The American Journal of Pathology, Vol. 183, No. 6, December 2013

The American Journal of
PATHOLOGY
ajp.amjpathol.org

CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

TRPC4 Inactivation Confers a Survival Benefit in Severe Pulmonary Arterial Hypertension

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Accepted for publication August 12, 2013.

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Pulmonary arterial hypertension (PAH) is characterized by elevated pulmonary arterial pressure with lumen-occluding neointimal and plexiform lesions. Activation of store-operated calcium entry channels promotes contraction and proliferation of lung vascular cells. TRPC4 is a ubiquitously expressed store-operated calcium entry channel, but its role in PAH is unknown. We tested the hypothesis that TRPC4 promotes pulmonary arterial constriction and occlusive remodeling, leading to right ventricular failure in severe PAH. Severe PAH was induced in Sprague–Dawley rats and in wild-type and *TRPC4*-knockout Fischer 344 rats by a single subcutaneous injection of SU5416 [SU (semaxanib)], followed by hypoxia exposure (Hx; 10% O_2) for 3 weeks and then a return to normoxia (Nx; 21% O_2) for 3 to 10 additional weeks (SU/Hx/Nx). Although rats of both backgrounds exhibited indistinguishable pulmonary hypertensive responses to SU/Hx/Nx, Fischer 344 rats died within 6 to 8 weeks. Normoxic and hypertensive *TRPC4*-knockout rats recorded hemodynamic parameters similar to those of their wild-type littermates. However, *TRPC4* inactivation conferred a striking survival benefit, due in part to preservation of cardiac output. Histological grading of vascular lesions revealed a reduction in the density of severely occluded small pulmonary arteries and in the number of plexiform lesions in *TRPC4*-knockout rats. *TRPC4* inactivation therefore provides a survival benefit in severe PAH, associated with a decrease in the magnitude of occlusive remodeling. *(Am J Pathol 2013, 183: 1779–1788; http://dx.doi.org/10.1016/j.ajpath.2013.08.016)*

Pulmonary arterial hypertension is characterized by increased pulmonary vascular resistance and arterial pressure, processes that culminate in right ventricular failure and death.[1] Intractable pulmonary vasoconstriction and occlusive vascular remodeling are integral to the pathogenesis of this syndrome.[2] These processes are causally linked to perturbations in the normal balance of vasoactive, mitogenic, and apoptotic factors in pulmonary vascular cells.[3] Currently available therapeutic options (such as calcium channel blockers, prostacyclin analogs, endothelin-1 receptor antagonists, phosphodiesterase inhibitors, and nitric oxide) seek principally to reduce vasoconstriction.[4] These therapeutic options only minimally decrease pulmonary arterial pressure and, with the possible exception of calcium channel blockers in a certain population of patients,[5] they do not affect disease progression. Despite recent improvements in patient outcomes, the persistently high morbidity and mortality for pulmonary arterial hypertension has prompted a search for signaling nodes within vascular cells that are mechanistically linked to disease progression and that, as such, represent novel therapeutic options with the potential for improved patient functional status and survival.[6]

Cytosolic calcium is a second messenger that represents one such signaling node, or convergence site.[7] In pulmonary arterial hypertension, multiple inflammatory cytokines, growth factors, and vasoactive autocoids act on transmembrane receptors and chronically elevate cytosolic calcium; cytosolic calcium is constitutively elevated in both endothelial[8] and smooth muscle cells[9] from pulmonary arterial hypertension patients. In endothelial cells, increased cytosolic calcium is a stimulus for proliferation, and has

Supported by NIH grants P01-HL066299 and R37-HL060024 (T.S.), R21-HL106101 (M.O.), and R01-OD010944 (M.A.).

http://dx.doi.org/10.1016/j.ajpath.2013.08.016

been incriminated in the formation of occlusive lesions within small pulmonary arterioles.[10] In smooth muscle cells, increased cytosolic calcium represents a stimulus for both contraction and proliferation, and has been incriminated in hypercontraction, hypertrophy, and hyperplasia of the medial wall.[11] However, the source of elevated calcium in endothelial and smooth muscle cells obtained from pulmonary arterial hypertensive patients is poorly understood. Sustained elevations in cytosolic calcium require calcium influx through transmembrane channels. The principal calcium channels in pulmonary arterial endothelial cells are receptor- and store-operated calcium entry channels, whereas smooth muscle cells also possess voltage-gated calcium channels.[12] Data from Yuan and colleagues[9] and Lin et al[13] suggest that, among these mechanisms of calcium influx, store-operated calcium entry critically contributes to vascular cell proliferation.

Transient receptor potential proteins of the canonical TRPC subfamily comprise store-operated calcium entry channel subunits.[14,15] TRPC1 and TRPC4, in particular, interact to form a heterotetramer calcium channel,[16] although the molecular basis of this channel signalplex (ie, signaling complex) is incompletely resolved. Activation of calcium entry through the TRPC1/4 channel requires TRPC4 binding to protein 4.1 near the pore within the carboxy-terminus.[15] Protein 4.1 in turn binds to the spectrin membrane skeleton that links the TRPC1/4 channel signalplex to the endoplasmic reticulum. Few ion channels are gated by protein 4.1.[17] However, another store-operated calcium entry channel, Orai1, also possesses a putative protein 4.1 binding domain and can be resolved within the TRPC1/4 signalplex, where it constitutively interacts with TRPC4.[18] Orai1 forms the molecular basis for a highly calcium selective channel responsible for the calcium release-activated calcium current, I_{CRAC},[19] and it also contributes to calcium selectivity of the TRPC1/4 channel,[20] suggesting that TRPC4 is essential for proper organization of the store-operated calcium entry signalplex. Given that TRPC4 is essential to the molecular make-up of store-operated calcium entry channels, and because it has been incriminated in the activation of endothelial nitric oxide synthase,[21,22] endothelial cell barrier disruption,[23] and smooth muscle cell contraction, hyperproliferation, and mitogenesis,[11] we sought to determine whether TRPC4 contributes to development of severe pulmonary arterial hypertension. To this end, we tested the hypothesis that TRPC4 promotes pulmonary arterial constriction and occlusive remodeling leading to right ventricular failure in severe pulmonary arterial hypertension.

