Arteriosclerosis, Thrombosis, and Vascular Biology

TRANSLATIONAL SCIENCES

Identification of Emetine as a Therapeutic Agent for Pulmonary Arterial Hypertension

Novel Effects of an Old Drug

Mohammad Abdul Hai Siddique, Kimio Satoh, Ryo Kurosawa, Nobuhiro Kikuchi, Md. Elias-Al-Mamun, Junichi Omura, Taijyu Satoh, Masamichi Nogi, Shinichiro Sunamura, Satoshi Miyata, Hirofumi Ueda, Hidetoshi Tokuyama, Hiroaki Shimokawa

OBJECTIVE: Excessive proliferation and apoptosis resistance are special characteristics of pulmonary artery smooth muscle cells (PASMCs) in pulmonary arterial hypertension (PAH). However, the drugs in clinical use for PAH target vascular dilatation, which do not exert adequate effects in patients with advanced PAH. Here, we report a novel therapeutic effect of emetine, a principal alkaloid extracted from the root of ipecac clinically used as an emetic and antiprotozoal drug.

APPROACH AND RESULTS: We performed stepwise screenings for 5562 compounds from original library. First, we performed high-throughput screening with PASMCs from patients with PAH (PAH-PASMCs) and found 80 compounds that effectively inhibited proliferation. Second, we performed the repeatability and counter assay. Finally, we performed a concentration-dependent assay and found that emetine inhibits PAH-PASMC proliferation. Interestingly, emetine significantly reduced protein levels of HIFs (hypoxia-inducible factors; HIF-1α and HIF-2α) and downstream PDK1 (pyruvate dehydrogenase kinase 1). Moreover, emetine significantly reduced the protein levels of RhoA (Ras homolog gene family, member A), Rho-kinases (ROCK1 and ROCK2 [rho-associated coiled-coil containing protein kinases 1 and 2]), and their downstream CyPA (cyclophilin A), and Bsg (basigin) in PAH-PASMCs. Consistently, emetine treatment significantly reduced the secretion of cytokines/chemokines and growth factors from PAH-PASMCs. Interestingly, emetine reduced protein levels of BRD4 (bromodomain-containing protein 4) and downstream survivin, both of which are involved in many cellular functions, such as cell cycle, apoptosis, and inflammation. Finally, emetine treatment ameliorated pulmonary hypertension in 2 experimental rat models, accompanied by reduced inflammatory changes in the lungs and recovered right ventricular functions.

CONCLUSIONS: Emetine is an old but novel drug for PAH that reduces excessive proliferation of PAH-PASMCs and improves right ventricular functions.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: emetine ■ hypertension ■ mitochondria ■ pulmonary artery ■ reactive oxygen species

The pathogenesis of pulmonary arterial hypertension (PAH) includes the thickening of intima, media, and adventitia, with abundant inflammatory cells.[1] Excessive proliferation and apoptosis resistance of pulmonary artery (PA) smooth muscle cells (PASMCs) are unique characteristics of PAH.[2] The elevation of circulating pro-inflammatory cytokines, such as TNF (tumor necrosis factor)-α, IL (interleukin)-1β, and IL-6 in PAH patients contributes to the generation of reactive oxygen species (ROS) in pulmonary vasculature, leading to enhanced oxidative stress.[3] We previously demonstrated that oxidative stress induces the secretion of CyPA (cyclophilin A) from PAH-PASMCs.[4] CyPA and its receptor Bsg (basigin) promote the secretion of cytokines/chemokines and growth factors from PAH-PASMCs and augment inflammatory cell migration and PASMC proliferation.[4] Additionally, we

Correspondence to: Hiroaki Shimokawa, MD, PhD, Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku Sendai 980-8574, Japan. Email shimo@cardio.med.tohoku.ac.jp

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Nonstandard Abbreviations and Acronyms

α-SMA	α-smooth muscle actin
BRD4	bromodomain-containing protein 4
Bsg	basigin
CoA	coenzyme A
CyPA	cyclophilin A
FGF2	fibroblast growth factor-2
HIF	hypoxia-inducible factor
HSP90	heat shock protein 90
IL	interleukin
MMPs	matrix metalloproteinases
mPAP	mean pulmonary artery pressure
PAH	pulmonary arterial hypertension
p-Akt	phosphorylated protein kinase B
PASMC	pulmonary artery smooth muscle cells
PCNA	proliferating cell nuclear antigen
PDGF-BB	platelet-derived growth factor BB
PDH	pyruvate dehydrogenase
PDK1	pyruvate dehydrogenase kinase 1
p-ERK1/2	phosphorylated extracellular signal-regulated protein kinases 1 and 2
p-MBS	phospho-myosin-binding subunit
RhoA	ras homolog gene family, member A
ROCK	rho-associated coiled-coil containing protein kinase
ROS	reactive oxygen species
RVSP	right ventricular systolic pressure
t-Akt	total protein kinase B
TGF	transforming growth factor
t-MBS	total myosin-binding subunit
TNFα	tumor necrosis factor-α
VEGF	vascular endothelial growth factor

demonstrated that Rho-kinase (ROCK1 and ROCK2 [Rho-associated coiled-coil containing protein kinase 1 and 2]), especially ROCK2, is substantially involved in the pathogenesis of PAH and pulmonary hypertension (PH) due to left heart diseases.[5–8] Interestingly, we found that CyPA is secreted in response to Rho-kinase activation.[9–11] Importantly, plasma levels of CyPA were significantly elevated in PAH patients compared with healthy controls.[4] Thus, the Rho-kinase/CyPA/Bsg signaling pathway in PAH-PASMCs can be a therapeutic target of PAH.

Mitochondrial dysfunctions have been reported in the pathogenesis of PAH, leading to proliferation, inflammation, and apoptosis resistance in PAH-PASMCs.[2,12] Mitochondrial oxidative phosphorylation is switched to glycolysis for alternative sources of energy production in PAH-PASMCs, which is known as Warburg effect.[2] Hyperproliferative PAH-PASMCs show enhanced expression of HIF (hypoxia-inducible factor)-1α even under normoxia, which is a key player of mitochondrial dysfunction.[13] Additionally, apoptosis resistance is another character of PAH-PASMCs,[14,15] like cancer cells with excessive proliferation.[16] These abnormal phenotypes of proliferation and apoptosis resistance in PAH-PASMCs are major hallmarks of PAH, which can be a target for the development of novel drugs.

Highlights

- Emetine has been identified as a novel drug that inhibits the proliferation of pulmonary arterial hypertension–pulmonary artery smooth muscle cells in a dose-dependent manner with little effects on control pulmonary artery smooth muscle cells with anti-inflammatory effects.
- Consistent with the results of emetine-mediated inhibitory effects on pulmonary arterial hypertension–pulmonary artery smooth muscle cell proliferation with anti-inflammatory effects in vitro, emetine successfully ameliorated monocrotaline-induced and Sugen/hypoxia–induced pulmonary hypertension in rats with anti-inflammatory effects.
- Based on the results in vivo and in vitro, emetine could be regarded as a promising drug for the treatment of pulmonary arterial hypertension patients with right ventricular failure.

Emetine is a principal alkaloid used as an emetic and antiprotozoal, which is extracted from the root of ipecac, a small plant in the tropical rain forest of Brazil.[17] Emetine has been used as an anticancer and antiviral drug with no side effects.[18–20] In the present study, we screened the original library of Tohoku University using a high-throughput screening system and discovered that emetine inhibits its PAH-PASMC proliferation with anti-inflammatory effects, leading to the recovery of mitochondrial function and amelioration of PH in rat models with different mechanisms. Our data suggest that emetine, an old emetic and antiprotozoal drug, could be a novel therapeutic agent for the treatment of PAH and can be translated into clinical use smoothly as it is already used in humans.

MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Approval

All protocols using human specimens were approved by the Institutional Review Board of Tohoku University, Sendai, Japan (No. 2013-1-160). All animal experiments were performed in accordance with the protocols approved by the Tohoku University Animal Care and Use Committee (No. 2015-Kodo-007) based on the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guideline.

Human Lung Samples

Lung tissues were obtained from patients with idiopathic pulmonary arterial hypertension at the time of lung transplantation or from control patients at the time of thoracic surgery for lung cancer at a site far from the tumor margins. Before surgery, all patients provided written informed consent for the use of their lung tissues for the present study. For ex vivo culture, fresh lung samples obtained during surgery were minced into ≈200 mg blocks. We maintained an equal wet weight of minced tissue in each well of 6-well plates with DMEM.

Isolation of PASMCs From PAH Patients

Human small PAs were obtained at the time of lung transplantation from PAH female patients. PAH-PASMCs were isolated from PAs <1.5 mm in outer diameter as previously described.[4] PASMCs were cultured in DMEM containing 10% FBS at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. PASMCs of passages 4 to 8 at 70% to 80% confluence were used for experiments.

High-Throughput Screening

We used the Tohoku University original libraries with 5562 unique compounds in the Drug Discovery Initiative in Japan. This compound library is an in-house library, which is owned by 6 research groups in the area of Natural Products and Synthetic Organic Chemistry, Tohoku University Graduate School of Pharmaceutical Sciences. The library collects unique compounds, including naturally occurring compounds and synthetic compounds.[21] Types of compounds included in the library are heterocyclic compounds, alkaloids, cyclic peptides, flavones, macrolides, and bioactive natural products and their synthetic intermediates. Structural features and content ratios in the library are as follows; 48% of heterocyclic compounds, 34% of compounds with stereocenters, 6% of natural products and their analogs, 12% of compounds with bridging structure, and 3% with spirocyclic structures. PAH-PASMCs were used for the first (proliferation assay) and second (repeatability and concentration-dependent assays) screenings, and control PASMCs were used for counter assay (proliferation assay). We optimized screening conditions (cell number, time-course of plating cells, and adding stimulus) beforehand. PAH-PASMCs were grown in DMEM with 10% FBS up to 80% confluency, which were plated at 1000 cells/45 µL mediums in each well of a 384-well plate (Greiner Bio-One, Austria) using the Multidrop Combi (Thermo Fisher Scientific, Waltham, MA). They were then placed in the automated incubator at 37°C for 24 hours. Diluted compounds (final concentration, 5 µmol/L) were added to columns of every plate by the Biomek NX^P (Beckman Coulter, Brea, CA). The plates were incubated for additional 48 hours and evaluated by CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) and the SpectraMax Paradigm (Molecular Devices). The intraplate and interplate variability showed a coefficient of variance of 5.9% and 4.0%, respectively.

Cell Proliferation Assay

For the assessment of cell proliferation, PASMCs were seeded in 96-well plates (3000 cells per well) in DMEM with 10% FBS and were incubated with compounds at each concentration for 48 hours. Cell proliferation was measured by the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI).[22]

Measurements of ROS

PAH-PASMCs were plated on 96-well plate in 3000 cells per well concentration maintained in DMEM containing 10% FBS at 37°C in a humidified atmosphere with 5% CO_2. PAH-PASMCs were allowed to adhere for 24 hours and treated with vehicle or emetine (1 and 5 µmol/L, Tokyo Chemical Industry, Co, Ltd., Tokyo, Japan) for 24 hours. After the treatment, 2,7-dichlorofluorescein diacetate (final concentration 10 µmol/L, Sigma-Aldrich), CellROX Deep Red (final concentration 5 µmol/L, Thermo Fisher Scientific), or MitoSOX Deep Red Reagent, for oxidative stress detection (final concentration 5 µmol/L, Thermo Fisher Scientific), were added to the culture medium according to manufacturer's instruction. After the incubation for 30 minutes at 37°C, cells were washed twice with warmed PBS, and the fluorescence intensity was measured with SpectraMax i3 (Molecular Devices, Sunnyvale, CA).

