Potassium Channel Subfamily K Member 3 (KCNK3) Contributes to the Development of Pulmonary Arterial Hypertension

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Background—Mutations in the *KCNK3* gene have been identified in some patients suffering from heritable pulmonary arterial hypertension (PAH). *KCNK3* encodes an outward rectifier K^+ channel, and each identified mutation leads to a loss of function. However, the pathophysiological role of potassium channel subfamily K member 3 (KCNK3) in PAH is unclear. We hypothesized that loss of function of KCNK3 is a hallmark of idiopathic and heritable PAH and contributes to dysfunction of pulmonary artery smooth muscle cells and pulmonary artery endothelial cells, leading to pulmonary artery remodeling: consequently, restoring KCNK3 function could alleviate experimental pulmonary hypertension (PH).

Methods and Results—We demonstrated that KCNK3 expression and function were reduced in human PAH and in monocrotaline-induced PH in rats. Using a patch-clamp technique in freshly isolated (not cultured) pulmonary artery smooth muscle cells and pulmonary artery endothelial cells, we found that KCNK3 current decreased progressively during the development of monocrotaline-induced PH and correlated with plasma-membrane depolarization. We demonstrated that KCNK3 modulated pulmonary arterial tone. Long-term inhibition of KCNK3 in rats induced distal neomuscularization and early hemodynamic signs of PH, which were related to exaggerated proliferation of pulmonary artery endothelial cells, pulmonary artery smooth muscle cell, adventitial fibroblasts, and pulmonary and systemic inflammation. Lastly, in vivo pharmacological activation of KCNK3 significantly reversed monocrotaline-induced PH in rats.

Conclusions—In PAH and experimental PH, KCNK3 expression and activity are strongly reduced in pulmonary artery smooth muscle cells and endothelial cells. KCNK3 inhibition promoted increased proliferation, vasoconstriction, and inflammation. In vivo pharmacological activation of KCNK3 alleviated monocrotaline-induced PH, thus demonstrating that loss of KCNK3 is a key event in PAH pathogenesis and thus could be therapeutically targeted. **(*Circulation*. 2016;133:1371-1385. DOI: 10.1161/CIRCULATIONAHA.115.020951.)**

Key Words: cell proliferation ■ electrophysiology ■ hypertension, pulmonary ■ ion channels ■ pulmonary artery

Pulmonary artery (PA) hypertension (PAH) is a severe and progressive condition characterized by increased mean pulmonary arterial pressure >25 mm Hg at rest, with a normal PA wedge pressure in the absence of chronic respiratory, cardiac, or thromboembolic disease.[1] PAH is a complex disease associated with endothelial cell (EC) dysfunction and

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endothelial-to-mesenchymal transition, endothelial and pulmonary arterial smooth muscle cell (PASMC) proliferation, pulmonary vasoconstriction, and inflammation. The nature of the initial trigger that promotes pulmonary vascular cell proliferation is still unclear. During the last decade, genetic studies have identified several predisposing genes associated with PAH, mainly within the transforming growth factor-β signaling pathway.[2]

Clinical Perspective on p 1385

Recently, using whole-exome sequencing, Ma and colleagues[3] identified *KCNK3* (which codes for the potassium channel subfamily K member 3) as a new predisposing gene for PAH. *KCNK3* mutations were identified in 2 of 92 unrelated patients (3.2%) with familial PAH and in 3 of 230 patients (1.3%) with sporadic PAH. PAH caused by a *KCNK3* mutation is an autosomal-dominant disease with incomplete penetrance, as has been previously described for *BMPR2* mutations. A *KCNK3* mutation causes the first channelopathy identified in PAH.[4]

The *KCNK3* gene encodes for an outward K^+ channel characterized by the presence of 4 transmembrane domains and 2 pore domains per subunit[5] and is a member of 2-pore-domain K^+ channels (K2P). KCNK3, also called Twik-related acid-sensitive K^+ channel (TASK1), shares several characteristics with the background K^+ current, including minimal voltage sensitivity, extracellular pH sensitivity, resistance to classic K^+ channel inhibitors, and insensitivity to cytoplasmic Ca^{2+}.[6] Electrophysiological experiments have demonstrated a loss of function in all identified mutations of the channel.[3] However, additional experiments are required to decipher the pathophysiological role of KCNK3 channels in the development of nonheritable forms of PAH.

In the present study, we investigated the expression and function of KCNK3 in patients with idiopathic PAH (iPAH) and heritable PAH (hPAH) who do not carry PAH-causing mutations by assessing the associated biochemical and molecular biology. We also evaluated the role of KCNK3 in the development of pulmonary hypertension (PH) using the well-established PH rat model induced by monocrotaline exposure (MCT-PH). We measured the involvement of KCNK3 in pulmonary arterial tone using organ bath studies. We analyzed the consequences of long-term in vivo inhibition of KCNK3 in rats using a selective KCNK3 channel blocker (ie, A293, Sanofi, France) at the pulmonary vascular level.

Our overall hypothesis is that loss of function of KCNK3 is a hallmark for all forms of PH and contributes to PASMC and PAEC dysfunction and that stimulating KCNK3 function in vivo with ONO-RS-082[3] could alleviate experimental PH.

Methods

The online-only Data Supplement provides further details of our methods, including the suppliers and chemicals used.

Patients

Human lung specimens were obtained at the time of lung transplantation from 11 patients with PAH and at the time of a lobectomy or pneumonectomy for localized lung cancer from 12 control subjects. PAH patients underwent genetic counseling and gave their written informed consent for genetic analysis. Five patients with PAH were carriers of *BMPR2* mutations (p.Arg321X, p.Arg491Trp, p.Tyr113X, deletion of exon 3, and a splice defect in intron 3: c.418+3A>T) and corresponded to hPAH. No *BMPR2* mutations were identified in 6 patients, who were also included in the genome-wide association study analysis of Ma and collaborators,[3] and were not carriers of a *KCNK3* mutation. These patients corresponded to iPAH. In the lung specimens from control subjects, pulmonary arteries were studied at a distance from tumor areas. Transthoracic echocardiography was performed preoperatively in control subjects to rule out PH. The patients studied were part of the French Network on Pulmonary Hypertension, a program approved by our institutional Ethics Committee, who had given their written informed consent (protocol N8CO-08- 003, ID RCB: 2008-A00485-50, approved June 18, 2008).

Materials

Tetraethylammonium, glibenclamide, Bay-K8644, papain, dithiothreitol, and monocrotaline were obtained from Sigma. ONO-RS-082 was obtained from Enzolife Sciences. U44619 was purchased from Interchim. The selective blocker of KCNK3, A293 (2-(butane-1-sulfonyl-amino)-N-[1-(R)-(6-methoxypyridin-3-yl)-propyl]-benzamide, was kindly provided by Sanofi-Aventis.[7]

Hemodynamic Measurements, Evaluation of Right Ventricular Hypertrophy, and Tissue Collection

Rats were anesthetized with isoflurane (Minerve, Esternay, France). Hemodynamic measurements were recorded as previously described.[8] For the Fulton index of right ventricular (RV) hypertrophy, the ratio of RV weight to left ventricular plus septum weight was calculated.

Isolation of PASMCs

Hearts and lungs were rapidly excised into ice-cold physiological Ca^{2+}-free Hanks media. First- to third-order intrapulmonary arteries, with diameters between 2 and 0.2 mm, were dissected; the endothelium of vessels was disrupted by rubbing the luminal surface with a cotton swab; the fibroblast-containing adventitial layer was removed; and the media containing PASMC was dissociated with a cocktail containing papain, dithiothreitol, and BSA, as previously described by Hong et al.[9]

Isolation of PAECs

After dissection of the pulmonary arteries, PAECs were enzymatically dissociated according to an adapted protocol previously described by Paffett et al.[10]

Electrophysiological Recordings

K^+ currents were recorded with the whole-cell patch-clamp technique at room temperature (22°C –24°C) in normoxic conditions with an Axopatch 200B amplifier, a Digidata 1440A, and pClamp 10 software (Molecular Devices, Sunnyvale, CA). Borosilicate glass pipettes (Harvard Apparatus) were pulled with a Sutter puller and fire polished; they had a resistance of 3 to 5 MegaΩ. The pipette solution contained (in mmol/L) 130 KCl, 1 $MgCl_2$, 10 HEPES, and 1 EGTA, pH 7.2 (adjusted with KOH). The bath solution contained (in mmol/L) 124 NaCl, 5 KCl, 0.5 NaH_2PO_4, 0.5 KH_2PO_4, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, and 10 glucose, pH 7.4 (adjusted with NaOH). Cell capacitances (9–15 pF for PASMCs and 4–5 pF for PAECs) were obtained for each cell measured, and the current was normalized to the cell capacitance.

The cell was clamped at 0 mV for 3 minutes, allowing I_{Kv} inactivation. The residual steady-state current was measured as the noninactivating current, I_{KN}. The KCNK3 current was recorded by applying a 1.2-second voltage ramp from 60 mV to –100 mV. I_{KV} was elicited by applying a 400-millisecond voltage step to 0 mV from a holding potential of –40 mV. Then, we applied A293 (KCNK3 inhibitor) and measured the A293-sensitive current corresponding to I_{KCNK3}. Resting membrane potentials (E_m) were assessed with the current clamp (I=0) mode. The junction potential of ≈4.6 mV between the pipette and bath solution was corrected offline.