Materials and Methods

Animals

All experimental procedures were performed in accordance with current provisions of the U.S. Animal Welfare Act and were approved by the Institutional Animal Care and Use Committee of the University of South Alabama. Severe occlusive pulmonary arterial hypertension in rats was induced by a single subcutaneous injection of 20 mg/kg SU5416 [SU (semaxanib)] (Cayman Chemical, Ann Arbor, MI) on day 1, followed by exposure to 3 weeks of normobaric hypoxia (Hx; 10% O_2) and then re-exposure to normoxia (Nx; 21% O_2) for 3 to 10 additional weeks (SU/Hx/Nx). The hemodynamic and histopathological parameters of pulmonary arterial hypertension were compared among three experimental groups of age- and weight-matched rats: male Sprague-Dawley (SD), male Fischer 344 (F344), and male *TRPC4*-knockout (KO) F344. Each experimental group included a set of normoxic time-control rats. *TRPC4*-KO F344 rats were generated by Transposagen Biopharmaceuticals (Lexington, KY), as part of the Knockout Rat Consortium (*Trpc4*^{tm1Bni}, targeted mutation 1, Bernd Nilius), and were bred and genotyped both at Transposagen and at the University of South Alabama.

Genotyping of the *TRPC4*-KO Rats

Rat tail snips were collected according to the guidelines of the University of South Alabama Animal Care and Use Committee. DNA was extracted from tail snips, as described previously,[24] and 2 μL of the resulting DNA solution was subjected to PCR analysis using three primers (primer A, 5′-GTGTTGGTCTCCATTACTTCAGCT-3′; primer B, 5′-ATTCTTCCCTTTGAGCCCACT-3′; and transposon primer, 5′-CTGACCTAAGACAGGGAATT-3′) in a total volume of 20 μL containing 1× GoTaq Green PCR master mix (Promega, Madison, WI) and 1 μmol/L of each primer. The cycling parameters were denaturation at 94°C for 5 minutes; then 30 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 1 minute; and extension at 72°C for 7 minutes.

Basal Calcium Dynamics

Rat pulmonary artery segments (~500 mm in length) were dissected, cut open longitudinally, and mounted intima side up on Sylgard blocks (Dow Corning, Midland, MI) for endothelial imaging, as described previously.[25,26] Arteries were incubated with Ca^{2+} indicator loading solution (Life Technologies, Carlsbad, CA) containing 15 μmol/L Fluo-4 AM and 0.06% Pluronic F-127 in HEPES (pH 7.45) at room temperature for 40 minutes in the dark. After a wash, blocks were placed face down on two parallel 100-μm supporting pins in a glass-bottom chamber containing HEPES. The chamber was mounted on an inverted microscope fitted with a PerkinElmer (Waltham, MA) spinning disk RS-3 confocal unit. Ca^{2+}—dependent fluorescence (488 nm excitation, 510 nm emission) was measured at 8 frames per second at 25°C (20× objective) using UltraVIEW software version 1.1.0.9 (PerkinElmer). Only recordings with >90% of total viewable area in focus were processed

and analyzed. Dynamic basal data were processed using a custom algorithm[25,26] implemented as a plug-in with ImageJ software version 1.47 (NIH, Bethesda, MD) specifically designed to i) detect sites of dynamic Ca^{2+} change above statistical noise ($P < 0.01$), ii) define regions of interest (ROI; 5 pixel or 1.7 μm diameter) at active site centers, and iii) analyze average fluorescence intensities at ROIs to determine specific event parameters (amplitude, duration, and spread). Fluorescence data are expressed as the F/F_0 ratio, where F_0 is determined by a linear regression of base data at each ROI. For acetylcholine (ACh) responses, average Ca^{2+}−dependent fluorescence was assessed over a field of 2.8×10^4 μm (approximately 80 cells).

Echocardiography

Echocardiography was performed using a Vevo 770 imaging system (VisualSonics, Toronto, ON, Canada) at baseline and at the end of weeks 5 and 8, to evaluate the progression of cardiac dysfunction. Rats were anesthetized but were kept spontaneously breathing using isoflurane 2.0% in a 1:1 O_2/air mixture. Two-dimensional, M-mode, and Doppler flow imaging was performed with a 30-MHz probe. Measurements of cardiac output (CO), left ventricular end-diastolic volume (LVEDV), tricuspid annular plane systolic excursion (TAPSE), and pulmonary artery acceleration time (PA-AT) were acquired as previously reported.[27]

Hemodynamic Measurements in Catheterized Rats

Rats were anesthetized with 30 mg/kg i.p. pentobarbital sodium. A polyvinyl catheter (PV-1; internal diameter, 0.28 mm) was inserted into the right ventricle via the right jugular vein for measurement of right ventricular systolic pressure (RVSP). A microtip P-V1 catheter (SPR-838; Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the left ventricle. Left ventricular systolic pressure (LVSP) and CO were measured. Cardiac index (CI) was calculated by dividing CO by body weight.

