Pulmonary Hypertension

TWIK-2 Channel Deficiency Leads to Pulmonary Hypertension Through a Rho-Kinase–Mediated Process

Lavannya M. Pandit, Eric E. Lloyd, Julia O. Reynolds, William S. Lawrence, Corey Reynolds, Xander H.T. Wehrens, Robert M. Bryan

Abstract—TWIK-2 (KCNK6) is a member of the 2-pore domain (K_2p) family of potassium channels, which are highly expressed in the vascular system. We tested the hypothesis that TWIK-2 deficiency leads to pulmonary hypertension. TWIK-2 knockout mice and their wildtype littermates at 8 weeks of age had similar mean right ventricular systolic pressures (24±3 and 21±3 mm Hg, respectively.) Significantly, by 20 weeks of age, the mean right ventricular systolic pressures in TWIK-2 knockout mice increased to 35±3 mm Hg (P<0.036), whereas mean right ventricular systolic pressures in wildtype littermates remained at 22±3 mm Hg. Elevated mean right ventricular systolic pressures in the TWIK-2 knockout mice was accompanied by pulmonary vascular remodeling as determined by a 25% increase in the cross-sectional area of the vessels occupied by the vessel wall. Additionally, secondary branches of the pulmonary artery from 20-week-old TWIK-2 knockout mice showed an enhanced contractile response to U46619 (10^{-6} moles/L), a thromboxane A2 mimetic, which was completely abolished with the Rho-kinase inhibitor, Y27632 (10^{-6} and 10^{-5} moles/L). Treatment of TWIK-2 knockout mice with the Rho-kinase inhibitor, fasudil, in the drinking water for 12 weeks, abolished the development of pulmonary hypertension and attenuated the vessel remodeling. We concluded that mice deficient in the TWIK-2 channel develop pulmonary hypertension between 8 and 20 weeks of age through a mechanism involving Rho-kinase. Our results suggest that downregulation of TWIK-2 in the pulmonary vasculature may be an underlying mechanism in the development of pulmonary hypertension. (Hypertension. 2014;64:1260-1265.) • Online Data Supplement

Key Words: KCNK6 • potassium channel • pulmonary hypertension • Rho-kinase • TWIK-2

Pulmonary hypertension (PH) is a pathological lung condition that occurs because of vascular remodeling. Although normal mean pulmonary arterial pressure varies from 10 to 20 mm Hg, PH is defined as mean pulmonary arterial pressure ≥25 mm Hg. Although the precise incidence and prevalence of PH is uncertain and underestimated, it is being diagnosed at an increasing rate. The World Health Organization has stratified PH into 5 major groups based on the genetic, environmental, and pathogenic triggers. Although the underlying mechanisms may be different, increased pulmonary vascular resistance resulting from a combination of sustained vasoconstriction, thrombosis, inflammation leading to pulmonary vascular remodeling is common to all groups. An important consequence of PH is an increase in right ventricular afterload which, over time, leads to right ventricular hypertrophy, right heart failure, and ultimately death.

One of several hypotheses to explain PH involves dysfunction or downregulation of potassium (K+) channels in the pulmonary circulation. K+ channels regulate a number of cellular functions that possibly contribute to the development of PH. In particular, K+ channels function to regulate membrane potential in pulmonary vascular smooth muscle cells. When K+ channels open during normal physiological conditions, K+ moves along its electrochemical gradient from the cytoplasmic space to the extracellular space. The efflux of positive K+ ions results in a more hyperpolarized membrane potential. When K+ channels close, become dysfunctional, or their expression is downregulated, the cell depolarizes and intracellular Ca2+ increases as a result of voltage-dependent calcium channel activation. Because intracellular free Ca2+ is a second messenger for smooth muscle contraction, K+ channel dysfunction can result in heightened constriction of pulmonary arteries, leading to increased pulmonary vascular resistance.

