BMP signaling controls PASMC $K_V$ channel expression in vitro and in vivo

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Young, Katharine A., Charles Ivester, James West, Michelle Carr, and David M. Rodman. BMP signaling controls PASMC $K_V$ channel expression in vitro and in vivo. Am J Physiol Lung Cell Mol Physiol 290: L841–L848, 2006. First published December 9, 2005; doi:10.1152/ajplung.00158.2005.—Bone morphogenetic proteins (BMPs) have been implicated in the pathogenesis of familial pulmonary arterial hypertension. The type 2 receptor (BMPR2) is required for recognition of all BMPs. Transgenic mice with a smooth muscle cell-targeted mutation in this receptor (SM22-tet-BMPR2delx4+) developed increased pulmonary artery pressure, associated with a modest increase in arterial muscularization, after 8 wk of transgene activation (West J, Fagan K, Steudel W, Fouty B, Lane K, Harral J, Hoedt-Miller M, Tada Y, Ozimek J, Tuder R, and Rodman DM. Circ Res 94: 1109–1114, 2004). In the present study, we show that these transgenic mice developed increased right ventricular pressures after only 1 wk of transgene activation, without significant remodeling of the vasculature. We then tested the hypothesis that the increased pulmonary artery pressure due to loss of BMPR2 signaling was mediated by reduced $K_V$ channel expression. There was decreased expression of $K_V$1.1, $K_V$1.5, and $K_V$4.3 mRNA isolated from whole lung. Western blot confirmed decreased $K_V$1.5 protein in these lungs. Human pulmonary artery smooth muscle cells (PASMC) treated with recombinant BMP2 had increased $K_V$1.5 protein and macroscopic $K_V$ current density, which was blocked by anti-$K_V$1.5 antibody. In vivo, nifedipine, a selective L-type Ca$^{2+}$ channel blocker, reduced RV systolic pressure in these dominant-negative BMPR2 mice to levels seen in control animals. This suggests that activation of L-type Ca$^{2+}$ channels caused by reduced $K_V$1.5 mediates increased pulmonary artery pressure in these animals. These studies suggest that BMP regulates $K_V$ channel expression and that loss of this signaling pathway in PASMC through a mutation in BMPR2 is sufficient to cause pulmonary artery vasoconstriction.

A potential role for bone morphogenetic protein (BMP) signaling in the pathogenesis of vascular disease was suggested by studies of hereditary pulmonary arterial hypertension (PAH), a disorder characterized by the pathological development of increased pressure and structural remodeling of the pulmonary circulation. Mutations in the BMP type 2 receptor (BMPR2) gene were found to be responsible for hereditary PAH (10, 17). Subsequently, sporadic cases of PAH were also found to be associated with mutations in BMPR2 (37).

Transgenic mice have demonstrated a critical role for BMP signaling in development. BMPR2(−/−) mice die early in development, before gastrulation. BMPR2(+/−) mice develop normally and were originally thought to have no cardiovascular phenotype (4). However, a recent report in BMPR2(+/−) mice suggested that they may have modest pulmonary hypertension, though that finding was not verified by a second group (18). Our laboratory constructed a conditional transgenic mouse expressing a dominant-negative BMPR2 (dnBMPR2) selectively in smooth muscle cells (SM22-tet-BMPR2delx4− mice). When the mutation was activated for 2 mo immediately after birth, the mice developed increased pulmonary artery pressure associated with a modest increase in arterial muscularization (39). This suggested that the increased pressures were due predominantly to vasoconstriction rather than remodeling, which was unexpected since the principal known activity of BMP signaling in smooth muscle cells was as an antimitogenic stimulus (26). Therefore, understanding the mechanisms contributing to this unusual increase in pulmonary artery tone is essential to understanding how mutations in BMPR2 might lead to the development of familial PAH.

Voltage-gated potassium ($K_V$) channels regulate resting membrane potential in vascular smooth muscle cells and have been implicated in pulmonary artery-selective hypoxic vasoconstriction. Although this response may involve contributions from both endothelial and smooth muscle cells, isolated smooth muscle cells demonstrate contraction to acute hypoxia, indicating an intrinsic smooth muscle cell response to hypoxia (19). Hypoxia inactivates $K_V$ channels, which results in membrane depolarization, activation of voltage-dependent Ca$^{2+}$ channels, and Ca$^{2+}$ influx (1, 8, 27, 31, 42). This model correlates with data from the intact circulation, implicating calcium influx via voltage-gated Ca$^{2+}$ channels and, more specifically L-type Ca$^{2+}$ channels, as the primary stimulus for hypoxic vasoconstriction (21).