Animal Experiments

We used 2 rat models of PH. Six-week-old male Sprague-Dawley rats (160–200 g, Charles River, Wilmington, St Massachusetts) were housed in room air (21% O_2) under a 12-hour light and dark cycle. To examine the development of PH, we measured right ventricular (RV) systolic pressure (RVSP), RV hypertrophy, and pulmonary vascular remodeling.[4,23] For right heart catheterization, a 1.2F pressure catheter (SciSense, Inc, Ontario, Canada) was inserted in the right jugular vein and advanced into the RV to measure RVSP.[7] All data were analyzed using the Power Lab data acquisition system (AD Instruments, Bella Vista, Australia) and were averaged over 10 sequential beats.[4] We followed up the protocols used in the previous studies.[24,25] For the monocrotaline-induced PH model, animals were allowed 1 week to adjust to the new environment. Then, rats were subcutaneously injected with monocrotaline (60 mg/kg; Sigma) and housed under climate-controlled conditions on a 12:12-hour light-dark cycle with access to normal laboratory diet and water. Monocrotaline (Sigma-Aldrich, Co, St. Louis, MO) was dissolved in 1 N HCl, and the pH was adjusted to 7.4 with 1 N NaOH. The solution was administered as a single subcutaneous injection (60 mg/kg) in a volume of 3 mL/kg. Control, age-matched rats received an equal volume of isotonic saline. The animals were treated with emetine or vehicle for 3 weeks. Next, in Sugen/hypoxia model, rats were injected subcutaneously with the VEGF (vascular endothelial growth factor)-receptor inhibitor SU5416 (Sigma-Aldrich, St Louis, MO; 20 mg/kg body weight) under isoflurane anesthesia and were then exposed to hypoxia (10% O_2) for 3 weeks followed by normoxia for 6 weeks, and then the animals were treated with emetine (0.05 mg/kg per day per os) or vehicle for 4 weeks.

Measurement of Blood Pressure

Blood pressure at baseline was measured by the tail-cuff system (MK-2000ST NP-NIBP Monitor; Muromachi Kikai Co, Ltd, Tokyo, Japan) without anesthesia. We recorded blood pressure 3× per rat in the morning before giving anesthesia under 2% isoflurane for invasive hemodynamic measurements.

Assessment of RV Hypertrophy

After RV pressure was recorded, the animals were exsanguinated, and the hearts were isolated and fixed with 10% formalin. Formaldehyde-fixed dry hearts were dissected, and the RV wall was removed from the left ventricle (LV) and septum. The ratio of the RV to the LV plus septum weight was calculated to determine the extent of RV hypertrophy.

Treadmill Exercise Test

Exercise capacity was evaluated by measuring maximal walking distance on a motor-driven treadmill (MK-680; Muromachi Kikai Co, Ltd, Tokyo, Japan). The initial treadmill speed was 5 m/min and gradually increased by 5 m/min in a stepwise manner every 5 minutes up to 30 m/min for rats and continued until the animals fatigued.[26]

Echocardiography

Echocardiography was performed using a Vevo 2100 (Visualsonics, Toronto, Canada) under inhalation of isoflurane (1.0%–1.5% v/v). Rats or mice were laid in a supine position on a heating platform with all legs taped to ECG electrodes for heart rate monitoring. The chest of each rat or mouse was shaved and treated with a chemical hair remover to reduce ultrasound attenuation. To provide a coupling medium for the transducer, a prewarmed ultrasound gel was spread over the chest wall. Transthoracic echocardiography was performed with a Vevo 2100 high-resolution imaging system. RV internal diameter was measured as the maximal distance from the RV free wall to the septum using the apical 4-chamber view. To determine tricuspid annular plane systolic excursion, the M-mode cursor was oriented to the junction of the tricuspid valve plane and the RV free wall using the apical 4-chamber view. PA diameter was measured at the level of the pulmonary outflow tract during midsystole using the superior angulation of the parasternal short-axis view. LV ejection fraction was measured at the parasternal long-axis view, the LV area was traced during end-diastole and end-systole, and the ejection fraction was calculated with the following formula: LV ejection fraction (%)=(LVEDV [left ventricular end-diastolic volume]–LVESV [left ventricular end-systolic volume])/LVEDV×100. Pulsed-wave Doppler was used to measure PA acceleration time and the PA flow velocity time integral. PA acceleration time was measured from the pulsed-wave Doppler flow velocity profile of the RV outflow tract in the parasternal short-axis view and was defined as the interval from the onset to the maximal velocity of forward flow.[27]

Histological Analysis

After hemodynamic measurements, the lungs were perfused with cold PBS and perfusion-fixed by 10% formaldehyde solution for 24 hours. The whole lungs were embedded in paraffin, and cross-sections (3 μm) were prepared. Paraffin sections were stained with Elastica-Masson. For the assessment of pulmonary vascular remodeling, PAs adjacent to an airway distal to the respiratory bronchiole were evaluated as reported.[28] Briefly, PAs were considered fully muscularized when they had a distinct double elastic lamina visible throughout the diameter of distinct double elastic lamina visible for at least half the diameter. The percentage of vessels with double elastic lamina was calculated as the number of muscularized vessels per total number of vessels counted. In each section, a total of 60 vessels were examined by use of a computer-assisted imaging system (BX51, Olympus, Tokyo, Japan). This analysis was performed for the small vessels with external diameters of 20 to 75 μm. For the assessment of pulmonary vascular remodeling in rats, we used medial wall thickness of PAs with an external diameter of 50 to 100 μm. Medial wall thickness was expressed as follows; medial wall thickness=([medial thickness×2]/external diameter)×100.[24] More than 30 PAs per animal were measured.

Measurement of Cytokines/Chemokines and Growth Factors

Protein levels of cytokines/chemokines and growth factors in the conditioned medium (CM) or the lungs were measured with a Bioplex system (Bio-Rad, Tokyo, Japan) according to the manufacturer's instructions. We measured cytokines/chemokines and growth factors in CM from PAH-PASMCs. For the preparation of CM, we treated human PASMCs in 10 cm dishes with 2 μmol/L emetine for 24 hours. After the incubation period, the medium was collected as CM from PAH-PASMCs in DMEM and filtered to remove cell debris.[4] Collected medium was concentrated 100-fold with an Amicon Ultra filter (Millipore Corporation) to yield concentrated CM.

To analyze the levels of cytokines/chemokines in serum samples from Sugen/hypoxia–induced PH rats, we collected whole blood. Whole blood was centrifuged (4°C, 2000 rpm, 15 minutes), and thereafter, clear supernatants were standardized for total protein content using the BCA protein assay kit (Pierce, Rockford).[28] Human cytokines/chemokines and growth factors were measured with commercially available kits (27-Plex, no. M50- 0KCAF0Y and 21-Plex, no. MF0-005KMII; Bio-Rad). Rat cytokines/chemokines and growth factors were measured with commercially available kits (23-Plex, no. 12005641; Bio-Rad). Each experiment was performed in duplicate.

Harvest of PASMCs From Sugen/Hypoxia–Induced PAH Rats

The lungs of Sugen/hypoxia–induced PH in male Sprague-Dawley rats were perfused with cold PBS and subsequently removed. The lungs were minced in small pieces and incubated with collagenase type 2 for 30 minutes in a 37°C water bath. The suspension was filtered through 70-μm cell strainers and then centrifuged at 400*g* for 5 minutes at 4°C. After removal of the supernatant, the cell pellet was resuspended and cultured in DMEM containing 10% FBS at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cultured PASMCs were confirmed the expression of SM22α. Rat-PASMCs of passage 4 to 7 at 70% to 80% confluence were used for experiments.

Western Blotting

Human PASMCs were seeded in 100-mm dishes in DMEM with 10% FBS. PASMCs were treated with emetine (2 μmol/L) or vehicle for 24 hours. These cells were washed with cold PBS and lysed with cell lysis buffer (Cell Signaling) and protease inhibitor cocktail (Sigma-Aldrich) after the incubation period. Total cell lysates from lung homogenates and human PASMCs were loaded on the SDS-PAGE and transferred to

polyvinylidene difluoride (PVDF) membranes (GE Healthcare), following blocking for 1 hour at room temperature with 5% bovine serum albumin in tris-buffered saline with Tween 20.[29] The primary antibodies used were as follows: proliferating cell nuclear antigen (1 µg/mL, Santa Cruz Biotechnology, Santa Cruz), p-Akt (phosphorylated protein kinase B; 1 µg/mL; Cell Signaling), t-Akt (total protein kinase B; 1 µg/mL; Cell Signaling), p-ERK1/2 (phosphorylated extracellular signal-regulated protein kinases 1 and 2; 1 µg/mL; Cell Signaling), t-ERK1/2 (total extracellular signal-regulated protein kinases 1 and 2; 1 µg/mL; Cell Signaling), cyclophilin A (1 µg/mL, BML-SA296-0100; Enzo), Bsg (1 µg/mL, AF772; R&D systems), ROCK1 (1 µg/mL; Cell Signaling), ROCK2 (1 µg/mL; BD Biosciences), RhoA (Ras homolog gene family, member A; 1 µg/mL; Cell Signaling), HSP90 (heat shock protein 90; 1 µg/mL; BD Biosciences), BRD4 (bromodomain-containing protein 4; 2 µg/mL; Abcam), PDGF-BB (platelet-derived growth factor BB; 1 µg/mL; AF-220-NA; R&D systems), FGF2 (fibroblast growth factor-2; 1 µg/mL; Santa Cruz Biotechnology, Santa Cruz), HIF-1α (2 µg/mL; Novus Biologicals, Littleton, CO), HIF-2α (2 µg/mL; Novus Biologicals, Littleton, CO), PDK1 (pyruvate dehydrogenase kinase 1; 1 µg/mL, Enzo Life Sciences, Farmingdale, NY), Survivin (1 µg/mL, Cell Signaling), and GAPDH (1 µg/mL, Cell Signaling). Proteins were visualized by the enhanced chemiluminescence system (ECL Western Blotting Detection Kit, GE Healthcare) as previously described.[29] Densitometric analysis was performed with Image J Software (National Institutes of Health, Bethesda).