Isolated Arterial Ring Studies

Extralobar first-branch pulmonary artery and 3-mm-long thoracic aorta rings were placed into isometric tension baths containing 10 mL of Earle's balanced salt solution bubbled with 21% O_2−5% CO_2−74% N_2 gas at 37°C. Isolated arteries were brought to a resting tension of 1 g (aorta), 0.75 g (normotensive pulmonary artery), or 1.5 g (hypertensive pulmonary artery) and were equilibrated at this tension for 60 minutes, as described previously.[28] Next, 80 mmol/L KCl was injected into the bath, to elicit a maximum contractile response. The bath was washed with Earle's balanced salt solution, and 1 μmol/L phenylephrine (PE) was subsequently added. The time to peak PE contraction was recorded, and then 10 μmol/L ACh was administered to evaluate endothelium-dependent relaxation.

Morphometric Analysis of Pulmonary Vascular and Cardiac Remodeling

Rat lungs were fixed for histological analysis by tracheal instillation of a mixture of 1% formalin and 0.5% agarose under constant pressure (20 cmH_2O). After immersion in 10% formalin for 48 hours, lung tissue was cut into sections 5 mm thick, placed in 70% ethanol, embedded in paraffin, serially mounted onto Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA), and stained with H&E. Vascular occlusion density (OD) was assessed in a masked fashion by five independent observers (A.A., P.A., K.O., M.O., and T.S.) by grading the small (<50 μm) pulmonary arteries in at least three lung tissue samples per group as OD = 0 (no occlusion), OD ≤50%, or OD >50%. The same vessels were then classified according to the Heath−Edwards pathological grading of pulmonary vascular remodeling.[29]

Formalin-fixed, paraffin-embedded heart tissue was stained with Masson's trichrome stain to assess the degree of fibrosis within the heart tissue. Images were acquired using a Nikon (Tokyo, Japan) E600 light microscope with a digital interface Q-imaging software. Fibrosis was estimated as the percentage of blue-stained tissue areas (collagen fibers stain blue) relative to the total surface area.

Statistical Analysis

Data are expressed as means ± SEM, unless specified otherwise. Unpaired nonparametric *t*-test or analysis of variance with Bonferroni post hoc test were used for comparisons among the experimental groups. Survival results were plotted on a Kaplan−Meier curve and analyzed using a Mantel−Cox log-rank test. For histological grading studies, we used a contingency test, with Cochran−Mantel−Haenszel adjustment, where likelihood ratio and Pearson's P value were calculated. Differences were considered statistically significant at $P < 0.05$.

Results

Both SD and F344 Rats Develop Severe Pulmonary Arterial Hypertension

The novel strain of genetically modified rats used in this study was bred on an F344 background. Because F344 rats have not previously been exposed to SU/Hx/Nx, we first sought to confirm that they respond to this treatment with development of pulmonary arterial hypertension. Baseline hemodynamic parameters were similar in SD and F344 rats (data not shown). Eight weeks of SU/Hx/Nx in both SD and F344 rats produced a similar range of changes in RVSP, CI, LVSP, and right ventricular hypertrophy index [RV weight/(LV + septum weight)] (Figure 1). SD and F344 rats therefore develop a similar hemodynamic response to induction of severe pulmonary arterial hypertension.

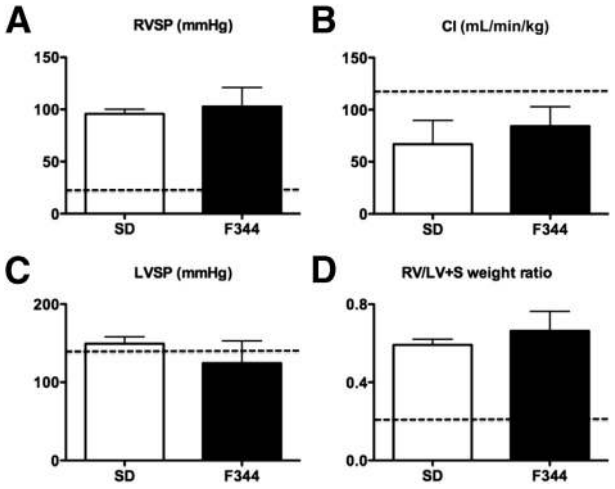

Figure 1 Rats of SD and F344 backgrounds develop severe pulmonary arterial hypertension. Eight weeks of SU/Hx/Nx exposure induced similar changes in RVSP (**A**), CI (**B**), LVSP (**C**), and the right ventricular hypertrophy index (**D**) in both strains of rats. For comparison, normotensive values from previous studies in SD rats (historic controls) are indicated by **dotted lines**. Data are expressed as means ± SEM. $n = 4$ per group.

TRPC4 Inactivation Causes a Calcium Signaling Defect in Pulmonary Artery Endothelium

F344 rats were genotyped to confirm the successful inactivation of *TRPC4* and to generate wild-type (WT) littermate controls (Figure 2A). WT rats expressed a fragment of 905 bp, KO rats expressed a fragment of 510 bp, and heterozygotic rats expressed both fragments.

Once animals were genotyped, we sought to determine whether TRPC4-deficient animals exhibited a calcium signaling defect characteristic of the absence of a functional store-operated calcium entry channel.[22] WT and KO F344 rats were selected after genotyping, and pulmonary arteries were harvested for study. Vessel segments were loaded with calcium dye and pulmonary artery endothelium was imaged *en face*. Gross inspection of the fluorescence signal obtained from dye-loaded endothelium revealed regular polyhedral endothelial organization, only slightly elongated toward the direction of flow, in WT animals (Figure 2B). In animals exhibiting *TRPC4* inactivation, however, endothelium was irregularly spaced and elongated in the direction of flow, an observation consistent across all of the vessels studied. These data suggest that *TRPC4* inactivation repatterns the pulmonary endothelium.

