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Expression of mutant bone morphogenetic protein receptor II worsens pulmonary hypertension secondary to pulmonary fibrosis

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Abstract: Pulmonary fibrosis is often complicated by pulmonary hypertension (PH), and previous studies have shown a potential link between bone morphogenetic protein receptor II (BMPR2) and PH secondary to pulmonary fibrosis. We exposed transgenic mice expressing mutant BMPR2 and control mice to repetitive intraperitoneal injections of bleomycin for 4 weeks. The duration of transgene activation was too short for mutant BMPR2 mice to develop spontaneous PH. Mutant BMPR2 mice had increased right ventricular systolic pressure compared to control mice, without differences in pulmonary fibrosis. We found increased hypoxia-inducible factor (HIF) 1-α stabilization in lungs of mutant-BMPR2-expressing mice compared to controls following bleomycin treatment. In addition, expression of the hypoxia response element protein connective tissue growth factor was increased in transgenic mice as well as in a human pulmonary microvascular endothelial cell line expressing mutant BMPR2. In mouse pulmonary vascular endothelial cells, mutant BMPR2 expression resulted in increased HIF1-α and reactive oxygen species production following exposure to hypoxia, both of which were attenuated with the antioxidant TEMPOL. These data suggest that expression of mutant BMPR2 worsens secondary PH through increased HIF activity in vascular endothelium. This pathway could be therapeutically targeted in patients with PH secondary to pulmonary fibrosis.

Keywords: idiopathic pulmonary fibrosis (IPF), pulmonary hypertension (PH), bone morphogenetic protein receptor II (BMPR2), hypoxia-inducible factor (HIF).

Pulm Circ 2015;5(4):681-690. DOI: 10.1086/683811.

Idiopathic pulmonary fibrosis (IPF) is a diffuse lung disease marked by an insidious onset and a poor overall prognosis. Pulmonary hypertension (PH) complicating IPF, also known as secondary PH (World Health Organization Group III), affects a significant number of patients, with some studies suggesting anywhere from one-third to one-half of all patients with IPF having concurrent elevated pulmonary pressures and right ventricle (RV) failure. Secondary PH is defined by excessive vascular remodeling beyond the degree expected for chronic hypoxic vasoconstriction and is associated with both a decreased exercise capacity and increased mortality.[1,2] Unfortunately, therapies approved for the treatment of idiopathic pulmonary arterial hypertension (PAH) have not proven effective in the treatment of PH associated with IPF, including ambrisentan[3] and sildenafil.[4] This patient population also fares worse with respect to lung transplantation, having increased instances of primary graft dysfunction.[5]

There is an urgent need to identify pathways involved in the development of secondary PH in order to develop novel therapeutics. One study examining genome-wide RNA expression profiling in lung samples from three populations (normal controls, PAH, and IPF with associated PH) identified a number of characteristic differences between these groups at the level of gene expression, among which was a significant change in the level of bone morphogenetic protein receptor II (BMPR2) in the secondary-PH group versus controls.[6] BMPR2, a member of the transforming growth factor-β family, has previously been shown to play a major role in the development of PAH, though its exact mechanism is unknown.[7] The importance of BMPR2 within vascular cells is known, with loss-of-function BMPR2 mutation resulting in increased endothelial cell apoptosis and development of PAH.[8,9] In addition, a recent case report details development of IPF in a BMPR2-mutation-carrying pa-

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Submitted June 10, 2015; Accepted July 23, 2015; Electronically published October 20, 2015.
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tient with PAH, suggesting at least some shared mechanistic pathways between the diseases.[10]

Another pathway that is potentially relevant to the development and progression of PH associated with IPF involves hypoxic signaling, which is known to cause worsened vasoconstriction and elevated pulmonary artery pressure in BMPR2 mutation carriers compared to non-BMPR2-mutation-carrying controls.[11] In addition, targets of hypoxia-inducible factor (HIF) signaling have been implicated in the development and progression of PH, including vascular endothelial growth factor.[12] HIF is an oxygen-sensing transcription factor that is stabilized in response to low-oxygen tension, resulting in increased expression of hypoxia response element (HRE)–associated genes.[13] Normoxic stabilization of HIF has previously been described in patients with PH,[14] possibly mediated through a BMPR2-facilitated increase in reactive oxygen species (ROS) production.[15] In addition, HIF has previously been implicated in the development of secondary PH in a chronic hypoxia murine model.[16-19]

Because of these previous reports of interaction between BMPR2 and HIF responsive signaling and because both pathways have been reported as important in the development of PH secondary to fibrosis, we investigated HIF and HIF target activity in the context of BMPR2 mutation in bleomycin-treated mice.