Two families of K+ channels, voltage-activated K+ (Kv), and 2-pore domain (K_2p) have been implicated in PH. Of the Kv channels that are responsible for setting the membrane potential of PAMSCs, K_{1,5} and K_{2,1} are of significance because they inactivate with decreases in O_2 tension. During hypoxic episodes, inactivation of these K_{1,5} channels produce depolarization to contract pulmonary vascular smooth muscle as described earlier. Absence of the K_{1,5} channels in mice lead to impaired vasoconstrictive responses to hypoxia. Furthermore,

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Kv1.5 expression and function are decreased in patients having idiopathic pulmonary arterial hypertension and have been implicated in the mechanism of PH associated with hypoxemia. Although Kv1.5 channel dysfunction is a provocative hypothesis underlying at least some forms of PH, mice lacking Kv1.5 channels demonstrated normal lung perfusion pressures compared with wildtype controls, without evidence for PH. Thus, dysfunctional or downregulation of Kv1.5 may be involved with the pathophysiology of some groups of PH, but cannot completely explain the underlying mechanism.

In addition to the Kv channel family, members of the K_{ap} family, TASK-1 (KCNK3) and TREK-1 (KCNK2), have also been implicated in the regulation of pulmonary vascular resistance and the possible development of PH. Recently, missense mutations in TASK-1 were identified as possible gene candidates causing idiopathic pulmonary arterial hypertension and familial PH. Alternatively, 1 study presented evidence against the involvement of TASK-1 dysfunction as a cause of PH in mice but left open the possibility that TASK-1 could be involved with PH in other species. Regardless of the evidence for and against TREK-1 and TASK-1 as the underlying mechanism for PH in mice, direct measurements of pulmonary arterial pressure (or its surrogate right ventricular pressure), the standard for diagnosing PH, have not been reported in mice lacking these K_{ap} channels.

TWIK-2 (KCNK6), another member of the K_{ap} family, is also expressed in the vascular system. Of the 15 known members of the K_{ap} family, TREK-1, TASK-1, and TWIK-2 show the greatest levels of expression for K_{ap} in the vascular system. Recently, our laboratory reported that mice lacking TWIK-2 are hypertensive at 8 weeks of age and have an altered contractile response in isolated aortic ring segments. Given the importance of TWIK-2 in the systemic circulation, we questioned whether it has a similar role in the pulmonary circulation. We hypothesized that (i) TWIK-2 is involved with regulating pulmonary vascular tone and (ii) TWIK-2 dysfunction in the pulmonary circulation leads to PH. After our initial results showed that TWIK-2 knockout mice develop PH and that branches of the pulmonary artery are hypercontractile to a thromboxane A2 mimetic, we sought to determine the mechanism involved with the onset of PH in TWIK-2 knockout mice.

Because Rho-kinase promotes contraction of vascular smooth muscle cells by inhibiting the dephosphorylation of myosin light chain, we tested the hypothesis that (iii) inhibition of Rho-kinase in mice lacking TWIK-2 attenuates hypercontractility of the pulmonary arteries and attenuates the development of PH.

**Results**

**TWIK-2 Expression in Lung Tissue and Pulmonary Vessels From Wildtype Mice**

TWIK-2 is expressed in the pulmonary artery and first-order branches and lung tissue from 20-week-old male knockout and wildtype mice (Figure S1A and S1B in the online-only Data Supplement). Kv1.5 and TASK-1 were included because they relate to previous studies involving PH. Expression of TWIK-2 (P<0.05; n=7) was absent in the knockout, whereas expression of Kv1.5 and TASK-1 was unaltered compared with the wildtype mice.

**Mice Lacking TWIK-2 Develop PH and Pulmonary Vascular Remodeling**

Right ventricular systolic pressure (RVSP), a surrogate measure of pulmonary pressures, was measured in TWIK-2 knockout male mice and wildtype male littermates (Figure 1A). At 8 weeks of age, there was no difference in mean RVSP between genotypes (21±3 and 24±3 mm Hg in wildtype and TWIK-2 knockout mice, respectively). However, at 20 weeks, mean RVSP in the TWIK-2 knockout increased to 35±3 mm Hg (P=0.036 compared with wildtype at 20 weeks [22±3 mm Hg] and TWIK-2 knockout at 8 weeks [n=4 for each group]). The results demonstrate that TWIK-2 knockout mice developed PH between 8 and 20 weeks.

