JNK2 regulates vascular remodeling in pulmonary hypertension

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Abstract

Pulmonary arterial (PA) wall modifications are key pathological features of pulmonary hypertension (PH). Although such abnormalities correlate with heightened phosphorylation of c-Jun N-terminal kinases 1/2 (JNK1/2) in a rat model of PH, the contribution of specific JNK isoforms to the pathophysiology of PH is unknown. Hence, we hypothesized that activation of either one, or both JNK isoforms regulates PA remodeling in PH. We detected increased JNK1/2 phosphorylation in the thickened vessels of PH patients' lungs compared to that in lungs of healthy individuals. JNK1/2 phosphorylation paralleled a marked reduction in MAP kinase phosphatase 1 (JNK dephosphorylator) expression in patients' lungs. Association of JNK1/2 activation with vascular modification was confirmed in the calf model of severe hypoxia-induced PH. To ascertain the role of each JNK isoform in pathophysiology of PH, wild-type (WT), JNK1 null (JNK1$^{-/-}$), and JNK2 null (JNK2$^{-/-}$) mice were exposed to chronic hypoxia (10% O_2 for six weeks) to develop PH. In hypoxic WT lungs, an increase in JNK1/2 phosphorylation was associated with PH-like pathology. Hallmarks of PH pathophysiology, i.e. excessive accumulation of extracellular matrix and vessel muscularization with medial wall thickening, was also detected in hypoxic JNK1$^{-/-}$ lungs, but not in hypoxia-exposed JNK2$^{-/-}$ lungs. However, hypoxia-induced increases in right ventricular systolic pressure (RVSP) and in right ventricular hypertrophy (RVH) were similar in all three genotypes. Our findings suggest that JNK2 participates in PA remodeling (but likely not in vasoconstriction) in murine hypoxic PH and that modulating JNK2 actions might quell vascular abnormalities and limit the course of PH.

Keywords

Background

Pulmonary hypertension (PH), a disease characterized by persistent high pulmonary arterial pressure, progressive increases in pulmonary vascular resistance, and vascular remodeling, causes right heart failure and mortality when untreated.[1,2] The etiology of PH is incompletely understood and multiple factors have been implicated in its pathogenesis. These include genetic predisposition[3] as well as a range of diverse endogenous and exogenous stimuli.[4] Pulmonary vascular abnormalities that involve aberrant vascular cell proliferation, survival, and migration are the key features of PH pathology.[1,2] Little is known about the mediators that initiate pulmonary vascular changes underlying PH, making the prevention and effective treatment of the disease challenging. Therefore, the identification of potential targets for treatment of PH remains a high priority.

C-Jun-N-terminal kinase (JNK) belongs to the mitogen-activated protein (MAP) kinase family. These kinases, which are encoded by three separate loci, JNK1–3, regulate cell proliferation, migration, survival, and cytokine

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production.[5] JNK1 and JNK2 are ubiquitously expressed, while JNK3 is restricted to the brain, testis, and heart. Although JNK1/2 are presumed to operate in a redundant fashion, it is noteworthy that they oppose each other functionally in several experimental systems.[6,7] Importance of either JNK1 or JNK2 in the pathogenesis of PH was described by us and others in the following four experimental paradigms: (1) JNK activation has been demonstrated in pulmonary artery (PA) in hypoxia, and monocrotaline-induced rat models of PH as well as in PA smooth muscle cells isolated from PH patients with hyperplastic growth;[8–10] (2) recently, Sala et al. have reported that JNK2 null mice are protected from the development of hypoxia-induced PH;[11] (3) JNK1, but not JNK2, regulates cultured PA adventitial fibroblast proliferation in response to various stimuli;[12,13] and (4) hypoxia-induced pathological angiogenesis is JNK1-dependent.[14] Although the abovementioned reports suggest the significance of JNK signaling in the pathological process of PH, the role of individual JNK isoforms in the development and progression of PH is unknown.

We hypothesized that activation of either JNK1 or JNK2, or both isoforms, will participate in the key molecular pathways involved in the vascular remodeling processes in PH. To test our hypothesis, we evaluated whether PH pathology is associated with the activation of JNK1/2 using lung tissues from healthy humans and PH patients as well as lung tissues from a well-established bovine model of severe hypoxic PH. Since loss-of-function studies in mice have provided major insights into the function of JNK isoforms in development and disease,[11,15] we then used JNK1 and JNK2 null mice to evaluate further the role of each individual isoform in the molecular mechanisms driving the development and progression of PH. For that, wild-type (WT), JNK1^{-/-}, and JNK2^{-/-} mice were exposed to either room air (normoxia) or 10% O_2 (hypoxia) for six weeks to induce PH.

Methods

Murine model of PH

Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and all protocols were approved by the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee. For the murine model of PH, 6- to 8-week-old male C57BL/6 mice of the following genotypes: wild-type (WT), JNK1 null (JNK1^{-/-}; http://jaxmice.jax.org/strain/004319.html), and JNK2 null (JNK2^{-/-}; http://jaxmice.jax.org/strain/004321.html) were purchased from the Jackson Laboratories. PH was induced in mice by six weeks of normobaric hypoxia exposure (10% O_2). The hypoxic environment was established by flushing a clear plastic polypropylene chamber with a mixture of room air and nitrogen, and the gas was recirculated. The oxygen concentration

(10%) was maintained using ProOx Oxygen controller (BioSpherix, Lacona NY, USA). The chamber has ventilation holes and a small quietly operating fan to provide air circulation for instant homogenization of the gases. Normoxic mice were kept in the same room as the hypoxic mice and both experienced identical light–dark cycle and ad libitum access to standard mouse chow and water.

Newborn calves model of PH

Studies with lung tissues from newborn calves were approved by the University of Colorado Institutional Animal Care and Use Committee, # 41702005(07)2A. Veterinary care of newborn calves was provided by the Department of Physiology at the Colorado State University according to the institutional guidelines of the School of Veterinary Medicine, Colorado State University (Fort Collins, CO, USA). Briefly, one-day-old Holstein calves were maintained at the Fort Collins altitude ($P_B = 640$ mmHg) (Neo-C) or in a hypobaric chamber at simulated altitude ($P_B = 445$ mmHg) (Neo-PH) for two weeks to develop a model of severe PH.[16] Calves were euthanized by overdose of sodium pentobarbital (160 mg/kg body weight). Animal handling and lung extraction occurred according to the previously described method.[16]

Human lungs

Dr. James West from Vanderbilt University provided formalin-fixed paraffin-embedded lung sections from healthy individuals and PH patients. Lung sections were from three Caucasian PH patients (two women, one man; age range = 22–35 years), all with heritable PH. One patient's specific genetic etiology is unknown, while the other two patients had mutations in the bone morphogenic protein receptor 2 (BMPR2), one in the ligand binding domain of BMPR2 and the other with the BMPR2 2579-2580deltT mutation deep in the cytoplasmic tail of the receptor.