Statistical Analyses

All results are shown as mean±SEM. Comparisons of means between 2 groups were performed by unpaired Student *t* test and 2-way ANOVA to check statistical significance for group comparisons. Comparisons of mean responses associated with some main effects of the different treatments and the severity of pulmonary vascular remodeling were performed by ANOVA with interaction terms, followed by Tukey honestly significant difference or Dunnett post hoc test for multiple comparisons, as appropriate. The normality assumption was tested by Shapiro-Wilk Normality test, and the equal variance assumption was tested by Bartlett test of homogeneity of variances. Once it failed to pass these tests to confirm the model assumptions, we applied Box-Cox transformation onto the response variables to improve the normality and to stabilize the variance. Statistical significance was evaluated with JMP 12 (SAS Institute, Inc, Cary, America) or R version 3.3.2 (http://www.R-project.org/). All reported *P* values are 2-tailed, with a *P* value of <0.05 indicating statistical significance.[4]

RESULTS

Identification of Emetine by High-Throughput Screening

The Drug Discovery Initiative was founded in Japan as a hub of the national collaborative research network for drug discovery, which provides consultation, technical assistance, and public chemical samples to researchers who begin chemical screening (http://www.ddi.u-tokyo.ac.jp/en/).[30,31] Tohoku University, one of the screening and library institutes of the Drug Discovery Initiative, has a unique library and automated machines to perform high-throughput screening (http://www.pford.med.tohoku.ac.jp/index.html). Here, to discover a novel drug for PAH patients, we used the screening system of the Drug Discovery Initiative with 5562 original compounds and derivatives in the original chemical library of Tohoku University. For the screening procedure, we established cell libraries of primary cultured PAH-PASMCs from 3 patients undergoing lung transplantation and evaluated their inhibitory effects on cell proliferation after treatment with each compound (Figure 1A, Figure I in the online-only Data Supplement). In the first screening, PAH-PASMCs were incubated with each compound in 384-well plates for 24 hours. Among the compounds, we initially selected 80 that effectively inhibited PAH-PASMC proliferation (Figure 1C). In the second screening, we performed repeatability assays and counter assays for the 80 compounds. Finally, based on the information on clinical use, we selected emetine as it inhibited PAH-PASMC proliferation with minimal effects on control PASMCs from healthy donors (Figure 1D). Importantly, several clinical studies demonstrated that emetine is a single promising agent to treat cancer cells, such as ovarian carcinoma, B-cell lymphoma, bladder cancers without side effects in humans.[32–34] Thus, emetine is a novel drug that inhibits PAH-PASMC proliferation in a dose-dependent manner without harmful effects on normal PASMCs.

Emetine Reverses the Imbalance Between Proliferation and Apoptosis in PASMCs

It has already been shown that PASMCs from patients with PAH (PAH-PASMCs) possess an imbalance between proliferation and apoptosis, which is regulated by several signaling pathways.[35] Here, we found that emetine treatment showed antiproliferative effects in both PAH-PASMCs and control PASMCs (Figure 2A). Interestingly, emetine has strong antiproliferative effects with low-doses in PAH-PASMCs compared with control PASMCs. Additionally, emetine treatment significantly reduced the proliferation of PASMCs harvested from rats (Rat-PASMCs) in a concentration-dependent manner (Figure 2A). Importantly, emetine treatment significantly reduced the activities of ERK1/2 and Akt in PAH-PASMCs compared with vehicle controls (Figure 2B). Consistently, emetine treatment significantly reduced the protein levels of proliferating cell nuclear antigen (explain in the first use) in PAH-PASMCs compared with vehicle controls (Figure 2B). Additionally, PAH-PASMCs showed higher levels of α-SMA (α-smooth muscle actin), TGF (transforming growth factor)-β1, calponin, and p-Smad 2/3 (phospho-Smad) and lower levels of p-Smad 1/5 in PAH-PASMCs compared with control PASMCs (Figure II in the online-only Data Supplement). Moreover, we

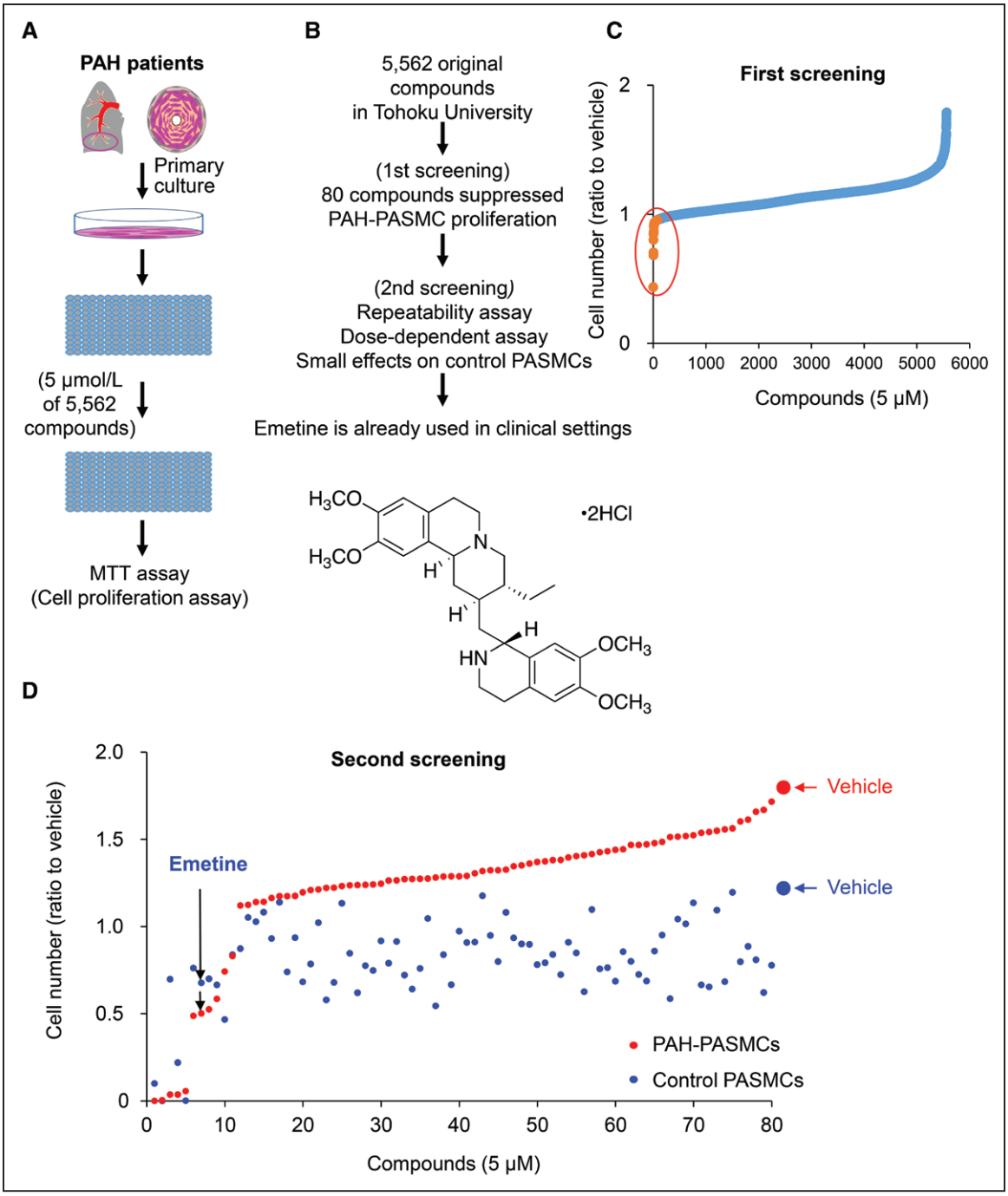

Figure 1. Identification of emetine by high-throughput screening.
A, The schema of the primary culture of pulmonary artery smooth muscle cells (PASMCs) from patients with pulmonary arterial hypertension (PAH; PAH-PASMCs) and screening of the Tohoku University Compound Library (5562 compounds). **B**, Schematic outline of high-throughput screening to identify emetine that inhibit PAH-PASMC proliferation with minimal harmful effects. **C**, Results of the first screening of 5562 compounds. The ratio of cell numbers after treatment with 5562 compounds (5 μM) for 24 h compared with day 0. Cell numbers were measured by 3-(4,5-di-methylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay. **D**, Results of the second screening of 80 compounds. The ratio of cell numbers of PAH-PASMCs or PASMCs from healthy donors (control PASMCs) after treatment with 80 compounds (5 μM) for 48 h compared with day 0. Cell numbers were measured by MTT assay.

found that PAH-PASMCs showed higher levels of RhoA and Rho-kinase activity p-MBS (phospho-myosin-binding subunit)/t-MBS (total myosin-binding subunit) compared with control PASMCs. Again, emetine treatment significantly reduced levels of RhoA and Rho-kinase activities in both cell lines compared with vehicle controls (Figure 2C). In contrast, emetine treatment significantly increased apoptosis assessed by TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay in PAH-PASMCs compared with vehicle

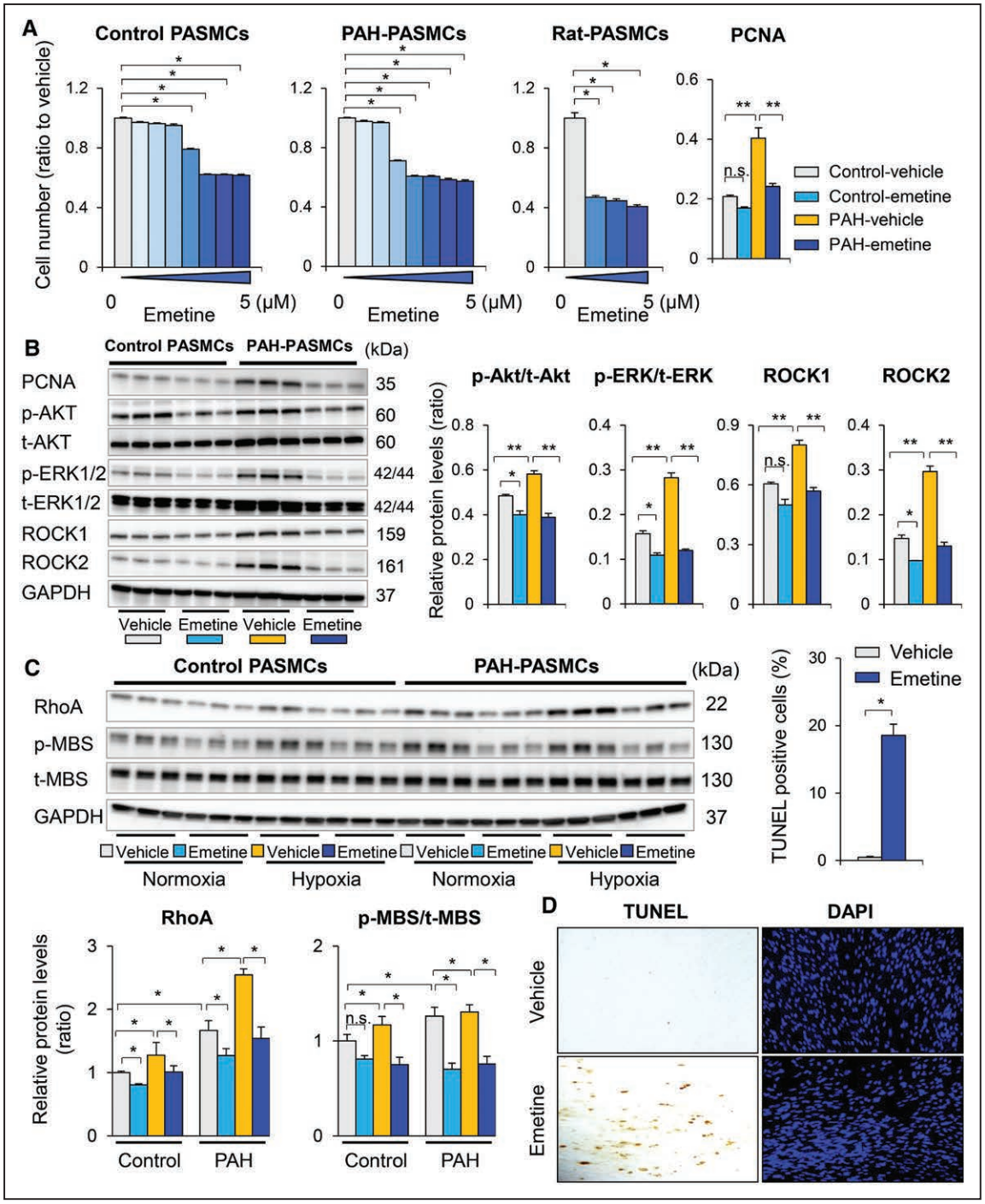

Figure 2. Emetine improves the proliferation/apoptosis imbalance in control and pulmonary arterial hypertension (PAH)–pulmonary artery smooth muscle cell (PASMCs).

