

Cardiovascular, Pulmonary, and Renal Pathology

Protein Kinase G-I Deficiency Induces Pulmonary Hypertension through Rho A/Rho Kinase Activation

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Protein kinase G (PKG) plays an important role in the regulation of vascular smooth cell contractility and is a critical mediator of nitric oxide signaling, which regulates cardiovascular homeostasis. PKG-I-knockout (*Prkg1*^{-/-}) mice exhibit impaired nitric oxide/cGMP-dependent vasorelaxation and systemic hypertension. However, it remains unknown whether PKG-I deficiency induces pulmonary hypertension. In this study, we characterized the hypertensive pulmonary phenotypes in *Prkg1*^{-/-} mice and delineated the underlying molecular basis. We observed a significant increase in right ventricular systolic pressure in *Prkg1*^{-/-} mice in the absence of systemic hypertension and left-sided heart dysfunction. In addition, we observed marked muscularization of distal pulmonary vessels in *Prkg1*^{-/-} mice. Microangiography revealed impaired integrity of the pulmonary vasculature in *Prkg1*^{-/-} mice. Mechanistically, PKG-I-mediated phosphorylation of Rho A Ser188 was markedly decreased, and the resultant Rho A activation was significantly increased in *Prkg1*^{-/-} lung tissues, which resulted in Rho kinase activation. The i.t. administration of fasudil, a Rho kinase inhibitor, reversed the hypertensive pulmonary phenotype in *Prkg1*^{-/-} mice. Taken together, these data show that PKG-I deficiency induces pulmonary hypertension through Rho A/Rho kinase activation-mediated vasoconstriction and pulmonary vascular remodeling. (*Am J Pathol* 2012, 180:2268–2275; <http://dx.doi.org/10.1016/j.ajpath.2012.02.016>)

Pulmonary hypertension (PH), characterized by progressive increases in pulmonary vascular resistance and vascular remodeling, causes right-sided heart failure and mortality without treatment.^{1,2} Increased pulmonary vascular tone and severe structural remodeling of distal pulmonary vasculature are primary determinants of increased pulmonary vascular resistance. Because the molecular mechanisms responsible for pulmonary vascular remodeling and vasoconstriction remain elusive, there are limited options available for the prevention and treatment of progressive PH.^{3,4}

Protein kinase G (PKG), a serine/threonine kinase, plays an important role in the regulation of vascular smooth cell contractility.^{5,6} Activation of soluble guanylyl cyclase by nitric oxide (NO) leads to increased synthesis of cGMP and activation of PKG. The PKG activation results in vasorelaxation via several mechanisms, including lowering of intracellular free Ca²⁺ levels and desensitization of the contractile apparatus to Ca²⁺.^{7,8} Two mammalian *Prkg* genes have been identified, encoding PKG-I and PKG-II.⁹ PKG-I is the prevalent PKG isoform expressed in the cardiovascular system, with a greater level in smooth muscle cells (SMCs) and a lower level in endothelial cells.^{10,11} The N-terminus (the first 90 to 100 residues) of PKG-I is encoded by two alternative exons generating two isoforms: PKG-I α and PKG-I β .⁹ The enzyme is activated at submicromolar to micromolar concentrations of cGMP. The PKG-I is composed of three functional domains: an N-terminal domain, a regulatory domain, and a catalytic domain. The regulatory domain contains two cGMP-binding sites with high and low affinity. The catalytic domain contains the Mg-ATP- and peptide-binding pockets. Binding of cGMP to the regulatory domain releases the inhibition of the catalytic center by the N-terminal autoinhibitory/pseudosubstrate site and allows the phosphorylation of serine/threonine residues in target proteins.^{6,12} The PKG-I phosphorylates Rho A at

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Ser188, which inhibits its membrane association and, thereby, prevents activation of its downstream targets, such as Rho kinase.^{13,14} Rho kinase-mediated phosphorylation of human myosin phosphatase target subunit 1 at Thr696 (corresponding to Thr694 in mouse) inhibits smooth muscle myosin phosphatase activity and induces SMC contraction.¹⁵

PKG-I-knockout (*Prkg1*^{-/-}) mice exhibit impaired NO/cGMP-dependent vasorelaxation and systemic hypertension in their early life.^{16–20} These mutant mice also exhibit a decreased life span (50% of these mice die before the age of 5 to 6 weeks),¹⁷ diminished ischemia-induced angiogenesis,²¹ and disturbed platelet adhesion and activation.²² Overexpression of PKG-I increases the sensitivity of cultured vascular SMCs to the anti-proliferative and pro-apoptotic effects of NO/cGMP,²³ whereas down-regulation of PKG-I induces the change of the SMC phenotype from a contractile, differentiated to a synthetic, dedifferentiated state,^{24,25} an underlying feature of pulmonary vascular remodeling. Although PKG dysfunction has been indicated in the pathogenesis of PH, there is no genetic evidence demonstrating that PKG-I deficiency induces PH. Herein, we report that deletion of *Prkg1* induces vascular remodeling and vasoconstriction and, thereby, PH in mice.

