Serotonin Increases Susceptibility to Pulmonary Hypertension in BMPR2-Deficient Mice

Lu Long, Margaret R. MacLean, Trina K. Jeffery, Ian Morecroft, Xudong Yang, Nung Rudarakanchana, Mark Southwood, Victoria James, Richard C. Trembath, Nicholas W. Morrell

Abstract—Heterozygous germline mutations in the gene encoding the bone morphogenetic protein type II (BMPR-II) receptor underlie the majority (>70%) of cases of familial pulmonary arterial hypertension (FPAH), and dysfunction of BMP signaling has been implicated in other forms of PAH. The reduced disease gene penetrance in FPAH indicates that other genetic and/or environmental factors may also be required for the clinical manifestation of disease. Of these, the serotonin pathway has been implicated as a major factor in PAH pathogenesis. We investigated the pulmonary circulation of mice deficient in BMPR-II (BMPR2−/− mice) and show that pulmonary hemodynamics and vascular morphometry of BMPR2−/− mice were similar to wild-type littermate controls under normoxic or chronic hypoxic (2- to 3-week) conditions. However, chronic infusion of serotonin caused increased pulmonary artery systolic pressure, right ventricular hypertrophy, and pulmonary artery remodeling in BMPR2−/− mice compared with wild-type littermates, an effect that was exaggerated under hypoxic conditions. In addition, pulmonary, but not systemic, resistance arteries from BMPR2−/− mice exhibited increased contractile responses to serotonin mediated by both 5-HT2 and 5-HT1 receptors. Furthermore, pulmonary artery smooth muscle cells from BMPR2−/− mice exhibited a heightened DNA synthesis and activation of extracellular signal-regulated kinase 1/2 in response to serotonin compared with wild-type cells. In vitro and in vivo experiments suggested that serotonin inhibits BMP signaling via Smad proteins and the expression of BMP responsive genes. These findings provide the first evidence for an interaction between BMPR-II–mediated signaling and the serotonin pathway, perturbation of which may be critical to the pathogenesis of PAH. (Circ Res. 2006;98:818-827.)

Key Words: pulmonary arterial hypertension • bone morphogenetic protein • serotonin

Idiopathic pulmonary arterial hypertension (IPAH) is characterized by narrowing and obliteration of the small arteries of the lung leading to increased pulmonary vascular resistance.1 Elevation of pulmonary arterial pressure leads to right ventricular failure. Patients present with dyspnea after exertion, and many died of right heart failure within 3 years of diagnosis, before modern therapies.2 Treatment with prostanoids or endothelin receptor antagonists improves symptoms and survival, although, for many, the long-term outlook remains poor.

Heterozygous germline mutations in the gene encoding the bone morphogenetic protein type II receptor (BMPR-II) occur in up to 70% of cases of familial PAH (FPAH).3,4 Similar mutations were found in up to 26% of cases of idiopathic PAH.5 However, the low disease gene penetrance suggests that other genetic or environmental factors are necessary to manifest clinical disease.

A series of studies have implicated serotonin (or 5-HT) as a key mediator of PAH.6–8 For example, treatment of rats with serotonin potentiates the effects of hypoxia on pulmonary arterial pressure, right ventricular hypertrophy and pulmonary vascular remodeling.9 More recently, a polymorphism in the 5-HT transporter (5-HTT) promoter, which increases expression of the 5-HTT, has been associated with IPAH,10 although this association has recently been challenged.11 Hypoxia, a common cause of pulmonary hypertension, increases the proliferative response of pulmonary artery smooth muscle cells to serotonin.12 The compelling data supporting the role of serotonin in PAH pathogenesis led us to question whether serotonin interacts with BMPR-II deficiency to increase susceptibility to pulmonary hypertension.

Here we report that right ventricular pressure, right ventricular weight, and pulmonary vascular morphometry are similar in the BMPR-II–deficient mouse (BMPR2−/−) and wild-type littermates under normoxic or chronic hypoxic conditions. However, chronic infusion of serotonin increased pulmonary artery pressure in the BMPR2−/− mouse, especially under conditions of chronic hypoxia. In addition, isolated pulmonary arteries, deficiency of BMPR-II increased the contractile response to serotonin.

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mechanism of this interaction includes increased serotonin-induced activation of extracellular signal-regulated kinase (ERK) 1/2 in BMPR-II–deficient cells and inhibition of BMP/Smad signaling by serotonin.