Hemodynamics and right ventricular hypertrophy

Hemodynamics as well as right ventricular hypertrophy (RVH) were measured in mice according to a previously described method.[17,18] Briefly, mice were anesthetized with intra-peritoneal injection of ketamine hydrochloride (60 mg/kg) and xylazine (8 mg/kg). A Miller 1.0-F pressure microtip catheter was inserted into the right ventricle (RV) via the right jugular vein to measure right ventricular systolic pressure (RVSP). Pressure waveforms were monitored to ensure the validity of the pressure measurements. Data were recorded using a Power-Lab data acquisition system (ADInstruments, Colorado Springs, CO, USA).

Post-hemodynamic measurements were conducted in mice euthanized by thoracotomy. The heart and lungs were removed from each mouse. The free wall of the RV was separated from the left ventricle plus septum (LV + S) and each was weighed. The ratio of RV/LV+S

weight was calculated as a measurement of RVH. The lungs were fixed in 10% formalin for 24 h and then embedded in paraffin.

Immunohistochemistry and immunofluorescence staining

Immunostaining was performed on 5-μm-thick formalin-fixed paraffin-embedded human, bovine, and murine lung sections. Immunohistofluorescent (IHF) detection of phosphoJNK1/2 and MAP kinase phosphatase-1 (MKP-1) was performed in lung sections using an anti-phosphoJNK1/2 antibody (Cell Signaling, Beverly MA, USA) and an anti-MKP-1 antibody (Novus Biologicals, Littleton, CO, USA), both at a 1:50 dilution. Lung sections processed for IHF were scanned at 20 × magnification to create high-resolution digital micrographs at the University of Arkansas for Medical Sciences Experimental Pathology Core facility using Aperio ScanScope FL driven by the Imagescope software.

To evaluate tissues for gross histological changes, formalin-fixed paraffin-embedded mouse lung sections were stained with hematoxylin and eosin (H&E). Trichrome staining was also performed on mouse lung sections to examine extracellular matrix (ECM) accumulation. Stained lung sections were scanned at 20 × magnification using Aperio ScanScope CS2.

Quantification of lung vascular remodeling

Vascular morphometric analysis in mouse lung sections was performed according to our previously described method.[19] Degree of vessel muscularization was assessed by two color IHF staining of lung sections with an anti-αSMA antibody (dilution 1:400, clone 1A4, Sigma-Aldrich Corp., St. Louis, MO, USA) and with an antibody targeting human von Willebrand factor (dilution 1:100, Abcam, Cambridge, MA, USA). IHF-labeled lung sections were scanned with Aperio ScanScope FL at 20 × magnification. To assess the degree of muscularization, vessels (20–100 μm in diameter) were categorized as partially muscularized (< 75% of the vessel circumference immunopositive for anti-αSMA antibody) or fully muscularized (> 75% of the vessel wall immunoreactive against anti-αSMA antibody) as previously described.[17] Vessel wall thickness was assessed in vessels immunopositive for αSMA (20–100 μm in diameter; n = 80–100 vessels) for each animal (n = 4/group). Vessel wall thickness was measured using Aperio ImageScope software and calculated using the following equation: Wall thickness = Wall width/Vessel width.

Data analysis

Data are expressed as mean ± SEM. Different groups were compared by one-way analysis of variance (ANOVA) and a subsequent Student-Newman-Keuls post-hoc test. A P value < 0.05 was considered statistically significant.

Results

High JNK1/2 phosphorylation along with distinct reduction in the level of MKP-1, key deactivator of JNK1/2, are observed in PH patients' lungs

To investigate whether JNK1/2 activation is modified in PH patients' lungs, we performed immunohistochemical localization of phosphoJNK1/2 in lung sections from PH patients and control individuals. Marked structural remodeling of PA was observed in PH patient's lungs as demonstrated by profound αSMA-positive thickened medial layer (red) in the vessel wall (Fig. 1a). PhosphoJNK1/2 immunopositive signal (green) in the PH-affected lung was higher compared to that in the control lung (Fig. 1a and Suppl. Fig. A1). JNK1/2 phosphorylation was mainly observed in the thickened adventitia of PA as well as in the interstitial cells of the disease-affected lung. In contrast, the staining intensity of MKP-1 (red), a key dephosphorylator of JNK1/2, was absent from the vascular wall of the PH patient's lung (Fig. 1b). MKP-1-immunopositive cells (red) within the vessel might represent the circulating cells in PH patient's lung (Fig. 1b). In contrast, very high MKP-1 expression was detected in the vascular wall as well as in the interstitial cells of the normal human lung (Fig. 1b). Taken together, these data demonstrate that PH affected lungs display a distinct pattern of JNK1/2 activation, which coincides with downregulation in the levels of JNK1/2 deactivator, MKP-1.

Hypoxia-induced vascular remodeling is associated with JNK1/2 activation in lungs of neonatal calves

To confirm the link between JNK1/2 activation and vascular remodeling in PH, we used lung tissues from control and PH calves and performed double IHF staining for phosphoJNK1/2 and αSMA. In contrast to the control lungs, marked structural remodeling in the vascular wall was observed in PH lungs (Fig. 2 and Suppl. Fig. A2). Although medial smooth muscle layer was clearly modified, the most noticeable alteration was found in the adventitial compartment of the vascular wall in the Neo-PH lungs (Fig. 2 and Suppl. Fig. A2; cells immunopositive for αSMA appear red). JNK1/2 phosphorylation was detected in the remodeled adventitial compartment of the Neo-PH lungs (Fig. 2; see the insert for phosphoJNK1/2-immunopositive cells that appear green). Initial examination of the medial layer of the remodeled vessel in Neo-PH lung also indicated some signal for phosphoJNK1/2 (green), which was concentrated at one focal area of the medial compartment (Fig. 2 and Suppl. Fig. A2). However, detailed examination of the medial compartment demonstrated that phosphoJNK1/2 expression was not associated with smooth muscle cells (SMCs) (see the left insert, the Neo-PH panel, Fig. 2 and Suppl. Fig. A2). These data support the idea that a correlation exists between the JNK1/2 activation and the structural modification of vascular wall in a well-established experimental model of hypoxia-induced PH.
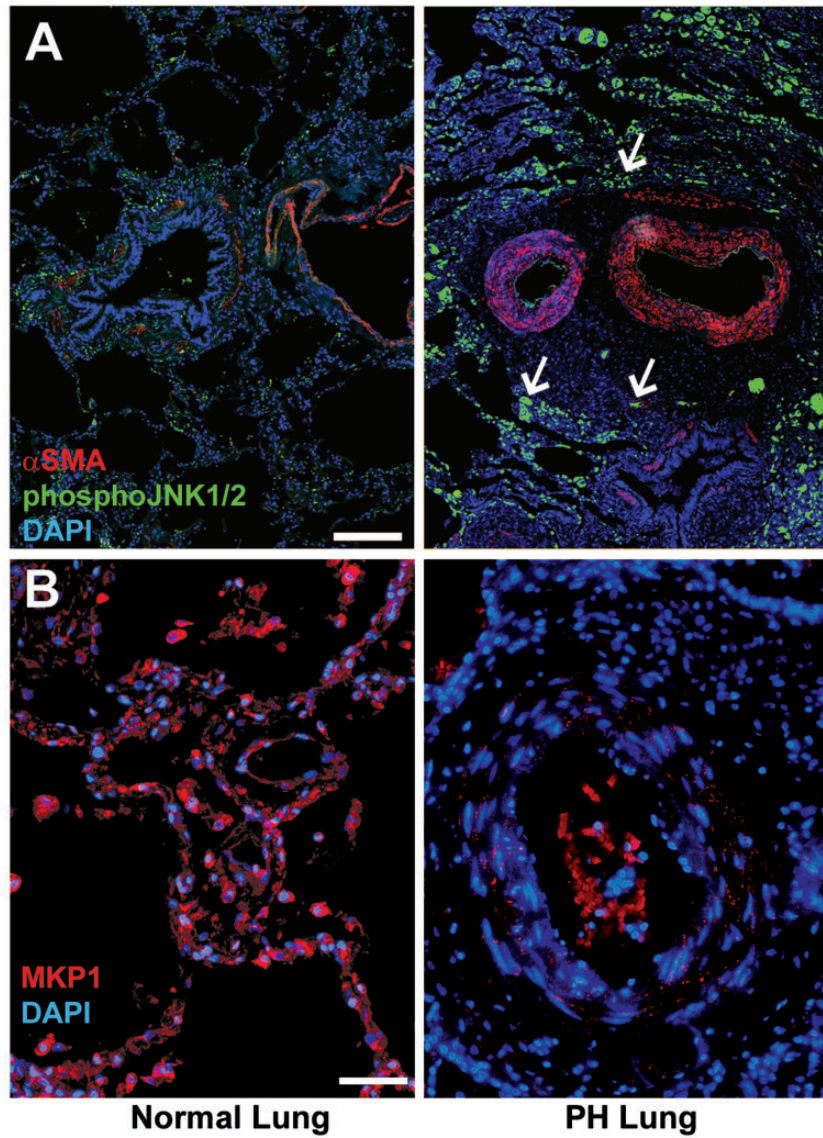
Fig. 1. Increased JNK1/2 phosphorylation in PH is associated with reduced expression of MKP-1 in the lungs of PH patients. (a) Marked increase in JNK1/2 phosphorylation is observed in the lungs of PH patients compared to the lungs of healthy individuals. Paraffin-embedded lung sections from healthy individuals and from PH patients were processed for immunohistofluorescent (IHF) detection of phosphoJNK1/2 (green) and αSMA (red), whereas the nuclei were stained with DAPI (blue). Arrows point to examples of phosphoJNK1/2-positive adventitial cells. Representative IHF images of lung sections are shown. (b) Expression of MAP kinase phosphatase-1 (MKP-1), a dephosphorylator of JNK1/2, is distinctly downregulated in PH lungs as compared with normal lungs. Representative photomicrographs of lungs (normal lungs: n = 2 and PH lungs: n = 3) depicting cells immunopositive for MKP-1 (red) and nuclei stained with DAPI (blue). Scale bars: (a) = 200 μm and (b) = 50 μm.