Isometric Tension Measurement

Contractile responses of the intrapulmonary artery were measured with a small-vessel wire myograph (Danish Myotechnology, Arhus, Denmark), as reported previously.[11] Briefly, second-order

intrapulmonary arterial segments (length, 1.4–2 mm) were mounted on the jaws of the myograph using a 25-µm-diameter tungsten wire. Data acquisition was performed with PowerLab 8/30 and LabChart Pro version 7.1 software (AD Instrument). The protocols are detailed in the online-only Data Supplement.

Quantitative Polymerase Chain Reaction

Total RNA was extracted with the classic Trizol procedure. One µg total RNA was reverse-transcribed with a QuantiTect Reverse Transcription Kit (Qiagen). For gene expression quantification, we used Applied Biosystems TaqMan gene expression assays with the TaqMan Universal PCR Master Mix. The results were analyzed using the $2^{-\Delta\Delta CT}$ method. The primers used in this study are listed in Table I in the online-only Data Supplement.

Multiplex Data

We performed simultaneous quantification of recognized serum biomarkers of cardiovascular diseases in sera from rats using the Millipore Milliplex (RV1MAG-26K and RV2MAG-26K, Millipore, Molsheim, France). Data were analyzed with Bioplex manager software, version 6.1 (Biorad), at Plate-forme Cardiex-Therassay (University of Nantes, Nantes, France).

Western Blot Analysis

Human or rat lung tissue samples were prepared as previously described.[12] The Western blot protocol is described in the online-only Data Supplement.

Immunofluorescence Staining

Frozen lungs were prepared as previously described.[13] Immunofluorescence labeling is described in the online-only Data Supplement.

Statistical Analyses

Data are expressed as the mean±SEM of n observations. Comparisons were made with the Mann-Whitney (comparison between 2 groups) or Kruskal-Wallis test (comparison between >2 groups). Differences were considered statistically significant at $P<0.05$. All statistical tests were performed with GraphPad Prism software (GraphPad, version 6.0 for Windows).

Results

KCNK3 Is Functionally Expressed in Freshly Isolated (Noncultured) PASMCs From Control Rats

Multiple immunofluorescence labeling revealed that KCNK3 was expressed in situ in PASMCs and PAECs (Figure 1A) and was maintained in freshly isolated PASMCs from control rats (Figure 1B).

Whole-cell patch-clamp experiments to measure endogenous KCNK3 current in isolated rat PASMCs showed that the KCNK3 channel was insensitive to standard K^+ channel blockers (tetraethylammonium and glibenclamide).[14] PASMCs were clamped at 0 mV for 3 minutes to inactivate the voltage-dependent K^+ channels (Kv). We then applied a voltage ramp protocol between 60 and –100 mV for 100 milliseconds, as shown in Figure 1C. Residual Kv and KATP channels were inhibited by simultaneous applications of tetraethylammonium (10 mmol/L, general K^+ channel blockers) and glibenclamide (10 µmol/L, K_{ATP} channel blocker). In agreement with the KCNK3 channel properties,[15] the K^+ current was blocked by decreasing external pH to 6.3, whereas an external pH of 8.3 highly increased the current. This effect was reversed by returning to the external solution pH to 7.3 (Figure 1C and 1D).

KCNK3 Current and KCNK3 Expression Decreased Progressively During the Development of PH

MCT-PH is the standard model for severe PH. The progressive neomuscularization and obstruction of precapillary resistance in arteries that occurred in the MCT-PH rat model led to a delayed and progressive increase in mean pulmonary arterial pressure and RV hypertrophy, as measured by the Fulton index, developing 14 days after monocrotaline exposure and becoming established and severe by day 21 (Figure 2A and 2B).

To discriminate the KCNK3 current, we measured the difference in K^+ current at external pH values of 8.3 and 6.3. We named this current the acidic-K^+-sensitive current. Our results showed a progressive decrease in the acidic-K^+-sensitive current with the change in PH (ie, at 7, 14, and 21 days after exposing Wistar rats to monocrotaline). At 7 days after exposure to MCT, the acidic-K^+-sensitive current was significantly reduced by 30%; it was reduced by 80% at 14 days and by 90% at 21 days (Figure 2C and 2D).

Expression of lung KCNK3 protein was reduced by 80% to 90% at 14 and 21 days after monocrotaline exposure (Figure 2E and 2F). However, it was not affected at 7 days even though the acidic current was already significantly reduced at this early time point. Moreover, transcription of KCNK3 was also impaired by 80% in isolated PAs (not shown), whereas the KCNK3 mRNA level was normal in pulmonary veins (not shown).

Using the current clamp mode, we measured significant and progressive depolarization of the resting membrane potential (Em). Em values were –35±1.52, –26±4.0,–20±1.1, and –21.6±2.6 mV in the controls at 7, 14, and 21 days after monocrotaline exposure, respectively (Figure 2G).

Decreased KCNK3 function/expression was also observed in another strain of rats, Sprague-Dawley rats. In Sprague-Dawley rats, the acidic-K^+-sensitive current was reduced by 62% and 85% at 7 and 21 days, respectively, after monocrotaline exposure (Figure 2H). KCNK3 protein level was also reduced by 90% in Sprague-Dawley rats after monocrotaline exposure (Figure 2I). Surprisingly, the acidic-K^+-sensitive current was 2-fold higher in PASMCs from Sprague-Dawley rats compared with Wistar rats, which could be explained by the differential expression of TASK2, another pH-sensitive channel (Figure I in the online-only Data Supplement).

Moreover, we confirmed (by immunostaining) the plasma-membrane localization of KCNK3 in freshly isolated PAECs. Isolated PAECs were positive for the EC marker CD31 (Figure 2J), positive for von Willebrand factor, and negative for α-smooth-muscle actin (not shown). To measure KCNK3 current specifically in isolated PAECs, we previously labeled ECs with a Dylight 594–conjugated tomato lectin from *Lycopersicon esculentum* (a specific marker for EC). At 7 days after monocrotaline exposure, the acidic-K^+-sensitive current was reduced by 80% (Figure 2K) and was associated with membrane depolarization (–26.6±1.38 mV in controls versus –18.7±2.8 mV after monocrotaline exposure; Figure 2L).

Correlative electron microscopy experiments performed on controls or MCT-PH lung samples from rats nicely

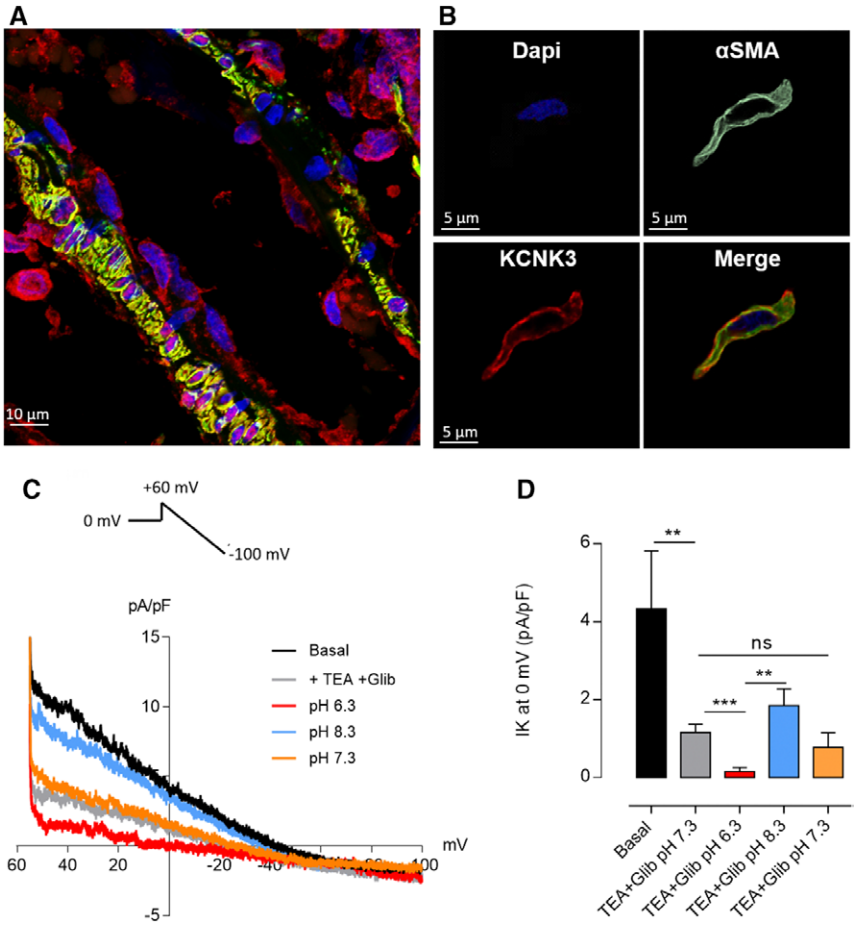

Figure 1. Potassium channel subfamily K member 3 (KCNK3) is functionally expressed in freshly isolated (noncultured) pulmonary artery (PA) smooth muscle cells (PASMCs) from control rats. **A**, Localization by immunofluorescence labeling and confocal imaging of KCNK3 in PAs from control rat lungs. KCNK3 expression and α-smooth-muscle actin (α-SMA) expression are shown in red and green, respectively, and nuclei are shown in blue (DAPI). Scale bar, 10 µm. **B**, Localization of KCNK3 by immunofluorescence labeling in freshly isolated PASMCs from control rats. α-SMA expression and KCNK3 expression are shown in green and red, respectively, and nuclei are shown in blue (DAPI). Scale bar, 5 µm. **C**, Typical current-voltage relationship corresponding to recordings from a 1.2-second voltage ramp protocol (60 to –100 mV) from a holding potential of 0 mV and maintained for 3 minutes. Current was sequentially recorded (1) under basal conditions (black curve) and in the presence of (2) tetraethyl-ammonium (TEA) plus glibenclamide (Glib; 10 mmol/L and 10 µmol/L, respectively) at pH 7.3 (gray curve), (3) TEA+Glib at pH 6.3 (red curve), (4) TEA+Glib at pH 8.3 (blue curve), and (5) TEA+Glib at pH 7.3 (orange curve). The current was normalized by the capacitance of the cell and is represented in pA/pF. **D**, Current density of I_K measured at 0 mV in myocytes isolated from 3 control PAs (3 different rats, 5<n<8 cells). Bar graph mean±SEM. *P<0.05; **P<0.01.

demonstrated that KCNK3 channels were localized mainly at the plasma membrane of PASMCs and PAECs in control rats (Figure IIA in the online-only Data Supplement). According to our Western blot and quantitative polymerase chain reaction experiments, in MCT-PH rats, the numbers of gold particles (KCNK3 channel) were severely impaired in PASMCs and PAECs (Figure IIB in the online-only Data Supplement). Moreover, the KCNK3 channels accumulated in the endoplasmic reticulum compartment and were absent of the plasma membrane of MCT-PASMCs (Figure IIB in the online-only Data Supplement).