Although endothelial cell cytosolic calcium averages approximately 100 nmol/L over extended time periods, there are dynamic fluctuations in cytosolic calcium from moment to moment. We therefore measured basal cytosolic calcium dynamics for 2 minutes at 8 Hz frequency. ROIs were identified within individual cells based on a threshold excursion criterion of greater than two-fold transient increases in cytosolic calcium,[25,26] and cytosolic calcium in

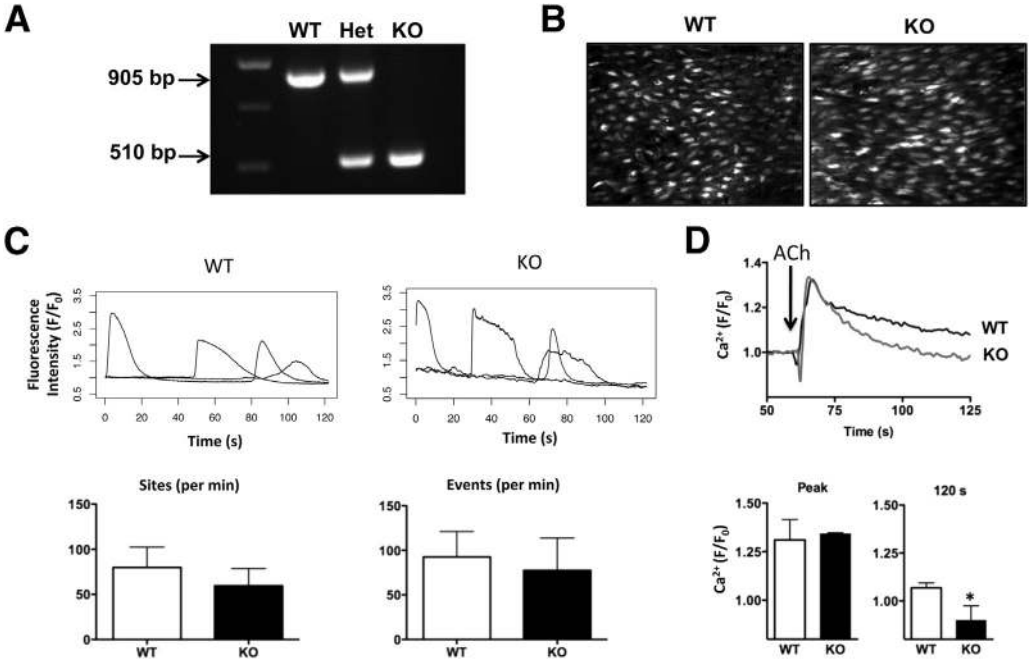

Figure 2 *TRPC4*-KO rats exhibit a defect in ACh-induced calcium influx. **A**: Genotyping of *TRPC4* WT, heterozygotic (Het), and KO F344 rats. **B**: Pulmonary artery segments were isolated and loaded with the Fluo-4 AM cytosolic calcium indicator. Endothelium was then imaged *en face*. Evaluation of endothelial cell shape revealed a distinct anatomical pattern among WT and *TRPC4*-KO vessels, with elongated endothelium in *TRPC4*-KO rats. **C**: Basal cytosolic Ca^{2+}—dependent fluorescence recorded in open artery preparations of WT and *TRPC4*-KO rats revealed no difference in basal cytosolic calcium dynamic oscillations. **Top panels**: Representative tracings of dynamic cytosolic Ca^{2+} events occurring in individual cells over 2 minutes. Each line represents the ROI (5 μm diameter) from a different cell. **Bottom panels**: Total sites and events per field per minute ($n = 4$ per group). **D**: Average whole-field cytosolic Ca^{2+} signal stimulated by 2 μmol/L ACh in pulmonary artery endothelium of WT and *TRPC4*-KO rats over time, as well as peak Ca^{2+} response and steady-state signal after 120 seconds. Data are expressed as means ± SEM. $n = 4$. $^*P < 0.05$ versus WT.

