Sildenafil Potentiates Bone Morphogenetic Protein Signaling in Pulmonary Arterial Smooth Muscle Cells and in Experimental Pulmonary Hypertension

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Objective—Mutations in the bone morphogenetic protein type II receptor (BMPR-II) are responsible for the majority of cases of heritable pulmonary arterial hypertension (PAH), and BMPR-II deficiency contributes to idiopathic and experimental forms of PAH. Sildenafil, a potent type-5 nucleotide-dependent phosphodiesterase inhibitor, is an established treatment for PAH, but whether sildenafil affects bone morphogenetic protein (BMP) signaling in the pulmonary circulation remains unknown.

Methods and Results—Studies were undertaken in human pulmonary arterial smooth muscle cells (PASMCs) and in vivo in the monocrotaline rat model of PAH. In PASMCs, sildenafil enhanced BMP4-induced phosphorylation of Smad1/5, Smad nuclear localization, and Inhibitor of DNA binding protein 1 gene and protein expression. This effect was mimicked by 8-bromo-cyclic GMP. Pharmacological inhibition or small interfering RNA knockdown of cyclic GMP-dependent protein kinase I inhibited the effect of sildenafil on BMP signaling. In functional studies, we observed that sildenafil potentiated the antiproliferative effects of BMP4 on PASMC proliferation. Furthermore, sildenafil restored the antiproliferative response to BMP4 in PASMCs harboring mutations in BMPR-II. In the monocrotaline rat model of PAH, which is characterized by BMPR-II deficiency, sildenafil prevented the development of pulmonary hypertension and vascular remodeling, and partly restored Smad1/5 phosphorylation and Inhibitor of DNA binding protein 1 gene expression in vivo in monocrotaline exposed rat lungs.

Conclusion—Sildenafil enhances canonical BMP signaling via cyclic GMP and cyclic GMP-dependent protein kinase I in vitro and in vivo, and partly restores deficient BMP signaling in BMPR-II mutant PASMCs. Our findings demonstrate a novel mechanism of action of sildenafil in the treatment of PAH and suggest that targeting BMP signaling may be beneficial in this disease. (Arterioscler Thromb Vasc Biol. 2013;33:34-42.)

Key Words: BMP signaling pathway | cGKI | PDE5 inhibitor | pulmonary hypertension | smooth muscle cell

Pulmonary arterial hypertension (PAH) develops as a result of profound remodeling of the lung vasculature, characterized by heightened proliferation and apoptosis resistance of vascular cells in the media and neointima. A reduction in cross-sectional area of the small pulmonary arteries and stiffened large pulmonary arteries increase pulmonary vascular resistance and elevate pulmonary arterial pressure. Although the molecular and cellular mechanisms that initiate vascular remodeling remain unclear, mutations in the bone morphogenetic protein type II receptor (BMPR-II) provide the strongest known risk factor for disease, being responsible for the majority of cases of heritable PAH.1-3 Mutations in BMPR-II lead to a loss of the growth-suppressive effects of BMPs in pulmonary artery smooth muscle cells via a reduction in downstream Smad1/5 phosphorylation and reduced transcription of Inhibitor of DNA binding protein 1 (IId1), which is a major target gene in response to BMP signaling.4-5 Recently, we reported that prostacyclin analogues enhance BMP/Smad pathway through cAMP, but knowledge of the impact of other existing PAH therapies on BMP signaling remain unclear.6

The production of endothelial nitric oxide and the stimulation of smooth muscle-soluble guanylyl cyclase to generate cGMP is an important vasodilator and antiproliferative pathway in the pulmonary circulation.7 Phosphodiesterase type 5 (PDE5) is largely responsible for the hydrolysis of

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cGMP and is expressed at a high level in the pulmonary circulation compared with systemic vessels. Decreased endothelial nitric oxide production and increased PDE5 expression and activity are 2 important pathologic features of PAH. The resulting decrease in the level of intracellular cGMP contributes to vasoconstriction and proliferation of vascular smooth muscle cells. Sildenafil, a specific PDE5 inhibitor, increases intracellular cGMP levels and promotes vasodilatation. It is widely used as an effective treatment for clinical PAH. Recently, Schwappacher et al reported that cGMP-dependent kinase I (cGKI) modulates BMP signaling in a mouse myoblast cell line. These authors found that cGKI interacts with BMPR-II, and that on BMP stimulation, cGKI is released to associate with activated Smad proteins and regulates gene expression as a nuclear cofactor for Smads. cGKI is one of the major mediators of nitric oxide/cGMP-triggered signal transduction. It is highly expressed in vascular smooth muscle cells and plays an important role in the regulation of vascular tone. Although cGKI may be involved in the growth-suppressive effects of aortic smooth muscle cells by BMPs, it remains unknown whether sildenafil exerts some of its beneficial actions through regulating the BMP signaling pathway in human pulmonary arterial smooth muscle cells (PASMCs).