However, we also measured a strong reduction in the intensity of other K^+ currents and a decrease in mRNA level of Kv1.2, Kv2.1, Kv1.4, Kv4.2, and Kv4.3 in severe MCT-PH, whereas other K2P channels (TASK2, TWIK1) were not affected (Figure IIIA–IIIC in the online-only Data Supplement).

A293 Inhibits Endogenous KCNK3 Current in Freshly Isolated PASMCs and Modulates the Vascular Tone of the PA

External acidic pH is known to inhibit other members of the K2P family.[16] To characterize I_{KCNK3} more specifically, we used a selective KCNK3 blocker: A293 (supplied by Sanofi). The dose response of A293 on global K^+ current caused a maximal decrease in global K^+ current at 1 µmol/L (Figure IVA in the online-only Data Supplement). To minimize any nonspecific effects, we also used A293 at 200 nmol/L. At 200 nmol/L, A293 reduced K^+ current by 30% (Figure IVA and IVB in the online-only Data Supplement). As expected, in MCT-PH, the A293-sensitive current was reduced by 90% in PASMCs (Figure 3A). In the presence of tetraethylammonium (TEA) plus glibenclamide (Glib), A293 reduced K^+ current similarly to acidic pH (Figure 3B and Figure IVA–IVC), thus confirming the ability of A293 to inhibit KCNK3. Using this specific

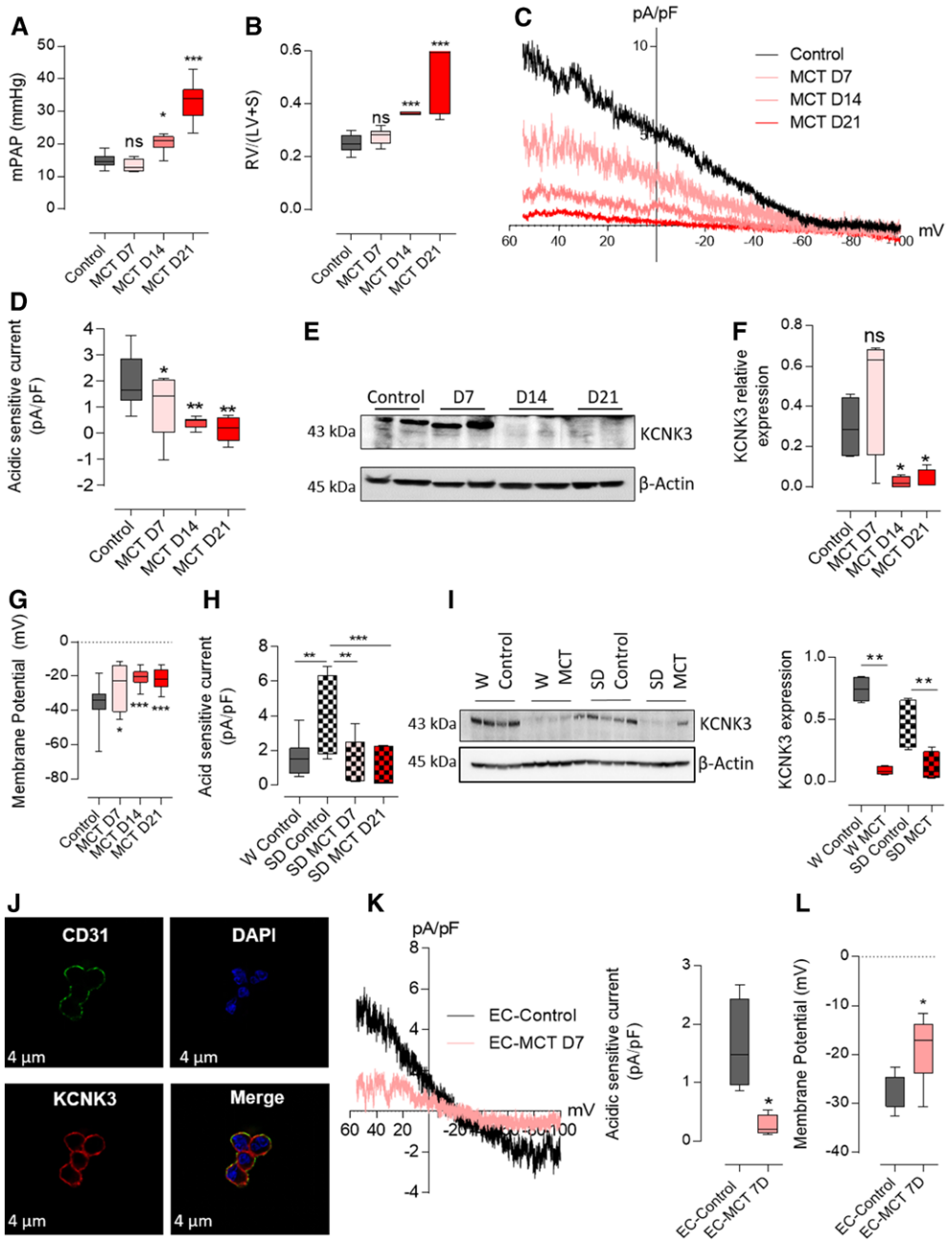

Figure 2. Potassium channel subfamily K member 3 (KCNK3) current and KCNK3 expression are decreased progressively during the development of monocrotaline (MCT)-induced pulmonary hypertension (PH) and correlate with resting plasma-membrane depolarization. **A**, Mean pulmonary artery (PA) pressure (mPAP; in mm Hg) and (**B**) the Fulton index (calculated as the ratio of right ventricular weight to left ventricular plus septal weight [RV/LV+S]) in controls and at 1, 2, and 3 weeks after MCT injection. **C**, Representative inward ramp current in control and MCT-PH PA smooth muscle cells (PASMCs; at 1, 2, and 3 weeks after MCT exposure), corresponding to the difference between the currents recorded at pH 8.3 and at pH 6.3, here called acid-sensitive K^+ current. **D**, Quantification of I_K measured at 0 mV recorded in freshly isolated PASMCs (controls and MCT-PH, n=5–13 cells). (**C** and **D**, 3 different rats per condition, 5<n<13 cells.) **E**, Representative Western blot image of KCNK3 protein expression in the lungs of control and MCT-PAH rats (1, 2, and 3 weeks after MCT exposure). β-Actin was used as the loading control (4 different rats per condition). **F**, Quantification of membrane potential (Em; 3 different rats per condition, 6<n<30 cells). **G**, Resting Em of PASMCs that were freshly isolated from control and MCT-PAH rats (1, 2, and 3 weeks after MCT exposure). **H**, Acid-sensitive current density measured at 0 mV in freshly isolated PASMCs from control and MCT-PAH Wistar (W) and Sprague-Dawley (SD) rats at 1 and 3 weeks after MCT exposure (3 different rats per condition, 6 ≤ n ≤18 cells). **I**, Representative Western blot image of KCNK3 protein expression in lungs from control and MCT-PAH in Wistar and Sprague-Dawley rats at 3 weeks after MCT exposure. β-Actin was used as the loading control. **Right**, Quantification of **I** (4 different rats per condition). **J**, Localization by immunofluorescence labeling and confocal imaging of KCNK3 in freshly isolated PA endothelial cells (ECs) from control rats. **K**, Variation in the acid-sensitive current as a function of time after MCT injection in freshly isolated PAECs (n=3). **L**, Quantification of resting Em in PAECs freshly isolated from control and 1 week after PH induction. **A** through **G** and **J** through **L**, Experiments performed with Wistar rats. Dot plots with min and max. *P<0.05; **P<0.01; ***P<0.005.