Materials and Methods

Experimental Design

BMPR2 knockout mice were generated as previously described and kindly provided by Dr Beppu (Massachusetts General Hospital, Boston).13 Heterozygous null BMPR2+− mice or wild-type littermate controls were used in these studies (strain C57BL/6J). Adult male mice (8 to 12 weeks of age; 25 to 30 g body weight [BW]) were used throughout. Animal experiments were reviewed and approved by the local animal care committee. Groups of wild-type and BMPR2+− mice were maintained in room air or in the hypoxic chamber.

Hemodynamic Evaluation

Following treatments, mice were anesthetized using Hypnorm (fentanyl and fluanisone 0.25 mL/kg and midazolam 25 mg/kg by intraperitoneal injection). BW was recorded, and right ventricular systolic pressure (RVSP) was measured via direct cardiac puncture, as previously described.14 To assess right ventricular hypertrophy, systolic pressure (RVSP) was measured via direct cardiac puncture, and its agonists or phenylephrine were expressed as a percentage of the response to 50 mmol/L KCl to calculate the maximum contraction (Fmax). The sensitivity to the various agonists (pECE50 values) was calculated from individual cumulative concentration response curves by graphical interpolation (Graphpad Prism).

Quantitative RT-PCR

Total RNA was extracted from BMPR2+− and wild-type mouse lungs (n=3 of each) then reverse transcribed. Quantitative PCR was performed using Eurogent SYBR1 green core reagents and samples were run on a Stratagene MX4000 multiplex QPCR system. Further quantitative PCR analysis was performed for expression of the inhibitor of DNA binding 3 (Id3) gene in mouse PASMCs treated with BMP2 and/or serotonin and normalized to β-actin. All samples were analyzed in the same sample run for Id3 and β-actin.

Western Immunoblotting

Protein was extracted from wild-type and BMPR2+− lung and subjected to immunoblotting. Blots were probed with a polyclonal antibody to BMPR-II as previously described,17 then stripped and reprobed with a monoclonal antibody to β-actin (AC-15, Sigma, Poole, UK). In protein derived from lungs and PASMCs, we determined the phosphorylation of Smad1/5 (951b, Cell Signaling, Hitchin, UK) or ERK1/2 (4377, Cell Signaling) using rabbit monoclonal antibodies as described previously.18 An antibody to total ERK1/2 was used as a loading control (Cell Signaling). The activation of Smad1/5 and ERK1/2 was studied in cell monolayers following stimulation by BMPs or serotonin, as previously described.19 Some studies were performed in the presence of diphenyleneiodonium (DPI), an inhibitor of the NAPDH oxidase.

Statistics

Data are presented as means±SE. Data between groups were compared using a 2-tailed t test or a 1-way analysis of variance followed by Tukey’s HSD test, whichever was appropriate. P<0.05 indicated a statistically significant result.

Results

Phenotyping of BMPR2+− and Wild-Type Mice Under Normoxia and Chronic Hypoxia

The mean RVSP in BMPR2+− mice maintained in room air was indistinguishable from wild-type littermate controls (Figure 1a). In addition, the ratio RV/LV + S was similar between genotypes (Figure 1b). We questioned whether BMPR2+− mice would develop more severe PAH than littermate controls in hypoxia. Exposure of animals to 2 or 3 weeks of continuous hypoxia increased RVSP and right ventricular hypertrophy (RVH) in all animals, with no significant differences between wild-type and BMPR2+− mice (Figure 1a and 1b). To ensure that measurements were not missing subtle changes in pulmonary vascular resistance between genotypes at baseline, we examined the pressure-flow characteristics of wild-type and BMPR2+− mouse lungs. Experiments were performed under both normoxic and acute hypoxic conditions (2% oxygen) to assess pulmonary vascular resistance under...
resting and elevated tone, respectively. Pressure-flow curves for wild-type and \textit{BMPR2}~\textsubscript{H11001}/\textsubscript{H11002} mice were similar, indicating no difference in pulmonary vascular resistance between genotypes (Figure 1c and 1d).