Figure 1. Sildenafil increases bone morphogenetic protein 4 (BMP4)-induced Smad1/5 signaling and Inhibitor of DNA binding protein 1 (Id1) gene expression in human pulmonary arterial smooth muscle cells (PASMCs). Immunoblots show the phosphorylation of Smad1/5 in PASMCs in response to sildenafil (1 µmol/L and 5 µmol/L) or BMP4 (10 ng/mL) after 24 hours (A). Quantification by densitometry in 3 separate experiments confirms the effects of sildenafil on BMP4-stimulated phospho-Smad1 levels (B). 10% FBS– (Con) and sildenafil (Sil)-treated cells stain negatively for phospho-Smad1/5 (green), BMP4-treated cells (3 hours) shows positive nuclear staining for phospho-Smad1/5, increased further by sildenafil. 4′,6-diamidino-2-phenylindole (DAPI) staining indicates the nuclei of cells (C). Quantification of nuclear localization is shown in D. Real-time PCR demonstrates increased Id1 gene expression induced by sildenafil (1 and 3 µmol/L) in the presence of BMP4 (10 ng/mL) in PASMCs (E). *P<0.05 compared with control; #P<0.05 compared with BMP4 treatment alone.
In the present study, we demonstrate that sildenafil enhances Smad1/5 phosphorylation after BMP4 stimulation via a cGMP/cGKI-dependent mechanism. Sildenafil also enhanced Smad1 binding on the Id1 promoter and further elevated Id1 gene expression induced by BMP4. Moreover, sildenafil partly rescued the defect in Smad1/5 activation and Id1 gene expression in human PASMCs harboring mutations in BMPR-II and restored BMP4-mediated growth-suppression in BMPR-II mutant cells. In vivo studies confirmed that sildenafil inhibited the progression of pulmonary hypertension and enhanced BMP signaling in the monocrotaline rat model, which is characterized by BMPR-II dysfunction.

Materials and Methods

An expanded version of the Methods section is available in the online-only Data Supplement.

Human PASMC Culture

Cells were derived from peripheral pulmonary arteries (<2 mm external diameter) or lobar arteries, as previously described.

Immunoblotting

PASMCs were treated with or without BMP4 or BMP2 (1 to 10 ng/mL) in 0.1% FBS/Dulbecco modified Eagles medium for up to 24 hours. Sildenafil citrate was generously provided by Pfizer (Pfizer Ltd, Walton Oaks, UK). Sildenafil was used over the concentration range 10 nmol/L to 5 μmol/L. In some experiments, the selective competitive inhibitor of cGKI, Rp-8-Br-PET-cGMP was used at 30 μmol/L. To assess activation of canonical BMP signaling, blots were incubated with polyclonal rabbit antiphospho-Smad1/5 or Smad6 (cell signaling) antibody or polyclonal rabbit anti-Id1 (CalBioreagents), as previously described. To confirm equal protein loading, blots were stripped and reprobed using an antibody against β-actin (Sigma).

Cell Proliferation Assay

PASMCs were seeded in 24-well plates (2×10⁴ cells per well) overnight in Dulbecco modified Eagles medium containing 10% FBS, then exposed to sildenafil or BMP4 in 10% FBS/Dulbecco modified Eagles medium or 10% FBS/Dulbecco modified Eagles medium alone. Medium was changed daily, and cells were counted on day 3 with a hemocytometer.

Small Interfering RNA Knockdown of cGKI

PASMCs were transfected with cGKI small interfering RNA (siRNA; Dharmacon) or control siRNA (siCP nontargeting pool, Dharmacon) using Dharmafect transfection reagent, following the manufacturer’s instructions.

Chromatin Immunoprecipitation

Assays were performed following the manufacturer’s instructions (Active Motif) with some modifications. DNA samples were analyzed by real-time PCR amplification using KAPA SYBR FAST qPCR reagents (Kapabiosystems).

The Monocrotaline Rat Model and Sildenafil Treatment

Male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) either received a single injection of monocrotaline (MCT, 60 mg/kg, s.c.) or saline. Three weeks after MCT injection, animals were treated for an additional 2 weeks with either 50 mg sildenafil/kg bodyweight daily or vehicle by gavage. Six animals were used per
At the end of the treatment protocol, rats were anesthetized for hemodynamic assessment, and then euthanized for measurement of right ventricular hypertrophy. Lung tissue preparation, staining, and vascular morphometry were performed as described. Intra-acinar arteries were analyzed by categorizing them as fully muscular, partially muscular, and nonmuscular. All analyses were done in a blinded fashion, and the experiments were performed according to institutional guidelines that complied with national and international regulations.

