the Pfaffl method (62). Efficiency for each gene was determined from 5-point serial dilutions of an unknown cDNA sample (PA or PASMCs). The expression of TRPC1 and TRPC6 were normalized to 18s in the same sample.

Western Blotting

De-endothelialized PA or PASMCs samples were homogenized by sonication in Laemmlie sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% protease inhibitor cocktail, 1 mM EDTA, and 200 μM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride. Total protein concentration in the homogenates was determined by the Dc method (Bio-Rad) using bovine serum albumin as a standard. Homogenates were denatured by adding dithiothreitol to 150 mM and heating at 95 °C for 3 min. Homogenate proteins were resolved by 10% SDS-PAGE calibrated with prestained protein molecular weight markers (Precision Plus, Bio-Rad). Separated proteins were transferred to polyvinylidene difluoride membranes (pore size 0.45 μM, Bio-Rad). Membranes were

blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.2% Tween 20, and blotted with affinity-purified rabbit polyclonal antibodies specific for TRPC1 (Sigma, St. Louis, MO), TRPC6 (Alomone Laboratories, Jerusalem, Israel), PKC-α (Cell Signaling Technology, Inc., Denvers, MA) or mouse monoclonal antibodies against α-actin (Sigma) and HIF-1α (Clone H1a67; Novus Biologicals Inc., Littleton, CO). The membranes were then washed for 15 min 3 times and incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Bound antibodies were detected using ECL HRP Chemiluminescent substrate reagent (Bio-Rad).

Drugs and materials

Unless otherwise specified, all reagents were obtained from Sigma-Aldrich. Stock solutions (30 mM) of CPA and nifedipine were made in pluronic dimethyl sulfoxide (DMSO, Invitrogen). Fura-2 AM (Invitrogen) was prepared on the day of the experiment as a 2.5 mM stock solution in DMSO.