A, The ratio of cell numbers after treatment with different concentrations of emetine (0, 0.005, 0.01, 0.05, 0.1, 0.5, 1, and 5 μM) for 24 h compared with day 0 in PASMCs in PASMCs from control, PAH patients and (0, 1, 2, and 5 μM) for 24 h compared with day 0 in Sugen/hypoxia rats (n=8 each). **B**, Quantifications of protein levels of p-Akt (phosphorylated protein kinase B), t-Akt (total protein kinase B), p-ERK1/2 (phosphorylated extracellular signal-regulated protein kinases 1 and 2) and t-ERK1/2 (total extracellular signal-regulated protein kinases 1 and 2), and proliferating cell nuclear antigen (PCNA) in control and PAH-PASMCs after treatment with emetine (2 μM) for 24 h compared with vehicles (n=3 each). **C**, Quantification of RhoA (Ras homolog gene family, member A), p-MBS (phospho-myosin-binding subunit), and t-MBS (total myosin-binding subunit) in control and PAH-PASMCs after treatment with emetine (2 μM) or the control vehicle for 24 h under normoxia and exposed to 1% hypoxia (n=3 each). **D**, Quantification of apoptosis by TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay after treatment with emetine (2 μM) for 24 h in PAH-PASMCs compared with vehicle controls (5 images in each group). The percentage of cells with a positive nuclear staining for TUNEL was assessed. All results are shown as mean±SEM. Comparisons of means between 2 groups were performed by unpaired Student *t* test and ANOVA with interaction terms, followed by Tukey honestly significant difference or Dunnett post hoc test for multiple comparisons, as appropriate. The normality assumption was tested by Shapiro-Wilk normality test, and the equal variance assumption was tested by Bartlett test of homogeneity of variances. **P*<0.05. DAPI indicates 4′,6-diamidino-2-phenylindole.

controls (Figure 2D). Additionally, emetine treatment tended to reduce the protein levels of RhoA and Rho-kinases (ROCK1 and ROCK2) in control aortic smooth muscle cells as well (Figure III in the online-only Data Supplement). These results indicate that emetine treatment reverses the imbalance between proliferation and apoptosis, resulting in reduced number of PAH-PASMCs.

Emetine-Mediated Downregulation of the CyPA-Bsg Signaling Pathway in PASMCs

We demonstrated that CyPA and its receptor Bsg are substantially involved in the pathogenesis of cardiovascular diseases, including PAH.[11,36,37] Thus, we hypothesized that emetine may have inhibitory effects on the signaling pathway. Interestingly, hypoxia significantly increased the protein levels of CyPA and Bsg compared with normoxic vehicle controls in control PASMCs and PAH-PASMCs. Importantly, we found higher expression of CyPA in PAH-PASMCs compared with control PASMCs (Figure 3A). Here, emetine treatment significantly reduced the protein levels of CyPA and Bsg compared with vehicle controls (Figure 3A). Moreover, emetine treatment significantly reduced the secretion of CyPA from PAH-PASMCs compared with vehicle controls (Figure 3B). Here, because the secretion of CyPA from PASMCs is regulated by Rho-kinase,[10,38] we next hypothesized that emetine treatment may inhibit the expressions of Rho-kinase isoforms, ROCK1 and ROCK2. Indeed, emetine treatment significantly reduced the protein levels of ROCK1 and ROCK2 in PAH-PASMCs compared with vehicle controls (Figure 3B). Here, we showed that extracellular CyPA and Bsg play a crucial role in the secretion of cytokines/chemokines and growth factors in a paracrine/autocrine manner.[4,22] Consistently, the secretion of cytokines/chemokines and growth factors (eg, IL-1β, IL-6, and TNF-α) was significantly reduced by emetine treatment compared with vehicle controls (Figure 3C). These results indicate that emetine downregulates ROCK1 and ROCK2, resulting in the reduced secretion of CyPA, Bsg, and inflammatory cytokines (Figure 3D), all of which promote cell proliferation and antiapoptotic effects in PAH-PASMCs.

Emetine-Mediated Inhibition of Signaling Hubs in PASMCs

Because emetine affected multiple inflammatory pathways, we hypothesized that emetine may act on signaling hubs affecting all cytokines/chemokines and growth factors. Indeed, emetine treatment significantly downregulated the protein levels of RhoA in PAH-PASMCs compared with vehicle controls (Figure 3C). In addition to RhoA, several hubs have been described, including BRD4, a key epigenetic modulator that promotes the development of PAH.[39] Additionally, HSP90 has been demonstrated to regulate the cell cycle in PAH-PASMCs.[40] Thus, we performed additional experiments to examine whether emetine affects BRD4 and HSP90 in PAH-PASMCs. Importantly, emetine treatment significantly reduced protein levels of BRD4 and HSP90 in PAH-PASMCs compared with vehicle controls (Figure 4A). Consistently, emetine treatment significantly reduced the production of PDGF-BB and FGF2 in PAH-PASMCs (Figure 4A), both of which are upregulated in PAH.[41] Altogether, emetine-mediated multiple anti-inflammatory and antiproliferative effects are based on its inhibitory effects on signaling hubs, RhoA, BRD4, and HSP90 in PAH-PASMCs.

Emetine Improves Mitochondrial Energy Metabolism in PAH-PASMCs

The expressions of signaling hubs are closely associated with the activities of transcription factor, HIF-1α.[39] Since the excessive activation of HIF-1α leads to a proliferative, apoptosis resistant phenotype through mitochondrial dysfunction in PAH-PASMCs,[13] we assumed that emetine may inhibit the abnormal proliferation via HIF-1α inhibition. Interestingly, hypoxic exposure significantly increased the expression of HIF-1α in control PASMCs and PAH-PASMCs compared with normoxic controls (Figure 4B). In contrast, emetine treatment significantly reduced the protein levels of HIF-1α compared with vehicle controls under both normoxia and hypoxia in control PASMCs and PAH-PASMCs (Figure 4B). In addition to HIF-1α, HIF-2α has been demonstrated to play a crucial role in the development of PAH.[42,43] Indeed, hypoxic exposure significantly increased the expression of HIF-2α compared with normoxic controls in control PASMCs and PAH-PASMCs (Figure 4B). Again, emetine treatment significantly reduced the protein levels of HIF-2α compared with vehicle controls under both normoxia and hypoxia (Figure 4B). We assessed the effects of emetine on mitochondrial respiration in control and PAH-PASMCs. Interestingly, we found that emetine improved mitochondrial respiration in PAH-PASMCs (Figure 5A), but not in control PASMCs (Figure IV in the online-only Data Supplement). Moreover, we assessed the effects of emetine on mitochondrial respiration in human pulmonary arterial endothelial cells. Importantly, emetine had no significant effects on mitochondrial respiration in control pulmonary arterial endothelial cells (Figure V in the online-only Data Supplement). In consistent with the mtROS (mitochondrial reactive oxygen species) data, emetine increased oxidative phosphorylation in mitochondria and produced mtROS as byproducts of the tricarboxylic acid cycle in PAH-PASMCs (Figure 5A). Indeed, emetine inhibited proliferation of PAH-PASMCs isolated from 3 different donors (Figure I in the online-only Data Supplement). Interestingly, knockdown of HIF-1α by siRNA (small interfering RNA) increased mitochondrial oxidative phosphorylation in PAH-PASMCs,

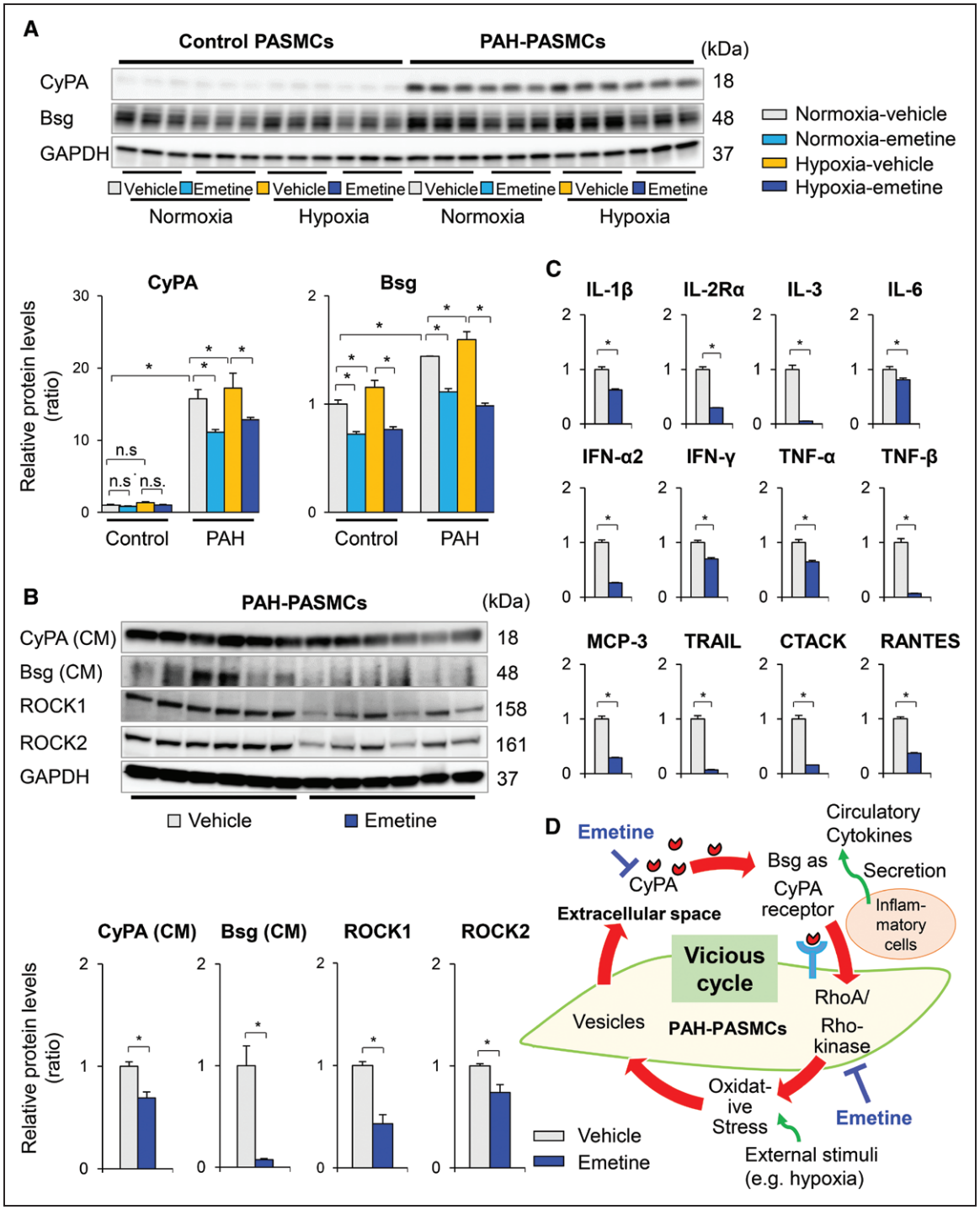

Figure 3. Emetine inhibits the CyPA (cyclophilin A)/Bsg (basigin) signaling pathway in control and pulmonary arterial hypertension (PAH)–pulmonary artery smooth muscle cell (PASMCs).