Table 1. List of primer sequences

Species and gene	Sequence
Mouse:	
18S, forward	ACCTGGTTGATCCTGCCAGTAG
18S, reverse	TTAATGAGCCATTCGCAGTTTC
Bmpr2, forward	TGGGCGCACCAGCCGATTTC
Bmpr2, reverse	CAGCTGGCCAGGCAGCCAAC
Id-1, forward	GTGAGCAAGGTGGAGATCCTG
Id-1, reverse	GGTGGTCCCGACTTCAGACTC
Hif1-α, forward	TGGAGATGCTGGCTCCCTAT
Hif1-α, reverse	TGGAGGGCTTGGAGAATTGC
Ctgf, forward	GGGAGAACTGTGTACGGAGC
Ctgf, reverse	AGTGCACACTCCGATCTTGC
Human:	
18S, forward	GTAACCCGTTGAACCCCATT
18S, reverse	CCATCCAATCGGTAGTAGCG
CTGF, forward	GTGAGCCTCGTGCTGGAC
CTGF, reverse	GAAGAGGCCCTTGTGCGG

METHODS

Transgenic mice

$TetO_7CMV$-$BMPR2^{Mut}$ transgenic mice were generated as previously described.[20] These mice have a dominantly acting BMPR2 mutation driven by a doxycycline-sensitive promoter. Transgenic mice were on the FVB/N background. Rosa26-rtTA2 and $TetO_7CMV$-$BMPR2^{Mut}$ mice were crossed to produce double-transgenic mice with doxycycline-inducible expression of transgene in all tissue types.[21] Mice were housed in animal care facilities at Vanderbilt University (Nashville, TN) with food/water ad lib. and provision of standard recommended room temperature (20°–26°C) and day : night cycle (12 : 12 hour cycle). The animal care facility is certified by the Association for Assessment and Accreditation of Laboratory Animal Care International, and all procedures were performed in compliance with the National Research Council for ethical handling of laboratory animals. The experimental protocol was approved by Vanderbilt's Institutional Animal Care and Utilization Committee.

Primers and antibodies

Primer sequences used are itemized in Table 1. Antibodies used were as follows: α-smooth muscle actin (α-SMA), rabbit polyclonal (Abcam, Cambridge, MA); von Willebrand factor, rabbit polyclonal (DakoCytomation, Glostrup, Denmark); HIF1-α and connective tissue growth factor (CTGF), both rabbit polyclonal (Novus Biologicals, Littleton, CO); and BMPR2, goat polyclonal (Abcam).

Experimental design

Twelve-week-old 20–30-g Rosa26-rtTA2 mice (controls) and Rosa26-rtTA2 × $TetO_7CMV$-$BMPR2^{Mut}$ mice had transgene expression induced by addition of doxycycline (Sigma-Aldrich, St. Louis, MO) in chow (1 g/kg) for 1 week. Mice then underwent intraperitoneal injection with 0.018 U/g bleomycin (reconstituted under sterile conditions with sterile phosphate-buffered saline and stored in 4°C for no more than 1 week after reconstitution; obtained from Bedford Laboratories, Bedford, OH) or vehicle twice weekly for 4 weeks before undergoing euthanasia.[22] The mode of delivery of bleomycin and time point of pulmonary hemodynamic measurement were chosen based on previous work that detailed more consistent fibrosis with this method versus intratracheal bleomycin delivery. The time point chosen was too early for the mutant BMPR2 mice to spontaneously develop PAH.[23] On induction with doxycycline, mice do not develop spontaneous PH until at least 6 weeks. One week after the last injection (approximately 40 days after doxycycline administration), mice were euthanized.

Hemodynamic measurements: right ventricular systolic pressure (RVSP), RV hypertrophy, and transthoracic echocardiogram

Transthoracic echocardiograms were collected 1 day before euthanasia using the Vivo 770 high-resolution image system (VisualSonics, Toronto, Canada).[24] Invasive hemodynamic measurement was conducted as described in previous studies.[20] In brief, mice were given 0.75 mg/g of 2.5% avertin (a mixture of *tert*-amyl alcohol and 2,2,2-tribromoethanol; Sigma-Aldrich) to induce anesthesia. Mice were then placed on a heating pad, and systemic blood pressure and pulse were measured via a tail cuff and transducer. The jugular vein was dissected and catheterized with a 1-French pressure catheter (SPR-1000; Millar Instruments, Houston, TX). The RV pressure tracing was then recorded utilizing Chart 5.3 software (AD Instruments). After completion of the measurements, blood was collected. The heart was then excised with removal of the atria, and the

RV and left ventricle (LV) plus septum were isolated for measurement of the Fulton index (RV : (LV + S)) as previously described.[25]

Histology, immunostaining, Western blot, semiquantitative scoring, and collagen content

On harvest, the left lung was inflated and placed in 10% formalin for histological processing, and the right lung was divided and snap frozen in liquid nitrogen for RNA and protein processing. Sections were prepared for immunohistochemistry and immunofluorescence staining. Immunostaining was performed for α-SMA to identify muscularized pulmonary vessels, which were then counted per high-powered field as previously described.[26] Western blots were performed on tissue and cell lysates as previously described.[27] Semiquantitative lung fibrosis scoring[28] and hydroxyproline microplate assay were performed as previously described.[29]

Quantitative reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from frozen whole-lung tissue and human cell lysate using a RNEasy kit (Qiagen, Venlo, Netherlands) per the manufacturer's recommendations, DNase treated, and prepared for quantitative RT-PCR. Specific transcript levels for HIF1-α were determined by normalization to 18S. Values are presented as mean normalized transcript level using the comparative C_t method ($2^{-\Delta\Delta C_t}$).