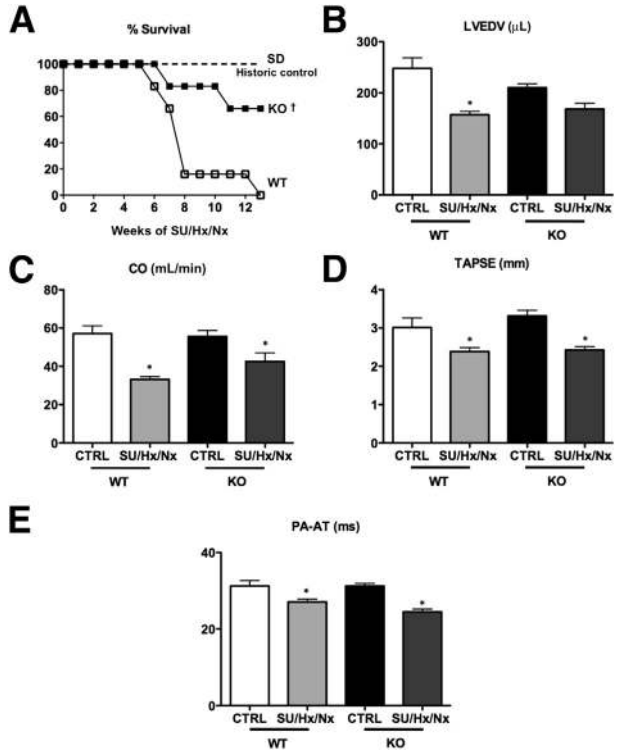

Figure 3 *TRPC4*-KO rats exhibit a survival benefit that cannot be explained by improved hemodynamic response to SU/Hx/Nx. **A**: Survival over 12 weeks of SU/Hx/Nx exposure. SD rats (**dotted line**) serve as historic controls. *TRPC4*-KO rats ($n = 12$) exhibited a clear survival benefit versus WT rats ($n = 6$) after induction of severe pulmonary arterial hypertension. **B**: M-mode echocardiography of the left ventricle revealed a smaller LVEDV in WT rats after SU/Hx/Nx exposure. **C**–**E**: Development of pulmonary arterial hypertension was associated with decrements in CO (**C**), TAPSE (**D**), and PA-AT (**E**) in WT ($n = 8$) and KO ($n = 12$) rats. Echocardiography was performed at baseline and after 8 weeks of SU/Hx/Nx exposure. Data are expressed as means ± SEM. $^{*}P < 0.05$ versus control. $^{\dagger}P < 0.0006$ versus WT, Mantel–Cox log-rank test.

each of the ROIs was recorded over time (Figure 2C). Individual endothelial cells from both WT and *TRPC4*-KO rats exhibited one or two oscillations within the 2-minute time period tested. Neither the total number of oscillatory cells (sites) per minute nor the number of oscillatory events per minute differed between groups (Figure 2C). In total, we recorded 450 oscillatory events from 419 ROIs in WT rats and 352 oscillatory events in 297 ROIs in KO rats; four different animals were studied in WT and KO rats. Thus, *TRPC4* inactivation did not alter basal cytosolic calcium oscillations in pulmonary artery endothelium.

ACh induces calcium store depletion from the endoplasmic reticulum and activates store-operated calcium entry channels. Next, therefore, we examined whether *TRPC4* inactivation impairs ACh-induced calcium influx. ACh application induced a transient, abrupt rise in cytosolic calcium, which decayed to a plateau level over the 2-minute time course of the experiment (Figure 2D). In *TRPC4*-KO animals, the peak ACh-induced rise in cytosolic calcium was normal, but the sustained increase in cytosolic calcium was reduced, indicating that *TRPC4* inactivation reduces the sustained rise in cytosolic calcium. These results establish that *TRPC4*-KO rats harbor a prominent calcium entry defect.

TRPC4 Inactivation Confers a Survival Benefit in Severe Pulmonary Arterial Hypertension

We next assessed the responses of WT and *TRPC4*-KO rats to long-term (13 weeks) SU/Hx/Nx exposure. Based on previous reports,[30] SD rats tolerate severe pulmonary arterial hypertension and right ventricular dysfunction for extended durations. In stark contrast, none of the WT F344 rats survived the 13-week experimental period; however, 70% of the *TRPC4*-KO rats survived for 13 weeks, illustrating a clear survival benefit after *TRPC4* inactivation (Figure 3).

With such unexpectedly high mortality in F344 rats, hemodynamic parameters were obtained using echocardiography in these animals only at baseline and at 8 weeks. Normotensive KO rats tended to have smaller LVEDV, compared with control WT rats ($P = 0.21$) (Figure 3B). Nonetheless, 8 weeks of SU/Hx/Nx exposure resulted in similar decreases in CO (Figure 3C), TAPSE (Figure 3D), and PA-AT in both WT and *TRPC4*-KO rats, relative to control (Figure 3E).

In these studies, we noted that pulmonary hypertensive F344 rats were highly sensitive to the 1.5% isoflurane used to anesthetize animals for echocardiography measurements; this sensitivity was especially evident in WT animals. A precipitous decrease in CO (acute decompensation) in WT animals was observed before death, an effect that was not

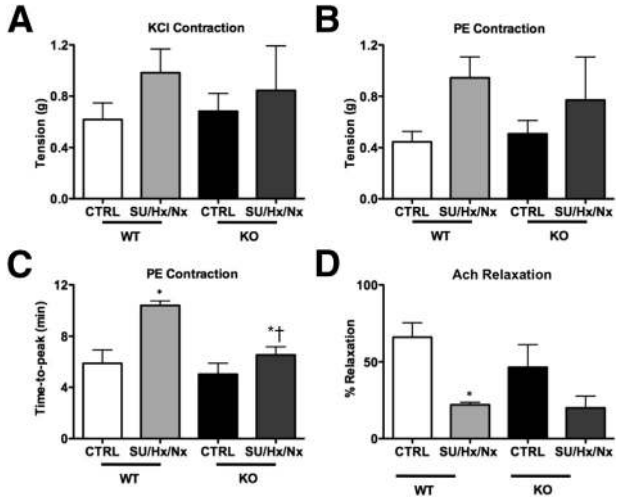

Figure 4 *TRPC4* inactivation did not significantly impair either the maximal smooth muscle cell constriction or endothelium-dependent dilation. Pulmonary arterial ring responses to treatment with KCl (**A**), PE (**B**), time to peak contraction induced by PE (**C**), and ACh-induced dilation of the KCl contraction (**D**) in WT and *TRPC4*-KO rats under control and SU/Hx/Nx conditions. Data are expressed as means ± SEM. $n = 4$ per group. $^{*}P < 0.05$ versus control; $^{\dagger}P < 0.05$ versus WT SU/Hx/Nx group.