Materials and Methods

Mice

All mice were bred and maintained at the University of Illinois at Chicago facility, according to NIH guidelines. The generation of *Prkg1*^{-/-} mice by homologous recombination has been described previously.¹⁷ *Prkg1*^{-/-} mice were obtained from Dr. Xiaoping Du (University of Illinois College of Medicine). Littermates of *Prkg1*^{-/-} and WT mice in C57BL/6 background were used. Approval for animal care and use in these experiments was granted by the Animal Care and Use Committee.

Primary Cultures of Mouse Pulmonary SMCs

Primary cultures of mouse lung SMCs from wild-type (WT) or *Prkg1*^{-/-} mice were isolated as previously described.²⁶ Briefly, a 21-gauge needle was inserted into the right ventricle to perfuse the pulmonary circulation with hot HBSS (50°C) and then with 2- μ m irons in 0.5% agarose. The lung was then dissected, minced, and collected in a 15-mL tube. After incubation with collagenase A (1 mg/mL in HBSS; Roche Applied Sciences, Indianapolis, IN) at 37°C for 45 minutes with shaking, the rosetted cells were isolated and washed using a magnetic particle concentrator (DynaL MPC-15; Dynal, Oslo, Norway). The freshly dispersed SMCs were plated onto a 60-mm cell culture dish and incubated with Dulbecco's modified Eagle's medium containing 10% newborn calf serum, penicillin (100 U/mL), and streptomycin (100 μ g/mL) in a humidified atmosphere of 5% CO₂ in air at 37°C and grown to 90% to 95% confluence. These primary SMCs were used at passage 2 for all experiments.

Hemodynamic Measurements

Right ventricular systolic pressure (RVSP) was determined with a 1.4F pressure transducer catheter (Millar Instruments, Houston, TX) and AcqKnowledge software (Biopac Systems Inc., Goleta, CA), as previously described.²⁶ Briefly, the 1.4F pressure transducer was inserted through the right external jugular vein of anesthetized mice (100 mg ketamine/5 mg xylazine/kg body weight, i.p.) and threaded into the right ventricle. RVSP was then recorded and analyzed with AcqKnowledge software. To inhibit Rho kinase, fasudil (60 mmol/L in 25 μ L of saline) was administered into each mouse through tracheal instillation. RVSP was monitored continuously for 30 minutes after administration of fasudil. Carotid artery pressure was also determined with a 1.4F pressure transducer catheter.

Echocardiography

Transthoracic two-dimensional targeted M-mode echocardiography was performed with a 15-MHz linear array transducer attached to a commercially available Sequoia C256 system (Siemens, Mountain View, CA). Mice were anesthetized with methoxyflurane (Metofane; Dow Chemical Company, Hawthorn Woods, IL), intubated, and maintained on isoflurane anesthesia. Images of the left ventricle (LV) were taken from the parasternal short-axis view at the level of the papillary muscles. Interventricular septal and LV posterior wall thickness and LV internal dimensions at the end of diastole and systole were measured by the American Society of Echocardiography leading-edge method from at least three consecutive cardiac cycles on the M-mode tracings, with a paper speed of 200 mm/second. The percentage fractional shortening of the LV was calculated from digital images by subtracting the LV systolic internal dimension from the LV diastolic internal dimension and dividing by the LV diastolic internal dimension.

Fluorescent Microangiography

A 1% low melting point agarose (Sigma-Aldrich, St. Louis, MO) solution containing 0.2- μ m yellow-green fluorescent microspheres was prewarmed and injected into the pulmonary artery through the left atrial appendage. The lungs were then fixed in 4% paraformaldehyde in PBS for 48 hours at 4°C, and sections (150 to 200 μ m thick) were prepared for imaging. Confocal optical sectioning was used to produce a Z-stack of images spanning 150 μ m of section depth. Projection images were generated from the Z-series to demonstrate vessel architecture throughout the entire thickness of the section. Fluorescence intensity was quantified in three separate sections per animal and three representative regions per section by a blinded observer. For this analysis, a single confocal optical section was taken at a predefined depth (10 μ m) using fixed gain and power settings. The perfusion index was calculated as the area ratio of the image surpassing the threshold value. The perfusion index represents the proportion of the optical section filled by vascular perfusion.²⁷