Cell culture

Given the method of intraperitoneal delivery of bleomycin in this model and previous literature linking vascular endothelial BMPR2 function to the onset of PH,[30] we chose to examine the effects of BMPR2 mutation in endothelial cells. Primary murine and human pulmonary microvascular endothelial cells (mPMVECs and hPMVECs, respectively) with and without BMPR2 mutation transfection were grown in culture as previously described[31] (EBM-2 media; Lonza, Basel, Switzerland) and maintained in standard cell culture incubators (37°C, humidified, 5%, CO_2). Native/wild-type and mutant PMVECs were grown to 80% confluence. In experiments with hypoxic exposure (fraction of inspired oxygen [FiO_2]: 5%), cells were place in a hypoxia cabinet (Coy Lab Products, Grass Lake, MI) for either 6 or 24 hours, as stated below. The content of oxidized lipids was determined by the thiobarbituric acid reactive substances assay (R&D Systems, Minneapolis, MN), with and without the addition of 1 mM TEMPOL (Sigma-Aldrich).[32]

HRE activity was determined using a luciferase-based system. Pulmonary endothelial cells with BMPR2 mutation and native cells with no BMPR2 mutation were seeded at 70% confluency in 96-well plates. Cignal lenti HIF reporter was obtained from Qiagen. The lenti HIF reporter is made from vesicular stomatitis virus pseudotyped lentivirus particles expressing the firefly luciferase gene under the control of a minimal cytomegalovirus promoter and tandem repeats of the HRE. Cells were transfected with 10 multiplicity of infection lenti HIF reporter in antibiotic-free conditions using SureENTRY transduction reagent (Qiagen; 5 μg/mL) as per the manufacturer's instructions. Twenty-four hours after transfection, the cells were switched into medium containing 10% fetal bovine serum. Doxycycline (300 ng/mL) was added to the medium to induce BMPR2 expression. Ninety-six hours after transfection, activity of firefly luciferase (indicative of HIF activity) was measured as relative light units using the Dual-Luciferase reporter assay system (Promega).

Statistics

Statistical analyses were performed using GraphPad Prism (GraphPad, La Jolla, CA). Differences among groups were assessed using one-way analysis of variance (ANOVA) or a Kruskal-Wallis rank ANOVA. Differences between pairs were assessed using a Mann-Whitney test. Survival differences were evaluated using a Fisher's exact test. Results are presented as mean ± standard error of the mean; $P < 0.05$ was considered significant.

RESULTS

Mice exposed to bleomycin have decreased levels of BMPR2 and inhibitor of differentiation 1 (ID-1) expression compared to vehicle exposure

In order to examine the response of BMPR2 and downstream signaling to bleomycin exposure, control wild-type mice were given intraperitoneal bleomycin and then euthanized to evaluate whole-lung homogenate levels of BMPR2 and ID-1, a canonical BMPR2-signaling cascade protein.[33] Both BMPR2 and ID-1 RNA expression levels were decreased in response to bleomycin exposure (Fig. 1*a*), and BMPR2 protein was decreased by 48% in the bleomycin-exposed group (Fig. 1*b*).

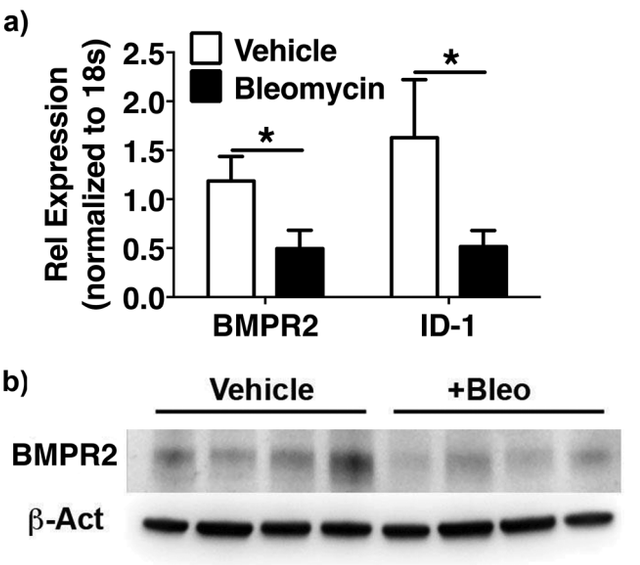


Figure 1. Bleomycin-induced pulmonary fibrosis results in decreased expression of bone morphogenetic protein receptor II (BMPR2) and downstream signaling molecule inhibitor of differentiation 1 (ID-1). *a*, Relative expression of BMPR2 and ID-1 was decreased in lungs of wild-type mice exposed to 33 days of bleomycin (Bleo; normalized to 18S). *b*, Immunoblot detailing decreased BMPR2 in the lungs of wild-type mice exposed to bleomycin. β-Act: β-actin. Asterisk indicates $P < 0.05$. $N = 4$ per group.