$K_V$ channels comprise tetramers of α-subunits, which form the functional channel, and auxiliary β-subunits that have been shown to regulate functional expression or modify channel properties. Although the molecular identity of the oxygen-sensing channel regulating hypoxic vasoconstriction is unsettled, evidence supports a role for $K_V$1.5 in regulating resting pulmonary artery smooth muscle cell (PASMC) membrane potential and hypoxic depolarization. Homotetramers of $K_V$1.2 or $K_V$1.5 form oxygen-sensitive channels in heterologous expression systems (3). Chronic hypoxia resulted in a decrease of $K_V$1.5 channels in PASMCs (30, 34). Whole cell recordings using anti-$K_V$1.5 antibody depolarized the membrane and reduced the increase in cytosolic Ca$^{2+}$ caused by hypoxia in cultured PASMCs (3). Transgenic mice lacking this subunit have impaired hypoxic vasoconstriction (2).

In the present study SM22-tet-BMPR2delx4+ mice were used to test the hypothesis that increased pulmonary artery pressure due to loss of BMPR2 signaling is mediated by reduced $K_V^{+}$. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
channel expression. If loss of BMPR2 specifically in PASMC results in vasocstriction, then pulmonary hypertension in these mice might occur soon after the activation of the dominant-negative transgene. RV systolic pressure was elevated after only 1 wk of transgene activation. A decrease in mRNA for Kv channel α-subunits Kv1.1, Kv1.5, and Kv4.3 was found by RT-PCR to probe lung tissue. Western blot showed decreased Kv1.5 protein in these lungs. Consistent with a direct regulatory effect of BMP signaling, human PASMCs treated with recombinant BMP2 showed increased Kv1.5 protein and macroscopic Kv current density, which was blocked by anti-Kv1.5 antibody. In vivo, nifedipine, a selective L-type Ca\(^2+\) channel blocker, reduced RV systolic pressure in SM22-tet-BMPR2\(^{△}\) mice to levels seen in control animals, suggesting that activation of L-type Ca\(^2+\) channels caused by reduced Kv1.5 mediates increased pulmonary artery pressure in these animals.

**METHODS**

**Cell culture.** Human PASMCs (Clonetics, San Diego, CA) were grown in SmBM media supplemented with SmGM-2-SingleQuot (Clonetics, San Diego, CA) in a humidified incubator at 37°C and 5% CO\(_2\). Cells were used after passages 4 and 9. Cells were plated and grown to ~80% confluence on 10-cm\(^2\) plates. The medium was changed on all the plates at the start of the experiment. We added 50 ng/ml BMP2 (RDI, Flanders, NJ) for 24, 8, 6, 4, and 2 h, and then cells were all harvested at the same time for mRNA and protein expression assays.

**RT-PCR in cultured human PASMCs and whole lungs from dnBMPR2 mice.** Primers were designed using GenBank sequences and the Perkin Elmer ABI Primer Express program (Table 1). Each primer (all are species specific) was searched against Basic Local Alignment Search Tool to ensure that it did not match any known gene, aside from that for which it was designed, especially other family members. The primers were designed specifically for quantitative RT-PCR to produce products of comparable size. RNA was made using a Qiagen RNAeasy mini kit (Valencia, CA). Mouse lungs and brains were isolated from adult SM22-tet-BMPR2\(^{△}\) mice. Because mouse pulmonary arteries are too small to provide sufficient quantities of tissue, whole lungs were used. cDNA was made using Superscript II RT with oligo (dT)\(_{12-18}\) primers (both from Invitrogen, Carlsbad, CA) from this RNA. For screening gels, PCR was carried out in a GeneAmp Sequence Detection System 5700 (Perkin Elmer, Norwalk, CT) using 40 cycles of 95–60°C PCR with a 10-min 95°C initial soak. For quantitative PCR, the same equipment was used, but the fluorescent indicator Sybgreen was intercalated to allow real-time light detection. Each sample was tested individually for the housekeeping gene hypoxanthonine guanine phosphoribosyl transferase (HPRT), Kv\(_{α}\) subunits (Kv1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 3.1, 4.3, 9.3), Kv\(_{β}\) subunits (β1, β2, β3), and Cav1.2 (this L-type Ca\(^{2+}\) channel was used as a control). A second housekeeping gene, β-actin, was used to verify that HPRT expression did not change. Linear increases in fluorescence were confirmed, and the cycle number at detection was expressed relative to that for detection of HPRT. Each measurement was made in triplicate and averaged, with three individual replicate experiments used for statistical analysis.