Figure 1C shows hematoxylin and eosin staining (top panels) and immunostaining of smooth muscle α-actin (bottom panels) from lung sections of 20-week-old TWIK-2 knockout mice and their wildtype littermates. Note the increase in wall thickness (arrows) in lung vessels from TWIK-2 knockout mice compared with wildtype mice (Figure 1C, top). Figure 1C (bottom) demonstrates that the vessel wall (arrows in the top 2 panels) consists of vascular smooth muscle (red in the image). Figure 1B shows the percent of the cross-sectional

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**Figure 1.** A, Mean right ventricular pressures in 8- and 20-week-old TWIK-2 knockout (KO) and their wildtype (WT) littermates (n=4 per group). \( P \leq 0.036 \) compared with WT at 20 weeks and TWIK-2 KO at 8 weeks. B, The percent of the cross-sectional area in lung vessels occupied by the vessel wall in TWIK-2 KO mice and their WT littermates at 20 weeks of age. This measure provides an index of wall thickness relative to the vessel size. \( P \geq 0.001 \) compared with all other groups (n=4 per group). C, Hematoxylin and eosin-stained sections (top) and immunostained sections of smooth muscle α-actin (bottom) from the lung of a 20-week-old TWIK-2 KO and its WT littermates. The arrows in the top panels indicate the vessel wall. Red in the bottom panels denotes smooth muscle α-actin and blue represents cell nuclei.
area in lung vessels occupied by the vessel wall in TWIK-2 knockout mice and their wildtype littermates. This measure provides an index of wall thickness relative to the vessel size. Note that the percent area occupied by the vessel wall is significantly increased in the pulmonary vessels of 20-week-old, but not 8-week-old TWIK-2 knockout mice. Although the results in Figure 1B were derived from the hematoxylin and eosin–stained images, similar results were obtained when measurements were made using the immunohistochemical images of smooth muscle α-actin (data not shown). The vascular remodeling seemed to correlate with the increase in mean RVSP Figures 1A and 1B).

**Mice Lacking TWIK-2 Have an Increased Right Ventricular End-Diastolic Volume, But No Alterations in Left Ventricular Size or Function**

During the development of PH, right ventricular end-diastolic volume is often increased initially as a result of a pressure overload followed later by a decrease in right ventricle ejection fraction (EF%) as the right ventricle remodels to the pressure overload. Figure S2A shows that 20-week-old TWIK-2 knockout mice have an increased right ventricular end-diastolic volume (P=0.02, n=4) when compared with their wildtype littermate controls. Representative MRI images of the right ventricle at end diastole in a wildtype and a TWIK-2 knockout mouse is shown in Figure S2E. Right ventricular contractility, as measured by the ejection fraction, was not altered in TWIK-2 knockout mice compared with wildtype littermates (Figure S2B).

Given that the TWIK-2 knockout mice have increased systemic blood pressures, it was important to determine whether increased mean RVSP was related to the systemic hypertension. With systemic hypertension, the increase in right ventricular pressures could result indirectly from left ventricular dysfunction or increased left ventricular filling pressures. Figure S2C and S2D shows left ventricular end-diastolic volume and ejection fraction (EF%) in 20-week-old TWIK-2 knockout mice. There were no significant differences in either when compared with wildtype littermates. Furthermore, the left ventricular wall thickness was not significantly different in the wildtype and TWIK-2 knockout mice. At its widest point, the ratio of the left ventricle wall to the total cross-sectional area of the left ventricle was 0.50±0.05 and 0.53±0.06 (P=0.66, n=4 each group) in TWIK-2 knockout mice and their wildtype littermates, respectively. An additional consideration is that systemic hypertension can lead to elevated left ventricular end diastolic pressures, which could be a cause of PH in the TWIK-2 knockout mice. We found no significant increase in the left ventricular end-diastolic pressures of 18- to 22-week-old TWIK-2 knockout and wildtype mice (data not shown).