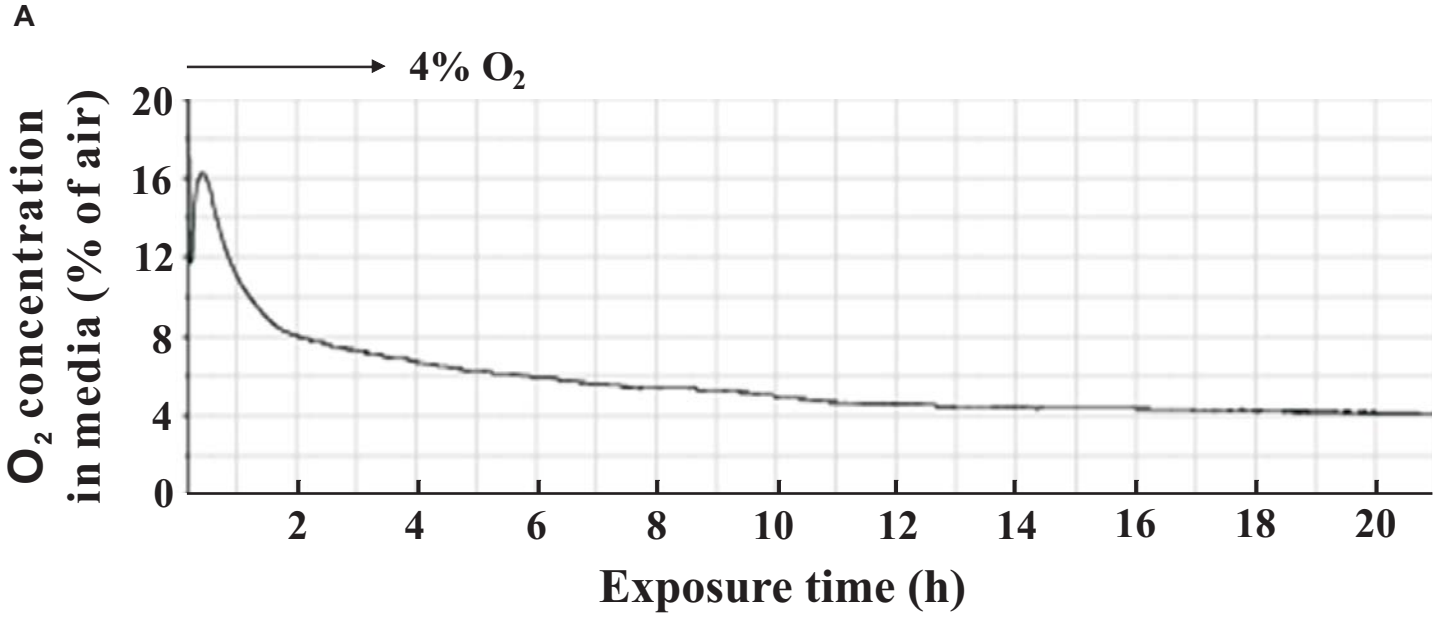
SUPPLEMENTAL FIGURES

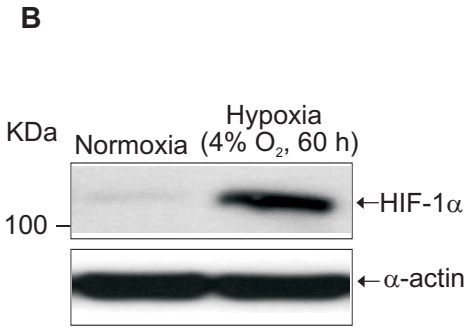
Supplemental figure 1: Media oxygen level change and HIF-1α expression in PASMCs culture during prolonged hypoxia exposure (4% O_2 for 60h). (**A**): Representative curve showing the time-course change of O_2 level in PASMCs culture media post exposure to 4% O_2. (**B**) and (**C**): Western blotting demonstrating that HIF-1α protein was greatly accumulated in cells exposed to prolonged hypoxia comparing to normoxia control cells. Data are means ± SEM; n = 3, * indicates $p<0.05$ versus normoxia.

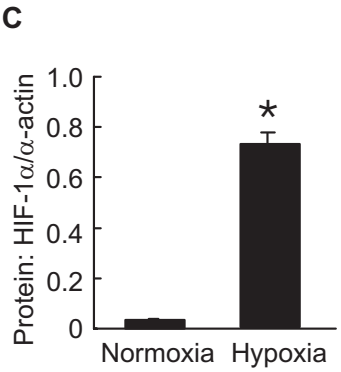
Supplemental figure 2: Treatment of TRPC1 siRNA (siT1) and TRPC6 siRNA (siT6) attenuated prolonged hypoxia-induced proliferation and migration of PASMCs. (A), (B) : Proliferation (A) and migration (B) of cells treated with non-targeting control siRNA (siNT) + Normoxia, siNT + Hypoxia, siT1 + hypoxia, siT6 + hypoxia. Data are means ± SEM, n = 3 in each group. * $p<0.05$ versus siNT + normoxia, and ** $p<0.05$ versus siNT + hypoxia.

Supplemental figure 3: STS inhibited the upregulation of PKC-α protein in PASMCs exposed to prolonged hypoxia. (A): Representative western blots of PKC-α and α-actin in cells exposed to prolonged hypoxia exposure (4% O_2 for 60h). (B): Mean intensity of PKC-α and α-actin blots relative to α-actin. Bar values are mean ± SEM, n = 3 in each group. * $p<0.05$ versus normoxia control, and ** $p<0.05$ versus hypoxia control.

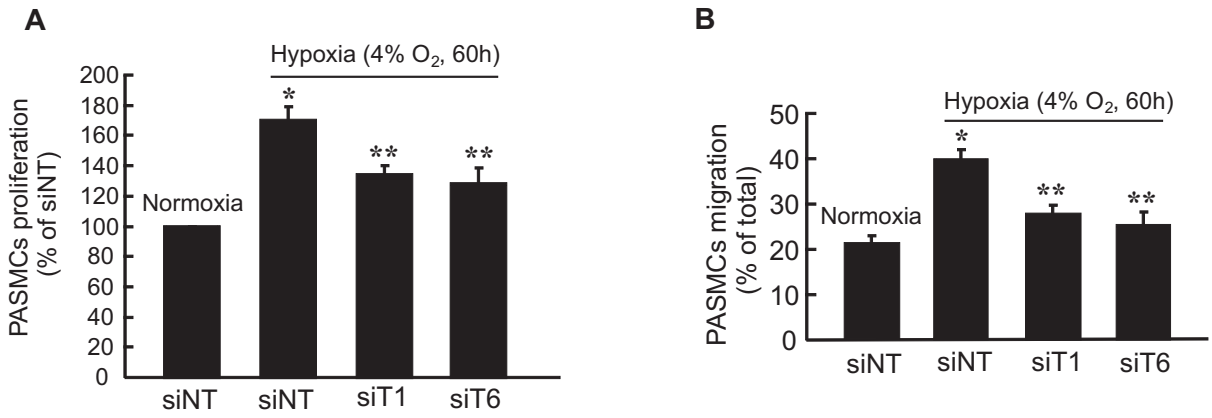
Supplemental figure 4: The chemical structure of sodium tanshinone IIA sulphonate.