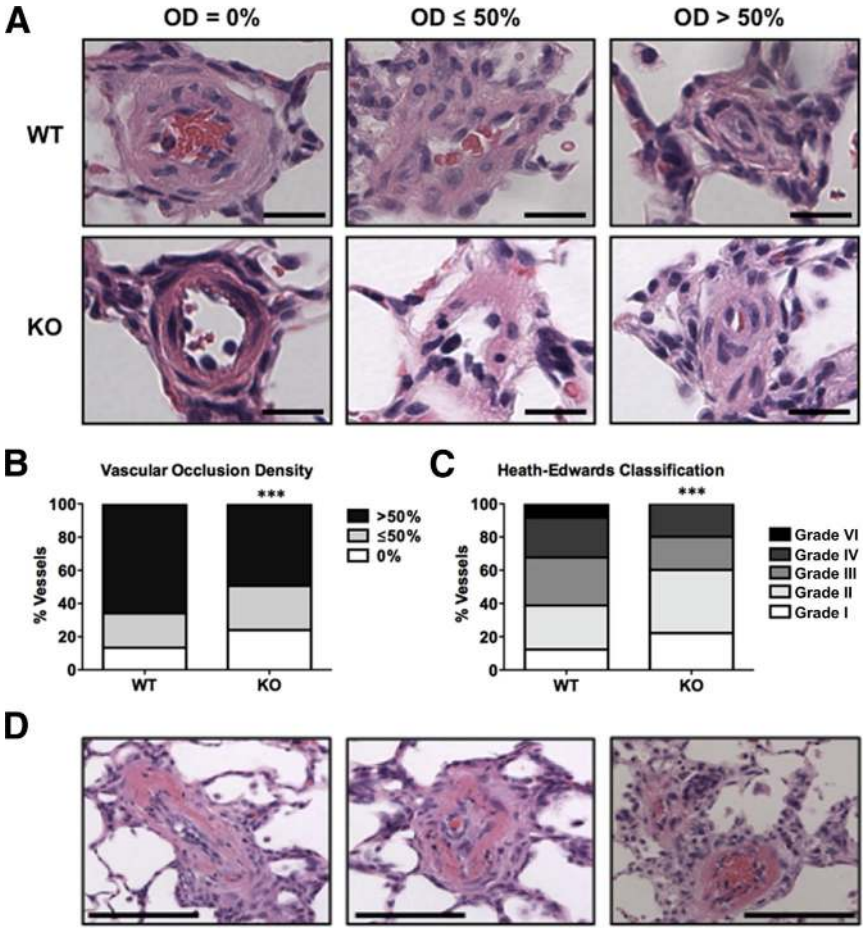

Figure 5 *TRPC4* inactivation reduced the magnitude of the arteriopathy after SU/Hx/Nx exposure. **A** and **B:** Luminal occlusion density (OD) of the small pulmonary arteries (<50 μm) was graded in pulmonary hypertensive lungs of WT and *TRPC4*-KO rats. The frequency of severely (>50%) occluded vessels was higher in WT than in KO rats. **C:** Heath—Edwards classification of pulmonary hypertension-induced vascular lesions. **D:** Examples of severe fibrinoid necrosis and perivascular inflammation (grade VI, necrotizing arteritis) of the pulmonary arteries in WT rats. Grading of lesions was performed in 50 arbitrarily chosen, but consecutive, small pulmonary arteries per sample, in three samples per experimental group. Significance was analyzed by likelihood ratio and Pearson's *P*. ***$P < 0.001$. Scale bar = 50 μm.

observed in the KO rats. Therefore, although CO was similar in WT and KO rats at baseline and after 5 weeks of pulmonary hypertension (data not shown), WT rats had an abrupt 40% reduction, compared with 7% reduction in KO animals, in CO at the 8-week time point.

In an attempt to more definitively examine the hemodynamic adaptations to SU/Hx/Nx, we repeated the exposure and catheterized a subsequent set of rats after only 6 weeks of pulmonary arterial hypertension. However, there was no significant difference in the average RVSP, CO, LVSP, or right ventricular hypertrophy index between WT and *TRPC4*-KO rats at 6 weeks (data not shown). Thus, echocardiography and catheterization data taken together indicate that the baseline hemodynamic adaptation to SU/Hx/Nx is similar in WT and *TRPC4*-KO rats, but that WT rats are more prone to acute cardiac decompensation and death.

TRPC4 Channels Are Critical for Pulmonary and Systemic Vasoreactivity

Calcium influx via TRPC4 channels contributes to smooth muscle contraction[31] and leads to increased production of nitric oxide in vascular endothelial cells.[22] We therefore determined the pulmonary arterial response to vasoactive agents in WT and *TRPC4*-KO rats. Pulmonary artery vasoreactivity responses to KCl (Figure 4A) and PE (Figure 4B) were similar in normotensive WT and *TRPC4*-KO rats. KCl- and PE-induced constriction was slightly augmented after development of pulmonary arterial hypertension, but the difference did not reach statistical significance. Interestingly, although the recorded PE-induced pulmonary vasoconstriction was almost equivalent in SU/Hx/Nx-exposed WT and *TRPC4*-KO rats, the time to peak contraction was lower in KO animals, suggesting that the contractile mechanism is more efficient after *TRPC4* inactivation (Figure 4C). ACh caused an approximate 50% relaxation to the KCl-induced constriction in normotensive rats, irrespective of TRPC4 status (Figure 4D). This endothelium-dependent relaxation was impaired in Su/Hx/Nx-exposed animals, and ACh induced only a 20% relaxation in vessels obtained from either WT or *TRPC4*-KO rats.