**TWIK-2 Knockout Mice Pulmonary Arteries Are Hypercontractile to a Thromboxane A2 Mimetic Through Activation of Rho-Kinase**

Isometric contractions to phenylephrine, an α-adrenoreceptor agonist, or U46619, a thromboxane A2 (TXA$_2$) mimetic, were measured in rings of first-order branches of pulmonary arteries from 20-week-old TWIK-2 knockout mice and their wildtype littermates (Figure 2A and 2B). Although the contractile responses to phenylephrine were similar between genotype, the response to U46619 was increased at a concentration of 10–6 mol/L (P=0.04, n=8 each group) in arterial rings from TWIK-2 knockout mice compared with those observed in arterial rings from wildtype mice. In addition, contractile responses to 60 mmol/L KCl (n=9) were similar between rings of the first-order branches of pulmonary arteries from TWIK-2 knockout mice and their wildtype littermates (data not shown).

Because stimulation of the TXA$_2$ receptor can activate downstream rho-kinase signaling, we determined whether the enhanced contractile response to U46619 could be blocked by inhibition of Rho-kinase. Figure 2C shows the contractile responses to a single dose of 10–6 mol/L U46619 in arterial rings from 20-week-old wildtype and TWIK-2 knockout mice in the absence and presence of the Rho-kinase inhibitor, Y27632 (10–6 and 10–5 moles/L). Y27632 had no significant effects on the force generated in ring from wildtype mice; however, it significantly attenuated the contractile response in a dose-dependent manner in pulmonary artery rings from TWIK-2 knockout mice. With Y27632 pretreatment, the contractile responses to 10–6 mol/L U46619 were decreased to levels in rings from wildtype mice. Thus, it seems that Rho-kinase activity was responsible for the hypercontractile response to U46619 in first-order branches of the pulmonary artery in TWIK-2 knockout mice.
Treatment With a Rho-Kinase Inhibitor Reduced RVSP and Pulmonary Vascular Remodeling in TWIK-2 Knockout Mice

Having demonstrated enhanced contractility with Rho-kinase activation in first-order branches of pulmonary arteries and increased Rho-kinase activity in whole lungs from 20-week-old TWIK-2 knockout mice, we determined whether inhibition of Rho-kinase in vivo could be an effective treatment for the hypertension that develops between 8 and 20 weeks in TWIK-2 knockout mice. Eight-week-old TWIK-2 knockout mice and wildtype littermates were provided with regular drinking water or drinking water with 1 mg/mL fasudil, a Rho-kinase inhibitor. Fasudil was used for these studies because it has been demonstrated as an effective inhibitor of Rho-kinase when administered orally.27 At 20 weeks of age, mean RVSP was measured by right heart catheterization, and lungs were harvested for histological analysis. Fasudil treatment abolished the increase in mean right ventricular pressure in TWIK-2 knockout mice (Figure 3A) and diminished pulmonary vascular remodeling. Figure 3B shows that fasudil treatment in the TWIK-2 knockout mice attenuated vessel remodeling as determined by the percent of the cross-sectional area in lung vessels occupied by the vessel wall. Figure S3 shows hematoxylin and eosin–stained lung sections with the arrow pointing to the arterial wall. Thus, the development of PH in TWIK-2 knockout mice seems to be mediated by the Rho-kinase pathway and can be attenuated by Rho-kinase inhibition.

Discussion

The results of our study demonstrate that loss of function of the 2-pore domain K+ channel, TWIK-2, produces PH. Given the importance of the TWIK-2 channel, in the systemic circulation,24 we questioned whether it has a similar role in the pulmonary circulation. Consistent with previous studies conducted in multiple species,21,26–30 we report that TWIK-2 is expressed in the lung and pulmonary circulation of mice (Figure S1). By using a TWIK-2 knockout mouse in the present study, we conclude the following: (i) loss of function of TWIK-2 in the pulmonary circulation leads to the development of PH (Figure 1A and 3A) and vascular wall remodeling (Figures 1B, 1C, and 3B and Figure S3), (ii) PH and remodeling occur between 8 and 20 weeks of age in the TWIK-2 knockout mice, (iii) the mechanism of this PH phenotype seems possibly mediated by hypercontractility as a result Rho-kinase pathway activation (Figure 2), and (iv) treatment of TWIK-2 knockout mice with a Rho-kinase inhibitor before the onset of PH significantly attenuates pulmonary arterial pressures (Figure 3A) and vascular remodeling (Figure 3B and Figure S3). Our studies suggest that TWIK-2 regulates pulmonary vascular tone by inhibiting the activity and expression of Rho-kinase.