A, Quantification of CyPA and Bsg in total cell lysate (TCL) obtained from control and PAH-PASMCs after treatment with emetine (2 μM) or vehicle controls for 24 h under both normoxia and hypoxia (n=3 each). **B**, Quantification of CyPA and Bsg in conditioned medium (CM) and ROCK (rho-associated coiled-coil containing protein kinase) 1 and ROCK2 in TCL of PAH-PASMCs after treatment with emetine (2 μM) or vehicle controls for 24 h (n=6 each). **C**, Quantification of protein levels of inflammatory cytokines and chemokines in CM from PAH-PASMCs after treatment with emetine (2 μM) or vehicle for 24 h (n=6 each). **D**, The schematic representation of molecular mechanisms implicating Rho-kinase and CyPA/Bsg signaling in the pathogenesis of pulmonary arterial hypertension. All results are shown as mean±SEM. Comparisons of means between 2 groups were performed by unpaired Student *t* test and ANOVA with interaction terms, followed by Tukey honestly significant difference or Dunnett post hoc test for multiple comparisons, as appropriate. The normality assumption was tested by Shapiro-Wilk normality test, and the equal variance assumption was tested by Bartlewtt test of homogeneity of variances. *P<0.05. CTACK indicates cutaneous T-cell–attracting chemokine; IFN, interferon; IL, interleukin; MCP, monocyte chemotactic protein; RANTES, regulated on activation, normal T-cell–expressed and secreted; and TRAIL, TNF-related apoptosis-inducing ligand.

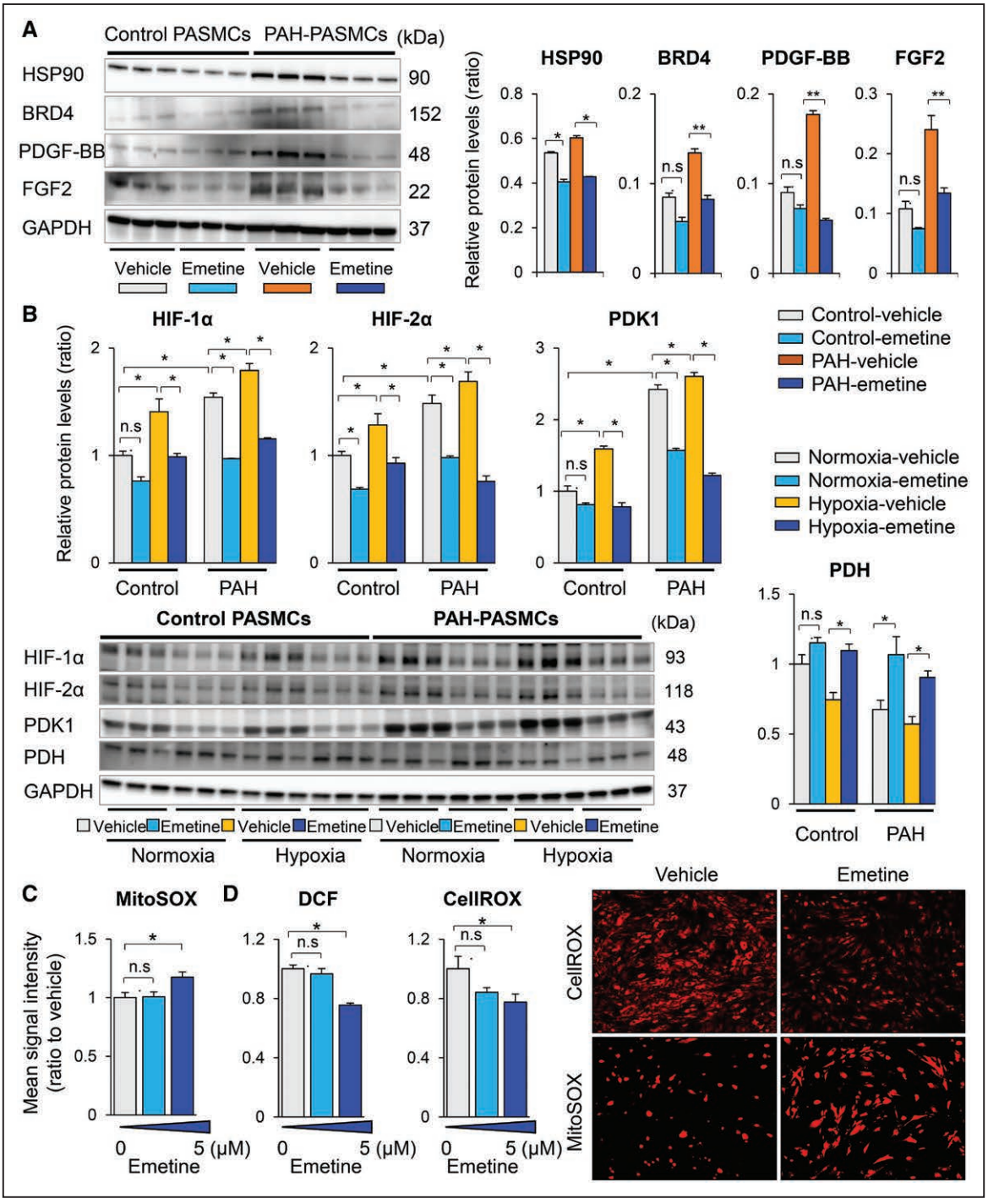

Figure 4. Emetine inhibits signaling hubs in pulmonary arterial hypertension (PAH)–pulmonary artery smooth muscle cell (PASMCs).

A, Quantification of protein levels of HSP90 (heat shock protein 90), BRD4 (bromodomain-containing protein 4), PDGF-BB (platelet-derived growth factor BB), and FGF2 (fibroblast growth factor-2) relative to internal control GAPDH in control and PAH-PASMCs after treatment with emetine (2 μM) or vehicle for 24 h (n=3 each). **B**, Quantification of HIF (hypoxia-inducible factor)-1α and HIF-2α, PDK1 (pyruvate dehydrogenase kinase 1), and PDH (pyruvate dehydrogenase) in control and PAH-PASMCs after treatment with emetine (2 μM) or vehicle under both normoxia or hypoxia for 24 h (n=3 each). **C**, Quantification of MitoSOX fluorescence intensity in PAH-PASMCs after treatment with emetine (0, 1, and 5 μM) or vehicle (n=8 each). **D**, Quantification of 2,7-dichlorodihydrofluorescein (DCF), CellROX Deep Red fluorescence intensity in PAH-PASMCs after treatment with emetine (0, 1, and 5 μM) or vehicle (n=8 each). All results are shown as mean±SEM. Comparisons of means between 2 groups were performed by unpaired Student *t* test and ANOVA with interaction terms, followed by Tukey honestly significant difference or Dunnett post hoc test for multiple comparisons, as appropriate. The normality assumption was tested by Shapiro-Wilk normality test, and the equal variance assumption was tested by Bartlett test of homogeneity of variances. **P*<0.05.

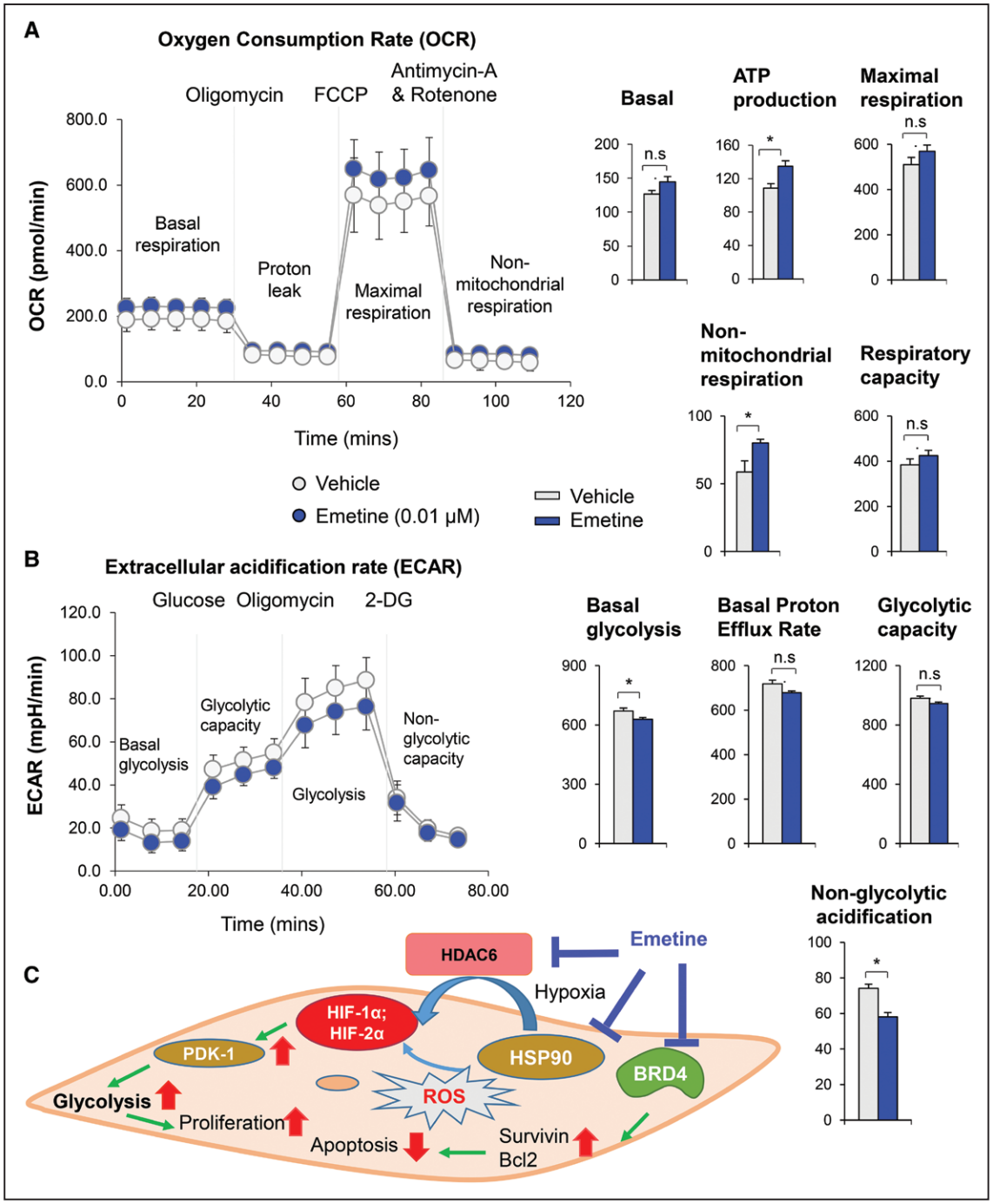

Figure 5. Emetine improves mitochondrial dysfunctions in pulmonary arterial hypertension (PAH)–pulmonary artery smooth muscle cell (PASMCs).