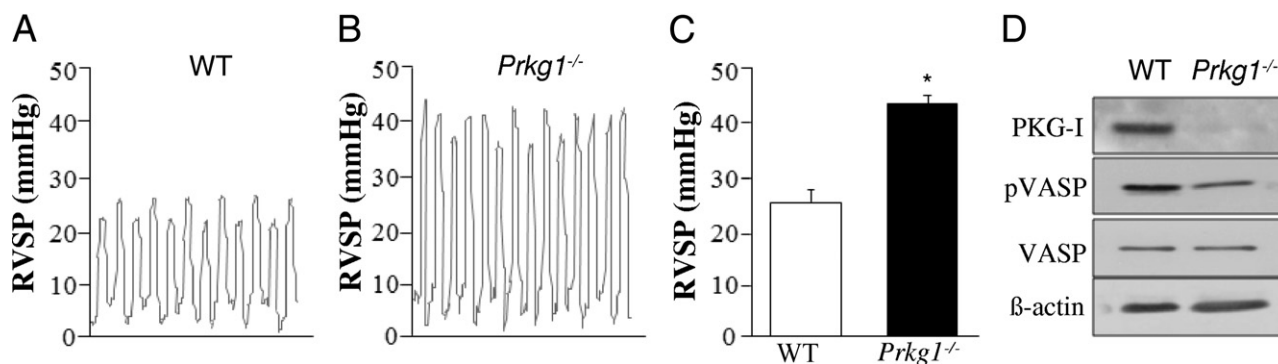


Figure 1. PKG-I deficiency induces PH in *Prkg1*^{-/-} mice. Representative RVSP tracings in WT (A) or *Prkg1*^{-/-} mice (B). C: Marked increase of RVSP in *Prkg1*^{-/-} mice. RVSP was measured in age- and sex-matched 7- to 8-week-old mice. Data are expressed as mean ± SD. **P* < 0.01 versus WT (*n* = 4 to 6). D: Western blot analysis of PKG-I expression and phosphorylation of VASP in lungs from 7-week-old mice. Lung lysate (30 μg per lane) was loaded and immunoblotted with antibodies against PKG-I, phosphorylated VASP (pVASP), VASP, and β-actin (loading control). Consistent with deletion of *Prkg1*, phosphorylation of VASP was significantly decreased in *Prkg1*^{-/-} lungs compared with WT lungs.

Immunofluorescent Staining

Lung tissues were fixed for 5 minutes by instillation of 10% PBS-buffered formalin through tracheal catheterization at a transpulmonary pressure of 15 cm H₂O, and then overnight at 4°C with agitation.²⁶ After paraffin processing, the tissues were cut into semithin sections (4 to 5 μm thick). For immunofluorescent staining, antigen retrieval was performed by incubating the slides in 10 mmol/L sodium citrate (pH 6.0) at 95°C for 10 minutes. After 1-hour incubation at room temperature in a blocking solution containing 2% bovine albumin serum (Sigma-Aldrich), 0.1% Triton X-100, and 2% normal goat serum, the sections were incubated for 2 hours at room temperature with anti-α smooth muscle actin (α-SMA) monoclonal antibody (1:400; Sigma-Aldrich). Nuclei were counterstained with DAPI. The anti-α-SMA-positive pulmonary arterial vessels per field (original magnification, ×200) were counted based on the diameter (<40 versus >40 μm). Twenty fields per section were randomly identified and counted.

Western Blot Analysis

Western blot analysis was performed using the following antibodies: anti-Rho A phosphorylated Ser188 (1:500; Santa Cruz, Santa Cruz, CA), anti-PKG-I (1:1000; Assay Designs, Inc. Ann Arbor, MI), anti-vasodilator-stimulated phosphoprotein (VASP; 1:1000; Axxora, San Diego, CA), and anti-VASP phosphorylated Ser239 (1:200; Axxora). Anti-β-actin (1:4000; BD Biosciences, San Jose, CA) was used as a loading control. Signals from immunoreactive bands were visualized by fluorography using an enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific, Waltham, MA). The intensity of each band was quantified using NIH ImageJ software (Bethesda, MD).

RhoGTPase Activity Assay

The GTP-bound active forms of Rho A were determined by pull-down assay.²⁸ Cells were washed with ice-cold PBS five times and lysed in a lysis buffer (50 mmol/L Tris,

pH 7.4; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; 500 mmol/L NaCl; 10 mmol/L MgCl₂; 10 μg/mL each of aprotinin and leupeptin; and 1 mmol/L phenylmethylsulfonyl fluoride). Similarly, lung tissues were homogenized with the same lysis buffer. After centrifugation at 18,000 × *g* at 4°C for 2 minutes, the extracts were incubated at 4°C for 60 minutes with glutathione-Sepharose beads coupled with glutathione-S-transferase-rhotekin fusion protein for determination of Rho A activity, following the manufacturer's instructions (Cytoskeleton Inc., Denver, CO). Bound Rho A proteins were quantified by using Western blot analysis, as previously described. Total Rho A levels were also determined by using direct Western blot analysis.

Statistical Analysis

Differences between groups were examined for statistical significance using the Student's *t*-test. *P* < 0.05 was considered significant.