Although decreased K+ channel activity has been hypothesized to underlie the development of PH, the TWIK-2 knockout model is the first K2P+ channel knockout mouse model, to our knowledge, known to develop PH. A recent study reported the important link between the missense mutation of another member of the 2-pore domain K+ channel family, TASK-1 or KCNK3, and a familial form of human PH.17 Given that the pulmonary circulation express a number of 2-pore domain K+ channels24,31 and that 2 members of this family have been implicated with PH to date (present study included), it is reasonable to consider that dysfunction of other members of this family could also result in PH.

One potential confounding factor involved with our model of PH is the systemic hypertension, which is present by 8 weeks of age in TWIK-2 knockout mice.24 Because the pulmonary and systemic circulations function at independent pressures, PH should be secondary to systemic hypertension only when there is dysfunction in the left side of the heart (classified as World Health Organization Group 2 PAH). We measured left ventricular function and morphology in TWIK-2 knockout mice at 20 weeks of age, a time when PH was fully developed. Our studies demonstrate that left ventricular end-diastolic volume and ejection fraction were similar in 20-week-old TWIK-2 knockout mice and their wildtype littermates (Figure 2C and 2D). Furthermore, we found no indication of left ventricular remodeling as determined by wall thickness in the TWIK-2 knockout mice at 20 weeks of age (see results) and no changes in left ventricular pressures as measured by left cardiac catheterization (see Methods and Supplementary materials). Thus, we conclude that PH, developing between the ages of 8 and 20 weeks in male TWIK-2 knockout mice, is not a result of left-sided heart failure. Instead, our model confers a unique hypertensive phenotype in the pulmonary vasculature resulting from TWIK-2 dysfunction in the pulmonary vessels.

Figure 3. A, Mean right ventricular systolic pressure in 20-week-old TWIK-2 knockout (KO) mice and their wildtype (WT) littermates. Mice were provided with either 1 mg/mL fasudil in the drinking water or drinking water alone (control; n=3–4 per group). *p<0.007 and 0.02 compared with TWIK-2 KO with fasudil and WT control, respectively. B, The percent of the cross-sectional area in lung vessels occupied by the vessel wall in TWIK-2 KO mice and their WT littermates at 20 weeks of age with and without 12 weeks of fasudil treatment. This measure provides an index of wall thickness relative to the vessel size. *p<0.001 compared with WT control and TWIK-2 KO mice treated with fasudil. **p=0.004 compared with WT control.
Our studies demonstrate a potentially important role for Rho-kinase in the development of PH in male TWIK-2 knockout mice. Rho-kinase, through its inhibition of myosin light chain phosphatase, helps to maintain a contracted state in vascular smooth muscle by preventing the dephosphorylation of myosin light chain. Additionally, Rho-kinase is involved with remodeling in the vessel wall, in part, through enhancing vascular smooth muscle motility and smooth muscle proliferation. At the arterial level, Rho-kinase activity was enhanced in the TWIK-2 knockout mice and seems to be at least partially responsible for PH in the TWIK-2 knockout mice by increasing pulmonary vascular resistance through alterations in arterial contractility. A recent publication addressed the complexity of the varying factors involved in pulmonary vasoconstriction and remodeling, including the contributions of hypoxia. Although we do not believe Rho-kinase activation is the only mechanism behind TWIK-2 deficiency leading to hypercontractility, the interactions between TWIK-2 and Rho-kinase signaling and their combined roles in intracellular calcium homeostasis are potentially revealing and require further study. Enhanced Rho-kinase activity is commonly associated with cardiovascular diseases, in general, including other forms of PH. For example, long-term inhibition of Rho-kinase attenuated chronic hypoxia- and monocrotaline-induced PH in rodents. In addition, fasudil acutely decreased pulmonary vascular resistance in humans with PH, although mean pulmonary arterial pressure only slightly decreased (<3 mmHg) in 1 study and was not significantly affected in another.