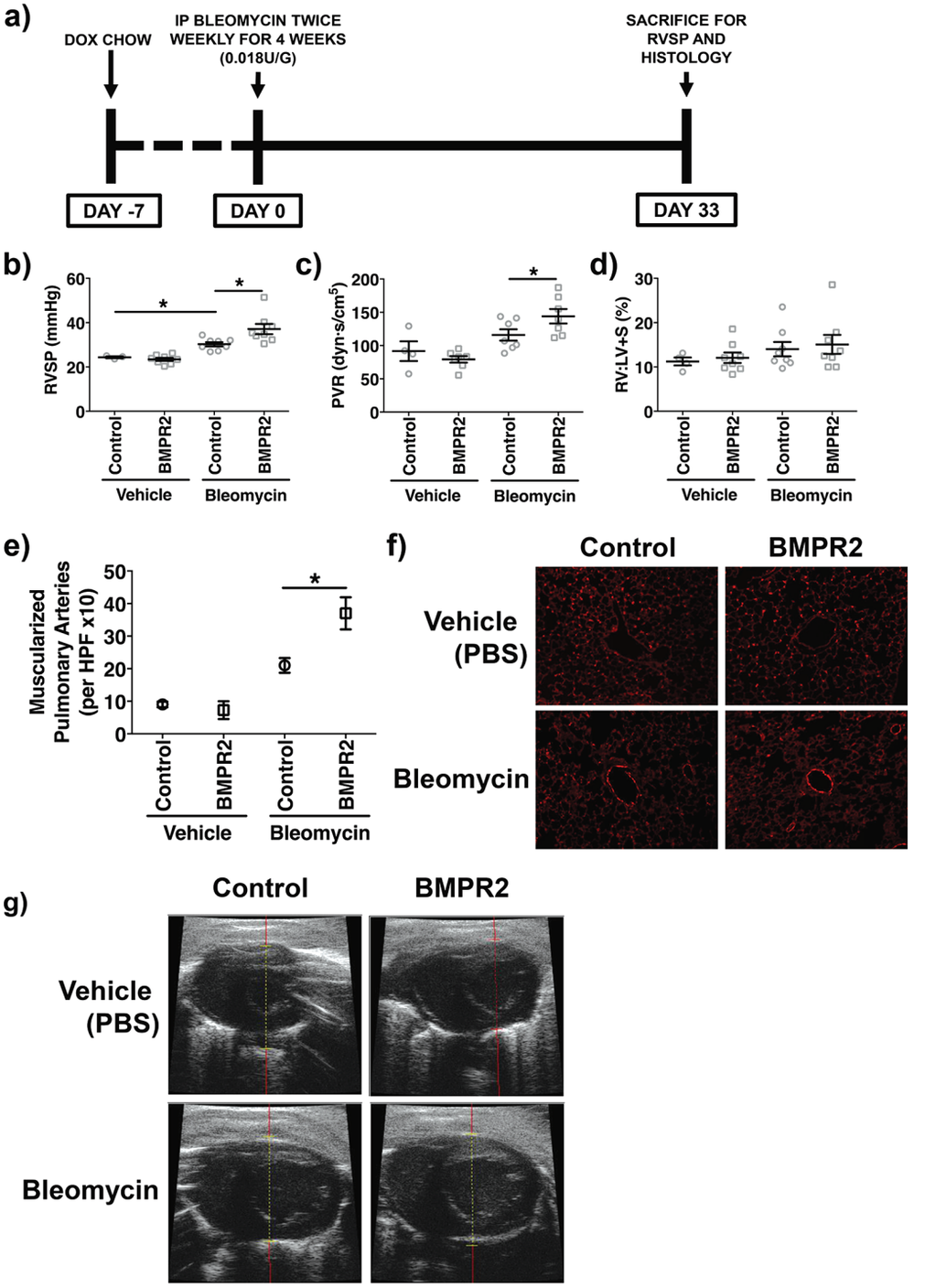


Figure 2. Mutant bone morphogenetic protein receptor II (BMPR2) mice exposed to bleomycin develop worse pulmonary hypertension compared to control, with no evidence of cardiovascular remodeling but with increased vascular remodeling. *a*, Time line of animal experiment with initiation of doxycycline (Dox) chow 7 days before starting twice weekly intraperitoneal (IP) bleomycin injections for 4 weeks. Echocardiogram was performed on day 32, with euthanasia for hemodynamic and histological data on day 33. *b*, Right ventricular systolic pressure (RVSP) was greater in mutant BMPR2 mice on exposure to bleomycin compared to bleomycin-treated controls. *c*, A similar change was noted in measurement of pulmonary vascular resistance (PVR). *d*, However, no change was noted in cardiac remodeling as determined by the right ventricle to left ventricle plus septum ratio (RV : (LV + S); $P = 0.70$). *e*, *f*, There was an increase in muscularization of pulmonary vessels within the bleomycin-exposed mutant BMPR2 mice lungs compared to control through counting of muscularized pulmonary arteries per 10 high-powered fields (HPF). *g*, Qualitative two-dimensional echocardiogram images of parasternal short-axis view in vehicle- and bleomycin-exposed mice demonstrating RV enlargement in both groups of mice exposed to bleomycin compared to vehicle. No difference was noted between bleomycin-treated groups in RV wall thickness or cavity dimensions. PBS: phosphate-buffered saline. Asterisk indicates $P < 0.05$. $N = 8$ per group, except $N = 4$ for control vehicle.