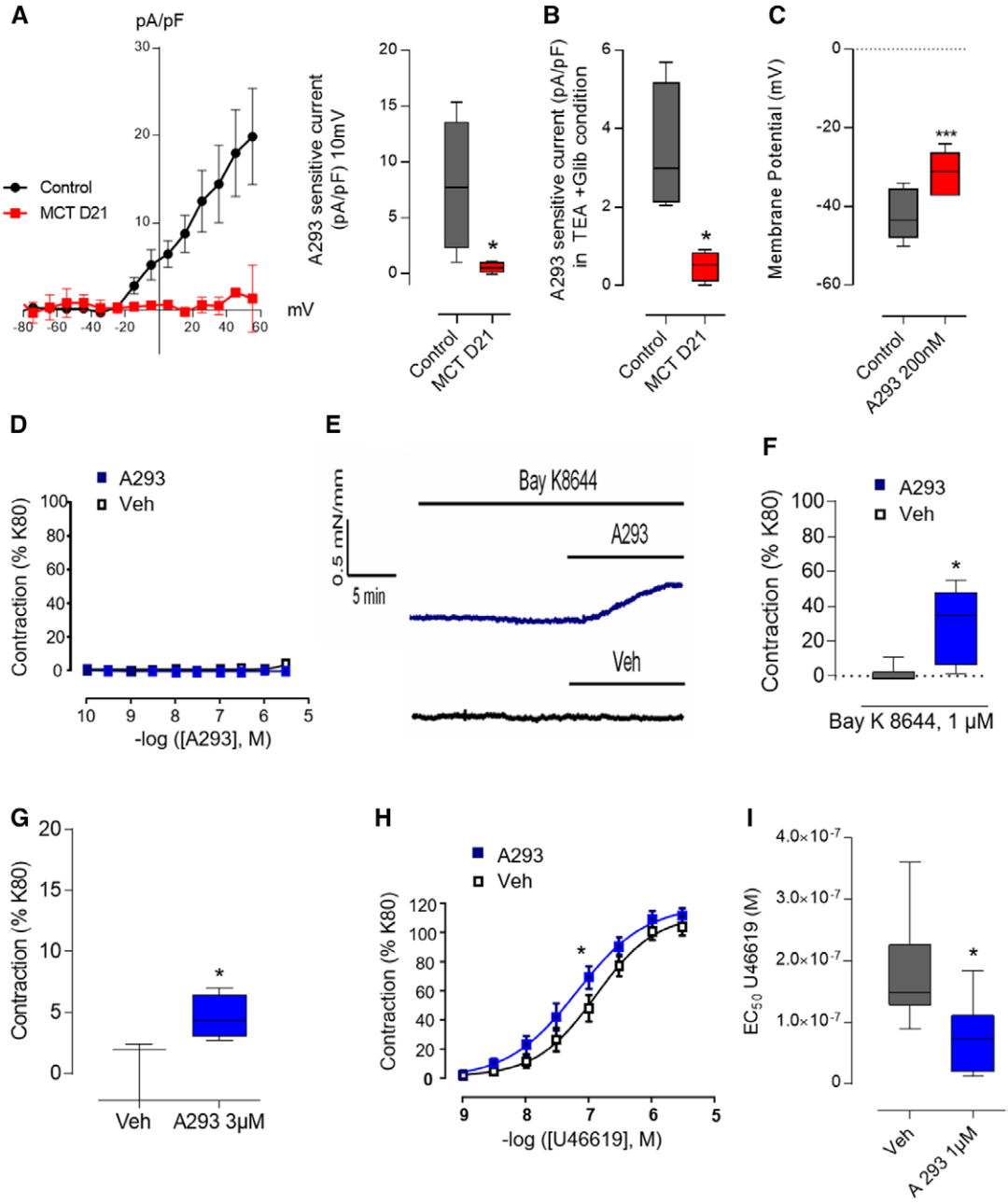

Figure 3. A293 inhibits endogenous potassium channel subfamily K member 3 (KCNK3) current in freshly isolated pulmonary artery (PA) smooth muscle cells (PASMCs) and modulates pulmonary arterial tone. **A**, Current-voltage relationship of the A293-sensitive current in control and monocrotaline (MCT)-induced pulmonary hypertension (PH; 21 days) PASMCs. **Right**, Quantification of the A293-sensitive current (2 different rats, n=6). **B**, Current density of the A293-sensitive current in the presence of tetraethyl-ammonium (TEA) plus glibenclamide (Glib; 2 different rats n=3). **C**, Bar graph represents quantification of resting membrane potential (Em) under basal conditions and after addition of A293 (200 nmol/L; 2 different rats, n=7). **D**, Concentration-response curves established by applying increasing concentrations of A293 (0.1 nmol/–3 µmol/L) or vehicle to resting PA segments mounted on the myograph to record isometric tension (4 and 5 different rats per conditions). **E**, Representative traces showing the effect of A293 or vehicle on PA resting tone in the presence of 1 µmol/L Bay-K8644 (L-type Ca^{2+} channel agonist; 6 different rats). **F**, Quantification of contractile responses induced by the addition of A293 or vehicle in the presence of 1 µmol/L Bay-K8644 (7 and 8 different rats). **G**, Quantification of contractile responses induced by the addition of 10 mmol/L TEA in the presence of A293 or vehicle. **H**, Dose-response curve established by applying increasing concentrations of U46619 (thromboxane A2 mimetic, 1–3 µmol/L) in the presence of 1 µmol/L A293 or vehicle. **I**, Half-maximal effective concentration (EC50) of U46619 in the presence of A293 or vehicle (**H** and **I**, 8 different rats). Dot plots with min and max. **P*<0.05; ***P*<0.01; ****P*<0.005.

pharmacological approach, we confirmed that KCNK3 function was severely reduced in PASMCs from MCT-PH rats and that KCNK3 was involved in the maintenance of resting Em (–40 to –30 mV; Figure 3C).

Using isolated vessels in organ bath experiments, we assessed the involvement of KCNK3 in controlling rat pulmonary arterial tone. We hypothesized that if KCNK3 significantly contributed to the resting tone of PA, then one would

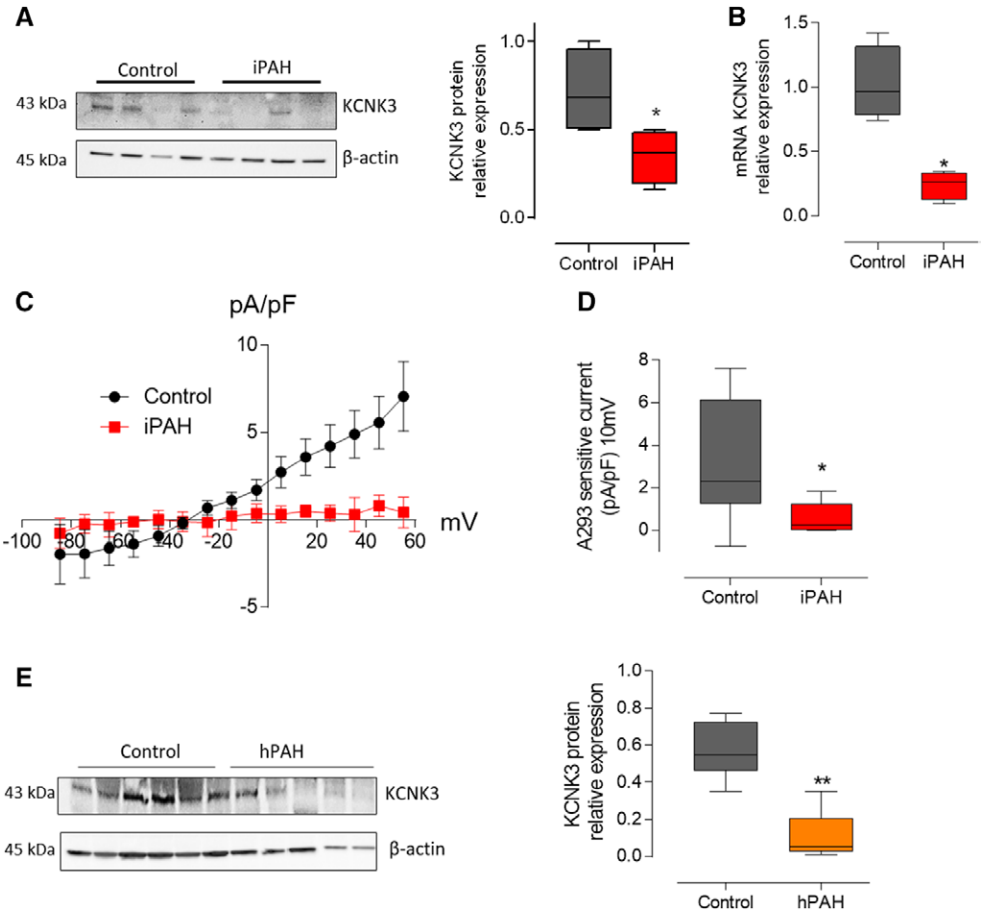

Figure 4. Pulmonary expression of potassium channel subfamily K member 3 (KCNK3) is reduced in patients with pulmonary arterial (PA) hypertension (PAH). **A**, Representative Western blot image to quantify KCNK3 expression in the lungs from control and patients with idiopathic PAH (iPAH; n=4). β-Actin was used as a loading control. **Right**, Mean values of **A** (n=4 for control subjects and patients with PAH). **B**, mRNA level expression of KCNK3 on PAs isolated from control subjects and patients with iPAH (n=4 for both control subjects and patients with PAH). **C**, Current-voltage relationship of the A293-sensitive current in human control and iPAH PA smooth muscle cells. **Right**, Quantification of the A293-sensitive current (n=10 and 7 cells). **E**, Representative Western blot image to quantify KCNK3 expression in lungs from control subjects (n=6 patients), patients with heritable PAH (hPAH) who were carriers of a *BMPR2* mutation (n=5 patients). β-Actin was used as a loading control. **Right**, Quantification of **D**. Dot plots with min and max. **P*<0.05; ***P*<0.01.

expect that KCNK3 inhibition would bring resting Em to a value close to the opening threshold of L-type Ca^{2+} channels, thus facilitating the contraction of arteries. Unexpectedly, increasing the concentration of A293 (0.1–3 µmol/L) did not contract quiescent PAs (Figure 3D). However, if isolated arteries were pretreated with Bay-K8644, a drug that potentiates voltage sensitivity of L-type Ca^{2+} channels,[17] A293 (at 1 µmol/L) induced significant contraction of PAs (35% of the response to the solution with $[K^+]$ at 80 mmol/L [K80]; Figure 3E and 3F). Used at 200 nmol/L, A293 also induced significant contraction of Bay-K8644–prestimulated PAs (8%–10% of the K80 response) and shifted the concentration-effect curve to the left compared with vehicle (Figure IVD and IVE in the online-only Data Supplement). Additionally, in the presence of A293, the addition of 10 mmol/L tetraethylammonium, known to block other K^+ channels, including BK_{Ca} and some Kv,[14] evoked significant PA constriction (20% of the K80 response; Figure 3G). Finally, 1 µmol/L A293 significantly potentiated the contractile response of the thromboxane A_2 mimetic U46619, shifting the concentration-effect curve to the left compared with vehicle (Figure 3H and 3I). Taken together, these results demonstrate that KCNK3 inhibition with 1 µmol/L A293 predisposes PAs to constrict.