A, Quantification of the mitochondrial oxygen consumption rate (OCR) in PASMCs from patients with pulmonary arterial hypertension (PAH-PASMCs) after treatment with emetine (0.01 μM) or the control vehicle for 24 h (n=5). First, oligomycin was injected to inhibit ATP synthase (complex V) followed by reduction of ATP production. Second, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) were injected to disrupt the mitochondrial membrane potential. Finally, rotenone and antimycin A were injected to inhibit the flux of electrons through complex I and III, respectively, which block mitochondrial oxygen consumption. **B**, Quantification of the extracellular acidification rate (ECAR) of PAH-PASMCs after treatment with emetine (0.01 μM) or the control vehicle for 24 h (n=5 each). Glucose enhances glycolysis; oligomycin enhances ATP synthase (complex V) and the increase in ECAR. Bar graphs of basal glycolysis, basal proton efflux rate, glycolytic capacity, and nonglycolytic acidification after 24 h treatment with emetine or vehicle (n=5 each). **C**, The schematic representation of molecular mechanisms implicating BRD4 (bromodomain-containing protein 4) and HSP90 (heat shock protein 90) as signaling hubs in pulmonary arterial hypertension. All results are shown as mean±SEM. Comparisons of means between 2 groups were performed by unpaired Student *t* test and ANOVA with interaction terms, followed by Tukey honestly significant difference or Dunnett post hoc test for multiple comparisons, as appropriate. The normality assumption was tested by Shapiro-Wilk normality test, and the equal variance assumption was tested by Bartlett test of homogeneity of variances. *$P<0.05$. Bcl2 indicates B-cell lymphoma 2; DG, deoxy-d-glucose; HDAC, histone deacetylase; HIF, hypoxia-inducible factor; ns, nonsignificant; PDK, pyruvate dehydrogenase kinase; and ROS, reactive oxygen species.

which was further enhanced by emetine treatment (Figure VI in the online-only Data Supplement). Furthermore, emetine treatment significantly reduced the protein levels of PDK1 in PAH-PASMCs (Figure 4B), which inactivates PDH (pyruvate dehydrogenase) and converts pyruvic acid to acetyl-CoA (coenzyme A). Consequently, emetine treatment increased the protein levels of PDH in control PASMCs and PAH-PASMCs (Figure 4B). Here, PDK1 and PDH regulate the mitochondrial function and ATP production.[13] Thus, we hypothesized that emetine treatment may recover the ATP production and thus increase the production of mitochondrial ROS. Indeed, emetine treatment significantly increased the levels of mitochondrial ROS in PAH-PASMCs compared with vehicle controls (Figure 4C). In contrast, emetine treatment significantly reduced the levels of cytosolic ROS assessed by 2,7-dichlorofluorescein diacetate and CellROX (Figure 4D). These results are consistent with the reports that CyPA and Bsg augment the production of cytosolic ROS in PAH-PASMCs.[4,37] Moreover, emetine treatment reduced extracellular acidification rate in PAH-PASMCs compared with vehicle controls (Figure 5B). Altogether, HIFs-mediated metabolic shift towards glycolysis was suppressed by emetine treatment in PAH-PASMCs (Figure 5C).[13] Thus, in terms of ROS production, emetine seems to have opposed effects between cytosolic and mitochondrial ROS production. Altogether, these results indicate that emetine treatment improves mitochondrial dysfunction in PAH-PASMCs.

Emetine Ameliorates PH in Rodent Models

Based on the emetine-mediated inhibitory effects on PAH-PASMC proliferation, we performed in vivo experiments in rodent models of PH. First, we examined the effect of emetine in a model of monocrotaline-induced PH in rats (Figure 6A). In this rat model, we started emetine treatment during the development of PH (prevention protocol). Daily oral administration of emetine for 3 weeks had no effect on body weight or blood pressure compared with vehicle controls (Figure 6B). However, emetine treatment significantly reduced RVSP and RV hypertrophy compared with vehicle controls (Figure 6C). Moreover, emetine significantly reduced medial wall thickness in large PAs and suppressed muscularization of distal PAs compared with vehicle controls (Figure 6D). Here, the protein levels of RhoA, ROCK1, ROCK2, CyPA, Bsg, BRD4, HSP90, and survivin were all significantly upregulated in vehicle group compared with controls (Figure 6E). However, in consistent with the in vitro studies, emetine treatment significantly reduced those protein levels compared with vehicle controls (Figure 6E). Next, to further evaluate the therapeutic potential of emetine for PAH, we used a second animal model of PH, in which rats were exposed to chronic hypoxia for 21 days in combination with an injection of SU5416 (Sugen/hypoxia model; Figure 7A). In this Sugen/hypoxia rat model, we started emetine treatment after the development of PH (treatment protocol). Daily oral administration of emetine for 28 days had no effect on body weight compared with vehicle controls (Figure 7B). Moreover, emetine significantly reduced the medial wall thickness in large PAs and suppressed the muscularization of distal PAs compared with vehicle controls (Figure 7C). Consistently, emetine treatment significantly reduced RV hypertrophy compared with vehicle controls (Figure 7D). Here, consistent with the results in monocrotaline-induced PH model, the protein levels of RhoA, ROCK1, ROCK2, CyPA, Bsg, BRD4, HSP90, and survivin in the lungs were all significantly upregulated in vehicle group compared with controls (Figure 7E). Additionally, emetine treatment significantly reduced these protein levels in the lungs compared with vehicle controls (Figure 7E). Moreover, serum levels of inflammatory cytokines (eg, IL-2, IL-6, and TNF-α) were significantly reduced by emetine treatment in the Sugen/hypoxia rat model (Figure VIIA in the online-only Data Supplement). Consistently, the expression of Bsg and MMP (matrix metalloproteinases) activities in RV tissues, assessed by DQ gelatin, were significantly reduced by emetine treatment in the Sugen/hypoxia rat model (Figure VIII in the online-only Data Supplement). Moreover, the levels of ROS production in RV tissues, assessed by DHE (dihydroethidium) and CellROX, were significantly reduced by emetine treatment in the Sugen/hypoxia rat model (Figure IX in the online-only Data Supplement). Here, RV functions are important for the survival of PAH patients. A follow-up study showed improved survival in PAH patients with improved RV functions.[44] Thus, we finally evaluated the hemodynamic parameters by cardiac catheterization and Doppler echocardiography. Importantly, emetine treatment significantly reduced RVSP, mean PA pressure (mPAP), cardiac output, and total pulmonary vascular resistance compared with vehicle controls (Figure 8A). These results indicate that emetine ameliorates PH in several different animal models. Additionally, emetine treatment significantly reduced RV end-diastolic diameter and improved hemodynamic parameters, such as PA acceleration time and tricuspid annular plane systolic excursion determined by echocardiography (Figure 8B and 8C). Altogether, emetine treatment significantly improved exercise capacity and increased treadmill walking distance (Figure 8B). These results indicate that emetine suppresses inflammation and improves systemic hemodynamics, ameliorating PH and RV failure (Figure 8D).

DISCUSSION

In the present study, we demonstrate that emetine inhibits PAH-PASMC proliferation through suppression of signaling hubs and ameliorates PH in rat models of different mechanisms. These concepts are based on the following: (1) we identified emetine as a compound that inhibits