**Western blot analysis.** Tissues were homogenized in 600 μl of homogenization buffer [RIPA buffer with 1% (vol/vol) phosphatase and protease inhibitor cocktail]. After centrifugation at 4°C (15 min, 10,000 g), the supernatant was used for determination of protein concentrations. Equal amounts of proteins were heated with denaturant sample buffer and separated by 8–16% Tris-Glycine gel electrophoresis. Proteins were then transferred to PVDF membrane in 20% MeOH. The membrane was blocked at room temperature with 5% nonfat dry milk in phosphate-buffered saline with 0.05% (vol/vol) Tween 20 and incubated with mouse polyclonal Kv1.5 antibody (1:100 dilution; USBiologicals, Swampscott, MA) overnight at 4°C. After being washed, the membrane was incubated with horseradish peroxidase-labeled donkey anti-mouse immunoglobulin secondary antibody (1:1,000 dilution) for 45 min at 37°C. Horseradish peroxidase was detected using the ECL+ Western blotting detection system (Amersham Bioscience, Piscataway, NJ). The amount of Kv1.5 protein was quantified by densitometry analysis.

**Heart catheterization.** All animal manipulations are approved by the University of Colorado at Denver Health Sciences Center Institutional Animal Care and Use Committee. Eight-week-old transgenic mice were fed doxycycline for 1 wk to activate the transgene. The mice, weighing 20–25 g, were anesthetized with intraperitoneal injection of 200 mg/kg ketamine and 10 mg/kg xylazine. If further anesthesia was necessary, repeat doses of 100 mg/kg ketamine and 5 mg/kg xylazine were administered. Mice were positioned supine on a heated operating table and studied in room air. Right ventricular (RV) pressure was directly measured with a 1.4 French Pressure Volume Conductance System SPR-839 (Millar Instruments, Houston, TX) inserted into the RV via the surgically exposed right jugular vein. Hemodynamics were continuously recorded with a Millar MPVS-300 unit coupled to a Powerlab 8-SP analog-to-digital converter, acquired at 1,000 Hz, and captured to a Macintosh G4 computer utilizing Chart5.3 software. Pressure-volume data were subsequently analyzed using PVAN3.3 (Miller Instruments) software on a Macintosh G4 emulating Windows 2000 via Virtual PC 6.0. Systolic pressure is a

**Table 1. Oligonucleotide sequences of primers used for RT-PCR**

<table>
<thead>
<tr>
<th>Standard Name</th>
<th>Sense Antisense</th>
<th>Location, nt</th>
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</thead>
<tbody>
<tr>
<td>α-Subunits</td>
<td></td>
<td></td>
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<td>K1.1 (NM_001595)</td>
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<td>883–903</td>
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<tr>
<td></td>
<td>5’-CGAGCTGAGAATGTTGAGT-3‘</td>
<td>351–371</td>
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nt, Nucleotides; K\(_v\), voltage-gated K\(_v\); Ca\(_v\), voltage-gated Ca\(_{\text{L-type}}\) channel. Numbers in parentheses are accession numbers in GenBank for sequences used in designing primers.
good measure of pulmonary vascular pressure, given that we know that the cardiac output didn’t change between dnBMPR2 mice and their control littermates and given that RV and left ventricular (LV) diastolic pressures were near 0.

After lethal injection of pentobarbital the heart and lungs were removed. Lungs were divided and processed for immunohistochemistry or molecular studies. The heart was divided into the RV and left ventricle and septum (LV+S), and the degree of right ventricular hypertrophy was assessed using the RV/LV+S ratio.