Bleomycin administration to mutant BMPR2 mice results in elevated RVSP with evidence of increased vascular remodeling

To evaluate the effect of bleomycin-induced fibrosis on the development of secondary PH, mutant BMPR2 and control mice were given intraperitoneal bleomycin and data collected on hemodynamic variables (Fig. 2*a*). After bleomycin treatment, there was a statistically significant increase in RVSP between control and mutant BMPR2 mice (30.0 ± 1.0 vs. 35.2 ± 1.9 mmHg, $P = 0.03$; Fig. 2*b*). The time point used was earlier than when mutant BMPR2 mice develop spontaneous PH. There was a significant increase in pulmonary vascular resistance (PVR) between the bleomycin-exposed groups, driven primarily by the difference in RVSP (111.1 ± 10.4 vs. 149.4 ± 11.3 dyn s/cm^5, $P = 0.04$; Fig. 2*c*). However, these changes were not associated with cardiac remodeling, given the lack of significant increase in RV hypertrophy based on the Fulton index (RV : (LV + S)) between control and BMPR2 bleomycin-exposed mice (13.5% ± 1.5% vs. 12.8% ± 1.9%, $P = 0.70$; Fig. 2*d*), as described in prior studies using BMPR2 transgenic mice.[34] In contrast to the cardiac remodeling measurements, increased RSVP and PVR were associated with an increase in muscularized pulmonary vessel counts in bleomycin-treated mutant BMPR2 mice (Fig. 2*e*, 2*f*). This finding was reinforced qualitatively by lack of significant change in RV wall thickness and end-diastole cavity dimension on echocardiographic images, as visualized in Figure 2*g*.

In order to determine whether there were differences in susceptibility to lung fibrosis in mutant BMPR2 mice compared to controls, Masson trichrome stains of histologic sections were evaluated for fibrosis scoring, and hydroxyproline assays were done from lung tissue. After bleomycin, both groups had marked fibrotic remodeling in the lung but to a similar degree. Specifically, there was no significant difference in observed fibrosis between control and mutant BMPR2 mice after exposure to bleomycin as determined by blinded scoring of trichrome-stained lung sections (1.3 ± 0.3 vs. 1.4 ± 0.2, $P = 0.90$; Fig. 3*a*, 3*b*). However, there was a significant difference between lung collagen content as determined by the hydroxyproline assay between control (453 ± 25 μg) and BMPR2 (575 ± 26 μg) mice (right middle lobe of the lung was utilized for all measurements; $P < 0.05$; Fig. 3*c*). Given the lack of difference in histologic evaluation of fibrosis, this collagen content difference may have been due to the differences in perivascular fibrotic remodeling. The role of BMPR2 signaling in vessel remodeling has been described previously.[35]

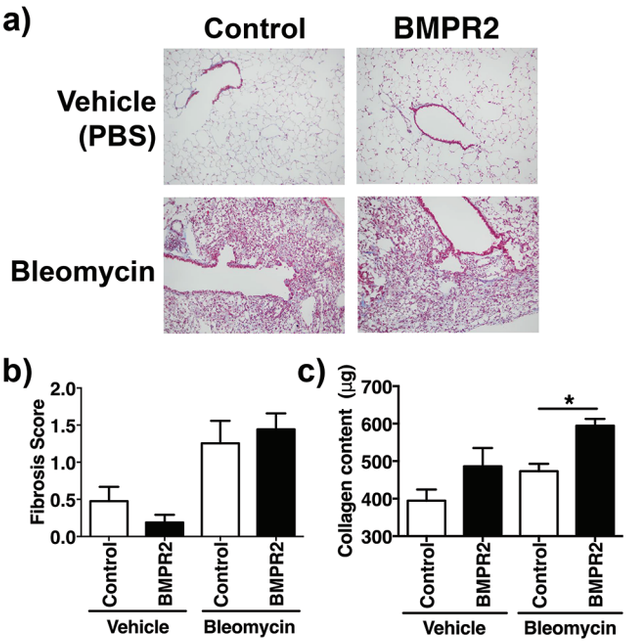


Figure 3. Mutant bone morphogenetic protein receptor II (BMPR2) mice had a similar level of lung fibrosis following bleomycin compared to control, with increased collagen content. *a*, Representative microscopy images of trichrome-stained lung sections of groups as listed, showing no difference in lung architecture at baseline and similar fibrosis after exposure to bleomycin. Magnification ×20. *b*, Graph representing no difference in control versus BMPR2 groups with respect to fibrosis score before and after intraperitoneal bleomycin treatment ($P = 0.90$). *c*, However, there was significantly increased collagen content in the right lower lobe of harvested lungs by microplate hydroxyproline assay in the bleomycin BMPR2 group compared to control. PBS: phosphate-buffered saline. Asterisk indicates $P < 0.05$. $N = 8$ per group, except $N = 4$ for control vehicle.

BMPR2 mutation results in increased HIF1-α activation in mice and cell culture

Whole-lung lysates from mutant BMPR2 mice exposed to bleomycin were found to have greatly increased HIF1-α protein (25-fold) compared to controls, without change in RNA expression, suggesting a posttranslational HIF stabilization in the bleomycin-exposed mutant BMPR2 mice (Fig. 4*a*, 4*c*, 4*d*). CTGF, a HIF-regulated mediator that is known to influence extracellular matrix remodeling, was found to have increased RNA and protein expression in response to bleomycin administration in lungs of mutant BMPR2 mice compared to controls (Fig. 4*b*–4*d*). We next sought to examine the upregulation of hypoxic-mediated CTGF RNA and protein expression in hPMVECs with BMPR2 mutation. The hPMVECs with BMPR2 mutation exposed to acute hypoxia (FiO_2 5% for 6 hours) displayed a similar significant increase in CTGF expression compared to native controls (Fig. 4*e*, 4*f*).