Results

PKG-I Deficiency Induces PH in *Prkg1*^{-/-} Mice

To determine the consequences of genetic deletion of *Prkg1*, we measured RVSP (to reflect the pulmonary artery systolic pressure) in *Prkg1*^{-/-} mice. *Prkg1*^{-/-} mice had significantly increased RVSP compared with WT mice (Figure 1, A–C). Genetic deletion of *Prkg1* was confirmed using Western blot analysis (Figure 1D). Accordingly, decreased phosphorylation of VASP, a substrate of PKG-I, was observed in *Prkg1*^{-/-} lungs compared with WT lungs. These data indicate that PKG-I deficiency induces PH in mice. Because structural remodeling of distal pulmonary arteries is the underlying feature of pulmonary vascular remodeling in the pathogenesis of PH, we stained lung sections with anti-α-SMA and quantified the number of muscularized distal pulmonary vessels. *Prkg1*^{-/-} lungs exhibited a >10-fold increase in muscularized distal arteries (<40 μm in diameter) compared with WT, whereas a similar number of muscularized large

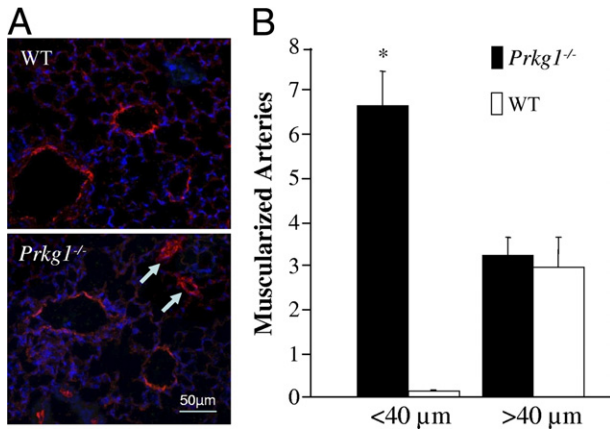


Figure 2. Increased muscularization of distal pulmonary arteries in *Prkg1*^{-/-} mice. **A:** Representative micrographs of immunostaining of WT and *Prkg1*^{-/-} lung sections from 7-week-old mice with anti- α -SMA (red). **Arrows**, muscularized distal pulmonary arterial vessels in *Prkg1*^{-/-} lung sections. **B:** Quantification of muscularized pulmonary arterial vessels in mouse lung sections. α -SMA-positive vessels were counted in 20 fields (original magnification, $\times 200$) of each lung section. Data are expressed as mean \pm SD. * $P < 0.001$ versus WT vessels with a diameter $< 40 \mu\text{m}$ ($n = 4$ mice in each group). *Prkg1*^{-/-} lungs exhibited a > 10 -fold increase in muscularized distal arteries ($< 40 \mu\text{m}$ in diameter) compared with WT.

vessels ($> 40 \mu\text{m}$ in diameter) was seen in *Prkg1*^{-/-} and WT lungs (Figure 2, A and B).

To assess the integrity of the pulmonary vasculature, we performed fluorescent microangiography by perfusing the

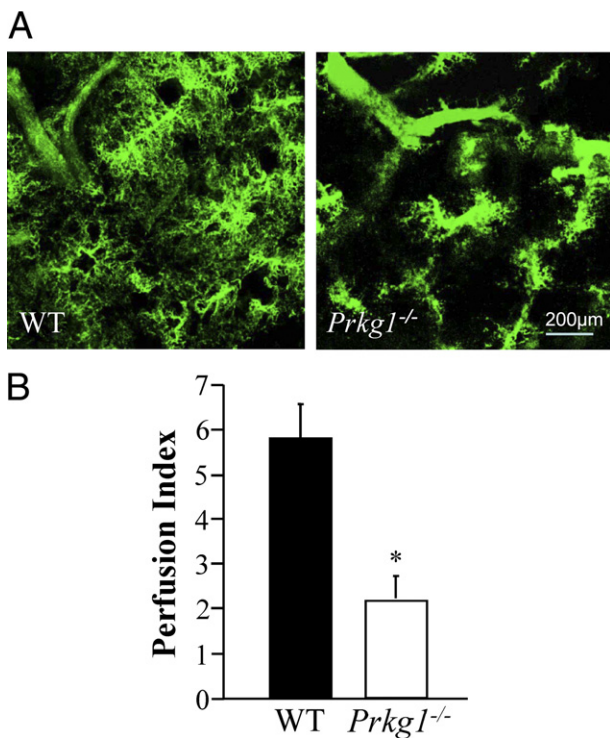


Figure 3. Impaired pulmonary vascular perfusion in *Prkg1*^{-/-} mice. **A:** Representative micrographs of fluorescent microangiography of lung sections perfused with fluorescent microspheres (green) suspended in agarose. Normal filling of the microvasculature was observed in WT lungs, whereas *Prkg1*^{-/-} lungs showed a marked loss of microvascular perfusion and widespread pre-capillary occlusion. **B:** Quantification of pulmonary microvascular perfusion. Data are expressed as mean \pm SD. * $P < 0.01$ versus WT ($n = 3$).