## Hypoxia induces an upregulation of JNK1/2 phosphorylation in mouse lung

To evaluate the role of JNK1 and JNK2 in the pathogenesis of PH, we utilized JNK1 and JNK2 null mice. WT, JNK1$^{-/-}$, and JNK2$^{-/-}$ mice were exposed to either room air or 10% O$_2$ for six weeks. At the end of the experimental period, lung sections from the normoxic and hypoxic mice were examined for JNK1/2 activation by IHF. A marked increase in JNK1/2 phosphorylation was observed in the hypoxic WT lungs compared to that of the control lungs (Fig. 3). Enhanced immunoreactivity against phosphoJNK1/2 was detected in the vascular wall as well as in the interstitial cells of WT lungs exposed to chronic hypoxia (Fig. 3; the lower panels demonstrating magnified areas from the normoxic and hypoxic lungs where phosphoJNK1/2-positive cells were identified by asterisks and arrows).

JNK1/2 phosphorylation was also observed in the JNK1$^{-/-}$ and JNK2$^{-/-}$ lungs (data not shown). There are two important
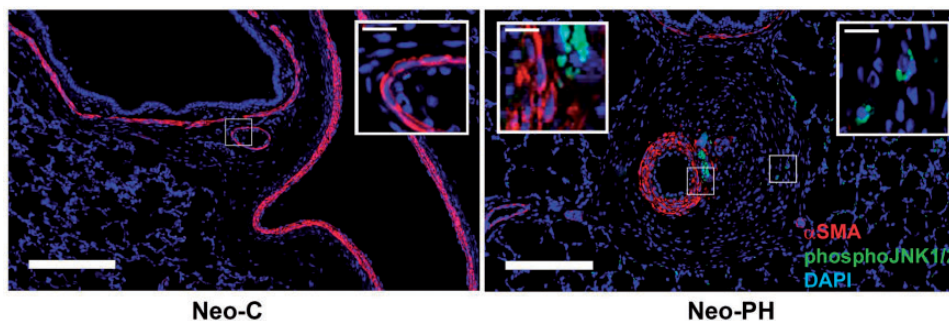
Fig. 2. Hypoxia-induced remodeling of the vascular wall is associated with activation of JNK1/2 in the lungs of neonatal calves. Immunohistochemical staining of paraffin-embedded lung sections from Neo-C and Neo-PH calves was performed using antibodies against phosphoJNK1/2 (green) as well as against αSMA (red). Cell nuclei were stained with DAPI (blue). Representative photomicrographs of lung sections from Neo-C and Neo-PH calves are shown. Staining was performed in lung sections of three different Neo-C and three different Neo-PH calves. Main scale bars = 200 μm; inset scale bars = 20 μm.
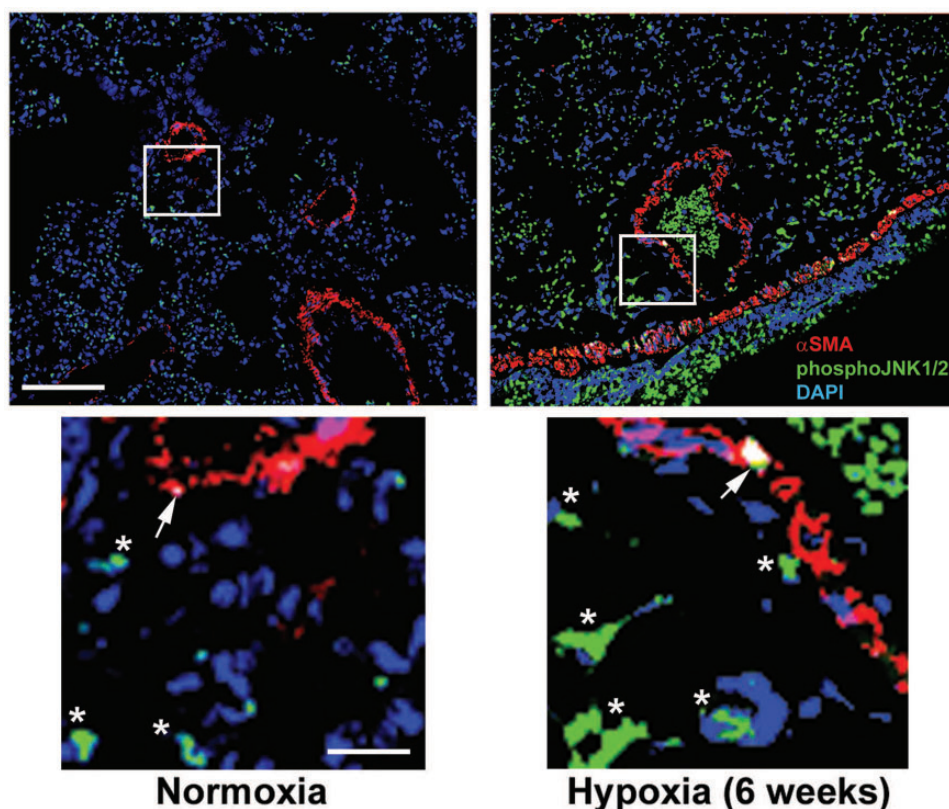
Fig. 3. Chronic hypoxia enhances JNK1/2 phosphorylation in mouse lungs. Mice were exposed to either room air (normoxia) or to 10% O_2 (hypoxia) for six weeks. At the end of the experimental period, lungs were isolated and fixed in 10% formalin. Paraffin-embedded lung sections were processed for immunodetection of phosphoJNK1/2 (green) and αSMA (red). Nuclei in the lung sections were detected with DAPI (blue). PhosphoJNK1/2-immunopositive lung cells are identified with asterisks. αSMA-positive cells which expressed phosphoJNK1/2 (white) are labelled with arrows in lung sections. Images shown are representative of six animals per group. Scale bar = 100 μm.