### PCR Methods

Quantitative-PCR was performed using SYBR1 GreenER qPCR SuperMix (sigma), and samples were run on an Applied Biosystems StepPlusOne Realtime PCR system.

### Statistics

Data are presented as mean±SEM. Means were compared between groups using a 2-tailed \( t \) test, or a 1-way analysis of variance followed by Tukey Honestly Significant Difference test, whichever was appropriate. \( P<0.05 \) was used to indicate a statistically significant result.

### Results

#### Sildenafil Enhances BMP-Stimulated Smad1/5 Phosphorylation and Nuclear Localization in PASMCs

To investigate the direct effect of sildenafil on BMP signaling, we first determined whether sildenafil alters BMPR-II expression or phosphorylation of Smad1/5 in human PASMCs. Sildenafil (1 μmol/L) alone had no effect on basal Smad1/5 phosphorylation in the absence of BMP4. As expected, BMP4 (10 ng/mL) induced Smad1/5 phosphorylation, but this effect was potentiated by cotreatment with sildenafil (1 and 5 μmol/L) in human PASMCs (Figure 1A and 1B) after 24 hours of exposure. This was not a result of a change in the level of BMP receptor expression, because over the same time period sildenafil had no effect on the expression level of BMPR-II mRNA (Figure 1 in the online-only Data Supplement). In addition, this was not a result of the suppression of the inhibitory Smad, Smad6 (Figure II in the online-only Data Supplement). Similar results were obtained with BMP2 and sildenafil (Figure III in the online-only Data Supplement). To determine whether sildenafil enhances nuclear localization of Smads, we examined the localization of phospho-Smad1/5 by confocal immunofluorescence microscopy in PASMCs. After stimulation with BMP4 (10 ng/mL) for 3 hours, phospho-Smad1/5 nuclear accumulation was observed in 38.2±3.2% of cells. BMP4 and sildenafil cotreatment increased nuclear accumulation to 70.3±13.2% of cells (Figure 1C and 1D). Incubation with sildenafil alone did not affect Smad1/5 nuclear localization.

#### Sildenafil Enhances Id1 mRNA Expression in PASMCs and Binding of Smad1 on the Id1 Promoter

Sildenafil alone had no effect on the level of Id1 gene transcripts at 24 hours. In the presence of BMP4, sildenafil significantly enhanced Id1 expression compared with BMP4 treatment alone (Figure 1E). It has been reported previously that there is a functional cGKI/Smad-binding site on the mouse Id1 promoter in C2C12 cells. We used endogenous chromatin immunoprecipitation assays with either untreated, or BMP4- or sildenafil-treated human PASMCs to examine whether sildenafil could enhance Smad1 binding on the human Id1 promoter. We found that sildenafil increased BMP4-induced Smad1 binding on the Id1 promoter by 35.3% compared with BMP4-induced Smad1-binding alone (Figure IV in the online-only Data Supplement).
cGKI Contributes to the Activation of BMP Signaling by BMP4 in Human PASMCs

It has been reported that the interaction between cGKI and BMPR-II could enhance Smad1/Id signaling in mouse C2C12 cells. Here, we confirmed the interaction between endogenous BMPR-II and cGKI by co-immunoprecipitation in human PASMCs (Figure V in the online-only Data Supplement). To determine whether the cGKI plays a role in the regulation of BMP/Smad signaling in PASMCs, we used siRNA knockdown. Compared with scrambled siRNA control, specific cGKI siRNA reduced cGKI protein expression by 59.1±4.8% (Figure 2A). In the presence of BMP4, sicGKI reduced phosphorylated Smad1/5 to 43.6±3.2% compared with scrambled siRNA, suggesting that cGMP/cGKI regulates pSmad1/5 activation by BMP4 in human PASMCs (Figure 2B). Similarly, sicGKI reduced Id1 protein expression in the presence of BMP4 to 49.1±5.3% compared with siCP control siRNA (Figure 2C).