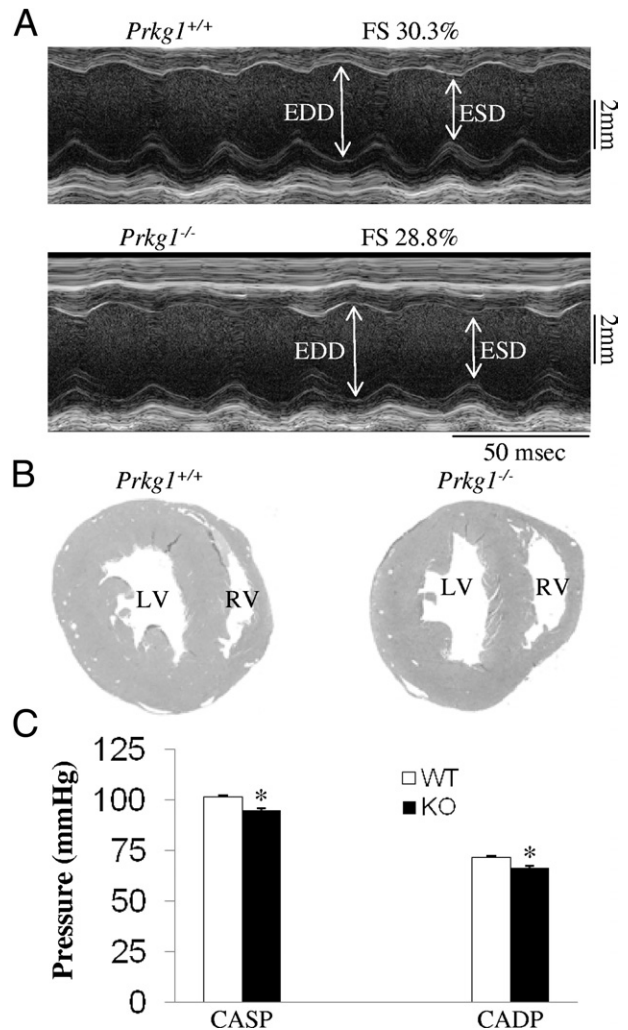


Figure 4. Normal left-sided heart function in *Prkg1*^{-/-} mice. **A:** Transthoracic M-mode echocardiographic tracings in a WT and *Prkg1*^{-/-} mouse (45-day-old littermate). **Double-sided arrows**, LV dimensions. EDD, end-diastolic dimension; ESD, end-systolic dimension; FS, fraction shortening, indicating cardiac contractility. **B:** Histological sections of hearts from 45-day-old littermates. Hearts were fixed in formalin and stained with H&E. Data are representative of three independent experiments with nearly identical results. **C:** Measurement of carotid artery pressure demonstrating no systemic hypertension in *Prkg1*^{-/-} mice. Data are expressed as mean \pm SD ($n = 3$). * $P > 0.05$. CADP, carotid artery diastolic pressure; CASP, carotid artery systolic pressure; KO, knockout.

lungs with fluorescent microspheres (green) suspended in agarose.²⁷ Normal filling of the microvasculature was observed in WT mouse lungs, whereas *Prkg1*^{-/-} lungs showed a marked loss of microvascular perfusion and widespread pre-capillary occlusion (Figure 3, A and B). These data suggest that microvascular remodeling and pre-capillary occlusion cause impaired pulmonary vascular integrity in *Prkg1*^{-/-} mice.

PH in Prkg1^{-/-} Mice Is Independent of Left-Sided Heart Disease and Systemic Hypertension

To determine whether left-sided heart dysfunction is involved in the pathogenesis of PH in *Prkg1*^{-/-} mice, echo-

Table 1. Analysis of *in Vivo* Cardiac Size and Function by Echocardiography in WT and *Prkg1*^{-/-} Mice

Mouse type	LVEDD (mm)	LVESD (mm)	FS (%)	EF (%)	SEPth (mm)	PWth (mm)	BW (g)
WT	3.93 ± 0.07	2.84 ± 0.04	28.9 ± 2.1	56.1 ± 3.5	0.75 ± 0.06	0.75 ± 0.07	24.7 ± 1.5
<i>Prkg1</i> ^{-/-}	3.87 ± 0.05	2.82 ± 0.03	28.5 ± 1.5	54.4 ± 2.1	0.73 ± 0.04	0.71 ± 0.03	20.0 ± 0.8*

Data are expressed as the mean ± SD (*n* = 3). Echocardiography was performed in anesthetized sex-matched 45-day-old mice. **P* < 0.05.

BW, body weight; EF, ejection fraction; FS, fractional shortening; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; PWth, left ventricular posterior wall thickness; SEPth, intraventricular septal wall thickness.

cardiography in anesthetized mice was performed to evaluate *in vivo* cardiac function of age-matched littermates. The *Prkg1*^{-/-} mice, at the age of 45 days, exhibited a similar LV function as the WT mice (Figure 4A and Table 1). There was no evidence of LV hypertrophy and hypertension in *Prkg1*^{-/-} mice compared with WT mice. A histological examination of the *Prkg1*^{-/-} heart revealed similar sizes of ventricular chambers and thickness of the posterior wall and septum (Figure 4B). Measurement of carotid artery pressure revealed no systemic hypertension in *Prkg1*^{-/-} mice at the age of 45 days (Figure 4C). These data suggest that PH in *Prkg1*^{-/-} mice is not secondary to LV dysfunction and systemic hypertension.