Nifedipine (Sigma) was solubilized in DMSO (Sigma) at a concentration of 4 mg/ml. The same dnBMPR2 animals with heart catheter in place were used as their own control. We injected nifedipine at 4 mg/kg intraperitoneally into stable animals and allowed 5 min for equilibration. Control experiments with DMSO alone were performed which showed no hemodynamic effects out to 20 min (data not shown). A time course with nifedipine was also performed, demonstrating that the maximal effect of nifedipine was seen at 5 min.

After 5 min of stabilization, hemodynamics was recorded.

Whole cell patch-clamp analysis. Cells were cultured as described above and plated on 35-mm² plates. The culture medium was changed, and BMP2 (50 ng/ml) was added 24 h before recording.

Currents were recorded by whole cell patch clamp. Data were acquired using pCLAMP9.0 software (Axon Instruments, Union City, CA) and filtered at 5 kHz. Voltage-dependent currents were corrected for linear leak and residual capacitance using an on-line P/4 subtraction program and series resistance compensation for linear leak and residual capacitance using an on-line P/4 subtraction program and series resistance compensation >90%. We recorded K⁺ currents by stimulating the cells in +20-mV steps from a holding potential of −80 mV. Extracellular solution (bath) contained (in mM): 141 NaCl, 4.7 KCl, 1.8 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 10 glucose (pH 7.4 with NaOH). Intracellular solution (ICF) contained (in mM): 125 KCl, 4 MgCl₂, 10 HEPES, 10 EGTA, and 5 Na₂ATP (pH 7.2 with KOH). All solutions were adjusted to 290–300 mosmol/kgH₂O with sucrose.

Anti-Kv1.5 antibody (USBiologicals), designed against an epitope in the COOH-terminal tail (located in the cytosol), was used at 1:125 in the pipette solution. Pipettes were filled with normal ICF in the tip, then backfilled with ICF containing antibody.

Statistical methods. All data are expressed as means ± SD. Comparisons between groups were made by independent Student’s t-test using Origin6 software (OriginLab, Northampton, MA). Multiple comparisons were done by ANOVA using MatLab6.5 software (The MathWorks, Natick, MA).

RESULTS

Phenotype of adult SM22-tet-BMPR2delx4+ mice fed doxycycline for 1 wk. SM22-tet-BMPR2delx4+ mice were fed doxycycline-HCl (0.5 mg/ml) in their water ad libitum for 1 wk to activate the transgene. The cardiovascular phenotype of these mice was evaluated in vivo by heart catheterization. RV and LV diastolic pressures were near zero in all mice studied (data not shown). Although RV relative weight was not significantly different between the two groups (Fig. 1A), RV systolic pressure in dnBMPR2 mice increased by almost 10 mmHg (or 26%) compared with doxycycline-fed control littermates without the transgene (Fig. 1B) (P = 0.011). The cardiac output for the dnBMPR2 mice was 4,330 μl/min (SD 762). There was no significant difference in the cardiac output for control littermates (data not shown).

Change in Kv channel expression in SM22-tet-BMPR2delx4+ mice. The increased tone seen in these mice after just 1 wk of transgene activation did not change in vascular constriction rather than remodeling are important in causing the pulmonary hypertension; therefore, Kv channel expression was examined in these animals. RT-PCR was used to test the functional expression of Kv and K<sub>Ca</sub> channels in these animals. Equal amounts of mRNA isolated from whole lung were reverse transcribed and amplified with subtype-specific oligonucleotide primers. Expression of these ion channels was compared with that of mouse HPRT, a common housekeeping gene.

Expression levels of Kv<sub>1.5</sub> subunits Kv<sub>1.2</sub>, Kv<sub>1.3</sub>, Kv<sub>1.4</sub>, Kv<sub>1.6</sub>, Kv<sub>2.1</sub>, Kv<sub>3.1</sub>, and of Kv<sub>β</sub> subunits Kv<sub>β1.1</sub>, Kv<sub>β2</sub>, and Kv<sub>β3</sub> did not change (Fig. 2A). The voltage-gated Ca<sup>2+</sup> channel α-subunit Cav<sub>1.2</sub> was used as a control, as increased amounts of this ion channel could result in increased pulmonary tension, and its level of expression did not change either (Fig. 2A).