Effects of Sildenafil on BMP Signaling Are cGKI-Dependent

Rp-8-Br-PET-cGMP is a potent, selective competitive inhibitor of cGKI. The enhanced expression of Id1 protein and increased Smad1/5 phosphorylation seen with sildenafil in the presence of BMP4 was completely reversed by Rp-8-Br-PET-cGMP (Figure 3A and 3B). Conversely, the cell permeable cGMP analogue, 8-Br-cGMP, also increased Smad1/5 phosphorylation and Id1 protein expression at 1 and 10 μmol/L in the presence of BMP4 (Figure VI in the online-only Data Supplement). In BMPR-II mutant PASMCs, BMP4 (100 ng/mL) failed to induce cell proliferation in Dulbecco modified Eagles medium/10% FBS after 72 hours of incubation. However, conincubation with sildenafil restored sensitivity to BMP4 growth suppression (D). ##P<0.01 compared with the cotreatment of sildenafil and BMP4. Results represent means of 3 separate experiments.

Sildenafil Partly Rescues Downregulated BMP Signaling and Restores Growth Inhibitory Effects of BMPs in BMPR-II Mutant Human PASMCs

We previously reported that Smad1/5 phosphorylation and induction of Id1 by BMP4 is reduced in PASMCs harboring mutations in BMPR-II.5,12 Here, we questioned whether...
sildenafil could rescue Smad1/5 phosphorylation and Id1 gene expression in mutant PASMCs. In 3 independent BMPR-II mutant PASMC lines (W9X, C347Y, and C347R), we observed that sildenafil enhanced BMP4 activation of Smad1/5 and Id1 protein expression after 24 hours coincubation (Figure 4A and 4B, and Figure IX in the online-only Data Supplement). The functional significance of the crosstalk between PDE5 inhibitor and BMP/Smad signaling was studied by assaying cell proliferation. In control PASMCs, BMP4 inhibited serum-induced cell proliferation over 72 hours, as expected (Figure 4C). The coincubation of BMP4 with sildenafil at a concentration of 100 nmol/L greatly reduced cell proliferation close to that observed in cells maintained in 0.1% FBS for 72 hours. In similar experiments conducted in PASMCs from patients with BMPR-II mutations, we confirmed that these cells are refractory to the growth-suppressive effects of BMP4, as we have previously described (Figure 4D). However, coincubation with sildenafil restored the antiproliferative effect of BMP4 in mutant PASMCs, even at lower concentrations of sildenafil (Figure 4D). To confirm that Id1 contributes to the combined antiproliferative effects of sildenafil and BMP4 in PASMCs, we used combined siRNA knockdown of Id1 and Id3. In cells exposed to control siRNA, BMP4 and sildenafil significantly inhibited the serum-stimulated proliferation of PASMCs. In contrast, after combined Id1/Id3 knockdown, sildenafil and BMP4 failed to inhibit PASMC proliferation (Figure X in the online-only Data Supplement). The antiproliferative effect of BMP4 and sildenafil did not appear to be involved in apoptosis, as we observed no increase in the number of apoptotic cells judged by nuclear morphology in sildenafil- or BMP4-treated cells (Figure XI in the online-only Data Supplement).

**Sildenafil Increases Lung Smad1/5 Activity and Id1 Expression in MCT-Induced Pulmonary Hypertension**

To determine whether the effects of sildenafil on BMP signaling are relevant in vivo, we used the rat MCT model of pulmonary hypertension. We have previously shown that this model is characterized by a marked reduction in BMPR-II protein expression and reduced phospho-Smad1/5 and Id1 mRNA expression after 3 weeks. In the present study, immunoblot analysis of lysates from rat lungs 5 weeks after MCT exposure confirmed this (Figure 5A). Sildenafil had little
Effect on the expression of BMPR-II protein in the lungs of rats exposed to MCT but significantly enhanced Smad phosphorylation (Figure 5A and 5C). Moreover, sildenafil significantly increased lung Id1 mRNA expression (Figure 5D). Furthermore, we used confocal microscopy to determine that MCT exposure is associated with reduced expression of phospho-Smad1/5 (Figure 5E) and Id1 (Figure XII in the online-only Data Supplement) in PASMCs in vivo. Moreover, sildenafil treatment restored PASMCs expression of phospho-Smad1/5 and Id1 in this model (Figure 5E and Figure XII in the online-only Data Supplement).