PKG-I Deficiency Results in Decreased Phosphorylation of Rho A Ser188 and Increased Activation of Rho A

Previous pharmacological studies^{13,29,30} have demonstrated that PKG phosphorylates Rho A at Ser188 and subsequently inhibits its membrane association, which prevents activation of its downstream targets, such as Rho kinase. To assess whether PKG-I deficiency results in decreased phosphorylation of Rho A, we performed Western blot analysis on lung tissues from WT or *Prkg1*^{-/-} mice using antibody against phosphorylated Ser188 of Rho A. As shown in Figure 5, A and B, a marked decrease of phosphorylation of RhoA Ser188 in *Prkg1*^{-/-} lungs was observed compared with WT lungs. To determine whether PKG-I deficiency promotes Rho A membrane association and activation, we performed a pull-down assay with glutathione-Sepharose beads, coupled with glutathione-S-transferase–rothekin fusion protein.²⁸ As shown in Figure 5, C and D, there was a more than twofold increase in GTP-bound Rho A in *Prkg1*^{-/-} lungs compared with WT lungs, whereas total Rho A expression was similar in both WT and *Prkg1*^{-/-} lungs, indicating Rho A activation in *Prkg1*^{-/-} lungs. Rho A activation was also markedly increased in SMCs isolated from *Prkg1*^{-/-} lungs compared with WT SMCs (Figure 6, A and B).

PKG-I Deficiency–Induced Vasoconstriction through Activation of Rho A–Rho Kinase Signaling Is a Critical Component of the Pathogenesis of PH

We next addressed the mechanistic role of PKG-I deficiency–induced activation of Rho A–Rho kinase signaling

in the pathogenesis of PH. After measurement of RVSP at the basal level, the mice were immediately treated with fasudil, a Rho kinase inhibitor, and RVSP was continuously monitored for an additional 30 minutes. There was a marked increase of RVSP in *Prkg1*^{-/-} mice compared with WT mice under basal conditions; however, fasudil treatment resulted in a significant decrease of RVSP in *Prkg1*^{-/-} mice but not in WT mice (Figure 7, A–C). These data suggest that Rho kinase–mediated vasoconstriction is an important component of the pathogenesis of PH in *Prkg1*^{-/-} mice.

Discussion

To our knowledge, this is the first study to demonstrate that genetic disruption of *Prkg1* causes PH in mice. Our data have shown that *Prkg1*^{-/-} lungs exhibit severe pulmonary vascular remodeling, as evidenced by muscular-

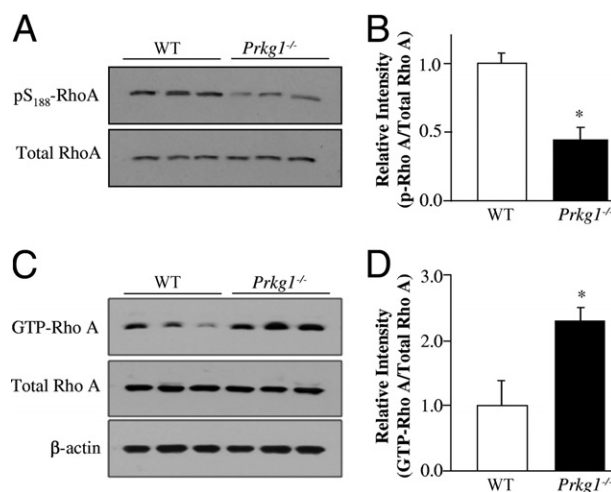


Figure 5. PKG-I deficiency activates Rho A signaling in *Prkg1*^{-/-} lungs. **A:** Western blot analysis demonstrating decreased phosphorylation of Rho A at Ser188 (pS₁₈₈-RhoA). Lung lysates, 30 μg, from 6-week-old mice per lane were loaded and immunoblotted with an anti-phosphorylated Ser188 of Rho A antibody. The experiment was repeated twice with similar data. **B:** Densitometric analysis of Rho A phosphorylation (p-Rho A) at Ser188. The intensity of each band of phosphorylated Ser-Rho A was normalized to the intensity of the respective total Rho A band. Data are expressed as mean ± SD (*n* = 3). **P* < 0.05 versus WT. **C:** PKG-I deficiency results in decreased phosphorylation of Rho A at Ser188. Prominent Rho A activation in *Prkg1*^{-/-} lungs. Lung lysates, 300 μg, from 6-week-old WT or *Prkg1*^{-/-} mice were used for pull-down assays to determine the GTP-bound active RhoA (GTP-Rho A). Each lung lysate, 30 μg, was loaded per lane for detection of total Rho A with an anti-Rho A antibody. Anti-β-actin immunoblotting was used as a loading control. **D:** Densitometric analysis of Rho A activation. The intensity of each band of GTP-Rho A was normalized to the intensity of the respective total Rho A band. Data are expressed as mean ± SD (*n* = 3). **P* < 0.01 versus WT. Rho A activity in *Prkg1*^{-/-} lungs was more than twofold greater than in WT lungs.