Pulmonary Expression of KCNK3 Is Reduced in Patients With PAH

We analyzed the pulmonary expression of KCNK3 in mRNA (isolated PA) and protein (lung tissues) from patients with iPAH and control subjects (Figure 4A and 4B). As shown in Figure 4, protein (n=4) and mRNA (n=5) levels of KCNK3 were significantly reduced in patients with iPAH compared with the control subjects (P<0.05; Figure 4A and 4B). Using patch-clamp technique, we then evaluated KCNK3 function in cultured human PASMCs (control and iPAH) plus the selective KCNK3 blocker A293 at 200 nmol/L. In iPAH PASMCs, the A293-sensitive current was reduced by 80% compared with non-PAH human PASMCs (Figure 4C and 4D). We showed that lung protein expression of KCNK3 was significantly reduced in patients with hPAH carrying a *BMPR2* mutation (n=5; Figure 4E).

Chronic In Vivo Inhibition of KCNK3 Induced Exaggerated Proliferation of PAECs, PASMCs, and Adventitial Fibroblasts With Subsequent Distal Artery Neomuscularization and Early Hemodynamic Signs of PH

To evaluate whether the loss of function of KCNK3 can initiate PH, we chronically inhibited KCNK3 in rats using A293 (10 $mg \cdot kg^{-1} \cdot d^{-1}$ IP). After 7 and 28 days, the global K^+ current was significantly reduced by 30% in isolated PASMCs (Figure 5A). Interestingly, the A293-sensitive current was strongly reduced after 7 and 28 days of A293 exposure (no significant differences were measured between 7 and 28 days; Figure 5B and 5C). Moreover, PASMCs isolated from A293-treated rats were significantly depolarized (–40 to –31 mV; not shown), whereas mRNA expression and protein expression of KCNK3 were not modified (Figure 5D–5F).

To evaluate the consequence of KCNK3 inhibition on in situ pulmonary cell proliferation, we visualized cells undergoing DNA replication in A293-exposed rats by measuring the incorporation of 5-ethynyl-2'-deoxyuridine (EdU; yellow arrows in Figure 5). As expected, we detected very few EdU-positive cells in control lung parenchyma (Figure 5G), whereas there were areas of intense microvascular EC proliferation ($CD34^+EdU$ cells) in A293-exposed rats (Figure 5Ga). Few PASMCs were EdU positive (α-smooth-muscle $actin^+EdU$ cells; Figure 5Gb). We also observed adventitial fibroblast cell proliferation in rats treated for 28 days with A293 ($vimentin^+EdU^+$ cells; Figure 5Gc).[18] To ascertain the overproliferation of adventitial fibroblasts in A293-treated rats, we performed coimmunostaining with the fibroblast markers CD90 and fibronectin. As shown in Figure V in the online-only Data Supplement, many EdU-positive cells were also positive for CD90 (Figure VA in the online-only Data Supplement) and fibronectin (Figure VB in the online-only Data Supplement), confirming the fibroblast phenotype of adventitial EdU-positive cells.

Interestingly, some EdU-positive cells were $CD4^+$ (T-helper cell markers) or $CD8^+$ (cytotoxic T-cell markers), denoting the presence of active inflammation in A293-exposed rats (Figure VIA and VIB in the online-only Data Supplement). Moreover, in rats treated for 7 days, the number of proliferative cells was significantly increased compared with controls but was still 2-fold less than in rats treated for 28 days (Figure 5Gd). Accordingly, A293-treated rats presented with significant distal neomuscularization (Figure 5H). Interestingly, A293-treated rats displayed significantly increased right ventricular systolic pressure (Figure 5I), whereas the RV was not yet hypertrophied (not shown) and cardiac output was not altered (Figure 5I).

Western blot analysis demonstrated increased expression of the fibroblast markers vimentin and phosphorylated vimentin (Ser55) in the lungs from A293-exposed rats (28 days) compared with control rats (Figure 6A). We also observed significantly increased phosphorylation of ERK1/2 in the lungs of A293-exposed rats (proliferative pathway; Figure 6A). At 28 days (but not 7 days), long-term KCNK3 inhibition led to significantly increased mRNA levels of platelet-derived growth factor (PDGF) receptor b, PDGFa, interleukin (IL)-17Ra, and α1 antitrypsin (A1AT; Figure 6B and 6C).

Moreover, the serum levels of monocyte chemoattractant protein-1 (MCP-1), tissue inhibitor of metalloproteinase-1 (TIMP-1), and Gro-1 (melanoma growth stimulating activity, alpha 1) were significantly higher in A293-treated rats compared with vehicle-treated rats (Figure 6D and 6E). This was related to an increase in lung mRNA expression of MCP-1, TIMP-1, and Gro-1. Other markers of endothelial dysfunction and inflammation were not modified (caveolin-1, CTGF, Interleukin 6, plasminogen activator inhibitor-1, tumor necrosis factor-α, adiponectin, soluble E-selectin, soluble intracellular adhesion molecule-1, von Willebrand factor; not shown). Kv1.5 mRNA expression was significantly reduced in A293-exposed rats (28 days), whereas Kv2.1 mRNA expression was not affected (Figure 6F).

In Vivo Pharmacological Activation of KCNK3 Interferes With MCT-PH Development

Because we had demonstrated that KCNK3 is downregulated in the lungs and PA of patients with PAH and MCT-PH rats, we next evaluated the consequences of in vivo pharmacological activation of the KCNK3 channel using ONO-RS-082. In 2007, a patent explored the molecules associated with phosphorylation of the human KCNK3 channel. The authors of the patent found several molecules that could modulate human KCNK3, including the phospholipase A2 inhibitor ONO-RS-082.[18a] In their work, which identified KCNK3 mutations in PAH, Ma and colleagues[3] proposed that ONO-RS-082 could be a beneficial therapeutic approach to target KCNK3.

In vitro, we demonstrated, using the patch-clamp technique, that ONO-RS-082 (10 μmol/L) improved KCNK3 current (tetraethylammonium-insensitive current; Figure VIIA and VIIB in the online-only Data Supplement). However, when administrated in vivo from days 14 to 21 to MCT-PH rats, ONO-RS-082 was inefficient at alleviating PH in MCT-PH rats (Figure VIIC in the online-only Data Supplement). Of relevance, we demonstrated that KCNK3 expression was totally abolished at these time points (Figure 2). Hence, we used a long-term treatment of ONO-RS-082 at 50 $mg \cdot kg^{-1} \cdot d^{-1}$ (from days 1–21; Figure 7A). At 3 weeks after treatment initiation, the hemodynamic measurements demonstrated that the long-term application of ONO-RS-082 significantly reduced PH induced by exposure to MCT. Compared with MCT-vehicle rats, a 50% reduction in RV systolic pressure (Figure 7B) and a 60% reduction in RV hypertrophy (Figure 7C) were observed in ONO-RS-082 rats receiving monocrotaline, whereas cardiac output was not improved by the ONO-RS-082 treatment (Figure 7D). Additionally, long-term administration of ONO-RS-082 induced a significant reduction in vascular wall thickness compared with MCT-vehicle rats (Figure 7E and 7F). Moreover, in the monocrotaline ONO-RS-082 group, Western blot analysis demonstrated lung KCNK3 expression similar to that in the control group (Figure 7G). These results demonstrate the potential efficacy and pharmacological therapeutic action on KCNK3 function to reduce PH when KCNK3 expression is still present.