To further dissect the BMPR2-HIF relationship, we transfected control and mutant BMPR2 mPMVECs with an HRE-dependent luciferase vector. Mutant-BMPR2-expressing cells had a doubling of luciferase activity under normoxic conditions, demonstrating that mutant BMPR2 expression alone can activate HRE pathways (Fig. 5*a*). In order to investigate the mechanism for BMPR2-mediated increase in HIF signaling, control and BMPR2 mPMVECs were exposed to hypoxia (FiO_2 5% for 24 hours) with and without the addition of the antioxidant TEMPOL (1 mM). HIF1-α level was increased in those cells expressing the mutant BMPR2 protein on exposure to hypoxia, an effect that was abolished with addition of TEMPOL, suggesting a role for increased ROS contributing to BMPR2-mediated HIF expression (Fig. 5*b*). This observation paralleled

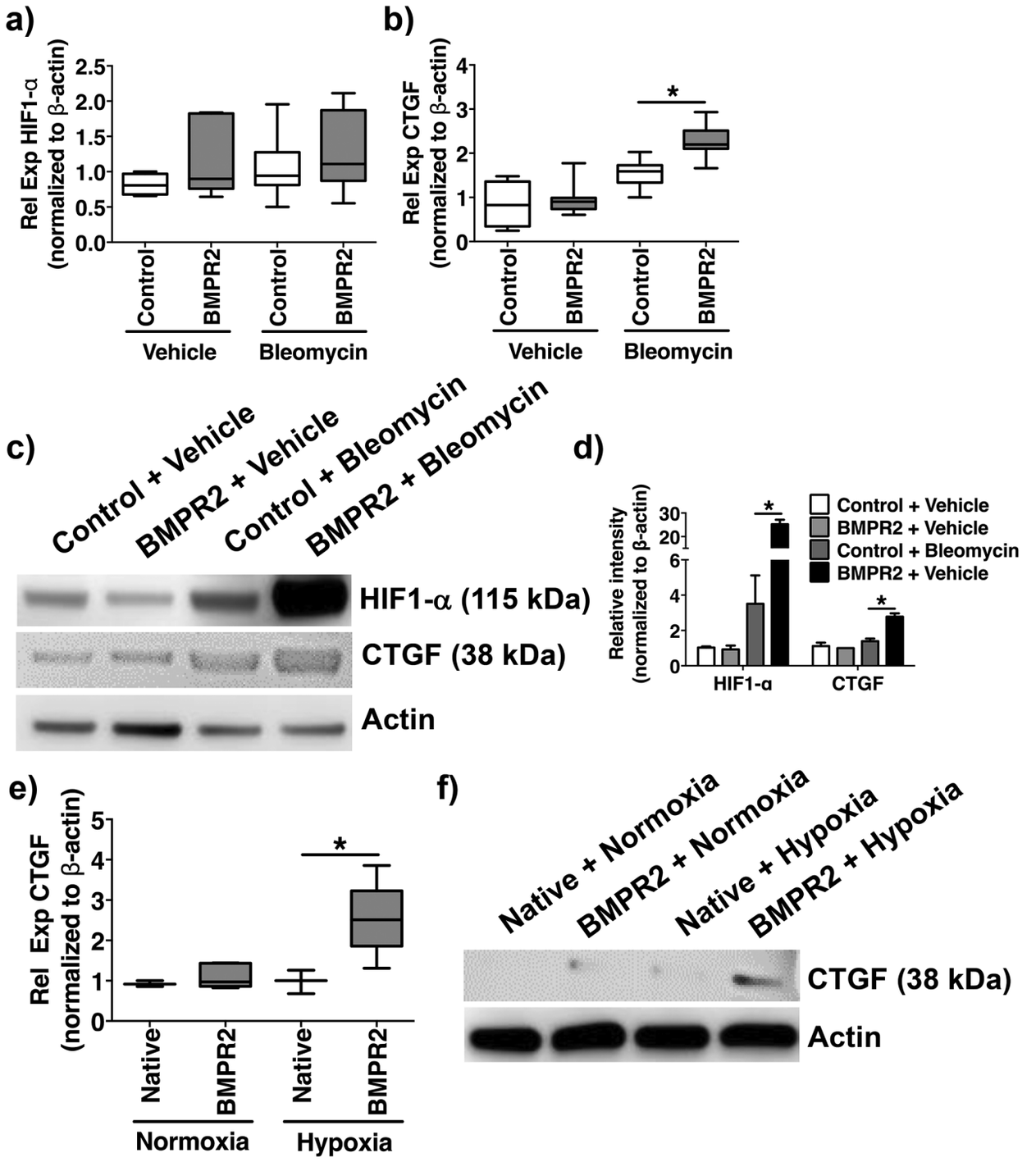


Figure 4. Mutant bone morphogenetic protein receptor II (BMPR2) mice exposed to bleomycin had increased hypoxia response element (HRE) gene expression, similar to human pulmonary microvascular endothelial cells carrying the BMPR2 mutation exposed to hypoxia. By quantitative polymerase chain reaction on whole-lung preparations, relative expression of hypoxia-inducible factor (HIF)1-α (*a*) was unchanged (normalized to 18S) on exposure to bleomycin, as contrasted with increased expression of connective tissue growth factor (CTGF; *b*). *c*, *d*, In whole-lung lysates, both proteins were significantly increased in mutant BMPR2 mice exposed to bleomycin (25-fold for HIF1-α). $N = 8$ per group, except $N = 4$ for control group. *e*, *f*, A similar association was seen in human pulmonary microvascular endothelial cells with BMPR2 mutation exposed to acute hypoxia (FiO_2 5% for 6 hours), with increased expression of CTGF. Asterisk indicates $P < 0.05$. $N = 4$ per group.