Expression of Kv<sub>α</sub>-subunits Kv<sub>1.1</sub>, Kv<sub>1.5</sub>, and Kv<sub>4.3</sub> all decreased in the dnBMPR2 mice (Fig. 2B). A decrease in protein expression was confirmed by Western blot for Kv<sub>1.5</sub> (Fig. 2C). Quantification of protein intensity from dnBMPR2 and control mice (n = 7) showed a decrease in Kv<sub>1.5</sub> expression of 29.4% in dnBMPR2 mice (P = 0.017). Protein expression levels for Kv<sub>1.1</sub> and Kv<sub>4.3</sub> did not change (Fig. 2D).

Nifedipine restored normal RV systolic pressure in SM22-tet-BMPR2delx4+ mice. The L-type Ca<sup>2+</sup> channel blocker nifedipine (4 mg/kg ip) was administered to SM22-tet-BMPR2delx4+ mice during heart catheterization to determine the contribution of these channels to the increased pressures in dnBMPR2 mice. As expected, if vasoconstriction in PASMC is regulated by L-type Ca<sup>2+</sup> channels, nifedipine restored RV systolic pressure in dnBMPR2 animals to the same level as control animals (Fig. 3). Nifedipine had no significant effect on...
This channel blocker also had no effect on cardiac output in neither the dnBMPR2 nor control animals (data not shown).

BMP increased expression of KV channels in cultured human PASMC.

To determine whether the BMP pathway directly regulates KV channel expression, recombinant BMP2 (RDI) was applied to human PASMCs grown in culture. Cells were treated with BMP2 for up to 24 h. As shown in Fig. 4, increased lengths of exposure to BMP2 resulted in increased amounts of KV1.5 expression, with the amount of protein at 24 h being 10-fold higher than in control, untreated cells. A second dose of BMP2 was applied at 4 h in one of the 8-h treatment groups (Fig. 4) to ensure that the exogenous BMP2 remained active. There was no difference in the amount of pressures in control animals. This channel blocker also had no effect on cardiac output in neither the dnBMPR2 nor control animals (data not shown).

**BMP increased expression of Kv channels in cultured human PASMC.** To determine whether the BMP pathway directly regulates Kv channel expression, recombinant BMP2 (RDI) was applied to human PASMCs grown in culture. Cells were treated with BMP2 for up to 24 h. As shown in Fig. 4, increased lengths of exposure to BMP2 resulted in increased amounts of Kv1.5 expression, with the amount of protein at 24 h being 10-fold higher than in control, untreated cells. A second dose of BMP2 was applied at 4 h in one of the 8-h treatment groups (Fig. 4) to ensure that the exogenous BMP2 remained active. There was no difference in the amount of

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**Fig. 2.** Decreased voltage-gated potassium (Kv) mRNA and protein expression in 1 wk doxycycline-fed SM22-tet-BMPR2<sup>de/h4</sup> mice. A: RT/PCR showing the mean number of copies of channel mRNA relative to hypoxanthine guanine phosphoribosyl transferase (HPRT). RNA was isolated from whole lung. B: Kv1.1, Kv1.5, and Kv4.3 mRNA were significantly decreased (<i>P</i> < 0.05). C: Western blot showing decreased Kv1.5 expression in SM22-tet-BMPR2<sup>de/h4</sup> mice. The band for Kv1.5 was at 66 kDa. Bar graph shows relative intensity of all Westerns (<i>n</i> = 7 for each case) with standard deviation (*<i>P</i> = 0.017). D: Western blot showing no change in Kv1.1 and Kv4.3 expression. dnBMPR2, dominant-negative bone morphogenetic protein type 2 receptor.

**Fig. 3.** Nifedipine restored normal RV systolic pressure in SM22-tet-BMPR2<sup>de/h4</sup> mice. Bar graph showing response of RV pressure to 4 mg/kg ip nifedipine in control (solid bars, <i>n</i> = 6) and transgenic mice (cross-hatched bars, <i>n</i> = 7). *<i>P</i> < 0.05 compared with baseline rTA pressure. Nifedipine lowered pulmonary pressure in SM22-tet-BMPR2<sup>de/h4</sup> mice to levels similar to control, without significantly affecting control pressures (<i>P</i> < 0.05 compared with baseline double transgenic pressure).