**Serotonin Increases Susceptibility to PAH in \textit{BMPR2}\textsuperscript{+/−} Mice**

Serotonin had no significant effect on RVSP or RVH in wild-type mice (Figure 2a and 2b). In contrast, \textit{BMPR2}\textsuperscript{+/−} mice exhibited a significant increase in RVSP when exposed to serotonin (22.4±0.9 mm Hg versus 17.7±0.8 mm Hg) (Figure 2a). In addition, serotonin infusion significantly increased the proportion of small arteries that became muscularized, although with no significant change in wall thickness of already muscularized arteries (Figure 3a and 3b). Infusion of serotonin for 2 weeks under hypoxic conditions further exaggerated the differences between genotypes (Figure 1c and 1d).

**Expression of BMPR-II and Smad Signaling in Mouse Lung**

Western blotting for BMPR-II protein confirmed the reduction in receptor expression in \textit{BMPR2}\textsuperscript{+/−} mice compared with controls (supplemental Figure I, available online at http://circres.ahajournals.org). Quantitative RT-PCR also confirmed that \textit{BMPR2}\textsuperscript{+/−} mice express BMPR-II mRNA at approximately 50% of levels found in wild-type littermates (supplemental Figure I). No change in BMPR-II mRNA transcript levels was observed in animals treated with serotonin. Therefore, we determined the phosphorylation of Smad1/5 proteins, the main signaling intermediaries downstream of BMP receptors, in mouse lungs exposed to 2 weeks of normoxia or hypoxia (Figure 4a and 4b), with no difference between genotypes. Infusion of serotonin in wild-type mice had no consistent effect on phosphorylation of Smad1/5 under normoxic conditions (data not shown), although the phosphorylation signal was low in normoxia. Thus, we examined the effect of serotonin infusion on \textit{Id3} mRNA expression in...
normoxic mouse lungs. Basal Id3 expression was similar in wild-type and \( B\)MPR2\(^{+/−}\) lungs, but serotonin depressed Id3 expression in both (see supplemental Figure II). Under hypoxic conditions serotonin infusion consistently reduced the elevated level of phosphorylated Smad1/5 (Figure 4c and 4d) in both wild-type and \( B\)MPR2\(^{+/−}\) mice. Taken together, these data suggest that serotonin inhibits BMP signaling pathways in the normoxic and hypoxic lung.

**Heightened Proliferation to Serotonin in \( B\)MPR2\(^{+/−}\) PASMCs**

To further explore the interaction between serotonin and BMPR-II deficiency, we performed experiments in isolated PASMCs. PASMC monolayers were >95% positive for smooth muscle \( \alpha \)-actin, whereas mouse fibroblasts did not stain when cultured under the same conditions (not shown). Serotonin increased \([H]\)-thymidine incorporation in

**Figure 2.** Serotonin potentiates susceptibility to pulmonary hypertension in \( B\)MPR2\(^{+/−}\) mice. a, Right ventricular systolic pressure in normoxic mice treated with saline (\( n=9 \)) or serotonin (\( n=12 \)) for 2 weeks. b, RV/LV+S ratios as an index of right ventricular hypertrophy. c and d, The same indices studied in mice treated with saline (\( n=8 \)) or serotonin (\( n=12 \)) for 2 weeks under hypoxic conditions. *\( P<0.05 \) compared with corresponding saline treated mice.

**Figure 3.** Serotonin exaggerates vascular remodeling in \( B\)MPR2\(^{+/−}\) mice. a, Percentage wall thickness in small muscular arteries accompanying terminal bronchioles in normoxic mice treated with saline (\( n=9 \)) or serotonin (\( n=12 \)) for 2 weeks. b, Percentage of muscularized arteries at the level of alveolar ducts. c and d, The same indices studied in mice treated with saline (\( n=8 \)) or serotonin (\( n=12 \)) for 2 weeks under hypoxic conditions. *\( P<0.05 \) compared with corresponding saline treated mice.
PASMCs derived from BMPR2−/− mice, compared with wild-type littermates (Figure 5a). The observed increase in DNA synthesis corresponded to increased cell number (Figure 5b). Increased [3H]-thymidine incorporation in response to serotonin in BMPR2−/− cells was blocked by the 5-HT2A antagonist, ketanserin, at both 0.1 μmol/L and 1 μmol/L concentrations, but not by a 5-HT2B antagonist or an inhibitor of the serotonin transporter (Figure 5c). Additional studies failed to show an exaggerated response to the 5-HT1 agonist, 5-carboxamidotryptamine, in BMPR2−/− PASMCs (not shown).