It is reasonable to consider that pulmonary artery vascular smooth muscle cells in TWIK-2 knockout mice are more depolarized and have an increase in intracellular-free Ca$^{2+}$ compared with that in wildtype mice. Because K$^+$ channels function to regulate membrane potential in pulmonary vascular smooth muscle cells, loss of function of TWIK-2 should depolarize the vascular smooth muscle and increase intracellular Ca$^{2+}$ through activation of voltage-dependent calcium channels. The depolarization and increased Ca$^{2+}$ in pulmonary vascular smooth muscle cells could produce PH through 2 possible mechanisms. First, intracellular-free Ca$^{2+}$ is a second messenger for smooth muscle cell contractions. With an increase in intracellular Ca$^{2+}$ resulting from the loss of TWIK-2, a persistent increase in the contractile state of pulmonary arteries and arterioles would serve to increase the pulmonary vascular resistance. Second, both membrane depolarization and increased cytoplasmic Ca$^{2+}$ are stimuli that activate Rho-kinase. Activation of Rho-kinase would both increase the sensitivity of the contractile machinery in the vascular smooth muscle to Ca$^{2+}$ and stimulate vessel remodeling.

**Perspectives**

This study is the first to demonstrate a role for the TWIK-2 K$^+$ channel in the regulation of pulmonary vascular tone. The observation that male TWIK-2 knockout mice spontaneously develop PH between 8 and 20 weeks of age makes the PH phenotype more physiological and comparable to human disease compared with other drug-induced models of PH. Of significance, the increase in right ventricular end diastolic volume seen in the TWIK-2 knockout mice paralleled the increase in right ventricular end diastolic volume without right ventricular remodeling that occurs during the early stages of PH in humans. The presence of right ventricular dilatation is notable because deaths and clinical deterioration in PH are directly related to alterations in right heart pressures and remodeling. We describe a potential role for enhanced Rho-kinase activity in the development of PH in the TWIK-2 knockout mice by increasing pulmonary vascular resistance through alterations in arterial contractility and by vascular remodeling. Given the recently described role of another K$_v$ channel in human PH, TWIK-2 dysfunction could be important in the pathogenesis of human PH through either a loss of function mutation in familial forms of PH or by downregulation of TWIK-2 from environmental factors (such as hypoxia) in other forms of PH.

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**Disclosures**

None.


**Novelty and Significance**

**What Is New?**

- These studies expand the current knowledge of potassium channels and their important contributions to the regulation of pulmonary vascular tone, cellular proliferation, and responses to environmental stimuli.

**What Is Relevant?**

- Human PH is a progressive disease resulting in severe right heart failure and death if left untreated. The disease is difficult to recognize and therapies are limited to vasodilators that often have significant adverse effects and poor tolerance.

- These studies present new mechanistic/genetic possibilities in the development of PH, thus offering new therapeutic targets.
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TWIK-2 channel deficiency leads to pulmonary hypertension through a Rho-kinase mediated process

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ONLINE SUPPLEMENTAL MATERIALS
ONLINE SUPPLEMENT

Methods & Materials
All studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine. Mice used in these studies were F1 hybrid C57/129 background strain as previously described (24;26). as well as back-crossed on C57BL6 background for ten generations.

RNA isolation and real-time polymerase chain reaction (RT-PCR)
RNA was isolated from the whole lung and pulmonary artery from TWIK-2 knockout (KO) and wild-type (WT) littermate male mice (20 weeks of age). The mice were anesthetized with a cocktail of ketamine (37.5 mg/ml)-xylazine (1.9 mg/ml)-acepromazine (0.7 mg/ml) followed by administration of heparin (50 units, Mckesson). Under sterile conditions, the thoracic cavity was opened and the pulmonary vasculature was flushed with ice-cold Hanks’ Balanced Salt Solution 1X (HBSS, Gibco) via cardiopuncture. The lung was removed, separated from the pulmonary artery, and immediately flash frozen. The pulmonary artery and the second order branches were placed in ice-cold HBSS, cleaned of connective tissue and fat, and flash frozen.