points to consider toward explaining the JNK1/2 phosphorylation in the lungs of null mice: (1) JNK1^{-/-} and JNK2^{-/-} mice express JNK2 and JNK1 isoforms, respectively; and (2) the antibody against phosphoJNK1/2 does not distinguish between phosphorylated forms of JNK1 and JNK2. In this context, it is notable that JNK1/2 double knockout mice are embryonically lethal,[20] ruling out their use in our adult mouse experiments.
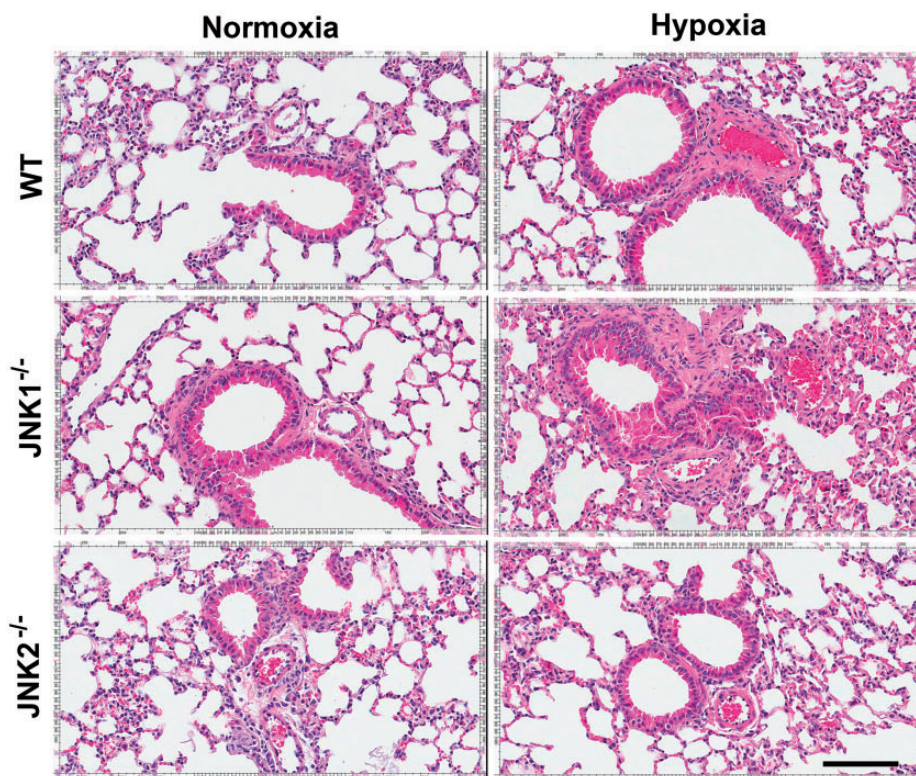
Fig. 4. Lung morphology of normoxic and hypoxic WT, JNK1$^{-/-}$, and JNK2$^{-/-}$ mice. Representative bright-field photomicrographs of H&E-stained lung sections. Scale bar = 100 μm.

Hypoxia upregulates ECM deposition in the lungs of WT and JNK1$^{-/-}$ mice

To investigate the pathological modifications induced by chronic hypoxia exposure in mouse lungs, we performed histological analysis of lungs of normoxia- and hypoxia-exposed WT, JNK1$^{-/-}$, and JNK2$^{-/-}$ mice. H&E-stained lung sections demonstrated an increase in the vascular wall remodeling as well as an increased ECM accumulation in hypoxic WT and JNK1$^{-/-}$ mice compared with corresponding normoxic animals (Fig. 4). In contrast, JNK2 gene deletion was associated with the absence of vascular wall thickening and lack of excessive ECM deposition, the two hallmark pathologies observed in the lungs of chronic hypoxia-exposed WT and JNK1-deficient mice (Fig. 4).

To confirm hypoxia-induced heightened deposition of ECM, lung sections from all three mouse genotypes were subjected to Masson's trichrome staining, which allows visualization of collagen accumulation in the tissue sections (Fig. 5). As expected from the results of the H&E staining, enhanced accumulation of ECM (blue) was observed in the vessel media and adventitia as well as in the parenchyma of hypoxic WT and JNK1$^{-/-}$ lungs, but not in hypoxia-exposed JNK2$^{-/-}$ lungs. These data confirm that chronic hypoxia exposure mimics the key pathophysiological features of PH, namely vascular wall remodeling and upregulation of ECM accumulation in the lungs of WT and JNK1$^{-/-}$, but selectively not in the JNK2$^{-/-}$ mice.

Vessels are modified in hypoxia-exposed WT and JNK1$^{-/-}$ lungs

Chronic hypoxia exposure is a well-known stimulus for the structural modification of vessels in the lung. To evaluate the vascular configuration, lung sections from normoxic and hypoxic WT, JNK1$^{-/-}$, and JNK2$^{-/-}$ mice were subjected to double immunofluorescent staining for detection of αSMA and Factor VIII. Increases in the number of αSMA-positive cells in the vessel wall (vessels 20–100 μm in diameter) were observed in hypoxia-exposed WT and JNK1$^{-/-}$ lungs (Fig. 6). In contrast, no changes in the number of αSMA-positive cells were noticed in the medial layer of vessels from hypoxia-exposed JNK2$^{-/-}$ mice (Fig. 6). These data suggest that in response to chronic hypoxia exposure, JNK2 might contribute to the maintenance of vascular structure in the lung.