**Potentiation of Serotonin Signaling in BMPR2-Deficient Cells**

To further investigate the basis of the exaggerated growth response to serotonin in BMPR2-deficient PASMCs, we determined the activation of ERK1/2, reported to be involved in the proliferative response to serotonin.19,20 Immunoblotting demonstrated that serotonin exposure caused a greater activation of ERK1/2 in BMPR2−/− PASMCs compared with wild-type cells (Figure 6a). In addition, activation of ERK1/2 by serotonin was inhibited by preincubation with ketanserin, consistent with the results of the [3H]-thymidine studies. The activation of ERK1/2 by serotonin is thought to be mediated via an increase in intracellular superoxide ($\text{O}_2^•$).20 Inhibition of intracellular $\text{O}_2^•$ generation by the inhibitor of NAD(P)H oxidase, DPI, significantly reduced the activation of ERK1/2 by serotonin in wild-type and BMPR2−/− cells (Figure 6b).

**Serotonin Interferes With BMP Signaling In Vitro**

Next we examined whether serotonin inhibited BMP signaling in PASMCs. We stimulated PASMCs with BMP2 (30

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**Figure 4.** Phospho-Smad1/5 expression in mouse lung. Immunoblots for phospho-Smad1/5 in wild-type (a) and BMPR2−/− (b) lungs under normoxic (n=3) and hypoxic (n=4) conditions. Two-week serotonin infusion reduced lung phospho-Smad1 levels under hypoxic conditions in wild-type (c) and BMPR2−/− (d) lungs (n=4 for serotonin treated and saline treated groups). Quantification of the phospho-Smad1/5 signal, compared with each β-actin loading control, is shown next to each blot. *P<0.05.
ng/mL) for 1 hour and, as expected, found that phosphorylation of Smad1/5 was reduced in BMPR2−/− cells (Figure 7a).

More importantly, preincubation of PASMCs with serotonin for 30 minutes before stimulation with BMP2 showed inhibition of Smad1/5 phosphorylation in wild-type and BMPR2−/− cells (Figure 7a). To investigate further the functional impact of serotonin on BMP signaling, we chose a well-documented transcriptional target of BMP/Smad signaling, Id3. Real-time quantitative RT-PCR studies revealed that serotonin powerfully inhibited the transcription of Id3 mRNA induced by BMP2 in wild-type and BMPR2−/− cells (Figure 7b and 7c), although the basal expression of Id3 was not significantly different in wild-type and BMPR2−/− cells. Interestingly, the transcription of Id3 mRNA in response to BMP2 was markedly reduced in BMPR2−/− cells (∼3.5-fold) compared with wild type (∼40-fold), although the inhibitory effect of serotonin was still evident (Figure 7c).

Pulmonary Arteries From BMPR2+/− Mice Demonstrate Increased Contractile Responses to Serotonin

Increased vascular tone, as well as vascular remodeling, contributes to pulmonary hypertension in animal models. Therefore, we investigated the contractile response of intrapulmonary arteries from wild-type and BMPR2−/− mice to serotonin. In BMPR2−/− mouse vessels, serotonin induced a more profound contraction, with the maximum response being increased nearly 2-fold to that observed in wild-type vessels (Figure 8a and supplemental Table I). The affinity of serotonin (pEC50) was not altered. In contrast, phenylephrine, an α1 receptor agonist, produced similar contractile responses in vessels from wild-type and BMPR2−/− mice, demonstrating pharmacological restriction of the exaggerated response to serotonin (Figure 8b).
Discussion

This study has demonstrated that deficiency of BMPR-II increases susceptibility to PAH induced by serotonin in mice. Although the pulmonary vascular phenotype of BMPR2<sup>−/−</sup> and wild-type littermates was similar under normoxic or chronic hypoxic conditions, infusion of serotonin increased RVSP, RVH, and pulmonary vascular remodeling in BMPR2<sup>−/−</sup> mice compared with controls. These data provide the first evidence for cross-talk between the BMP and serotonin pathways, both key systems implicated in the pathogenesis of pulmonary hypertension. Serotonin infusion was associated with a reduction in lung Smad1/5 activation in hypoxic mice. Serotonin inhibited BMP signaling in PASMCs, as evidenced by inhibition of Smad1/5 phosphorylation and inhibition of Id3 transcription. Furthermore, PASMCs isolated from BMPR2<sup>−/−</sup> mice exhibited a heightened DNA synthesis to serotonin and increased activation of ERK1/2 via O<sub>2</sub><sup>−</sup>. Moreover, we found that pulmonary, but not systemic, arteries from BMPR2<sup>−/−</sup> mice demonstrated an increased contractile response to serotonin, compared with arteries from wild-type littermates. Taken together, these data support the hypothesis that a deficiency of BMPR-II increases susceptibility to PAH and that serotonin may be a permissive factor in the manifestation of disease.