**Fig. 4.** Kv1.5 expression is increased in human pulmonary artery smooth muscle cells (PASMCs) treated with recombinant BMP2. A: Western blot showing increased Kv1.5 protein expression with longer exposure to BMP2. The band for Kv1.5 was at 66 kDa. Human PASMCs were treated with 50 ng/ml BMP2 for 2, 4, 6, 8, and 24 h. One group of cells treated for 8 h received a 2nd dose of BMP2 at 4 h (*). Untreated cells were used as the control. B: Group means for densitometry from blot in A (with SD). The increase in Kv1.5 expression at 24 h was significantly different from the untreated group (<i>P</i> = 0.009).
KV1.5 protein expressed between the two 8-h treatment groups. Data is averaged from two separate experiments.

Although there was a trend toward increased protein expression by 24 h for Kv1.1 and Kv4.3, the increase was not significant (data not shown). Given that there was no change in Kv1.1 and Kv4.3 protein expression in the dnBMPR2 transgenic mice and no significant increase in Kv1.1 and Kv4.3 protein expression in cultured PASMCs treated with exogenous BMP and that Kv1.5 is the most likely candidate for being involved in oxygen sensing in hypoxic vasoconstriction of these three Kv subunits (7, 30, 32), we focused on the possible contributions of Kv1.5 subunit to vasoconstriction.

**BMP2 increased Kv current in cultured human PASMC.** To examine a functional effect of BMP on cultured human PASMC, currents were recorded from cells treated with BMP for 24 h. Cultured PASMC displayed typical voltage-activated K⁺ currents (Fig. 5A). Treatment with exogenous BMP2 increased the current density in these cells approximately threefold at potentials greater than +50 mV compared with control cells (Fig. 5B).

To determine the contribution of Kv1.5 to the increased current, anti-KV1.5 antibody (USBiologicals) was applied via the pipette to BMP-treated cells. The BMP-stimulated current density was significantly reduced to levels seen in untreated cells.

**DISCUSSION**

It has been hypothesized that PAH represents a progression from predominantly pulmonary artery vasoconstriction to predominantly pulmonary artery remodeling, and patients with PAH who respond to L-type Ca²⁺ channel blocker therapy may represent the early phase of this progression, whereas patients who are refractory to vasodilator therapy represent the late phase where remodeling predominates (12, 35). Mechanisms contributing to the development of abnormal vasoconstriction in humans with PAH have been difficult to establish due the inaccessibility of pulmonary artery tissue, although loss of endothelial cell vasodilators, increased expression of vasoconstrictors, and loss of PASMC Kᵥ channels have all been postulated as potential mediators of pulmonary artery vasoconstriction (16, 22). Whether these mechanisms are relevant to familial PAH due to reduced BMP signaling is unknown.

We previously reported that SM22-tet-BMPR2delx4 transgenic mice have markedly increased pulmonary artery pres-
sure, with only a modest increase in pulmonary artery muscularization (39). This suggested the possibility that abnormal vasoconstriction contributed to the phenotype in these mice. In our original report mice were phenotyped after having the dominant-negative receptor activated from birth to age 8 wk. We reasoned that if loss of PASMC BMPR2 resulted in vasoconstriction, then pulmonary hypertension in these mice might occur soon after activation of the dominant-negative transgene. To test this hypothesis we treated adult transgenic mice with doxycycline to activate the transgene for 1 wk, after which hemodynamic phenotyping was performed. After only 1 wk of transgene activation, mice showed a 26% increase in RV systolic pressure compared with control littermates. This increase was less than that seen in animals that had received doxycycline from birth to age 8 wk (65%), consistent with a model of the early stage of BMPR2 mutation-mediated PAH. These results add two important pieces of information to that in our earlier report: 1) activation of the transgene in adult mice is capable of causing PAH and 2) loss of BMPR2 signaling rapidly results in pulmonary artery vasoconstriction.

A critical regulator of pulmonary artery tone is PASMC membrane potential, which is largely regulated by Kv channel activity (41). BMP2 signaling has not previously been reported to control SMC Kv channel expression. However, BMPs are potent SMC differentiation factors (5, 25, 26, 33), and since the differentiated phenotype of PASMC includes expression of a specific subset of Kv channels, it is possible that BMP2 signaling might control their expression. We therefore examined ion channel expression in the lungs of the dnBMPR2 mice, specifically determining the expression of Kva and Kvβ subunits, as these channel subtypes have been shown to be candidates involved in regulating hypoxic pulmonary hypertension (6, 9, 14, 15, 28, 29, 38, 43). Transcript levels of Kv1.1, Kv1.5, and Kv4.3 were decreased in these animals. We used Western blot to confirm that Kva1.5 protein was also decreased in whole lung isolated from adult SM22-tet-BMPR2delx4+ mice fed doxycycline for 1 wk. Protein levels of Kv1.1 and Kv4.3 were unchanged. Our subsequent efforts therefore focused on Kv1.5.