Discussion

The selective PDE5 inhibitor, sildenafil, is an effective therapy approved for the treatment of patients with severe PAH.14–17 The main mechanism of action is usually attributed to specific inhibition of PDE5 and increased levels of intracellular cGMP and stimulation of cGKI. Recently, cGKI was found to be a modulator of BMP signaling.10 Because reduced BMP signaling is now widely implicated in the pathobiology of

Sildenafil Treatment Inhibits the Development of PAH and Vascular Remodeling in the MCT Model of Pulmonary Hypertension

Five weeks after exposure to MCT, rats exhibited severe elevation of right ventricular pressure, and right ventricular hypertrophy, compared with unexposed animals. In contrast, rats treated with sildenafil for the final 2 weeks of the 5-week protocol demonstrated a reduction in all of these indices, compared with saline-treated rats (Figure 6A and 6B). In addition, MCT-exposed rats treated with sildenafil exhibited less severe distal muscularization of small pulmonary arteries compared with saline-treated animals (Figure 6C). However, there was no significant reduction of medial wall thickness in arteries of 100 μm diameter after treatment with sildenafil (Figure 6D). In the walls of small pulmonary arteries, MCT exposure was associated with an increase in the number of Ki67-positive cells, as a marker of cell proliferation. After treatment with sildenafil, fewer Ki67-positive PASMCs were seen in the walls of small arteries (Figure 6E).
idiopathic and heritable PAH, we questioned whether the mechanism of action of sildenafil could include enhancement of BMP signaling. We demonstrate that sildenafil increased the level of Smad1/5 c-terminal phosphorylation in response to BMP4, and enhances BMP-stimulated Id1 gene expression. We provide evidence that cGKI is central to this effect, because both pharmacological inhibition of cGKI with Rp-8-Br-PET-cGMP, or cGKI knockdown, could abolish the effect. Significantly, we demonstrated that sildenafil partially rescued the defect in Smad1/5 phosphorylation and Id mRNA expression that we have previously observed in BMPR-II mutant PASMCs. Moreover, sildenafil restored BMP4-mediated growth suppression in BMPR-II mutant cells from heritable PAH patients. To provide in vivo evidence for the beneficial effect of sildenafil on BMP signaling, we used the MCT rat model of pulmonary hypertension. We have previously shown that this model is characterized by reduced expression of BMPR-II and reduced BMP signaling.11 Sildenafil treatment partly restored Smad1/5 phosphorylation and Id1 gene expression in this model, with little impact on the expression of the BMPR-II receptor. To our knowledge, this is the first report that sildenafil enhances the activity of the Smad/Id axis in response to BPs in vascular cells. Our findings also reveal the critical involvement of cGKI during BMP signaling in the pulmonary circulation.

Sildenafil is a preferential inhibitor of PDE5 with an IC50 of 3.5 nmol/L. A single dose of sildenafil (100 mg) in patients with pulmonary arterial hypertension results in a plasma level of 1.2 µmol/L.9 Thus, our in vitro studies were conducted at physiologically relevant concentrations of sildenafil (≤1 µmol/L).

The interaction between cGKI and BMP receptors was recently demonstrated in C2C12 cells, but it was not known whether a similar interaction occurs in human PASMCs. cGKI is highly expressed in vascular smooth muscle cells. Under physiological conditions, cGKI mediates many of the effects of endothelial-derived NO, which is of crucial importance for smooth muscle cell function.21–23 Here, we confirmed an interaction between endogenous BMPR-II and cGKI in human PASMCs. To examine the direct effect of cGMP/cGKI on BMP signaling in PASMCs, we incubated cells with 8-Br-cGMP. The enhancement of BMP-stimulated Id gene expression by 8-Br-cGMP treatment supports a direct effect of cGMP/cGKI on BMP signaling in PASMCs. To further substantiate this observation, we used siRNA knockdown of cGKI. A reduction of cGKI expression to 50% was sufficient to reduce the phosphorylation of Smad1, and the induction of Id1 gene expression by BMP4. In addition, pharmacological inhibition of cGKI with Rp-8-Br-PET-cGMP totally abolished the enhanced Smad1/5 activity seen in the presence of sildenafil and BMP4.

Thus, the potentiation of BMP signaling by sildenafil appears to be a result of activated cGKI. The interaction between cGKI and BMP signaling may occur at several levels from receptor to gene transcription. Of note, we did not observe a significant change in BMPR-II expression in the presence of sildenafil in PASMCs, despite the enhancement of BMP signaling. It was previously demonstrated that cGKI contributes to the regulation of Id1 gene transcription by forming a Smad1–cGKI–TFII transcription complex on the Id1 promoter. Id proteins are the major downstream targets of BMP signaling, and our previous study has suggested an important role for Id1 protein in pulmonary vascular cell proliferation. In the present study, we confirmed in PASMCs that BMP4 promotes nuclear localization of cGKI, and sildenafil increased binding of Smad1 to the Id1 promoter in the presence of BMP4, consistent with the previously reported mechanism of action of cGMP/cGKI on BMP signaling.