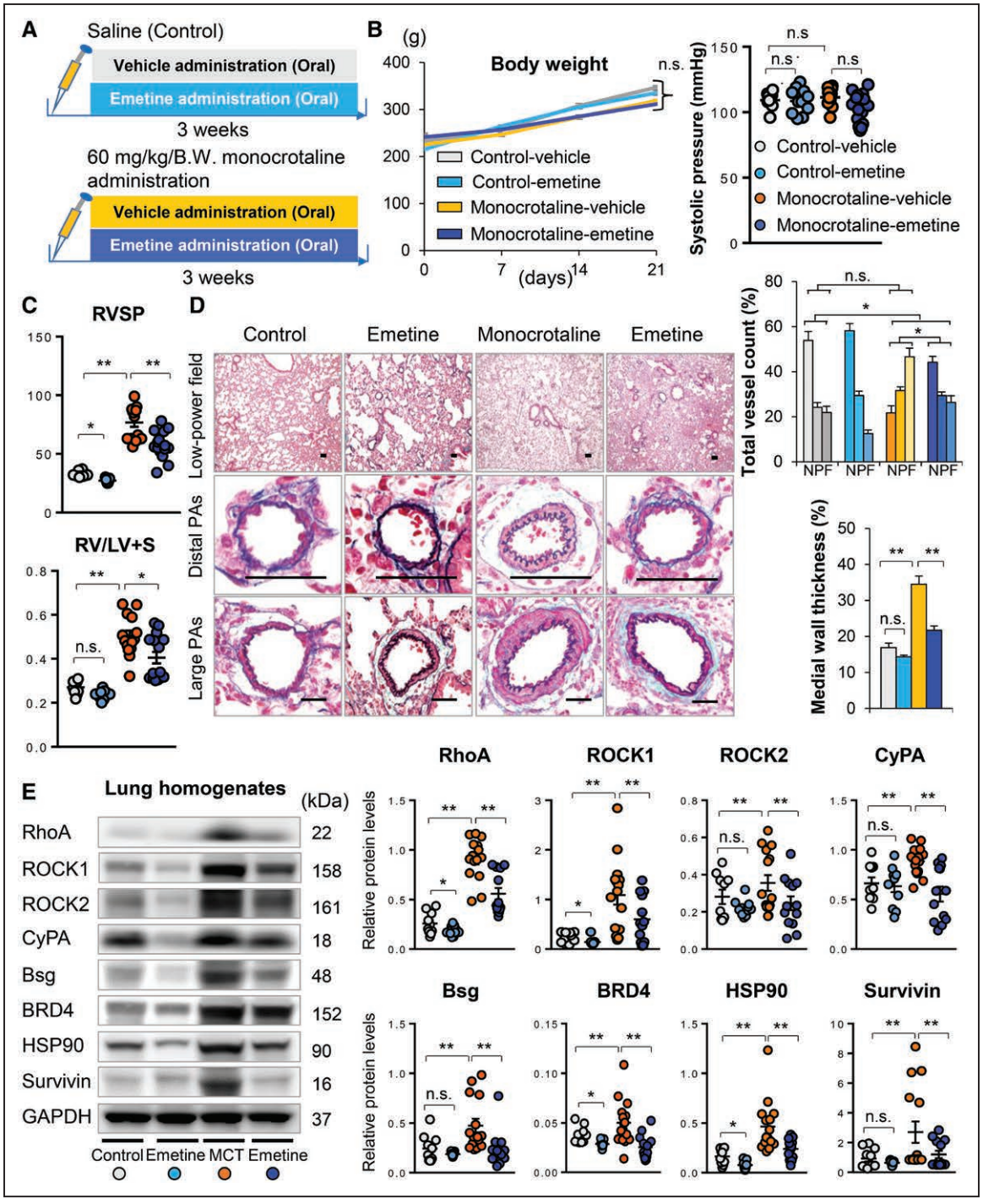

Figure 6. Emetine ameliorates monocrotaline-induced pulmonary hypertension in rats.
A, Schematic protocols for administration of emetine (0.05 mg/kg per day) or vehicle by oral gavage for 3 wk. **B**, Body weight follow-up and blood pressure measured by tail-cuff systems after treatment with emetine or control vehicle for 3 wk. **C**, Right ventricular (RV) systolic pressure (RVSP) and RV hypertrophy in rats after treatment with emetine or vehicle for 3 wk (n=10–15). **D**, Representative Elastica-Masson staining of the distal pulmonary arteries (PAs) and large PAs. **Upper-right**, Medial wall thickness of the large pulmonary arteries in rats (n=10–15 each). **Lower-right**, Muscularization of the distal pulmonary arteries in rats (n=10–15 each). Scale bars, 50 μm. **E**, Representative Western blots and quantification of protein levels of RhoA (Ras homolog gene family, member A), ROCK (Rho-associated coiled-coil containing protein kinase) 1, ROCK2, Bsg (basigin), CyPA (cyclophilin A), BRD4 (bromodomain-containing protein 4), HSP90 (heat shock protein 90), and Survivin in the lungs compared with GAPDH (n=10–15). All results are shown as mean±SEM. Comparisons of means between 2 groups were performed by unpaired Student *t* test and ANOVA with interaction terms, followed by Tukey honestly significant difference or Dunnett post hoc test for multiple comparisons, as appropriate. The normality assumption was tested by Shapiro-Wilk normality test, and the equal variance assumption was tested by Bartlett test of homogeneity of variances. **P*<0.05. BW indicates body weight; F, fully muscularized vessels; LV, left ventricle; MCT, monocrotaline; N, nonmuscularized vessels; ns, nonsignificant; P, partially muscularized vessels; and S, septum.

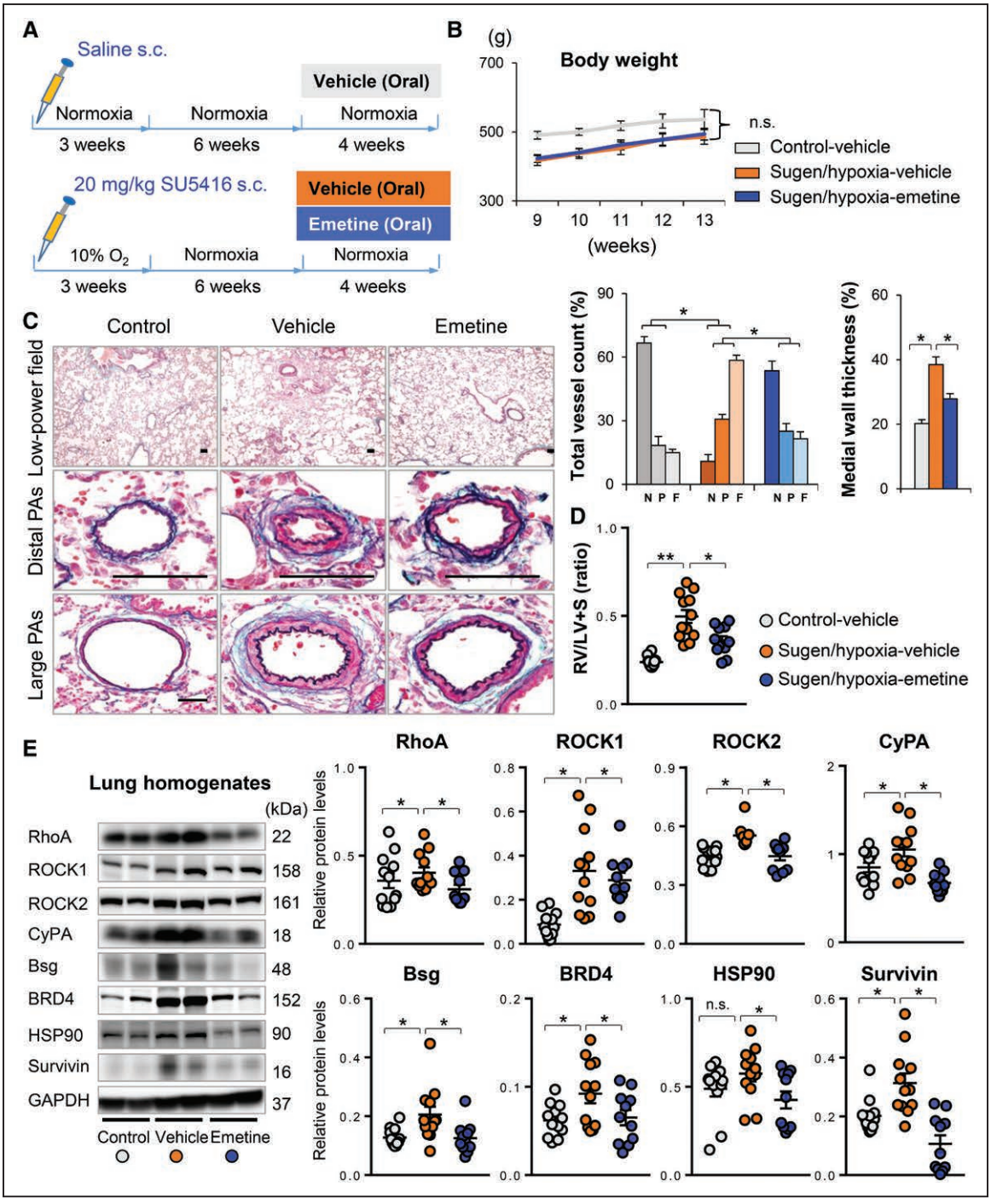

Figure 7. Emetine ameliorates sugen/hypoxia–induced pulmonary hypertension in rats.
A, Schematic protocols for emetine administration to the Sugen/hypoxia rat model, in which the animals were exposed to hypoxia (10% O_2) for 3 wk after SU5416 (20 mg/kg) injection followed by normoxia for 6 wk and then the animals were assigned to treatment with emetine (0.05 mg/kg per day) or vehicle for additional 4 wk. **B**, Changes in body weight in Sugen/hypoxia rats (n=11–12 each). **C**, Representative Elastica-Masson staining of the distal pulmonary arteries (PAs) and large PAs. **Upper-right**, Muscularization of the distal PAs in rats (n=11–12 each). **Lower-right**, Medial wall thickness of the large PAs in rats (n=11–12 each). Scale bars, 50 μm. **D**, The weight ratio of the right ventricle to the left ventricle plus septum (RV/LV+S; n=11–12). **E**, Representative Western blots and quantification of protein levels of RhoA (Ras homolog gene family, member A), ROCK (rho-associated coiled-coil containing protein kinase) 1, ROCK2, CyPA (cyclophilin A), Bsg (basigin), BRD4 (bromodomain-containing protein 4), and HSP90 (heat shock protein 90) in the lungs compared with GAPDH (n=11–12). All results are shown as mean±SEM. Comparisons of means between 2 groups were performed by unpaired Student *t* test and ANOVA with interaction terms, followed by Tukey honestly significant difference or Dunnett post hoc test for multiple comparisons, as appropriate. The normality assumption was tested by Shapiro-Wilk normality test, and the equal variance assumption was tested by Bartlett test of homogeneity of variances. *P<0.05. F indicates fully muscularized vessels; N, nonmuscularized vessels; ns, nonsignificant; P, partially muscularized vessels; and SC, subcutaneous.