Discussion

This study has 5 major findings. Using a combination of patch-clamp recording methods and biochemical and ex vivo

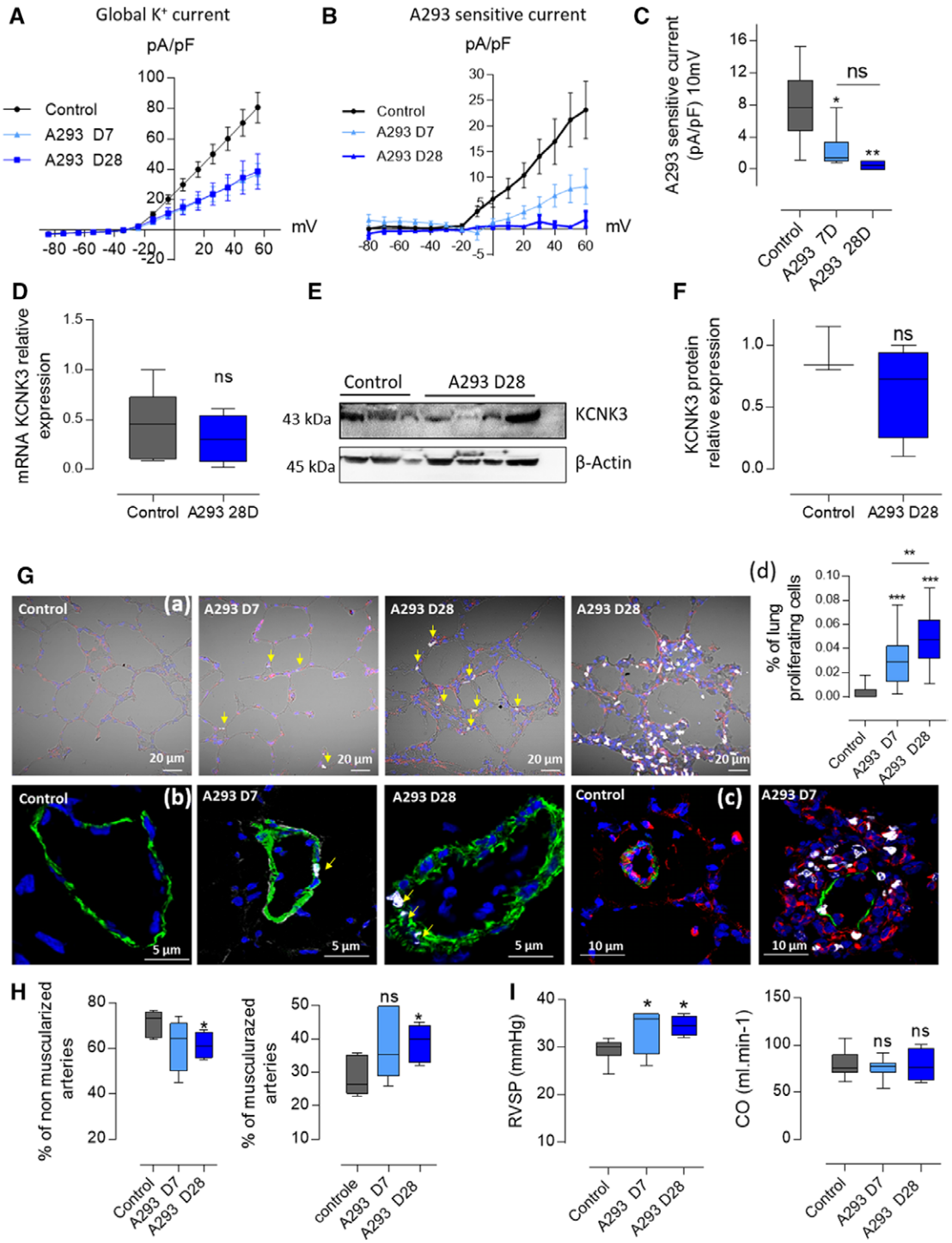

Figure 5. In vivo chronic inhibition of potassium channel subfamily K member 3 (KCNK3)–induced exaggerated proliferation of pulmonary artery (PA) endothelial cells (ECs), PA smooth muscle cells (PASMCs), and adventitial fibroblasts; distal artery neomuscularization; and early hemodynamic signs of pulmonary hypertension (PH). **A**, Current-voltage relationship curves for global K^+ current recorded in freshly isolated PASCMs from controls and A293-treated rats (at 7 and 28 days). **B**, Current-voltage relationship curves of A293-sensitive current in PASMCs from controls and A293-treated rats (2 different rats per conditions, 4<n<9 cells). **C**, Quantification of current density of A293-sensitive current measured in **B** (2 different rats per conditions, 4<n<9 cells). **D**, Lung mRNA expression of KCNK3 from control rats and A293-treated rats (28 days; n=4 different rats per condition). **E**, A representative Western blot image of lung protein expression of KCNK3 in control and A293-treated rats (28 days). β-Actin was used as a loading control. **F**, Quantification of **E** (n=4 different rats per condition). **G**, Immunofluorescent staining of frozen rat lung sections and confocal imaging with Click-iT 5-ethynyl-2'-deoxyuridine (EdU; white nuclei=EdU-positive nuclei=proliferating cells) in combination with (**a**) CD34 (ECs in red), (**b**) α-smooth-muscle actin (α-SMA; in green, and (**c**) vimentin (fibroblast marker in red). Counterstain was DAPI (blue). (**d**) Quantification of the percentage of lung proliferating cells. **H**, Quantification of the percentage of nonmuscularized arteries (**left**) and muscularized arteries (**right**; n=6 different rats per condition). **I**, Right ventricular systolic pressure (RVSP; **left**) and cardiac output (CO; **right**) in control and A293-treated rats (n=10 different rats per condition). Dot plots with min and max. **P*<0.05; ***P*<0.01.

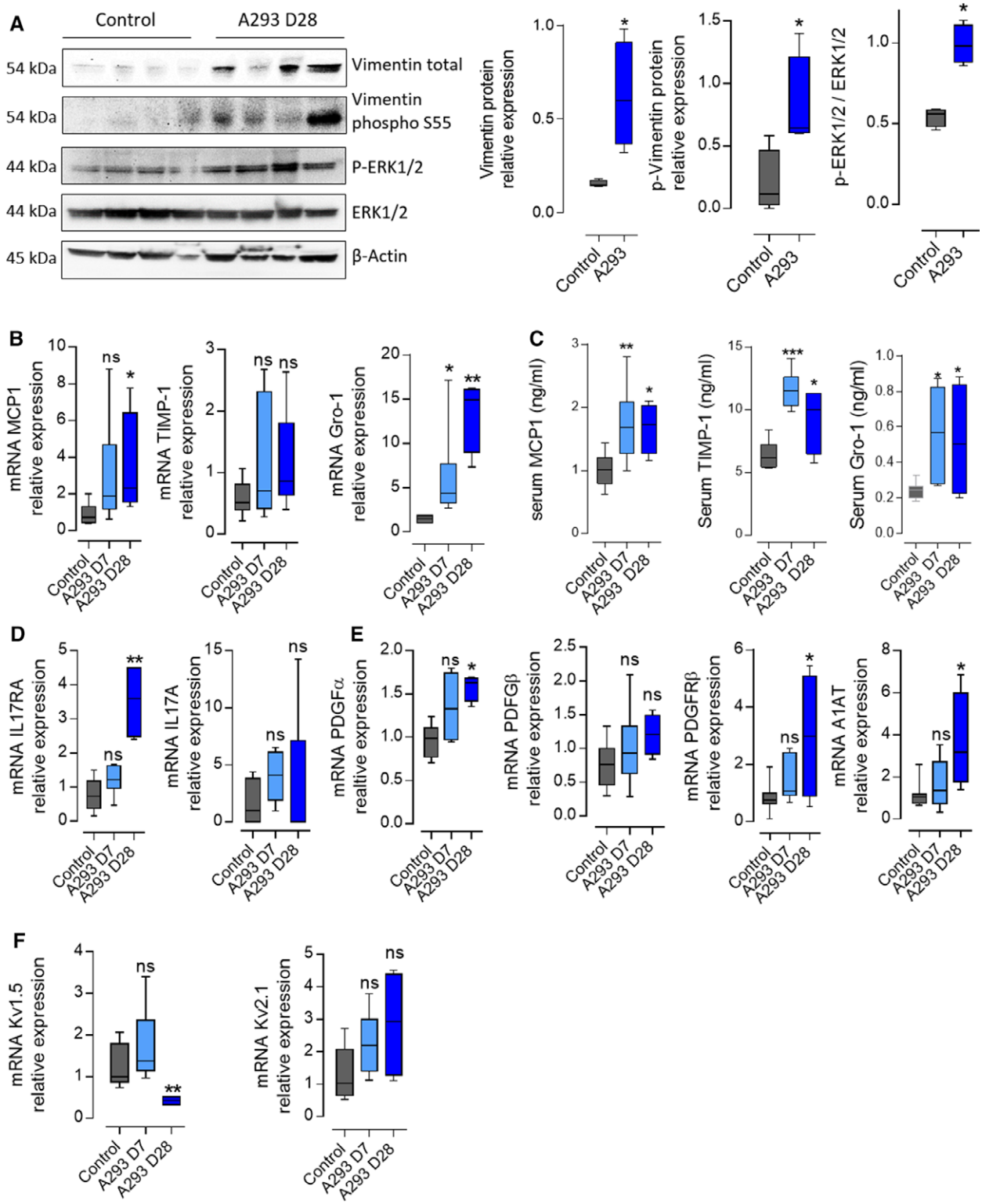

Figure 6. Molecular consequences of chronic potassium channel subfamily K member 3 (KCNK3) inhibition in lungs and sera. **A**, Vimentin, phosphorylated (p) vimentin (S55), phosphorylated extracellular signal-regulated kinase (ERK) 1/2, and ERK1/2 expression in the lungs of control and A293-treated rats (28 days). β-Actin was used as a loading control. **Right**, Quantification of Western blots (n=4 different rats per condition). **B**, mRNA expression of monocyte chemoattractant protein-1 (MCP-1), tissue inhibitor of metalloproteinase-1 (TIMP-1), and Gro-1 (melanoma growth stimulating activity, alpha 1) in lungs from control and A293-treated rats (after 7 and 28 days treatment; n=5 different rats per conditions). **C**, Serum concentrations of MCP-1, TIMP-1, and Gro-1 in control and A293-treated rats (at 7 and 28 days; n=5). **D**, mRNA expression of interleukin IL-17RA and IL-17a mRNA in lungs from control and A293-treated rats (at 7 and 28 days; n=5 different rats per condition). **E,** mRNA expression of platelet-derived growth factor (PDGF) α, PDGFβ, PDGF receptor (PDGFR) β, and α1 antitrypsin (A1AT) in lungs from control and A293-treated rats (at 7 and 28 days; n=5 different rats per condition). **F**, mRNA expression of KCNA5 (Kv1.5) and KCNB1 (Kv2.1) in lungs from control and A293-treated rats (at 7 and 28 days; n=5 different rats per condition). Dot plots with min and max. **P*<0.05; ***P*<0.01.