Vessel muscularization is selectively blunted in hypoxic JNK2$^{-/-}$ lungs

Since vascular remodeling is a critical pathophysiological marker of PH, we next examined the effect of chronic hypoxia exposure on one of the indexes common to hypoxic PH, namely the extent of distal vessel muscularization. To that end, we assessed the degree of vessel muscularization in all three genotypes of mice. Compared to normoxic lungs, muscularization of vessels was increased in the lungs
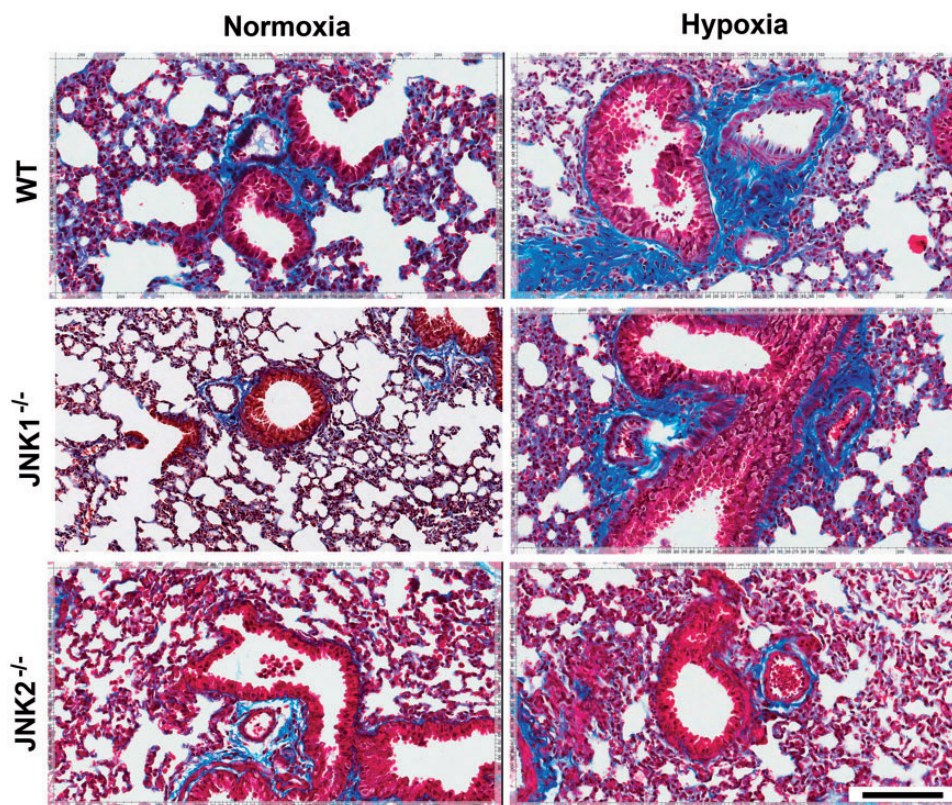
Fig. 5. Effects of JNK1 and JNK2 gene deletion on deposition of ECM in the lungs. Depicted are representative lung sections from WT, JNK1^{-/-}, and JNK2^{-/-} mice exposed to either normoxia or hypoxia, in which ECM deposition was evaluated with a Trichrome stain (blue). $n = 4$–6 mice/group. Scale bar $= 100\,\mu m$.

of hypoxic WT and JNK1^{-/-} mice as demonstrated by the morphometric analysis of immunoreactivity against αSMA in the vessels (Fig. 7a, b). In contrast, hypoxia failed to increase vessel muscularization in JNK2^{-/-} lungs (Fig. 7b) supporting our hypothesis that JNK2 might regulate the progression of pathology leading to vascular remodeling during the development of hypoxia-induced PH. The role of JNK2 in the regulation of vascular modifications in PH is further supported by vascular remodeling in hypoxia-exposed JNK1^{-/-} lungs (Suppl. Fig. A3), where presence of functional JNK2 is consistent with its contribution to the overall pathology of PH.

Vascular medial wall thickness is not affected by hypoxia in JNK2^{-/-} lungs

Morphometric analysis of lung sections immunostained for both αSMA and Factor VIII also revealed that the wall thickness (wall width/vessel width) of the vessels (20–100 μm in diameter) was similar in the lungs among all normoxic WT, JNK1^{-/-}, and JNK2^{-/-} mice (Fig. 8). However, WT and JNK1^{-/-} mice exposed to hypoxia showed significant increase in the vessels' medial wall thickness (Fig. 8). In contrast, the lungs of JNK2^{-/-} mice lacked such vascular remodeling in response to chronic hypoxia exposure (Fig. 8).

Taken together, our data strongly suggest that JNK2 regulates PA structural remodeling in hypoxia-induced PH.

Chronic hypoxia induces increase in RVSP and RVH among WT, JNK1^{-/-}, and JNK2^{-/-} mice

While total body weights increased over the six-week experimental period in mice of all genotypes under normoxic conditions, significant reduction in weight gain was observed only in hypoxia-exposed WT mice (Fig. 9a). Although total body weight increases in JNK1^{-/-} and JNK2^{-/-} mice appeared to be also negatively affected by chronic hypoxia exposure, the reduction in weight gain in JNK-deficient mice was not statistically significant (Fig. 9a).

To evaluate whether deficiency of either JNK1 or JNK2 gene would alter physiological responses to hypoxia, we measured RVSP in normoxic and hypoxic mice of both genotypes. Significantly higher RVSP was observed in hypoxic WT, JNK1^{-/-}, and JNK2^{-/-} mice compared to the respective normoxia controls (Fig. 9b).

We next assessed the ratio of RV to LV+S weight, to ascertain the impact of elevating RVSP on cardiac mass (Fig. 9c). There was no significant difference in (RV/LV+S) among hearts of normoxic WT, JNK1^{-/-}, and JNK2^{-/-} mice. However, the increased RVSP was accompanied by RVH as
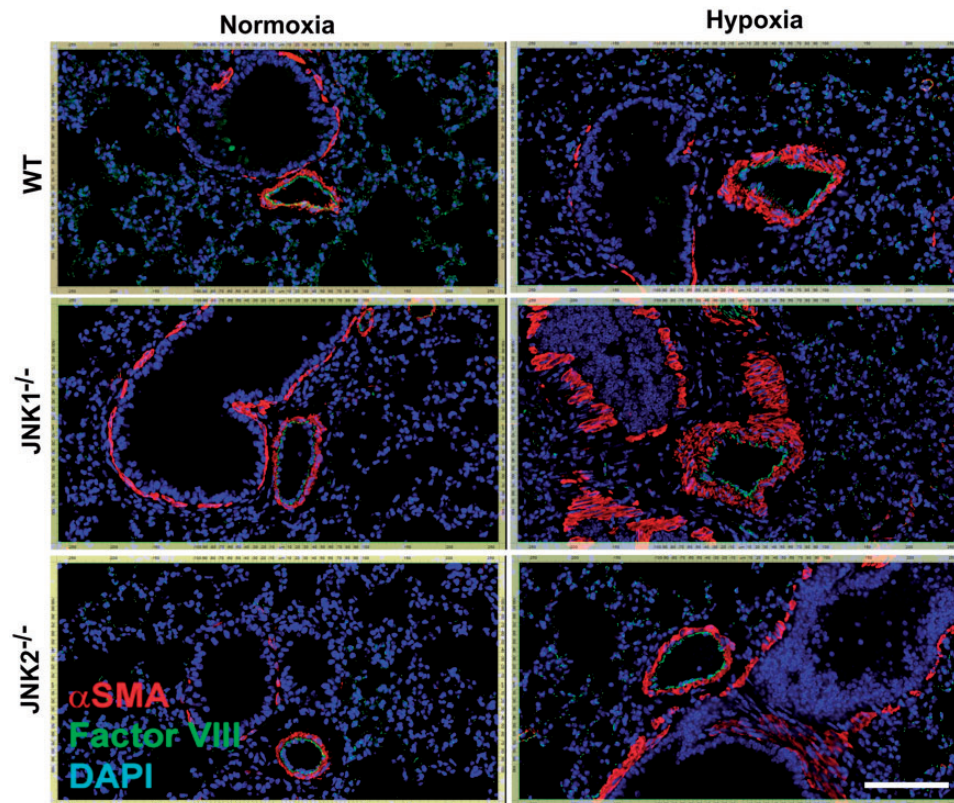
Fig. 6. Vessel wall structure in the lungs of WT and JNK1^{-/-} mice is altered in response to chronic hypoxia. Representative photomicrographs of lung sections in which αSMA (red) and Factor VIII (green) were immunodetected with IHF. Nuclei were identified with DAPI (blue). n = 4–6 mice/group. Scale bar = 100 μm.