TRPC4 Inactivation Reduces Severity of the Occlusive Pulmonary Arteriopathy and Cardiac Fibrosis in Severe Pulmonary Arterial Hypertension

To further gauge the role TRPC4 channels play in severe pulmonary arterial hypertension, we performed extensive histopathological analyses of pulmonary vascular and

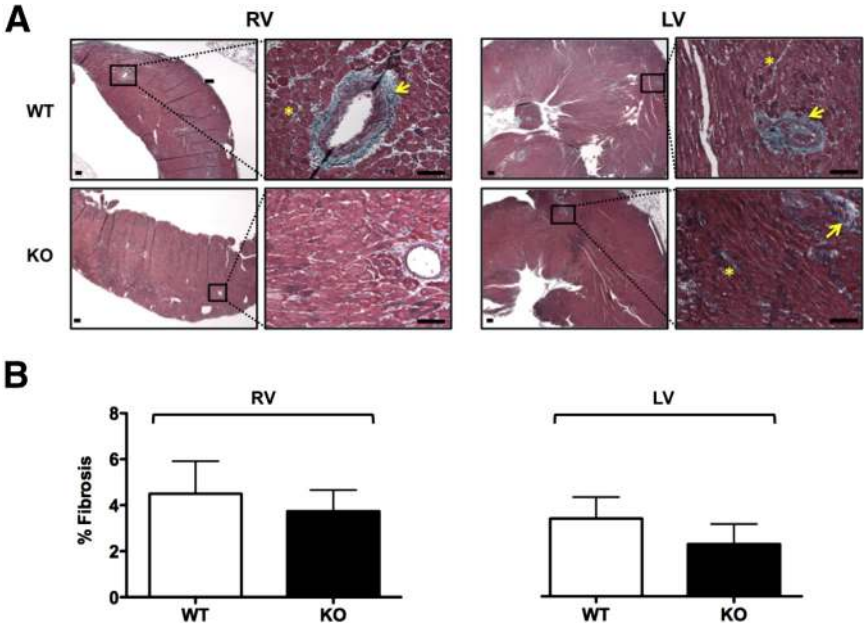

Figure 6 Both WT and *TRPC4*-KO rats exhibited cardiac fibrosis after SU/Hx/Nx exposure. **A**: Masson's trichrome staining of the RV and LV in WT and *TRPC4*-KO rats after 6 weeks of SU/Hx/Nx exposure; collagen fibers stain blue. Perivascular fibrosis (**arrows**) and interstitial fibrosis (**asterisks**) tended to be higher in both ventricles of the WT rats. **Boxed regions** correspond to the higher magnification images to the right. **B**: Quantification of the fibrotic zones in both ventricles revealed no significant difference between WT and *TRPC4*-KO hearts. Data are expressed as means ± SEM. $n = 4$ per group. Scale bar = 50 μm.

cardiac remodeling. Small pulmonary arteries (<50 mm) were first graded based on luminal occlusion (Figure 5A). The frequency of severely (>50%) occluded pulmonary arteries was lower in *TRPC4*-KO rats than in WT rats (Figure 5B; likelihood ratio and Pearson P value of <0.001). In addition, there was an increase in the density of fully patent pulmonary arteries (0% occlusion) in the KO animals. To acquire a better survey of pulmonary vascular remodeling in these rats, we categorized pulmonary arterial lesions according to the Heath–Edwards classification[29] (Figure 5C). Such a thorough approach revealed two key findings, that the number of mature plexiform lesions (grade 4) per lung was lower in KO rats and that some WT lungs exhibited a severe form of fibrinoid necrosis within the pulmonary arteries (necrotizing arteritis), coupled with perivascular inflammation (grade VI) (Figure 5D; likelihood ratio and Pearson P value <0.001). Thus, *TRPC4* inactivation impairs development of the occlusive arteriopathy prominent in severe pulmonary arterial hypertension.

We next evaluated the degree of cardiac fibrosis, because this is considered a terminal feature of maladaptive structural remodeling in the failing heart.[32] The prevalence of interstitial and perivascular collagen deposition within both ventricles tended to be higher in WT than in KO rats, although on average the difference did not reach statistical significance (Figure 6, A and B). Taken together with the hemodynamic results, WT animals exhibited severe pulmonary arterial hypertension characterized by maladaptive remodeling of both the small precapillary arterioles and the right and left ventricles, ultimately leading to heart failure and death. Although *TRPC4*-KO rats developed severe pulmonary arterial hypertension, occlusive vasculopathy was less severe, and these animals were not as prone to absolute heart failure.

Discussion

We report here for the first time the successful induction of severe occlusive pulmonary arterial hypertension that progresses to heart failure and death in F344 rats. The observed premature death of WT rats, and the reciprocal survival benefit in animals harboring a *TRPC4* inactivation, are the major highlights of the present study. The loss of TRPC4 channels in these rats corresponded to a milder form of pulmonary vascular remodeling, seemingly disproportional to their severe hemodynamic disturbances.

The importance of genetic mutations in the pathogenesis of severe pulmonary arterial hypertension has been highlighted in the most recent clinical classification of the disease, in which heritable forms were upgraded into a separate subcategory.[1] Germline mutations [eg, *BMPR2*, *ACVRL1* (alias *ALK1*), and endoglin (*ENG*)] have been evidenced in approximately 70% of families with pulmonary arterial hypertension,[33] as well as in almost 20% of sporadic cases.[34]

To model for these mutations and to examine the molecular basis of disease, mice have been the main species used, because of the ease and feasibility of genetic modification. However, critical anatomical, physiological, and pathological differences between humans and mice have hindered meaningful translational applications of the research outcomes.[35] Rats, in contrast, have shown promise in replicating certain hallmarks of human pulmonary arterial hypertension (eg, plexiform lesions), at least under certain conditions.[30,36] Unfortunately, the well-documented inter- and intrastrain variability of rats, in addition to several other technical challenges, has made these animals less favored for genetics-oriented research.[37] Our present study therefore represents a significant step forward. Here, we have provided evidence of a successful genetic modification in F344

rats, namely, *TRPC4* inactivation, which enables testing of the role of TRPC4 in a severe form of pulmonary arterial hypertension that is accompanied by reproducible induction of angio-obliterative lesions with abject heart failure.