Further demonstration of the potential importance of this mechanism is our demonstration that sildenafil enhanced BMP4-stimulated Smad1/5 activation and Id1 protein expression in BMPR-II mutant cells from heritable PAH patients. We have previously shown, and confirmed here, that mutant PASMCs are resistant to the growth-suppressive effects of BPs. The sensitivity to BMP4 growth inhibition could be restored in the presence of relevant concentrations of sildenafil. We have previously established that Id1 is a major regulator of cell proliferation downstream of BMPR-II. Thus, it is likely that rescue of Smad/Id1 expression by sildenafil is responsible for restoration of the antiproliferative effect. Indeed, we confirmed here that siRNA knockdown of both Id1 and Id3 abolished the antiproliferative effects of BMP4 plus sildenafil. Dysfunction of BMPR-II is now widely recognized as a central feature of heritable and other causes of PAH. Thus, the effect of sildenafil on BMP signaling may have broad implications for its mechanism of action in this and other conditions.

Finally, in an in vivo study, we provided evidence that the reduction of Smad/Id signaling in the rat MCT model could be partly reversed by sildenafil treatment. In this study, 50 mg/kg sildenafil had a marked inhibitory effect on the progression of PAH, when commenced 3 weeks after MCT exposure. Sildenafil markedly restored lung phospho-Smad1/5 activity and Id1 gene expression compared with saline-infused rats. This was concomitant with improved hemodynamic indices and reduced muscularization of small pulmonary arteries in sildenafil-treated animals. Moreover, we have shown that sildenafil treatment was associated with reduced proliferation of PASMCs in vivo and increased immunostaining for phospho-Smad1/5 and Id1 in small pulmonary arteries.

Taken together, our in vitro and in vivo data demonstrate a novel mechanism of action of sildenafil in vascular smooth muscle cells by enhancing the Smad/Id protein axis via cGMP/cGKI. This mechanism contributes to the antiproliferative and antiremodeling effect of sildenafil in PAH.

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Disclosures

None.
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Supplement Material

Methods

Expanded human PASMC Culture

Control human PASMCs were obtained from patients undergoing lung resection for suspected malignancy (n=5), derived from sites distant to the tumor. Cells were derived from peripheral pulmonary arteries (< 2 mm external diameter) as previously described or lobar arteries for growth studies\(^\text{12}\). Cells were also derived from the lungs of patients undergoing heart-lung transplantation for PAH (n=3, at Papworth Hospital), known to harbor a mutation in the BMPR2 gene. These included 2 patients with a mutation in the kinase domain of BMPR2 in which arginine or tyrosine is substituted for cysteine at position 347 (C347R and C347Y) and 1 patient with an exon 1 nonsense mutation at amino acid 9, W9X, predicted to lead to haploinsufficiency. The local ethics review committee approved the study, and subjects or relatives gave written informed consent.

Immunofluorescence Analysis

For quantification of positive phosphorylated Smad1 nuclear localization, each of the four treatments were viewed under fluorescent illumination on a Leica SPE confocal microscope, using a 40×, 1.15 NA oil immersion objective by two independent observers blinded to the treatments. Alexa Fluor donkey anti Rabbit 488 was used as secondary antibody to stain endogenous phosphorylated Smad1. To avoid bias, cell count was standardized with nuclear Hoescht staining. A beginning point was chosen at random and the next point was obtained by moving in a grid pattern across the section. This resulted in data from 10 unbiased and representative fields per section. The degree of immunofluorescence staining did not vary widely among samples. Background fluorescence was determined for sections stained with preimmune serum.
Chromatin Immunoprecipitation

Assays were performed following the manufacturer's instructions (Active Motif) with some modifications. In brief, 1 to 2.0 × 10^6 cells were used in each ChIP assay. Fragmented DNA following enzyme digest was analyzed by gel electrophoresis to ensure that 200- to 500-bp sized fragments were obtained before proceeding with ChIP with 3 to 5 µg of antibody using Rabbit anti Smad1 was purchased from Cell signalling. DNA pellets were resuspended in DNase-free water, and analyzed by real-time PCR amplification using KAPA SYBR FAST qPCR reagents (KAPABIOSYSTEMS), and the following cycling program: a first cycle of 3 min, a second cycle for 3s at 95 °C, and 30 s at 60 °C, which was repeated 50 times. Finally, one cycle of dissociation of 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s was performed to confirm that the melting curve indicated the presence of one PCR amplified DNA species.

Real-Time PCR

Quiescent PASMCs were incubated with BMP4 (1 to 10 ng/mL) or DMEM alone for 4 or 24 hours. Total RNA was extracted using TRIzol reagent, then reverse transcribed using High Capacity cDNA Reverse Transcription Kit from Applied Biosystems. Quantitative-PCR was performed using SYBR GreenER qPCR SuperMix (Sigma), and samples were run on Applied Biosystems StepPlusOne RealtimePCR system. Primers were used to allow amplification of Id1 and normalized to β-actin, which was included in each sample run.