At a later date, the frozen lungs were homogenized in TRIzol LS reagent (Invitrogen). RNA was extracted from both the pulmonary artery and the lung homogenate using RNeasy Micro kit (QIAGEN) according to the manufacturer’s instruction. The lungs were additionally treated with DNase (Invitrogen). RNA purity was assessed by nanodrop spectrophotometry with the acceptable 260/280 absorbance ratio being > 1.9. Reverse transcription was conducted on 0.1 μg and 1 μg total RNA for the pulmonary artery and lung respectively using random hexamers and Superscript III. Primers were designed using Primer Express 2.0 software and listed below. The gene abbreviation is followed by the NCBI accession number and amplicon size: Gapdh [NM_008084 (111 nt)], fwd 5’-AGCCTCGTCCCGTAGACAAAA and rev 5’-TGGCAACAATCTCCACTTTGC; TASK-1 (K2P3.1) [NM_010608 (135 nt)], fwd 5’-ACATGGACTCCCCTTGCTGT and rev 5’-CAAATGAATACGGAGGTGGCA; TWIK-2 (K2P6.1) [NM_001033525 (119 nt)], fwd 5’-AGGCATCGAAACCAGACGTGT and rev 5’-TCCCCGTGTGACTTTTCTACA.

Right and Left ventricular catheterization
Right ventricular systolic pressure (RVSP), an estimate of pulmonary arterial pressure, left ventricular systolic (LVSP) and end-diastolic pressures (LVEDP) was measured in male TWIK-2 KO and WT littermates at ages 8 and 20 weeks (RVSP) and 20-22 weeks (LVEDP & LVSP) Mice were anesthetized with 2% isoflurane in 100% O2. For RVSP measurements, a 1.4F high-fidelity micromanometer catheter (Millar Instruments, Houston, TX) was inserted into the right jugular and advanced through the right atria to the right ventricle. For LVEDP and LVSP measurements, 1.0F high-fidelity
micromanometer catheter (Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced through the aorta into the left ventricle. All pressure data were collected, digitized, and analyzed using the IOX data acquisition system (Emka Technologies, Falls Church, VA). The mean right ventricular and left ventricular systolic pressure was calculated as the average of $P_{max15}$ (where $P_{max15} = \text{mean maximum pressure}$) over 15 cardiac cycles where $P_{min15}$ (minimum pressure) was physiologic at 0-5 mmHg, confirmed by pressure-volume loop tracings. The left ventricular end-diastolic pressures (LVEDP) was measured at the Z-point on the left ventricular pressure (LVP) trace, at the time of the electrocardiographic R wave.

**Histopathology**

The chest cavity was opened in anesthetized mice and the lungs were perfused with cold physiologic saline through the right ventricle. The lung was excised and fixed in 10% neutral buffered formalin for 24 hours. After fixation, the lung was embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin-eosin (H&E). Images from two consecutive sections were obtained from five equally–spaced locations of the right lung using a Zeiss Axioplan I microscope with a 20x objective. Luminal and wall areas of vessels approximately between 50-100 µm in diameter were measured by a blinded observer using Adobe Photoshop CS3 Extended. The percentage of the total cross sectional area of a vessel occupied by the vessel wall provided an index of wall thickness relative to the vessel size $[(100 \times \text{wall area}) / (\text{luminal area} + \text{wall area})]$.

**Immunohistochemistry**

Paraffin sections (see above) were treated with Xylene, hydrated by sequentially washing in 100%, 90% and 75% ETOH in water, fixed in 4% paraformaldehyde, and washed in phosphate buffered saline (PBS). The slides were permeabilized and blocked with 10% goat serum, 0.5% BSA, and 0.1% Tween-20 in PBS for 30 minutes at room temperature. Tissues were incubated with mouse smooth muscle α-actin antibody (Sigma A5228, diluted 1:500) or mouse non-immune IgG for control (Jackson ImmunoResearch, #015-000-003) at room temperature for 2 hours, washed with PBS, followed by incubation with Alexa Fluor 594 Goat anti-mouse IgG (Molecular Probes, A11005, diluted 1:500) at room temperature for 30 minutes in the dark. Following three washes with PBS, Vectashield mounting medium with DAPI (to visualize nuclei) was added and the sections were covered with a glass coverslip.