evidenced by a significantly higher (RV/ LV + S) weight ratio in hypoxia-exposed mice among all genotypes compared to the respective normoxic control animals (Fig. 9c). Collectively, these data suggest that chronic hypoxia exposure induces an increase in RVSP and RVH of similar magnitude among WT, JNK1^{-/-}, and JNK2^{-/-} mice.
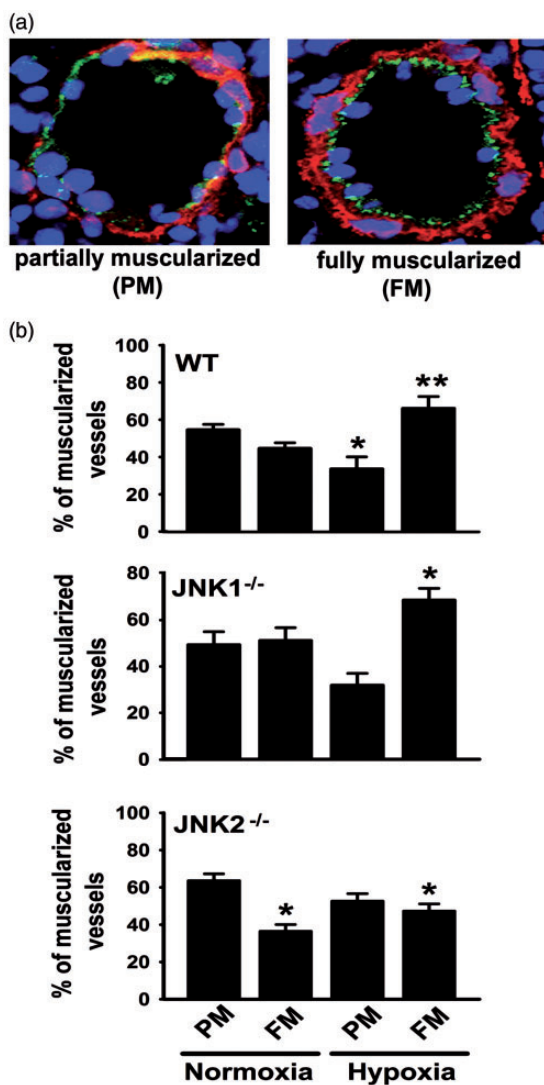
Discussion

In the present study, we demonstrated that marked vascular wall thickening is associated with heightened JNK1/2 phosphorylation and concomitant reduction in the expression of a phosphatase that deactivates JNKs, the MKP-1, in PH patients' lungs. Enhanced JNK1/2 phosphorylation also was observed in the remodeled vascular wall in the lungs of hypoxia-exposed neonatal calves, a well-established model of hypoxia-induced PH.[16] To evaluate the potential contribution of individual JNK isoforms to PH pathophysiology, focusing specifically on vascular remodeling process in the lungs, we modeled the hypoxia-induced PH in WT, JNK1^{-/-}, and JNK2^{-/-} mice. Exposure of WT mice to chronic hypoxia reproduced with fidelity our findings from human as well as bovine lung tissues, demonstrating that in mice hypoxia also induced a marked increase in JNK1/2 phosphorylation as well as other key features of PH, such as excessive accumulation of ECM, vascular muscularization,

and medial wall thickening. Adding credence to our hypothesis that JNK signaling contributes to PH pathology, are the observations that hypoxia also promoted PH-like pathophysiological changes in the JNK1^{-/-} lungs, whereas JNK2 gene ablation halted hypoxia-stimulated upregulation of ECM accumulation as well as vascular remodeling in the mouse lungs. Although both JNK1^{-/-} and JNK2^{-/-} mice were susceptible to hypoxia-induced increases in RVSP and RVH to a similar degree as observed in WT mice, unlike WT mice, hypoxia failed to reduce weight gain in these two types of JNK-null mice. Collectively, our data suggest that JNK2 might function as a gatekeeper that allows vascular remodeling, explaining why in the absence of JNK2 these key structural PH-associated pathologies are absent. Consequently, these findings suggest JNK2 as a potential therapeutic target for attenuation of vascular remodeling, which in combination with drugs targeting elevated PA pressure could aid development of a comprehensive therapy for PH. Inhibition of JNK2 might be of benefit in preventing progression of PH and we propose that it should be explored further.
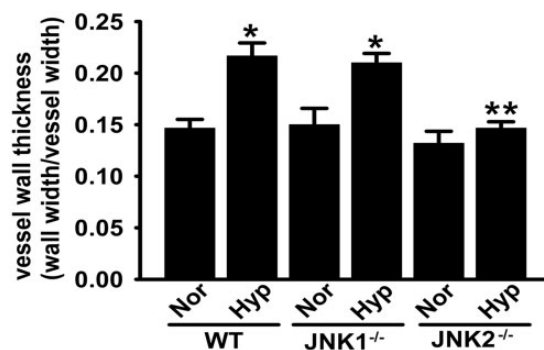
Even though JNK activation has been reported in the PA wall of hypoxia- and monocrotaline-induced rat models of PH as well as in cultured PA smooth muscle cells from PH patients,[8–10] JNK phosphorylation patterns in the lungs of patients with PH remained unexplored until the present

Fig. 8. JNK2$^{-/-}$ lungs are protected from hypoxia-induced increase in the vascular medial wall thickness. Changes in the ratio of vessel wall width to total vessel width in the lungs of WT, JNK1$^{-/-}$, and JNK2$^{-/-}$ mice exposed to normoxia or hypoxia were measured using Imagescope software in whole-section images captured on the Aperio ScanScope CS2. Data are expressed as mean $\pm$ SEM; n $=$ 4–6 mice/group. *$P < 0.002$ compared to normoxia in WT, JNK1$^{-/-}$, and JNK2$^{-/-}$; **$P < 0.001$ compared to hypoxia/WT and hypoxia/JNK1$^{-/-}$.