Immunoprecipitation

HPASMCs were washed with PBS and lysed directly by buffer (1%(v/v) Triton X-100, 150mM NaCl, 20mM Tris/HCl pH7.5, protease inhibitors (Roche Diagnostics), 1mM phenylmethylsulfonylfluoride. Immunoprecipitation was performed with 10µg of antibody
overnight under rotation at 4°C. After washing, the beads was boiled 10mins, precipitated proteins were separated by SDS-PAGE, transferred to PVDF membrane and analysed by immunoblotting.

cGMP concentration assay

hPASMCs were treated with sildenafil 1µM or 100µM, BMP4 10ng/mL or 100ng/mL for 10 min. The cell lysate were used for cGMP determination with a sensitive ELISA procedure (Parameter). The sensitivity of the assay was about 0.56 - 3.06 pmol/mL. The levels of cGMP were expressed as pmol/mL.

SiRNA knockdown of BMPRII

PASMCs were plated at 20,000/well in 8 well chamber slides, with DMEM containing 10% FBS and left overnight. Cells were then transfected with BMPRII siRNA (Smart pool, Dharmacon) or control siRNA (siCP non-targeting pool, Dharmacon) using Dharmafect transfection reagent, following the manufacturer’s instructions. 24 hours later, transfection media was changed to 200ng/mL BMP4 contained DMEM. After 30min, the medium was taken off. The cells were immersed in methanol for 10mins at 4°C before incubating with cGKI antibody (rabbit anti human, Assay designs) and α smooth muscle actin (mouse anti human, sigmal) for 1 hour, both 1:100 dilution. After incubating with Alexa 488 anti Rabbit and Alexa 586 anti mouse(both 1:100) as secondary antibody for 1 hours, slides were mounted with V E C T A S H I E L D® Mounting Medium. Each of the four treatments was viewed under fluorescent illumination on a Leica SPE confocal microscope.
Chromatin staining with Hoechst 33342

Control or staurosporine-treated cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) buffer for 20 min at 4°C, exposed to 5μg/mL Hoechst 33342 in PBS for 30 min at room temperature, washed three times with PBS and, finally mounted with VECTASHIELD® Mounting Medium. The cell preparations were examined under UV illumination with an Olympus fluorescence microscope.

Immunohistochemistry

All incubations were carried out at room temperature in a humid chamber, unless otherwise stated. Paraffin sections were dewaxed in xylene and rehydrated through descending series of ethanol concentrations. They were then washed in running tap water and in deionized water before incubation with 50μg/mL Proteinase-K (Roche Diagnostics Ltd, East Sussex, UK) in 0.1mol/L Tris-HCl buffered saline (TBS) at pH7.6 for 10 minutes for antigen retrieval before immunostaining. Both types of sections were then incubated with blocking buffer containing 0.5% bovine serum albumin (BSA) in TBS to suppress non-specific antibody binding. Excess blocking buffer was removed and sections were incubated onovernight at 4°C in primary antibody: rabbit anti Id1 (Cal Bioreagents) at 1:50 dilution and rabbit anti Ki67 (abcam) at 1:100, mouse anti α smooth muscle actin (sigma) 1:200 in blocking buffer. For phospho-Smad1/5 staining, antigen retrieval was performed by boiling slides for 10 minutes in 0.01mol/L sodium citrate, pH 6.0. Slides were incubated with anti phosphor-Smad1/5(cell signalling) 1:10 overnight at 4 °C in PBS with 1% bovine serum albumin. As a negative control, the primary antibody was replaced by normal rabbit serum. Positive controls included staining of sections of human lung sections for pSmad1/5 and Id1. After rinsing extensively with TBS, bound anti-Id1, anti-pSmad1/5 and anti-Ki67 was detected by secondary antibody conjugated with anti Rabbit NL493 conjugated Donkey IgG (R&D)
diluted 1:100 in blocking buffer, bound anti-α smooth muscle actin was detected by secondary antibody conjugated with anti mouse NL557 conjugated Dondey IgG (R&D) diluted 1:100 in blocking buffer with 10µg/mL Hoechst 33342(invitrogen). Slides were incubated with secondary antibody for 1 hour. After extensive rinsing in TBS, sections labelled with antibodies were further rinsed in deionized water and mounted in Vectashield Mounting Medium before viewing using a TCS-NT CONFOCAL Laser Scanning Microscope(Leica Microsystems Ltd, Knowhill, Milton Keynes, UK). The pulmonary artery from each group is comparable with 20µm scale bar at right corner of each image. The arteries are randomly selected on each section from a group of n=6 each.