**Isometric tension ring studies**

The first order branches of the pulmonary artery from 20 week old TWIK-2 KO mice and WT littermates were excised, cleared of fat and connective tissue and cut into 2 mm ring segments. Each segment was mounted on metal stirrups and placed in a myograph (Chuelteck, Houston, TX) to measure isometric force. The segments were bathed in Krebs buffer consisting of (in 10^{-3} moles/liter): 119 NaCl, 4.7 KCl, 1 MgSO4, 1.2 KH2PO4, 24 NaHCO3, 11 glucose, 2.5 CaCl2). The Krebs buffer was warmed to 37°C and equilibrated with a gas consisting of CO2 5% / O2 20% / N2 balance to obtain pH=7.4. Data was acquired and analyzed using Powerlab/8sp (AD Instruments,
Colorado Springs, CO) at 10 Hz with LabChart v4.2.4 (AD Instruments). Pulmonary arterial rings were allowed to equilibrate for 60 minutes with force gradually being increased to 7 millinewtons (mN), the optimum baseline tension identified in preliminary experiments. Rings were repeatedly contracted with 40 mmol/L KCl in Krebs buffer until the force generated from consecutive KCl contractions was similar. Each arterial ring was used for only one experiment.

**MRI and Cardiac Function**

Mice were initially anesthetized with 3% isoflurane (mixed with 100% oxygen) and maintained with 1–2% isoflurane during imaging. ECG, respiratory rate, and body temperature were measured using an animal-monitoring system (SA Instruments, Stony Brook, NY). Respiratory- and cardiac-gated images were acquired at end-diastole and end-systole using a 7.0T, Bruker Pharmascan, 22-mm to center-bore horizontal scanner. The imaging parameters to acquire cardiac- and respiratory-gated spin echo images were as follows: repetition time, 5.7 millisecond; echo time, 2.8 millisecond; field of view, 4.0 cm; number of slices, 6; slice thickness, 1.0 mm; matrix 128 × 128; and number of averages, 1. The multi-slice scan was performed in the axial orientation to best visualize the left and right ventricles. Data were analyzed using Amira 3D image processing software (Mercury Computer Systems, Chelmsford, MA).

**Chemicals and reagents**

U-46619, a thromboxane mimetic, and Y27632, a rho-kinase inhibitor, were purchased from Sigma-Aldrich and initially dissolved in ethanol. Phenylephrine (PE) (Sigma-Aldrich) was prepared as concentrated stock solutions in distilled water. On the day of experiments, aliquots of the stock solutions were diluted in Krebs buffer. Fasudil (LC laboratories) was added to drinking water at a concentration of 1 mg/ml.

**Statistical analysis**

The data are expressed as mean ± SEM or mean ± SE of the least square mean. Statistical analysis was performed using Student’s t-test for mRNA analysis (Supplementary Figure S1) and MRI cardiac function analysis (Supplementary Figure S2). Two-way ANOVA was used to analyze mean right ventricular systolic pressures (Figures 1A and 3A), percent of vessel cross sectional area occupied by the vessel wall (Figures 1B and 3B), and for isometric contraction studies involving Y27632 (Figure 2C). Two-way repeated-measures ANOVA were used to analyze concentration response contractions in Figure 2A and 2B. A post hoc Holms-Sidak test was used to compare individual groups if the ANOVA analysis showed significance. Differences were considered to be significant at p<0.05.
**SUPPLEMENTARY FIGURE S1**

**S1:** Pulmonary artery (A) and whole lung (B) mRNA expressed relative to GAPDH. *P<0.05 compare to corresponding WT, n=9 (4 WT/5 KO).
SUPPLEMENTARY FIGURE S2

S2: MRI measurement of (A) right ventricular end-diastolic volume (RV-EDV), (B) right ventricular ejection fraction (RV-EF), (C) Left ventricular end-diastolic volume (LV-EDV), and (D) Left ventricular ejection fraction (LV-EF) in 20 week old TWIK-2 KO mice and their WT littermates. (n=4 for each genotype, * p=0.02 compared to corresponding WT). (E): Representative MRI images of the right (RV) and left ventricles (LV) at end diastole from a 20 week old male TWIK-2 KO and a WT male littermate.
**SUPPLEMENTARY FIGURE S3**

**S3**: H&E stained lung sections showing small arteries in 20 week old TWIK-2 KO mice and their WT littermates with and without fasudil treatment. The arrows point to the vessel wall. Reference bar = 50 µm.