Although the dnBMPR2 is expressed in all smooth muscle cells, we have previously shown that lung structures are normal by immunofluorescence and morphometric phenotyping of 8-wk doxycycline-fed SM22-tet-BMPR2delx4+ mice (39). Although it is possible that there are paracrine effects from other smooth muscle structures in the whole lung, the most straightforward explanation of the change in vascular phenotype in these mice is that the loss of BMP signaling in the pulmonary arteries of these mice resulted in changes in ion channel expression in these vessels.

BMP causes transcriptional changes in the cell by binding to types 1 and 2 BMPR heterotetramers, which then phosphorylate and activate downstream effectors such as SMADs, ultimately affecting gene expression (11, 20, 24). To examine whether the BMP signaling pathway directly regulated Kv1.5 expression, we treated cultured human PASMCs with exogenous recombinant BMP2 protein. After 24 h of exposure to BMP there was a 10-fold increase in Kv1.5 protein expression. This increase in protein expression was mirrored by a threefold increase in current density that was blocked by including anti-Kv1.5 antibody in the recording solution. Thus we conclude that BMP signaling directly regulates maximum Kv current density in PASMC primarily through increased expression of Kv1.5.

In acute hypoxic vasoconstriction, inactivation of Kv channels results in depolarization of the plasma membrane, leading to activation of L-type voltage-dependent Ca2+ channels and activation of the contractile apparatus (21). We used nifedipine to test the hypothesis that increased pulmonary artery pressure seen in adult dnBMPR2 mice fed doxycycline for 1 wk was also due to activation of L-type Ca2+ channels. The selective L-type Ca2+ channel blocker nifedipine reduced pulmonary artery pressure in SM22-tet-BMPR2delx4+ mice to the same level as control animals. The administration of nifedipine failed to have any significant effect on cardiac output, thus demonstrating that the decrease in pulmonary pressure caused by nifedipine occurs through inactivation of L-type Ca2+ channels within the pulmonary vasculature.

These are the first studies to our knowledge linking loss of BMPR2 signaling to reduced expression of Kv channels. This finding is consistent with several previous studies in animal models of secondary PAH (23, 36, 38) as well as studies of PASMC cultured from the lungs of patients with idiopathic pulmonary arterial hypertension (40). In the latter study, Yuan et al. (40) reported reduced mRNA for a variety of Kv channels, including Kv1.5. Thus it appears that there is commonality in the mechanisms underlying multiple etiologies of PAH centered upon reduced expression of the Kv channels required to set resting membrane potential in PASMC.

Our studies in cultured human PASMC demonstrated a direct link between BMP signaling and expression of Kv channels. Thus it is likely that an early event in the development of PAH in humans with BMPR2 mutations causes increased pulmonary artery vasoreactivity. This is consistent with studies of apparently unaffected carriers of BMPR2 mutations, who developed abnormally high pulmonary artery pressure in response to exercise (13). Whether functional changes in BMP signaling underlie reduced Kv expression in patients with PAH and normal BMPR2 alleles is unknown. Also unknown is what additional genetic and environmental cofactors combine with reduced BMP signaling to stimulate progression of PAH in individuals harboring BMPR2 mutations.

In conclusion, these studies in SM22-tet-BMPR2delx4+ mice suggest that loss of BMPR2 signaling in PASMC is sufficient to cause pulmonary artery vasoconstriction. The vasoconstriction appears to be due to reduced expression of Kv1.5 leading to PASMC depolarization and activation of L-type Ca2+ channels. At this early stage of the disorder, increased pulmonary artery pressure is readily reversible by L-type Ca2+ channel blockade. Future studies will be directed at understanding additional mechanisms that result in progression in model of familial PAH.

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REFERENCES


24. Yuan JX, Aldinger AM, Juhazsowa M, Wang J, Conte JV Jr, Gaine SP, Orens JB and Rubin LJ. Dysfunctional voltage-gated K+ channelinin...