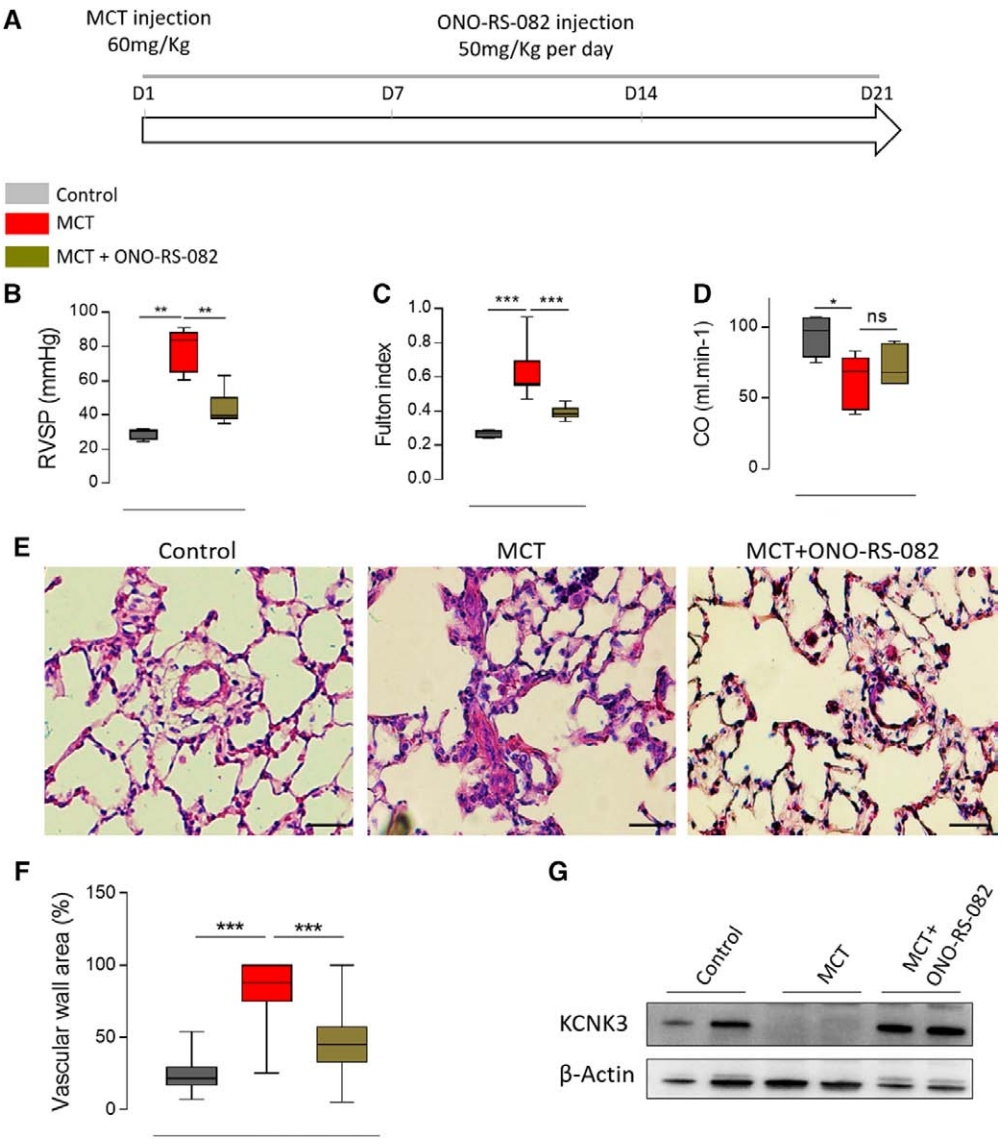

Figure 7. In vivo pharmacological activation of potassium channel subfamily K member 3 (KCNK3) interferes with monocrotaline (MCT)-induced pulmonary hypertension (PH) development. **A,** In vivo experimental design. ONO-RS-082 (50 mg·kg^{-1}·d^{-1} for 3 weeks) was administrated long term during MCT exposure by intraperitoneal injection. **B**, Right ventricular systolic pressure (RVSP; mm Hg; n=4-7 different rats per condition) **C**, Fulton index (n=5-10 different rats per condition). **D**, Cardiac output (CO; mL/min). **E**, Representative hematoxylin and eosin staining of paraffin-embedded lung sections from the control, MCT, and MCT+ONO-RS-082 groups. **F**, Vascular wall area (%) in the control, MCT, and MCT+ONO-RS-082 groups (n=6 different rats per condition). **G**, Representative Western blot image of protein KCNK3 expression in lungs from control, MCT, and MCT+ONO-RS-082 rats. β-Actin was used as a loading control (n=2 different rats per condition). Dot plots with min and max. Scale bar, 20 μm. ***P*<0.01; ****P*<0.001.

and in vivo approaches, we have provided, for the first time, strong evidence for the following. First, KCNK3 expression and function are severely reduced in iPAH, hPAH, and MCT-PH. Second, I_{KCNK3} current decreases progressively during the development of MCT-induced PH and was correlated with the depolarization of the resting plasma membrane. Third, KCNK3 modulates pulmonary arterial tone. Fourth, long-term inhibition of KCNK3 (with the compound A293) enhances distal artery neomuscularization, together with subsequent development of early hemodynamic signs of PH, and exacerbated proliferation of PAECs, PASMCs, and adventitial fibroblasts, in association with an increase in some markers of inflammation. Fifth, in vivo pharmacological activation of KCNK3 reduces the development of PH in the monocrotaline rat model.

Interestingly, we have shown that reduction in KCNK3 expression and function are concomitant to PH induction and precedes hemodynamic changes, thus suggesting that reduction of I_{KCNK3} is not a mere consequence of PH but rather an active player in its pathogenesis. At 1 week after PH initiation, I_{KCNK3} is reduced, whereas protein expression of KCNK3 is as yet unaffected, suggesting that KCNK3 may be not correctly addressed to the plasma membrane or inhibited. Correlated to the decrease of I_{KCNK3}, the plasma membranes of PASMCs and PAECs from MCT-PH rats are depolarized by ≈10 mV, confirming the role of KCNK3 in resting Em, which has

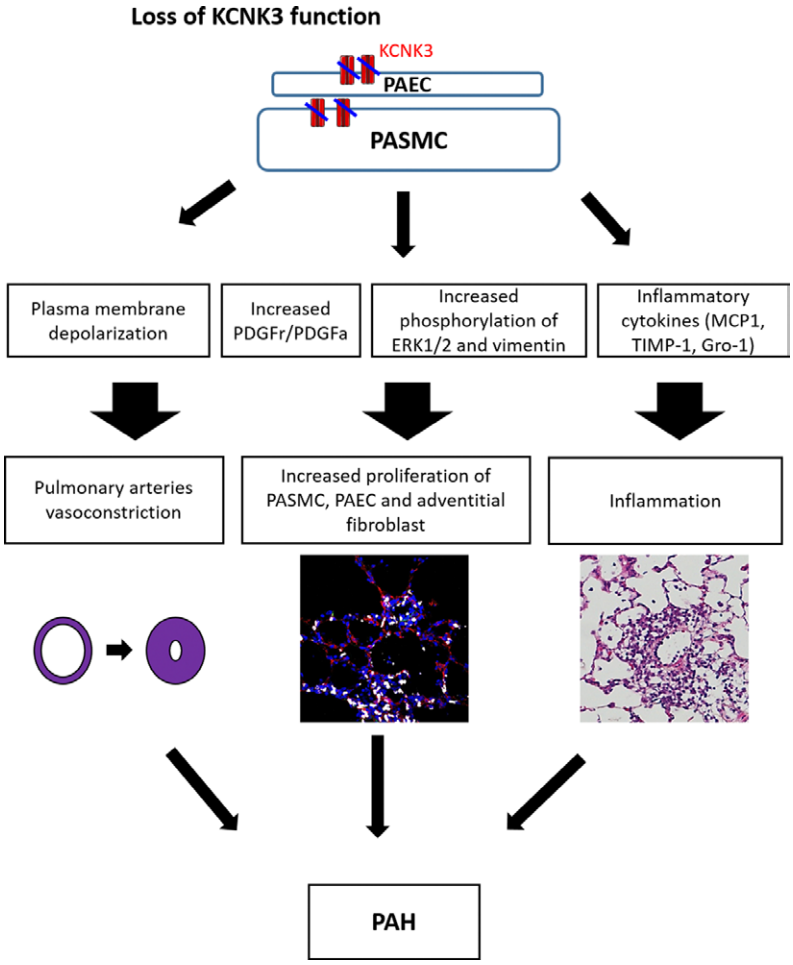

Figure 8. Proposed sequence of events arising from loss/inhibition of potassium channel subfamily K member 3 (KCNK3). Loss of KCNK3 function in pulmonary artery smooth muscle cells (PASMCs) and pulmonary artery endothelial cells (PAECs) results in plasma-membrane depolarization, leading to pulmonary arterial vasoconstriction, pulmonary vascular cell proliferation (partly explained by increased expression of platelet-derived growth factor [PDGF]), and inflammation. All these events are responsible for pulmonary vascular remodeling and pulmonary arterial hypertension (PAH). ERK indicates extracellular signal-regulated kinase; MCP-1, monocyte chemoattractant protein-1; PDGFr, platelet-derived growth factor receptor; and TIMP-1, tissue inhibitor of metalloproteinase-1.