We reasoned that BMPR2<sup>−/−</sup> mice may be a useful genetic model of FPAH in humans, because many disease causing mutations in human BMPR2 likely result in haploinsufficiency. However, we observed no difference between BMPR2<sup>−/−</sup> mice and wild-type littermates with regard to RVSP, RVH, or muscularization of peripheral pulmonary arteries. In addition, pressure-flow curves in the isolated perfused lung suggested no difference in pulmonary vascular resistance. These results are consistent with a recent report of the pulmonary vascular phenotype in the same BMPR2<sup>−/−</sup>
mice used in the present study,21 although they differ from earlier results in the same animal.22 Beppu et al22 reported that BMPR2−/− mice exhibit mild elevation of pulmonary vascular resistance and increased wall thickness of muscular pulmonary arteries compared with wild-type littermates under normoxic conditions. Taking these reports together the pulmonary vascular phenotype in unchallenged BMPR2−/− mice is, at best, only subtly different from wild-type controls. Song et al21 recently demonstrated that BMPR2−/− mice develop more severe pulmonary hypertension than wild-type controls in the setting of pulmonary inflammation induced by adenovirus-mediated overexpression of 5-lipoxygenase. However, overexpression of a dominant-negative BMPR-II in vascular smooth muscle appears to be sufficient for the development of pulmonary hypertension in transgenic mice.23 Taken together, the in vivo data suggest that BMPR2 haploinsufficiency is not in itself sufficient to generate significant pulmonary hypertension in mice but increases susceptibility to specific pulmonary hypertensive stimuli including serotonin and inflammation induced by 5-lipoxygenase. In contrast to these studies, mice deficient in the BMPR-II ligand BMP424 are protected from hypoxia-induced pulmonary hypertension. These studies suggest that deficiency of a single ligand is not the functional equivalent of receptor deficiency and may indicate a specific function of BMPs during hypoxia.

Because serotonin has been widely implicated in the pathogenesis of IPAH,8 we investigated the possibility that serotonin could influence the pulmonary vascular phenotype of BMPR2−/− mice. Serotonin was infused over a period of 2 weeks at a dose (5 nmol/h) previously shown to elevate blood serotonin levels in rats.9 In the present study, serotonin increased susceptibility to PAH in BMPR2−/−, but not wild-type, mice. This potentiation became more evident under chronic hypoxic conditions. Although numerous mechanisms might contribute to the effects of serotonin in BMPR2−/− mice, we hypothesized that serotonin may exacerbate a deficiency in BMP signaling. Interestingly, chronic hypoxia was associated with an increase in Smad1/5 phosphorylation in mouse lung, consistent with recent reports of increased lung BMP expression under similar hypoxic conditions.24 Hypoxia-induced activation of Smad1/5 occurred to a similar extent in wild-type and BMPR2−/− lungs. However, the in vivo environment is complex with multiple ligands and receptors capable of Smad1/5 activation. Nevertheless, immunoblotting of mouse lung protein after 2 weeks of serotonin infusion showed a reduction in hypoxia-induced Smad1/5 activation. Furthermore, serotonin infusion reduced Id3 mRNA expression in wild-type and BMPR2−/− mouse lungs. We have previously demonstrated that Smad1/5 activation

Figure 8. Increased contractile responses to serotonin in BMPR2−/− pulmonary arteries. a and b, Cumulative concentration-response curves to serotonin (a) and phenylephrine (b) in small muscular arteries from wild-type and BMPR2−/− mice. c and d, Cumulative concentration-response curves to 5-CT (c) and α-methyl-5-HT (d) in small muscular arteries from wild-type and BMPR2−/− mice. e, Cumulative concentration-response curves to serotonin in BMPR2−/− pulmonary arteries in the presence and absence of ketanserin (30 nmol/L) and SB224289 (200 nmol/L).
exerts antiproliferative effects on human PASMCs. Thus, a suppression of Smad1/5 phosphorylation during serotonin infusion might exert a permissive effect on vascular remodeling via Smad responsive genes.