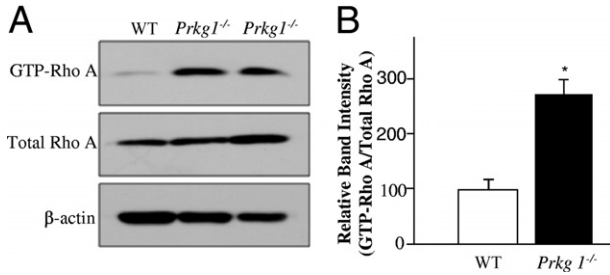


Figure 6. PKG-I deficiency induces Rho A activation in *Prkg1*^{-/-} pulmonary vascular SMCs. **A:** Representative Western blot analyses demonstrating Rho A activation in *Prkg1*^{-/-} pulmonary vascular SMCs. Pulmonary vascular SMCs isolated from 6-week-old mouse lungs were expanded in culture and lysed at passage 2 for detection of GTP-Rho A by pull-down assays. Total Rho A was detected by direct immunoblotting with an anti-Rho A antibody. β -Actin immunoblotting was used as a loading control. **B:** Densitometric analysis of Rho A activation. The intensity of each band of GTP-Rho A was normalized to the intensity of the respective total Rho A band. Data are expressed as mean \pm SD ($n = 3$ independent experiments). Rho A activity in *Prkg1*^{-/-} SMCs was threefold greater than in WT SMCs. * $P < 0.01$ versus WT.

ization of distal pulmonary vessels and impaired vascular integrity. PKG-I deficiency results in activation of Rho A-Rho kinase signaling, which causes vascular remodeling and vasoconstriction. Our data demonstrate that PKG-I deficiency-induced vasoconstriction is the critical component of the pathogenesis of PH in *Prkg1*^{-/-} mice.

The biological importance of NO/cGMP/PKG signaling was first appreciated for promoting vascular smooth muscle relaxation.^{31,32} NO stimulates cGMP synthesis and activates PKG in vascular SMCs, thereby relaxing small arteries and arterioles, resulting in a decreased blood pressure.³³ *Prkg1*^{-/-} mice exhibit defective relaxation of vascular, visceral, and penile smooth muscle¹⁷⁻²⁰; thus, *Prkg1*^{-/-} mice show impaired cGMP-dependent relaxation of large and small arteries and develop systemic hypertension as early as the age of 4 weeks.¹⁷ However, the elevated blood pressure in *Prkg1*^{-/-} mice is returned to a normal level at an older age (eg, 6 weeks old).^{17,34} The normalization of blood pressure in older *Prkg1*^{-/-} mice may be the result of cross activation of protein kinase A by the high cGMP levels that are poten-

tially generated in these mice.^{35,36} Herein, we show that *Prkg1*^{-/-} mice exhibit hypertensive pulmonary phenotypes at the age of 6 to 8 weeks, independent of systemic hypertension and LV dysfunction. The persistent hypertensive pulmonary phenotypes suggest that vasorelaxation via the cGMP/PKG-I pathway is essential to the regulation of basal pulmonary vascular pressure, which is in contrast to its role in regulation of basal blood pressure.^{17,34} Our recent studies^{26,37} have also shown that tyrosine nitration-mediated impairment of PKG-I activity, secondary to loss of caveolin-1, results in the development of PH in *Cav1*^{-/-} mice. Taken together, these data demonstrate the essential role of normal PKG-I activity in regulating the pulmonary vascular tone. Impaired PKG activity, through either decreased expression or post-translational modification, leads to PH.

Lincoln and colleagues^{38,39} first described a role for PKG in modulation of aortic SMC phenotype and implicated its role in vascular disease. They observed that constitutive expression of PKG-I α restores the contractile phenotype in cultured aortic SMCs, whereas inhibition of PKG reverses the phenotype.³⁹ Overexpression of PKG-I increases the sensitivity of cultured vascular SMCs to the anti-proliferative and pro-apoptotic effects induced by NO/cGMP,²³ whereas down-regulation of PKG-I induces the change of SMC phenotype from a contractile, differentiated to a synthetic, dedifferentiated state,²⁴ which is the characteristic feature of pulmonary vascular remodeling. We have observed a >10-fold increase of muscularization of distal pulmonary vessels, a drastic loss of microvascular perfusion, and widespread pre-capillary occlusion in *Prkg1*^{-/-} lungs. These data suggest that PKG-I deficiency induces pulmonary microvascular remodeling and pre-capillary occlusion, resulting in impaired pulmonary vascular integrity, and thereby contributes to the pathogenesis of PH in *Prkg1*^{-/-} mice.