Fig. 7. Hypoxia-induced increase in vessel muscularization is selectively blunted in JNK2$^{-/-}$ mice. (a) Representative images of partially muscularized (PM) and fully muscularized (FM) vessels in mouse lungs demonstrating IHF for αSMA (red) and Factor VIII (green); as well as DAPI-stained nuclei (blue). (b) Quantification of PM and FM vessels in the lung of normoxic and hypoxic WT, JNK1$^{-/-}$, and JNK2$^{-/-}$ mice. For the WT group: *$P < 0.02$ compared to normoxia/PM; **$P < 0.01$ compared to normoxia/FM data as well as to hypoxia/PM. For the JNK1$^{-/-}$ group: *$P < 0.002$ compared to hypoxia/PM. For the JNK2$^{-/-}$ group: *$P < 0.02$ compared to normoxia/PM. Data are expressed as mean $\pm$ SEM; n $=$ 4–6 mice/group.

study. Here, we report for the first time that JNK1/2 phosphorylation is upregulated in the remodeled vessel wall of PH-affected patients' lungs. Considering the role of JNK more broadly, disease-associated JNK activation can be affected differently by a variety of factors and conditions capable of modulating JNK phosphorylation status. Reduction in JNK phosphorylation seen in asthmatic patients is a case in point.[21] In contrast, our data demonstrate heightened JNK phosphorylation in human lungs

affected with PH – a finding that is consistent with the data from human abdominal aortic aneurysms, which display significant elevation in phosphorylated JNK in the vascular wall.[22] Interestingly, we also found that elevated JNK phosphorylation in PH patients' lungs is associated with marked reduction in the levels of its negative regulator, MKP-1. Since JNK dephosphorylation is regulated by MKP-1,[23] a decrease in MKP-1 expression in patients' lungs is consistent with, and permissive of, elevated JNK1/2 phosphorylation. Since MKP-1 can inactivate other members of MAP kinase family such as ERK1/2 and p38 by dephosphorylation,[24–27] we cannot exclude the possibility that reduction in MKP-1 levels might contribute to the heightened activation of ERK1/2 and p38 in PH lungs. The notion of disinhibition of JNK function in the absence of MKP-1 is supported by the observation that MKP-1 deficient mice develop amplified RVSP and an increase in both RVH and vascular remodeling in response to hypoxia exposure.[28] Unfortunately, the authors of the paper did not explore JNK phosphorylation status in these mice. Taken together, our data suggest that pathophysiology of PH is associated with increased JNK1/2 phosphorylation, which is linked with marked reduction in MKP-1 expression.

We have also observed a link between vascular wall thickening and elevated JNK1/2 phosphorylation in a well-established model of hypoxia-induced PH in neonatal calves. In the present report, relatively sparse detection of JNK1/2 activation in the remodeled vessels of Neo-PH lungs might be due to evaluation of JNK1/2 phosphorylation at the end of the two-week exposure to hypoxia. Since maximal JNK1/2 activation has been demonstrated at the beginning of the hypoxia exposure in another experimental model of hypoxia-induced PH,[8] detection of JNK1/2 phosphorylation in the lungs of neonatal calves at an earlier stage of the experimental period might have revealed a
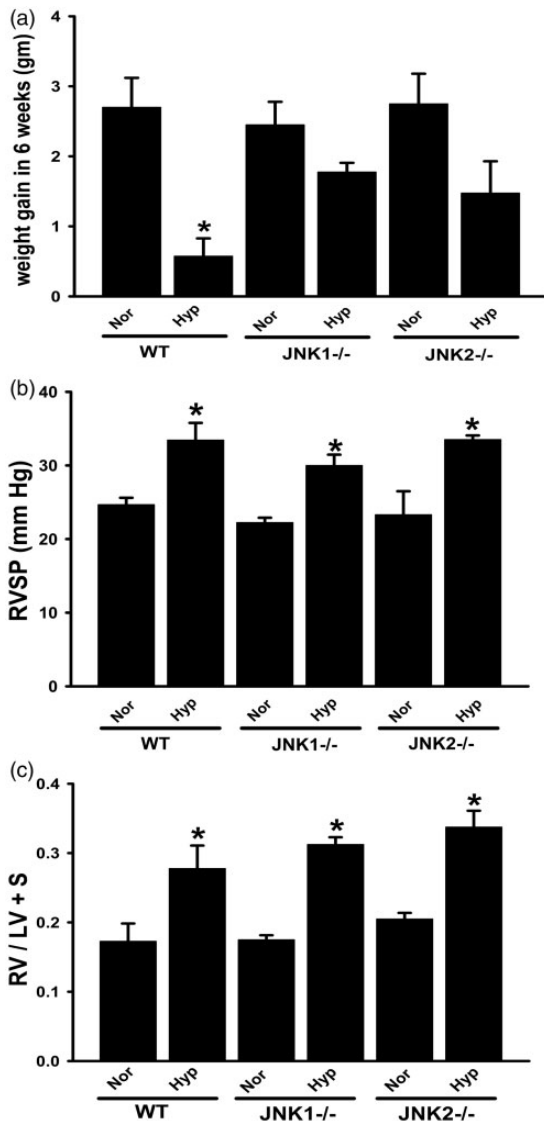
Fig. 9. Chronic hypoxia induces development of PH in WT, JNK1^{-/-}, and JNK2^{-/-} mice. (a) In WT mice, but not in the null mice, total body weight gain was significantly attenuated by chronic exposure to hypoxia as compared to normoxia. *$P < 0.004$. (b) Chronic hypoxia exposure induced an increase in RVSP in mice of all three genotypes. *$P < 0.05$ compared to normoxic values. (c) Right ventricular hypertrophy occurred in hypoxia-exposed WT, JNK1^{-/-}, and JNK2^{-/-} mice in a similar fashion. *$P < 0.01$ compared to normoxic values. Data are expressed as mean ± SEM; n = 4 mice/group.

different activation pattern. Nonetheless, neonatal calves' data also support the association between heightened JNK1/2 phosphorylation and vascular wall thickening in an experimental model of PH.

Despite the high homology among JNK family members, several reports validate unique isoform-specific functions in various physiological responses.[5] An example related to hypoxic signaling is that both JNK1 and JNK2 are involved in the regulation of hypoxia-inducible factor-1α (HIF-1α) expression, albeit each isoform controls HIF-1α expression via a different mechanism. JNK1 prevents the degradation of HIF-1α protein in a Von Hippel Lindau-independent manner,[29] whereas JNK2 regulates HIF-1α expression through maintaining its messenger RNA stability.[30] Another intriguing example of differential roles of JNK1 and JNK2 isoforms comes from our earlier studies in the neonatal calf model of hypoxia-induced PH demonstrating that PDGF-BB-induced heightened proliferation of PA adventitial fibroblasts is selectively mediated by JNK1 activation.[13] The above reports suggested to us the need for evaluation of the specific roles of JNK1 and JNK2 in PH pathogenesis. In the present study, we used JNK1^{-/-} and JNK2^{-/-} mice and compared the effects of chronic hypoxia on lung pathophysiology (particularly structural alterations of the vessels) in the presence or absence of specific JNK isoforms. Although mice lacking JNK1 or JNK2 appear morphologically normal, they are reportedly immunocompromised due to defects in T-cell function.[31] In our present studies, both WT and JNK1^{-/-} mice demonstrated hypoxia-induced remodeling of lung vessels. In contrast, JNK2^{-/-} mice lacked this hallmark pathological feature of PH. Although both JNK1^{-/-} and JNK2^{-/-} mice, like WT mice, demonstrated significant increases in RVSP and RVH in response to chronic hypoxia exposure, these null mice were less prone to hypoxia-induced reduction in total body weight gain compared to the weight loss observed in hypoxic WT mice. Since important interplay exists between JNK1 and JNK2 isoforms in the regulation of lipid and glucose metabolism,[32] JNK1^{-/-} and JNK2^{-/-} mice might be able to adjust their metabolism to maintain their total body weight in response to stresses such as six-week hypoxia exposure. This intriguing observation needs further exploration since other knockout mice such as MKP-1-deficient mice demonstrate more pronounced hypoxia-induced weight loss compared to WT mice.[28] Collectively, our data suggest that hypoxia-induced vascular remodeling in the lung might be selectively regulated by JNK2.