Our data from whole mouse lungs exposed to 2 weeks of serotonin infusion suggested that serotonin may inhibit BMP signaling in vivo. The effects in vivo are likely to be complex, with numerous inputs from diverse lung cellular compartments, BMPs and, BMP receptors. We, therefore, investigated this phenomenon further in isolated PASMCs from wild-type and BMPR2−/− mice. BMP4-induced Smad1/5 activation was reduced in BMPR2−/− cells. This is consistent with a recent report in BMPR2−/− PASMCs in which multiple BMP ligands showed reduced activation of Smad1/5. In that study, complete ablation or knockdown of BMP- II expression led to a gain of function in terms of increased activation of Smad 1/5 in response to BMP6 and -7. In the present study, exposure of PASMCs to serotonin inhibited the BMP2−/− induced phosphorylation of Smad1/5 in wild-type and BMPR2−/− cells. In addition, we determined the effect of serotonin on the transcription of a known target gene of BMP signaling, the helix-loop-helix factor inhibitor of DNA binding 3 (Id3), implicated in vascular smooth muscle growth and differentiation. The transcription of Id3 in response to BMP2 was reduced in BMPR2−/− PASMCs compared with controls, and serotonin exerted a marked inhibitory effect on Id3 transcription in wild-type and BMPR2−/− cells. Taken together, these findings suggest that serotonin inhibits the activation of BMP-dependent antiproliferative pathways in PASMCs.

A further key finding in this study was that PASMCs from BMPR2−/− mice exhibited an increased proliferative response to serotonin compared with wild-type cells. This proliferative response was inhibited by the 5-HT2A receptor antagonist, ketanserin, but not by inhibitors of the 5-HT1, 5-HT2B, or the 5-HT3. Serotonin caused an exaggerated activation of the proproliferative ERK1/2 pathway in BMPR2−/− PASMCs. This effect was also mediated via the 5-HT2A receptor and was at least partly dependant on O2 generation. Taken together, our in vitro results suggest that serotonin inhibits antiproliferative Smad1/5 signaling in wild-type and BMPR2−/− PASMCs and selectively enhances ERK1/2 proproliferative pathways in BMPR2−/− cells. We have previously suggested that an imbalance between antiproliferative Smad1/5 signals and proproliferative ERK1/2 pathways contribute to proliferation in BMPR2 mutant human PASMCs, a concept supported by the present study. These results provide a potential mechanism for the increased susceptibility to pulmonary hypertension induced by serotonin on a background of BMPR2 deficiency. Although our data confirm a role for O2 in the activation of ERK1/2 by serotonin, it remains to be determined whether deficiency of BMPR2 leads to intrinsic alterations in intracellular O2 generation.

Recent work has implicated the serotonin transporter, 5-HTT, in the proliferative response of human PASMCs to serotonin and mice overexpressing the 5-HTT develop spontaneous PAH in normoxic conditions. The 5-HT3 receptor has previously been shown to exert effects on vascular remodeling, matrix deposition, and transforming growth factor-β expression in hypoxic rats and mice. However, our data suggest that the functional interaction between serotonin and BMPR-II deficiency occurs mainly via the 5-HT2A receptor in the mouse.

In addition to vascular remodeling, vasoconstriction plays a role in the pathogenesis of PAH. Therefore, we studied the contractile response to serotonin in pulmonary arteries using wire myography. BMPR2−/− pulmonary arteries demonstrated an increased contractile response to serotonin. The efficacy, but not affinity, of serotonin was increased in the BMPR2−/− mice, suggesting that there may be an increase in serotonin receptor number. To investigate this further, we studied the effects of the 5-HT2A agonist α-methyl-5-HT and the 5-HT1 agonist 5-CT and observed an increase in the Emax of α-methyl-5-HT, suggesting an increase in 5-HT2A receptors in these vessels. The lack of contraction of mouse pulmonary artery to 5-HT2B agonists (M.R.M., unpublished observation, 2005) makes it likely that it is 5-HT2A mediating contraction in these experiments. The response to 5-CT was also increased. We know that contractile responses to 5-CT are inhibited by both acute and chronic treatment with 5-HT1B/D antagonists. The 5-HT1A receptor subtype mediates contraction in human small muscular pulmonary arteries. It is also the 5-HT1B receptor that mediates hypoxic pulmonary hypertension in rat and mouse models, and, hence, it is likely that there is an increased response to 5-HT1B receptors in the BMPR2−/− vessels studied here. The involvement of these specific receptor subtypes was confirmed with the use of selective inhibitors of 5-HT1A and 5-HT1B, both of which were able to inhibit the heightened contractile response to serotonin in BMPR2−/− arteries.