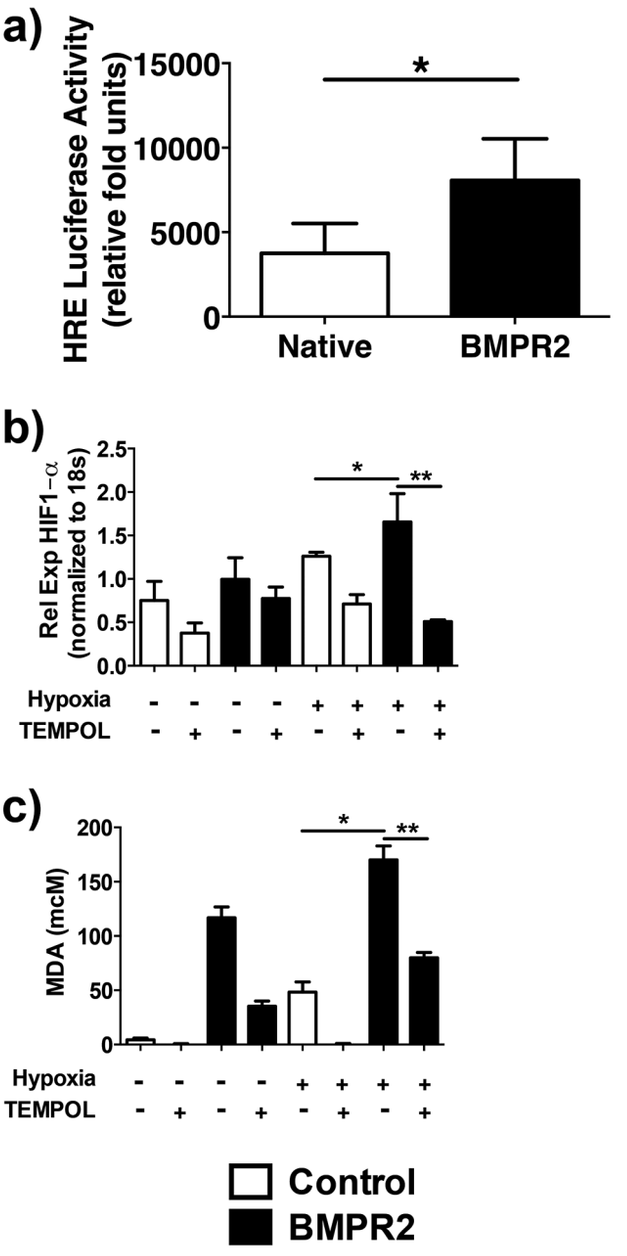


Figure 5. Mutant bone morphogenetic protein receptor II (BMPR2) expression promotes hypoxia response element (HRE) activity and hypoxia-inducible factor (HIF) expression, an effect that is attenuated with antioxidant administration. *a*, Using HRE luciferase systems, mutant BMPR2 cells had greater HRE activity than native cells. $N = 5$ in each group. *b*, Mutant BMPR2 cells have increased HIF expression upon exposure to hypoxia (FiO_2 5% for 24 hours) compared to controls, which is markedly decreased with exposure to the antioxidant TEMPOL. *c*, A similar trend is shown with respect to increased reactive oxygen species (ROS) production, as determined by malondialdehyde (MDA) concentration, in mutant BMPR2 cells exposed to hypoxia, which is also markedly decreased with exposure to the antioxidant TEMPOL. One asterisk indicates $P < 0.05$; two asterisks indicate $P < 0.001$. $N = 4$ per group.

the decrease in ROS production (as measured by malondialdehyde concentration) in mutant BMPR2 cells exposed to hypoxia (same conditions) with or without the addition of TEMPOL (Fig. 5*c*).

DISCUSSION

In these studies, mice expressing mutant BMPR2 had similar levels of bleomycin-induced lung fibrosis compared to control littermates but developed more severe PH with an associated increase in HIF expression. This increase in HIF activity related to mutant BMPR2 expression appears to be facilitated, at least in part, by production of ROS (Fig. 6). The downstream hypoxic signaling related to mutant BMPR2 expression was corroborated in hPMVECs, showing an increase in HRE activity in cells exposed to hypoxia. Together, our findings link BMPR2 and HIF signaling to secondary PH, adding to prior studies connecting BMPR2 signaling and the vascular endothelium with various other chronic lung diseases, including asthma,[36] sarcoidosis,[37] and chronic obstructive pulmonary disease.[38] It is important to note that at the harvest time point examined, the murine model of BMPR2 mutation would not be expected to develop spontaneous PAH—this usually occurs 6 weeks after doxycycline induction, at the earliest.[23]

HIF is the most important mediator of the cellular response to hypoxia and has been implicated in the development of PH in several models of PH such as monocrotaline-treated rats,[39] chronic hypoxia,[19] and fawn-hooded rats.[40] HIF has previously been shown to be expressed in immunohistochemistry stains of lung vasculature in patients with PH.[41] Additionally, epigenetic downregulation of superoxide dismutase 2 causing an increase in mitochondrial ROS production[42] and normoxic stabilization of HIF1-α has been speculated to play a role in the development of PAH.[15] Our finding of a large increase in HIF protein within the lung tissue of mutant BMPR2 mice exposed to bleomycin is a novel discovery that demonstrates HIF association with worsening development of PH associated with lung fibrosis.