Collective experimental evidence indicates that each distinct cell type contributing to the cytoarchitectural layout of the PA wall plays a specific role in the pathogenesis of PH.[33] For example, our data from PH patients' lungs demonstrate increased JNK1/2 phosphorylation in the thickened adventitial compartment and interstitial cells; however, the increase in phosphorylation is absent in SMCs in these patients (Fig. 1). Such JNK-cell type relationships are highly complex as demonstrated by data from cultured SMCs isolated from PH patients where a growth factor-independent proliferation does require JNK activation.[10] In the case of the newborn calf model of PH, our data demonstrate increased expression of activated JNK1/2 in the remodeled adventitial layer, interstitial cells, and occasionally in SMCs of Neo-PH lungs (Fig. 2). Furthermore, these data suggest the role for JNK1/2 in pathologically changing cellular composition of the vascular wall as PH progresses. In addition, our studies in a murine model of hypoxia-induced PH also demonstrated a

relationship between the vascular pathology and JNK1/2 activation involving the adventitial and medial layers as well as interstitial cells (Fig. 3). Therefore, the observation that in JNK2 null mice chronic hypoxia fails to induce medial thickening and muscularization of small PAs adds further credence to our idea that JNK2 is necessary for the remodeling process within the PA wall. Knowing this will enable us to conduct longitudinal studies in experimental models of hypoxia-induced PH aiming to address the relationships involving JNK2 activities in various cell types and at various time points in the progression of disease.

JNK1 is also a key regulator of lung remodeling in multiple models of fibrosis.[34–36] Hypoxia-induced pathological angiogenesis in the retina is also mediated by JNK1 via increased vascular endothelial growth factor (VEGF) production.[14] In our previous reports, we have found that JNK1 is a key regulator of hyperproliferative responses of bovine PA adventitial fibroblasts in response to various stimuli.[12,13] However, in the present study, JNK1^{-/-} mice demonstrated hypoxia-induced increase in RVSP and RVH in the fashion like that in WT mice. Vascular wall remodeling was detected in hypoxic lungs of JNK1^{-/-} mice as well as WT mice (Fig. 6 and Suppl. Fig. A3). Based on our data from the neonatal calf model of hypoxia-induced PH,[13] we speculated that JNK1^{-/-} mice would have blunted hypoxia-induced vascular remodeling process. Finding just the opposite in JNK1^{-/-} mice might be due to species-specific differences in JNK1 function in various physiological responses.

Although JNK1 is activated by a variety of extracellular stimuli leading to a range of cellular responses,[24] the importance of JNK2 in regulation of different physiological responses is beginning to be recognized. For example, in the present studies using hypoxia-induced PH models, we found that JNK2 might be a critical regulator of vascular remodeling since vessel wall thickening is selectively prevented in the hypoxic lung by the deletion of the JNK2 gene. Both vessel muscularization and wall thickness remained unchanged in hypoxic JNK2^{-/-} lungs compared to WT and JNK1^{-/-} lungs. JNK2 is also a key player in other vascular diseases such as atherosclerosis since atherosclerosis-prone ApoE^{-/-} mice simultaneously lacking JNK2 (ApoE^{-/-}JNK2^{-/-} mice), but not ApoE^{-/-} JNK1^{-/-} mice, develop less atherosclerotic plaques than do ApoE^{-/-} mice.[37] Interestingly, the physiological responses to hypoxia in JNK2^{-/-} mice such as RVSP and RVH were like those in WT and JNK1^{-/-} mice. Therefore, our data suggest that the mechanistic pathways involved in pressure responses (JNK2 – independent) and structural remodeling of the vascular wall (JNK2 – dependent) in the lungs of JNK2^{-/-} mice, are distinctly different – a finding corroborated by other published reports.[38,39] JNK2 actions in PH might relate to its interactions with several signaling molecules such as: (1) endothelial nitric oxide synthase, which is phosphorylated by JNK2 to regulate nitric oxide production;[40] (2) endothelin-1 and angiotensin II for blood pressure control and to promote structural alteration of PA;[41] and (3) TGFβ1 for induction of PDGF-BB expression.[42]

An important point to consider here is that JNK2 has been shown to promote hypoxia-induced mitophagy.[43] Damage in the heart and lungs of hypoxic JNK2^{-/-} mice is more prominent compared to their WT counterparts as mentioned in the above studies. However, our studies reported here demonstrate that JNK2 deletion results in protection of mouse lungs from hypoxia-induced vascular abnormalities. The variation in the results between our study and other studies could be explained by the differences in time of hypoxia exposure, oxygen concentration used to create hypoxic conditions, and the sex of the mice used for the experiments. Zhang et al. used female mice, which were exposed to hypoxia (7% O_2) for one, four, or seven days to induce stress conditions.[43] We used male mice in which PH was developed by exposing animals to hypoxia (10% O_2) for six weeks. However, Sala et al. recently reported that JNK2 may play a role in the development of hypoxia-induced PH since mice deficient in JNK2 are relatively (but not completely) spared from hypoxia-induced PH.[11]

PH pathogenesis has been ascribed to two distinct mechanisms, the initial event of vasoconstriction followed by remodeling of small- and medium-sized vessels, which is a hallmark of severe and advanced PH. Several reports demonstrated that vasoconstriction and vascular remodeling are mediated by distinct signaling pathways both of which contribute to PH pathophysiology. One such example is angiotensin-converting enzyme inhibitors, which prevent PA remodeling in rats exposed to chronic hypoxia, but do not attenuate the development of PH or RV hypertrophy.[44] Another study has reported that rosiglitazone, an antidiabetic drug, can attenuate and reverse PA remodeling and neomuscularization associated with hypoxic PH. Despite these remodeling-preventing attributes, this agent failed to block development of PH.[45] Similarly, even though in our studies in JNK2 null mice, hypoxia failed to induce vascular wall remodeling, increases in RVSP and RVH were still present. These observations in JNK2 null mice call for further assessment of other physiological parameters including the levels of endothelin, angiotensin, nitric oxide, and prostacyclin under both normoxic and hypoxic conditions. In conclusion, we demonstrated that JNK2 gene deletion prevents hypoxia-induced structural remodeling of PA wall and therefore, we propose that JNK2 might be a promising therapeutic target in PH, particularly considering a combination therapy with agents that reduce vasoconstriction itself.

Authors' contributions

MD designed and performed the experiments. JW provided human lung tissue sections from healthy and PH individuals. MD and WMZ analyzed the data and wrote the manuscript. JW and KRS reviewed the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The author(s) declare that there is no conflict of interest.

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