Our results indicate the presence of a relatively specific functional antagonism between the BMPR-II and serotonin signaling pathways, with deficiency of BMPR-II potentiating the contractile and growth response to serotonin in vitro and increasing susceptibility to PAH and pulmonary vascular remodeling in vivo. The molecular mechanism of this interaction includes inhibition of Smad signaling and BMP target gene transcription by serotonin and enhanced activation of proproliferative ERK1/2 pathways by serotonin in the setting of BMPR2 deficiency. These findings provide a link between 2 key systems widely implicated in the pathogenesis of pulmonary arterial hypertension.

Acknowledgments
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References


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Online Figure 1.

Expression of BMPR-II mRNA and protein. 

**a**, Western blot of BMPR-II protein expression in wild type (+/+) and BMPR2+/- mice (n=3 of each) showing reduced expression of BMPR-II in heterozygous mice. Beta-actin is shown as a loading control; 
b, levels of BMPR-II transcripts in control mice and BMPR2+/- mice and after exposure to serotonin for 2 weeks. *P<0.05 compared with wild type control mice, samples repeated in triplicate from n=3 mice; data represent means and 95% confidence intervals.
Online Figure 2. Real time RT-PCR expression of Id3 mRNA normalised to β-actin in wild type (+/+) and BMPR2 heterozygous (+/-) mouse lungs under control normoxic conditions or after 2 weeks of serotonin (5HT) exposure. Results represent the means of 3 separate experiments. *P<0.05 compared with control.
Online Figure 3. a, cumulative concentration response curves to serotonin and b, phenylephrine in small mesenteric arteries from wild type and BMPR2+/- mice.
Online Table 1

Table 1. Affinity (pEC50) and efficacy (Emax) of serotonin (5-HT), a-methyl-5-HT and 5-carboxamidotryptamine (5-CT) in small muscular arteries from wild type (+/+ ) and BMPR2+/- (+/-) mice

<table>
<thead>
<tr>
<th>Group</th>
<th>pEC50</th>
<th>Emax</th>
<th>n</th>
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<tr>
<td>+/+ 5-HT</td>
<td>7.23 ± 0.14</td>
<td>127 ± 11</td>
<td>6</td>
</tr>
<tr>
<td>+/- 5-HT</td>
<td>7.06 ± 0.13</td>
<td>240 ± 21***</td>
<td>5</td>
</tr>
<tr>
<td>+/- a-methyl-5-HT</td>
<td>6.78 ± 0.20</td>
<td>85 ± 17</td>
<td>5</td>
</tr>
<tr>
<td>+/- a-methyl-5-HT</td>
<td>7.09 ± 0.16</td>
<td>189 ± 32*</td>
<td>8</td>
</tr>
<tr>
<td>+/- 5-CT</td>
<td>nc</td>
<td>nc</td>
<td>5</td>
</tr>
<tr>
<td>+/- 5-CT + BMP4</td>
<td>5.62 ± 0.25</td>
<td>163 ± 28</td>
<td>8</td>
</tr>
<tr>
<td>+/- 5-HT + BMP4</td>
<td>7.29 ± 0.16</td>
<td>113 ± 10</td>
<td>4</td>
</tr>
<tr>
<td>+/- 5-CT + BMP4</td>
<td>7.61 ± 0.18</td>
<td>131 ± 8****</td>
<td>4</td>
</tr>
</tbody>
</table>

Data shown as mean ± SEM, n= number of mice. Statistical analysis was by ANOVA with Neuman-Keuls post test ***P<0.001, *P<0.05 cf +/+ ; ****P<0.001 cf +/- 5-HT nc. Not calculated