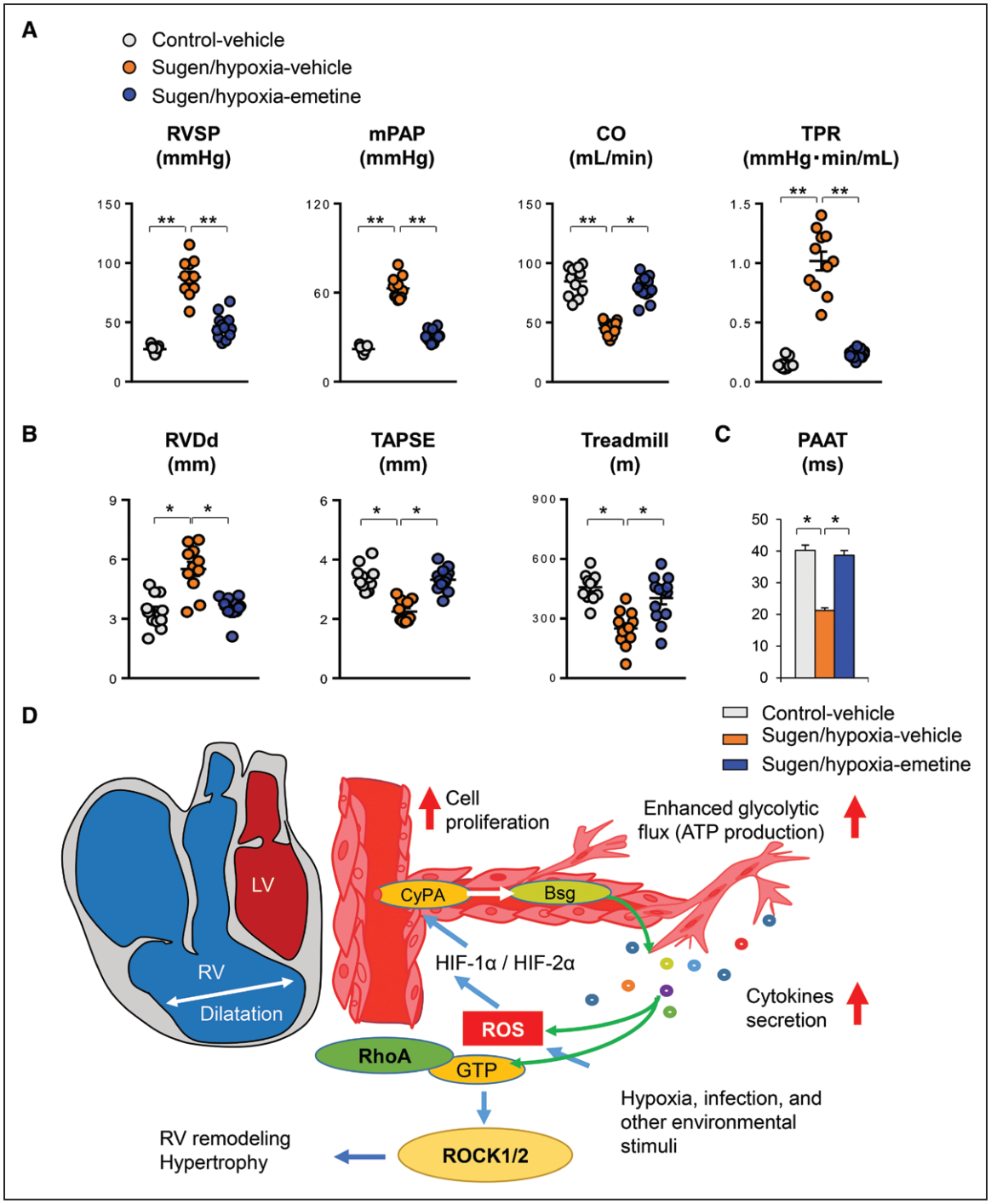

Figure 8. Emetine improved pulmonary hemodynamics in sugen/hypoxia pulmonary hypertension (PH) rats.
A, Right ventricular (RV) systolic pressure (RVSP), mean pulmonary arterial pressure (mPAP), cardiac output (CO), and total pulmonary resistance (TPR) in Sugen/hypoxia rats after treatment with vehicle or emetine for 4 wk (n=11–13). **B**, RV diastolic diameter (RVDd), tricuspid annular plane systolic excursion (TAPSE), and walking distance assessed by a treadmill test (n=11–13). **C**, Pulmonary artery (PA) acceleration time (PAAT; n=11–13). **D**, The schematic representation of molecular mechanisms implicating adverse RV remodeling in pulmonary arterial hypertension. Data represent the mean±SEM. Comparisons of parameters were performed with the unpaired Student *t* test or, followed by Tukey honest significant difference test for multiple comparisons. All results are shown as mean±SEM. Comparisons of means between 2 groups were performed by unpaired Student *t* test and ANOVA with interaction terms, followed by Tukey honestly significant difference or Dunnett post hoc test for multiple comparisons, as appropriate. The normality assumption was tested by Shapiro-Wilk normality test, and the equal variance assumption was tested by Bartlett test of homogeneity of variances. *$P<0.05$. Bsg indicates basigin; CyPA, cyclophilin A; HIF, hypoxia-inducible factors; LV, left ventricle; RhoA, Ras homolog gene family, member A; ROCK, rho-associated coiled-coil containing protein kinase; and ROS, reactive oxygen species.

PAH-PASMC proliferation dose-dependently with no harmful effects on control PASMCs; (2) emetine induced apoptosis in PAH-PASMCs; (3) emetine downregulated the expressions of RhoA/Rho-kinases and signaling hubs, such as HSP90 and BRD4; (4) emetine reduced the secretion of CyPA, Bsg, and inflammatory cytokines/chemokines and growth factors from PAH-PASMCs; and (5) emetine ameliorated PH and improved RV functions in rat models of PH.

Identification of Emetine as a Novel Drug for PAH

Despite the advances in medical therapies, the prognosis remains poor in patients with severe PAH with RV failure.[1] Nowadays, we can use 3 main types of drugs for PAH; all of them are vasodilators of the PAs.[45] Thus, it is practically difficult to stop this aggressive disease and to reverse the already-established pulmonary vascular remodeling by these medications alone.[46] As a result, lung transplantation is the last option for severe patients.[47] Thus, it is warranted to develop effective treatment that achieves reverse remodeling of PAs as an additional strategy for PAH.[48] Based on the background, we focused on the abnormal proliferative phenotype in PAH-PASMCs to discover a novel drug for PAH.[41] Hyperproliferation of PAH-PASMCs is the key in the development of pulmonary vascular remodeling and resultant elevated pulmonary vascular resistance in patients with PAH.[16] At present, there is no drug that specifically inhibits PASMC proliferation. In the present study, using the Tohoku University library with 5562 original compounds, we identified emetine that inhibits PAH-PASMC proliferation with anti-inflammatory effects, which is clinically used for the treatment of amebic infections in humans.[49] As emetine is also known as anticancer and antiviral agents,[18–20] we performed validation study in 2 experimental PH models in rats and found that it ameliorates experimental PH models with no adverse effects. Indeed, we used 0.05 mg/kg per day as a therapeutic dose in experimental PH rat models and 5 μmol/L as a highest concentration in in vitro experiments. The suspected peak plasma concentration (Cmax) after administration of emetine (0.05 mg/kg) in rats was 0.47 nmol/L based on the previous reports of single-dose toxicity studies.[50] Nair et al[51] provided information about the translation of doses between animals and starting dose for clinical trials in human. Thus, the dose of emetine we used in vivo was 1/20 000 and concentration in vitro was 1/2000 compared with those of inhibiting ribosomal 40S subunit.[52] It has been demonstrated that a selective Rho-kinase inhibitor, fasudil, significantly reduced mean PA pressure without any change in systemic blood pressure.[53–55] In the present study, we also found that oral administration of emetine in experimental PH rats causes a significant reduction in RVSP and mean PA pressure without affecting systemic blood pressure.

Emetine Inhibits the Rho-Kinase/CyPA/Bsg Signaling Pathway in PAH-PASMCs

We have previously demonstrated that CyPA and Bsg are 2 downstream targets of the RhoA/Rho-kinase system.[11] Rho-kinase is an important therapeutic target in cardiovascular diseases.[56] Excessive and continuous activation of Rho-kinase promotes secretion of CyPA from vascular smooth muscle cells, and extracellular CyPA stimulates vascular smooth muscle cell proliferation.[24] Additionally, extracellular CyPA induces endothelial cell adhesion molecule expression and endothelial apoptosis.[29] Bsg is an extracellular CyPA receptor. Bsg is also known as an essential receptor for malaria, which disrupts NO metabolism and causes harmful endothelial activation, including the Rho/Rho-kinase activation. Based on these backgrounds, we have demonstrated that CyPA and Bsg promote hypoxia-induced PH.[57] More importantly, plasma CyPA was significantly increased in patients with PAH and well correlated with the disease severity and long-term survival.[57] Thus, extracellular CyPA and its signaling through Bsg are novel therapeutic targets for PAH. We have previously reported that statins and Rho-kinase inhibitors reduce CyPA secretion from vascular smooth muscle cells.[58] Thus, inhibition of CyPA secretion by Rho-kinase inhibitors may have therapeutic efficacy in PAH. In addition, Bsg is strongly expressed in the PAs of patients with PAH.[57] Thus, pharmacological agents that prevent the interaction of extracellular CyPA and vascular Bsg could be useful for the treatment of PAH (Figure 3D). PASMCs in the remodeled PAs have special characteristics with proproliferative and antiapoptotic features. Thus, we have developed a screening system focusing on the inhibitory effects on PAH-PASMCs.[59,60] In the system, we used PAH-PASMCs and high-throughput screening to identify novel agents to inhibit their proliferation. Indeed, in the present study, our drug discovery research demonstrated that emetine significantly inhibits CyPA and Bsg, suppressing proliferation of PAH-PASMCs. Importantly, emetine suppressed CyPA and Bsg expressions in the lung and ameliorated PH in 2 rat models (prevention and treatment protocols). Taken together, inhibiting both CyPA and Bsg by emetine treatment may represent a novel therapeutic strategy for the treatment of PH (Figure 3D). Thus, emetine may target the CyPA/Bsg system which could be a master target to block the vicious cycle for oxidative stress augmentation and PAH-PASMC proliferation.

Emetine Improves Metabolic Abnormalities in PAH-PASMCs

Recent studies demonstrated the suppressed oxidative phosphorylation in mitochondria and increased glycolysis

in cytosol in PAH patients.[61] PDH has a gatekeeping mechanism that allows the conversion of pyruvate into acetyl-CoA in the mitochondria.[62] Activation of HIF-1α has been described in both animal and human PAH,[63] which upregulates PDK1 and thus downregulates PDH, which in turn causes inhibition of glucose oxidation in PAH.[64] Several studies pointed out the similarities between cancer and PAH-PASMCs, both of which manifest excessive proliferation and apoptosis resistance.[63,65] Activated HIF-1α in PAH-PASMCs contributes to the metabolic shift from oxidative phosphorylation to glycolysis by increasing PDK1 expression.[35] Here, PDK1 halts the tricarboxylic acid cycle progression resulting in a metabolic shift of mitochondrial glucose metabolism from oxidative phosphorylation to cytosolic glycolysis in PAH-PASMCs.[13] Mitochondrial metabolic abnormality reduces production of mitochondrial ROS and causes hyperpolarization and decreased function of oxygen-sensitive and voltage-gated K^+ channels.[66] Inhibition of voltage-gated K^+ channels promotes vascular remodeling and PASMC proliferation by increasing cytosolic calcium and cytosolic K^+, which inhibits caspase-dependent apoptosis.[66] In the present study, emetine treatment significantly reduced the levels of HIF-1α and downstream PDK1, and thus restored PDH and increased oxidative phosphorylation in mitochondria in control PASMCs and PAH-PASMCs.[13] Mitochondrial metabolic abnormality reduces production of mitochondrial ROS and causes hyperpolarization and decreased function of oxygen-sensitive and voltage-gated K^+ channels.[66] Inhibition of voltage-gated K^+ channels promotes vascular remodeling and PASMC proliferation by increasing cytosolic calcium and cytosolic K^+, which inhibits caspase-dependent apoptosis.[66] In the present study, emetine treatment significantly reduced the levels of HIF-1α and downstream PDK1 and thus restored PDH and increased oxidative phosphorylation in mitochondria in PAH-PASMCs.[13] It has been reported that metabolic shift toward glycolysis causes reduction of electron flux in the electron transport chain of tricarboxylic acid cycle, which consequently reduces mitochondrial ROS.[35] Indeed, we found that emetine treatment significantly increased mitochondrial ROS assessed by MitoSOX. Additionally, emetine treatment significantly increased apoptosis in PAH-PASMCs. Importantly, emetine treatment significantly reduced cell proliferation in PASMCs from patients with PAH and control donors, in which emetine showed antiproliferative effects. Indeed, we assessed the effects of emetine on mitochondrial respiration in control and PAH-PASMCs. Here, we found that emetine improved mitochondrial respiration in PAH-PASMCs (Figure 5A), but not in control PASMCs (Figure IV in the online-only Data Supplement). Moreover, we assessed the effects of emetine on mitochondrial respiration in pulmonary arterial endothelial cells from control donors. Importantly, emetine had no significant effects on mitochondrial respiration in control pulmonary arterial endothelial cells (Figure V in the online-only Data Supplement). Thus, one of the emetine-mediated antiproliferative and proapoptotic effects may be mediated by the recovery of mitochondrial functions in PAH-PASMCs.

Study Limitations

There are several limitations to the present study. First, we mainly evaluated signaling hubs and inflammation, but there might be other mechanisms involved through which the drug suppresses cell proliferation. Second, there were no obvious adverse events caused by emetine treatment. Third, we had a lack of samples, especially in vitro studies. However, adverse events will need to be examined in more details in the future.

ARTICLE INFORMATION

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Affiliations

From the Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan (M.A.H.S., K.S., R.K., N.K., M.E.-A.-M., J.O., T.S., M.N., S.S., S.M., H.S.); and Tohoku University Graduate School of Pharmaceutical Sciences, Sendai, Japan (H.U., H.T.).

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Disclosures

None.

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