It is known that BMPR2 can lead to production of ROS contributing to cell injury, angiogenesis, and aberrant fibrogenic repair in the development of membranous nephropathy.[33] However, it is only recently that we have grown to appreciate the role that BMPR2 plays in the development of pulmonary vascular oxidative injury, specifically through mitochondrial dysregulation,[43] with at least one study suggesting that ROS formation may play a direct role in the pathogenesis of human PAH.[44] mutant BMPR2 expression has also been shown to lead to stabilization of ROS in the setting of acute changes in oxygen tension,[45] with resulting worsening of the PH phenotype. Interestingly, in endothelial cells derived from patients with PAH, baseline DNA damage was higher compared to control patient samples, with evidence of increased ROS production.[46] Our data are thus consistent with prior work describing HIF stabilization in the presence of increased ROS production,[47,48] providing a potential mechanism that explains in part how BMPR2 signaling contributes to the development of secondary PH.[49-51]

To explore a potential mechanism linking BMPR2-mutation-mediated induction of HIF to increased PH, we looked for down-

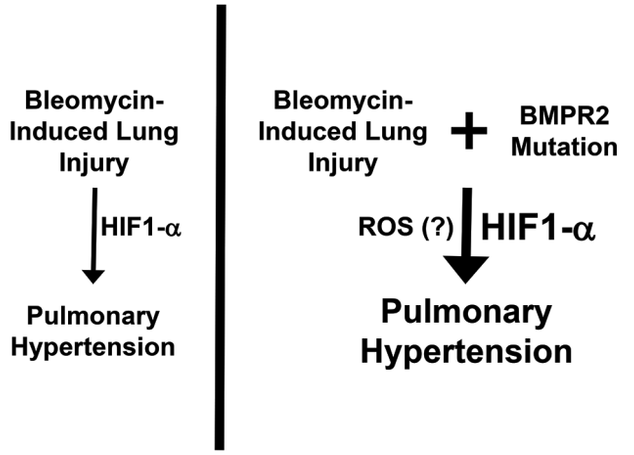


Figure 6. Summary of bone morphogenetic protein receptor II (BMPR2) mutation influence on development of pulmonary hypertension associated with pulmonary fibrosis, potentially mediated through reactive oxygen species (ROS) production and stabilization of hypoxia-inducible factor (HIF)1-*α*.

stream mediators of the HRE that may influence extracellular matrix remodeling. Since HIF is known to directly mediate CTGF,[52-54] a member of the CCN family of proteins that coordinates extracellular matrix remodeling on exposure to several stimuli of cellular stress, we demonstrate an increase in CTGF expression in our in vivo model, as well as in vitro endothelial cell culture experiments. In addition, previous mouse studies have implicated upregulation of CTGF signaling within vascular smooth muscle cells as playing a key role in the development of PH in both monocrotaline-treated rats[55] and pulmonary-artery-banded mice.[56]

Thus, our identification of HIF stabilization in both a mouse and a human model of BMPR2 mutation represents a new pathway for development of novel pharmacologic treatments for PH[57,58] associated with chronic parenchymal lung disease, such as pulmonary fibrosis. This discovery is an important first step in an evolving area of research, though it prompts several questions for future research, including the role of endothelial cell BMPR2 signaling in the in vivo model of fibrosis-associated PH, through either (*a*) influencing paracrine signaling to surrounding smooth muscle cells[59,60] or (*b*) progenitor cell recruitment and reprogramming.[61] In addition, this study suggests that therapy directed at hypoxia-mediated targets shown to influence the development of PH, such as BMP antagonist gremlin 1,[62] may prove beneficial in treating human subjects with disease. These future experiments will help define the agent and method of delivery for any potential HIF-targeting therapies for disease.

In conclusion, we have shown that bleomycin-exposed mice with mutant BMPR2 expression develop a more severe PH phenotype associated with increased HIF stabilization, which is likely facilitated by ROS production. Together, these findings represent a novel pathway for development of therapeutics for secondary PH.

ACKNOWLEDGMENT

The excellent professional assistance of John H. Newman, MD, is acknowledged and appreciated.

Source of Support: National Institutes of Health (NIH) grants NHLBI HL105479 (WEL), HL85317 (TSB), HL92870 (TSB), HL095797 (JDW), HL87738 (AJB), and HL121174 (JPF); NIH grant NCRR UL1 RR024975; and an American Thoracic Society/Pulmonary Hypertension Association Fellowship Research Grant (AJB).

Conflict of Interest: None declared.

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