Supplemental figure 1



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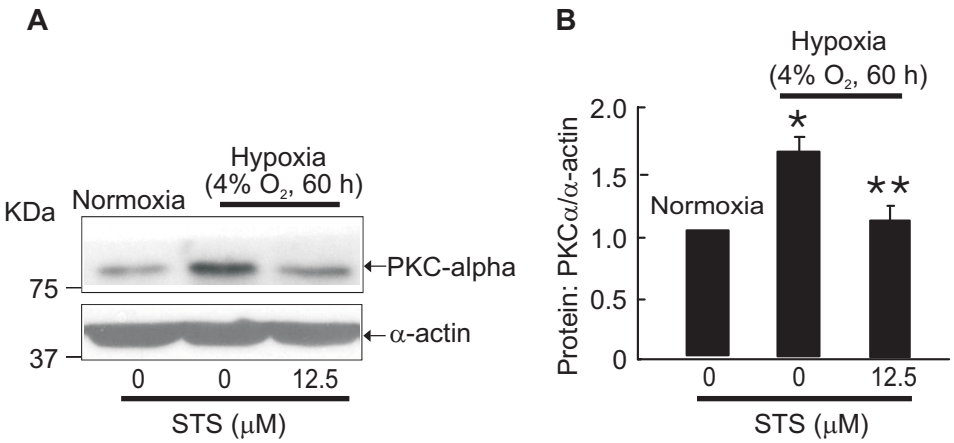

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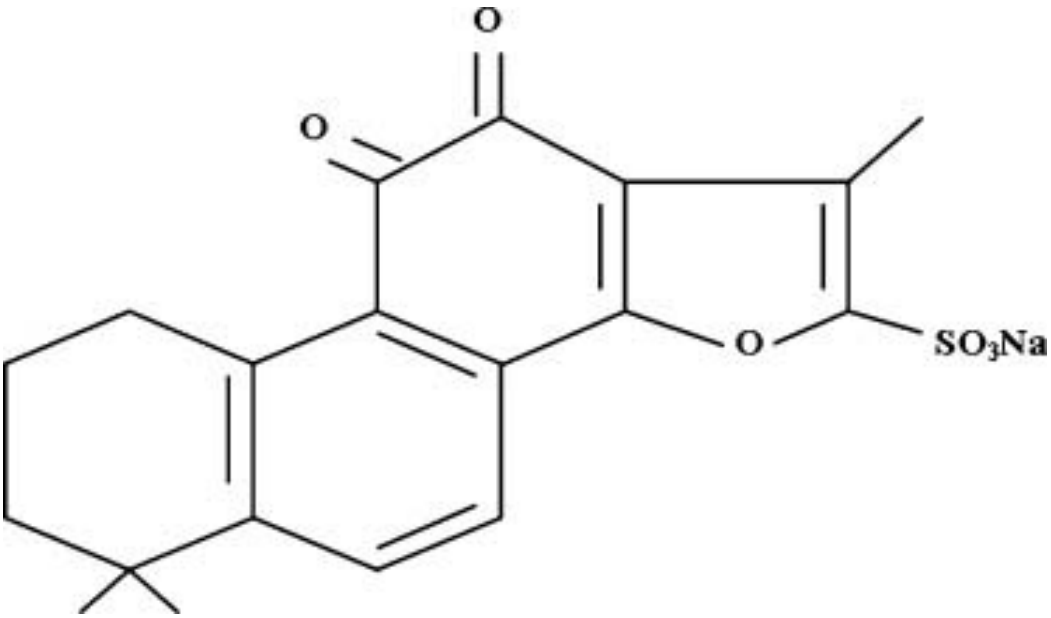

Supplemental figure 4