Recent studies^{14,40-42} have shown an important role of the PKG-regulated Rho A/Rho kinase signaling pathway in modulating pulmonary vascular remodeling and vasoconstriction. PKG-I phosphorylates Rho A at Ser188 and

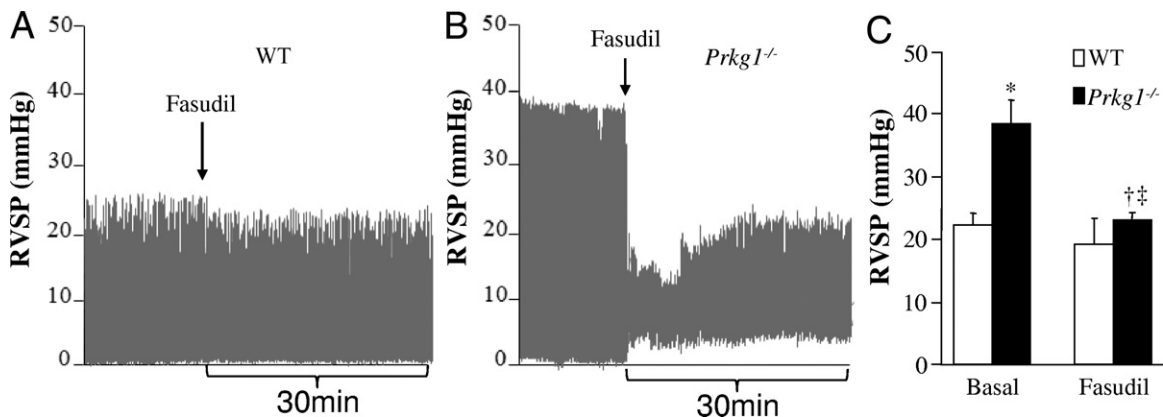


Figure 7. The causal role of Rho kinase activation, secondary to PKG-I deficiency, in the pathogenesis of PH in *Prkg1*^{-/-} mice. Representative RVSP tracings at the basal level and after fasudil treatment in WT (**A**) and *Prkg1*^{-/-} mice (**B**). After measurement of RVSP under basal conditions, the 7-week-old mice were treated with Rho kinase inhibitor fasudil (60 mmol/L in 25 μ L of saline/mouse, i.t.), and then RVSP was continuously monitored for 30 minutes. **C:** Normalized RVSP in *Prkg1*^{-/-} mice after fasudil treatment. Data are expressed as mean \pm SD ($n = 3$ to 5). * $P < 0.01$ versus WT at the basal level, [†] $P < 0.05$ versus *Prkg1*^{-/-} at the basal level, and [‡] $P > 0.05$ versus WT treated with fasudil.

inhibits its membrane association, thereby preventing Rho kinase activation.^{13,15} Consistently, we observed decreased phosphorylation of Rho A Ser188 and resultant activation of Rho A in *Prkg1*^{-/-} lungs. PKG-I deficiency also leads to marked Rho A activation in primary cultures of pulmonary vascular SMCs isolated from *Prkg1*^{-/-} lungs. However, the total protein levels of Rho A in *Prkg1*^{-/-} lungs and isolated pulmonary vascular SMCs are the same as in WT lungs. In contrast, other studies have shown a critical role of PKG in mediating NO-stimulated Rho A expression. NO from pulmonary artery SMCs of rat or human induces Rho A expression via an increase in Rho A protein stability and Rho A gene transcription through PKG.⁴³ Long-term inhibition of NO synthesis induces a significant decrease in Rho A mRNA and protein expression in nitro-L-arginine-treated rat aorta and pulmonary artery.⁴⁴ Rho A has played a major role in the regulation of multiple cellular functions, including actin cytoskeleton organization, contraction, gene expression, differentiation, and proliferation.^{14,45–47} Thus, Rho A activation, secondary to PKG-I deficiency, may be the major contributor of the severe pulmonary vascular remodeling leading to PH in *Prkg1*^{-/-} mice.

We have shown that the Rho A/Rho kinase signaling pathway plays a key role in the mechanisms of the pathogenesis of PH in *Prkg1*^{-/-} mice. Short-term inhibition of Rho kinase by i.t administration of fasudil completely inhibited the increase of RVSP in *Prkg1*^{-/-} mice. These data demonstrate that vasoconstriction secondary to impairment of PKG activity is an important component of the pathogenesis of PH. Several pharmacological studies have also suggested a role for Rho kinase in the development of PH. The i.v. or oral treatment with Rho kinase inhibitor (Y-27632 or fasudil) normalizes the high pulmonary arterial pressure in chronically hypoxic rats, attenuates the development of chronic hypoxia-induced PH in mice, and reduces pulmonary arterial lesions in monocrotaline-induced PH in rats.^{40,41,43} Inhaled Y-27632 or fasudil causes sustained and selective pulmonary vasodilation in monocrotaline-induced PH and in spontaneous PH in fawn-hooded rats, as well as in chronically hypoxic rats.⁴⁸ Rho kinase is one of the main downstream effectors of Rho A. On activation, Rho A activates Rho kinase, which phosphorylates the myosin phosphatase target subunit 1 of smooth muscle myosin phosphatase at Thr696 and leads to inhibition of its activity, an increased sensitivity of myosin light chain to calcium, and contraction.^{15,49} These data collectively suggest that activation of the Rho A/Rho kinase signaling pathway secondary to decreased PKG activity is a critical component of the underlying mechanisms of PH in *Prkg1*^{-/-} mice.

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