The use of SU/Hx/Nx exposure in SD rats is a landmark event in the field of pulmonary arterial hypertension research, because the resulting hemodynamic and histopathological changes in SD rats are highly conserved and are comparable to those observed in patients.[30,36] One major departure from the natural history of the clinical disease, however, is the surprising tolerance that these rats exhibit to severely compromised cardiopulmonary status, and thus their prolonged survival. We now report the first evidence of an animal model (ie, F344 rats exposed to SU/Hx/Nx) in which the natural history of severe pulmonary hypertension is accompanied by death, as occurs in the human disease. Furthermore, we show for the first time a mortality determinant in SU/Hx/Nx-exposed rats, namely, expression of a functional TRPC4 channel. The failure of currently available pulmonary arterial hypertension treatments to reduce patient mortality[38] points to the need for new drugs, and TRPC4 could be an auspicious target.

The mechanism or mechanisms by which *TRPC4* inactivation reduces mortality in severe pulmonary arterial hypertension requires further investigation. The past two decades of research on pulmonary hypertension have yielded several insights into the pathogenic mechanisms of the progressive and fatal disease. These include sustained pulmonary vasoconstriction,[39] occlusive vascular remodeling,[40] pulmonary and cardiac oxidative stress,[41] metabolic and endocrine disorders,[42] and inflammation.[43] At the subcellular and molecular levels, disturbances in ion channels[44] and mitochondrial function[45] have also been implicated in the pathogenesis of pulmonary hypertension, as have aberrations in signaling pathways, such as the Rho kinase pathway.[46] In light of these findings, we tested putative mechanisms that could explain the observed survival benefit in *TRPC4*-KO rats. Because activation of store-operated calcium entry is a stimulus for smooth muscle contraction, we initially considered the possibility that *TRPC4* inactivation may decrease pulmonary arterial pressure and provide a survival benefit. However, this effect on smooth muscle tone might be counterbalanced by TRPC4-induced release of endothelium-derived relaxing factors, particularly nitric oxide, as reported by Freichel et al.[22]

In the present study, both WT and *TRPC4*-KO rats developed the same degree of stable hemodynamic compromise, in the forms of severely elevated RVSP, diminished CI, and reduced TAPSE. Impairment of endothelial-dependent relaxation was not observed in isolated pulmonary arterial segments (ACh, 10 μmol/L). Although the magnitudes of receptor-dependent (PE, 1 μmol/L) and receptor-independent (KCl, 80 mmol/L) contractions were similar in WT and *TRPC4*-KO animals, the time to peak contraction was improved in KO animals, suggesting an increase in the efficiency of contraction. Our data therefore do not support the idea that *TRPC4* inactivation reduces (or worsens) the magnitude of pulmonary arterial hypertension. Rather, the present findings suggest that high pulmonary arterial pressures are not sufficient to cause heart failure and death in the absence of other exacerbating factors.

We next considered whether TRPC4 is an essential determinant of vascular remodeling. This idea is supported by reports linking TRPC4 channels to vascular endothelial and smooth muscle cell proliferation.[11,47] In support of this idea, the severity of pulmonary arterial occlusions and the number of plexiform lesions were substantially reduced in KO rats, especially the complex lumen-occluding lesions. Nonetheless, *TRPC4* inactivation did not abolish lesion formation; rather, it appeared to delay the onset and severity of lesion formation. Although such a decrease in the magnitude of occlusive remodeling undoubtedly reflects improved vascular function, it is unlikely that this improvement is sufficient to account for the observed survival benefit. Moreover, questions remain as to whether occlusive remodeling is sufficient to cause heart failure and death. Drugs such as prostacyclin analogs can improve functional status of pulmonary arterial hypertension patients, despite evidence from postmortem autopsies that they increase the number of plexiform lesions.[48] Furthermore, SU/Hx/Nx-exposed SD rats develop severe plexogenic arteriopathy with middle- and late-stage pulmonary arterial hypertension, but they survive long after these lesions become prominent.

We next considered whether *TRPC4* inactivation improves cardiac, renal, and hepatic adaptation to pulmonary arterial hypertension. In animals with severe pulmonary arterial hypertension that were hemodyamically stable, hemodynamic and echocardiographic signs of cardiac failure were similar in WT and *TRPC4*-KO rats. However, acute declines in CO predicted early mortality in WT rats, an effect that was not apparent in KO rats. Moreover, the degree of structurally maladaptive remodeling (ie, fibrosis) was more pronounced in WT rats. In fact, the biventricular nature of this fibrosis in WT rats was surprising, given our previous observation that left ventricles of SD rats with severe pulmonary arterial hypertension were consistently spared from significant structural changes.[49] Although WT rats exhibited evidence of cardiac failure, findings from kidney and liver function tests were inconclusive, because laboratory values mostly fell within normal ranges for both groups of rats (data not shown).

Overall, the present study offers novel insights into the role of TRPC4 channels in severe pulmonary arterial hypertension and uniquely validates the prospect of using genetically modified rats as a preclinical model of disease. Based on the present results, we conclude that *TRPC4* inactivation reduces mortality associated with severe pulmonary arterial hypertension, at least in part by decreasing both the magnitude of occlusive remodeling and the susceptibility to heart failure.

Acknowledgments

We thank Viktoriya Pastukh for her contribution to genotyping the *TRPC4*-KO rats and Dr. Chris Morris for his assistance with statistical analysis.

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