been previously suggested as a nonspecific KCNK3 inhibitor (acidic pH or anandamide)[15] or an siRNA strategy.[19]

In this study, we used a selective KCNK3 inhibitor (A293), which was originally described to inhibit 90% of KCNK3 current, 50% percent of KCNK9 current, and <10% of other K^+ channels tested (TASK2, TASK4, TREK-1, Kv1.1, Kv1.3, Kv1.4, Kv1.5, Kv4.3, and hERG) at 1 µmol/L.[7] Of note, KCNK9 is not expressed in rat PA[15] (data not shown) and hence cannot be part of the K^+ current. In line with the role played by KCNK3 in resting Em, we have demonstrated that KCNK3 inhibition modulates pulmonary arterial tone and contributes to vasoconstriction.

Manoury and coworkers[20] suggested a crucial role for KNCK3 function in PASMCs, which we have highlighted in our study. They demonstrated that KCNK3 is almost lost in culture conditions (a highly proliferative phenotype) compared with in situ conditions (contractile condition). In line with this observation, patch-clamp measurements of human cultured PASMCs revealed that the A293-sensitive current was smaller than the current recorded on PASMCs freshly isolated from rats (by 10-fold). Moreover, the A293-sensitive current was significantly reduced in human iPAH PASMCs compared with controls. Hence, in our study, we mainly used freshly isolated PASMCs or PAECs.

It is well established that the MCT-PH model is characterized by abnormal pulmonary vascular cell proliferation. In vivo long-term KCNK3 inhibition experiments have revealed an exaggerated proliferation of PAECs, PASMCs, and adventitial fibroblasts, which are hallmarks of PAH,[21] further demonstrating the role of KCNK3 in the loss of function and as an initial molecular trigger of PAH by promoting cell proliferation. Accordingly, knockdown of KCNK3 in mouse neuroblastoma N2A cells increases N2A cell proliferation rates by >25%,[22] thus illustrating our finding. At a mechanistic level, long-term inhibition of KCNK3 induced increased expression of A1AT, which could participate in the hyperproliferative phenotype of adventitial fibroblasts. Indeed, high levels of A1AT are, for instance, observed in lung adenocarcinoma cell line,[23] and A1AT stimulates fibroblast proliferation in vitro[24]; conversely, decreased expression of A1AT reduces cell migration and cell invasion.[23] Moreover, the vimentin overexpression observed in A293-treated lungs suggests the accumulation of pulmonary fibroblasts. Like A1AT, vimentin is a cancer marker involved in cell proliferation and migration processes[25] and in mesenchymal transition, which could potentially occur during the development of vascular pathologies.[26] We have recently demonstrated high expression of vimentin and of phosphorylated vimentin (Ser55) during the development of PAH.[13] Ser55 on vimentin is phosphorylated in various types of cells only during the early mitotic phase by Cdk1.[27] Vimentin has been shown to interact with phosphorylated extracellular signal-regulated kinase (ERK) 1/2 and to protect it against dephosphorylation,[28]

extending its mitotic potential. Accordingly, we observed increased phosphorylation of ERK1/2, which certainly supports vascular proliferation induced by the inhibition of KCNK3. Our results also suggest that PA remodeling, induced by KCNK3 inhibition, may occur though the PDGF/PDGF receptor axis, a crucial promitotic axis in PAH.[29,30]

We also observed an increase in MCP-1, TIMP-1, IL-17RA, and other inflammatory molecules known to be implicated in the immunopathology of PAH.[31] This highlights the fact that KCNK3 inhibition acts on the whole spectrum of PAH pathomechanisms, from vasoconstriction and vascular cell proliferation to PAH-associated chronic inflammation. For instance and in accordance with our results, (1) TIMP-1 is overexpressed by cultured PASMCs from patients with iPAH,[32] and in vivo TIMP-1 gene transfer occurs in rat-potentialized PAH induced by chronic hypoxia[33]; (2) MCP-1/CCL2 is strongly involved in the development of human and experimental PAH[34,35] and plays a key role in arterial remodeling though direct mitotic/chemotactic effects on PASMCs and PAECs but also recruits monocytes/mononuclear cells and populations of circulating progenitors[36,37]; and (3) there is an IL-17–dependent (Th17) immune polarization in PAH[38] and IL-17 mRNA, and protein expression is significantly increased in hypoxia-induced PH mouse models.[39] Interestingly, IL-17 binds to its receptor composed of IL-17RA and IL-17RC subtypes, leading to activation of the canonical ERK1/2, phosphoinositide 3-kinase–Akt, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinase pathways.[40]

We have shown that KCNK3 expression was reduced in patients with iPAH and hPAH and in MCT-PH rats. Interestingly, we have also previously demonstrated that PH induced by cyclophosphamide in rats is associated with a 70% reduction in KCNK3 expression.[12] Moreover, acute hypoxia blocks KCNK3 current in cultured PASMCs.[19] Together, these findings suggest that inhibition of KCNK3 is involved in the development of diverse causes of PH. Indeed, KCNK3 is known to be regulated by several mechanisms involved in PH pathobiology. For instance, endothelin-1, an extremely powerful vasoconstrictor that is involved in vascular remodeling, has been shown to inhibit KCNK3 via Rho kinase in human PASMCs.[41-42] Growth factor signaling, such as PDGF receptor and tyrosine kinase activity, act via a common pathway that influences the function and expression of K^+ channels[43–44] and could also partially explain the decrease in I_{KCNK3}. Moreover, downstream of the PDGF or tyrosine kinase receptors, Src kinase signaling is crucial for K^+ channel function, including TASK1/KCNK3.[45]

Our findings are summarized in Figure 8. We propose that the loss of function of KCNK3 is followed by the following sequence of events: (1) plasma-membrane depolarization; (2) PA vasoconstriction; (3) increase in pulmonary vascular cell proliferation (vascular ECs and PASMCs); (4) increase in adventitial fibroblast proliferation via upregulation of phosphorylated ERK1, PDGF, and vimentin; (5) pulmonary inflammation via upregulation of the MCP-1, Gro-1, IL-17 axes; and (6) increase in right systolic ventricular pressure

Limitations

ONO-RS-082 is a phospholipase A2 inhibitor. The impact of ONO-RS-082 treatment on phospholipase A2 activity could also have contributed to alleviate MCT-PH. We have demonstrated here that KCNK3 expression was abolished whereas expression of phospholipase A2 was known to be upregulated in the MCT-PH models (more precisely, 3 and 4 weeks after PH initiation by monocrotaline exposure).[46] If the improvement in PH by ONO-RS-082 was caused by decreased phospholipase A2 activity, we would have observed a partial correction of PH as a curative strategy. This was not the case, suggesting that the improvement in PH by long-term ONO-RS-082 treatment was caused mainly by its pharmacological activation of KCNK3 channels.

Conclusions

In conclusion, we show, for the first time, several lines of evidence that KCNK3 channel dysfunction participates in the development of PH and may act as one of its triggers. As recently supported by the discovery of *KCNK3* mutations that cause hPAH, we show that decreased KCNK3 expression is an emerging hallmark of PAH. Lastly, and as suggested by Ma and collaborators,[3] we show that in vivo KCNK3 activation with ONO-RS-082 is viable and effective at restoring/ameliorating PH in our monocrotaline experimental PH model when KCNK3 is expressed.

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Disclosures

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CLINICAL PERSPECTIVE

Pulmonary arterial hypertension (PAH) is a severe cardiopulmonary condition leading to right-sided heart failure and ultimately death. In the last 20 years, the development of drugs that specifically target pathways involved in disease pathogenesis has led to improvements in the quality of life and clinical outcomes in patients with PAH, but there is still no cure for this devastating disease. Recently, the identification of loss-of-function mutations in the gene encoding the potassium channel subfamily K member 3 (KCNK3) in some patients with PAH has highlighted a novel dysfunctional pathway and a potential therapeutic target. In the present study, we demonstrate that loss of function of KCNK3 is a hallmark of idiopathic and heritable PAH and experimental pulmonary hypertension. Moreover, we show that in vivo pharmacological activation of KCNK3 with the phospholipase A2 inhibitor ONO-RS-082 alleviates monocrotaline-induced pulmonary hypertension in rats when KCNK3 is still expressed in pulmonary arteries. This suggests that this strategy might be of interest in patients with PAH who retain a residual level of KCNK3 expression, possibly within the frame of personalized therapy. A major limitation of the ONO-RS-082 compound is its phospholipase A2 inhibitory activity, which can have deleterious side effects. Identification or development of more specific molecules is thus needed to envision KCNK3 activation in humans. Alternatively, targeted gene therapy allowing KCNK3 re-expression in affected pulmonary arteries could be another possible approach.