Supplemental Figures

Supplemental Figure I. The effect of sildenafil on BMPRII mRNA expression. Real-time qPCR result shows unchanged BMPRII expression by sildenafil at 24 hours, in the presence or absence of BMP4. Data are representative of 3 separate experiments.
Supplemental Figure II. Sildenafil does not alter levels of the inhibitory Smad, Smad6. Immunoblots show Smad 6 protein levels are increased by BMP4 (10ng/ml) but are not altered by sildenafil at 1 or 5µM in PASMCs (A). β-actin was used as a loading control. Densitometry from 3 separate experiments is shown in (B).
Supplemental Figure III. Sildenafil enhances BMP2-induced phosphorylation of Smad1/5. Immunoblots showing the phosphorylation of Smad1/5 in PASMCs in response to sildenafil (1µM and 5 µM) and/or BMP2(10ng/mL) after 24 hours.

Supplemental Figure IV. Sildenafil enhances Smad binding to the Id1 promoter. Chromatin immunoprecipitation assay demonstrates that Smad1/5 binding to the Id1 promoter was enhanced by sildenafil compared to BMP4 (10ng/mL) treatment alone for 24 hours. * P<0.05 compared with BMP4 alone.
Supplemental Figure V. BMPR-II interacts with cGKI in PASMCs. Endogenous complexes of cGKI and the BMP type II receptor was pulled down by BMPR-II or IgG antibody in unstimulated PASMCs. Whole-cell extracts were subjected to immunoprecipitation followed by Western blot analysis with cGKI antibody shown. The input corresponds to lysate prior to immunoprecipitation (IB, immunoblotting; IP, immunoprecipitation).

Supplemental Figure VI. Enhanced Id1 expression by 8-br-cGMP in the presence of BMP4. Real-time qPCR showed significantly enhanced transcription of Id1 by BMP4 in the presence of sildenafil at 24 hour. Data are representative of 3 separate experiments. # P<0.05 compared with BMP4 alone.
Supplemental Figure VII. BMP4 induces nuclear localization of cGKI, which is dependent on BMPR-II. Immunofluorescence staining of endogenous cGKI in PASMCs under basal conditions (SiCP – siRNA control) and following knockdown of BMPRII with siRNA, after 30mins BMP4 stimulation. BMP4 treatment leads to nuclear localisation of cGKI, which is inhibited by BMPR-II depletion. DAPI (blue), alpha-smooth muscle actin (red), and cGKI (green) staining and merged images.
Supplemental Figure VIII. BMP4 does not elevate intracellular cGMP levels. PASMCs were incubated with BMP4 (10 or 100 ng/mL), Sildenafil (1 or 100µM) in DMEM in the absence of serum for 10mins. Representative of 3 separate experiments.

Supplemental Figure IX. Sildenafil enhances pSmad1/5 activity in BMPRII mutant PASMCs. Immunoblots showing phospho-Smad1/5 levels in PASMCs from PAH patients with mutations in the kinase domain of BMPR-II (C347Y, C347R) showing the increase in BMP4-stimulated phospho-Smad1/5 level induced by sildenafil (1µM; 24hours).
Supplemental Figure X. Id1/3 knockdown prevents the growth inhibitory effects of sildenafil in PASMCs. Growth studies were performed in DMEM/10% FBS either alone or containing sildenafil (Sil; 100nM) and BMP4 (100 ng/mL) for 7 days before counting. BMP4 and sildenafil co-treatment inhibited 10% FBS induced growth in siCP (siRNA control) treated cells (*P < 0.05). Following combined siId1 and siId3 knockdown, PASMCs growth cannot be inhibited by BMP4 and sildenafil co-treatment (ns, not significant). Results represent means of 3 separate experiments.

Supplemental Figure XI. Sildenafil does not induce apoptosis in PASMCs. Hoechst 33342 (5µg/mL) nuclear staining demonstrates the lack of apoptosis of PASMCs after 24 hours in DMEM and serum free medium in the presence of BMP4 (10ng/mL) and/or sildenafil (Sil; 1µM). Staurosporine (0.1µM), a known inducer of apoptosis, was used as positive control (arrows show apoptotic condensed nuclei).
Supplemental Figure XII. Sildenafil restores Id1 protein expression in small pulmonary arteries of monocrotaline exposed rats. Confocal images of small pulmonary arteries in rat lung tissue from control, monocrotaline-exposed (MCT) and monocrotaline-exposed rats treated with sildenafil (MCT+Sil) stained with antibodies for Id1 and alpha-smooth muscle actin (α-SMA). In control tissue, α-SMA–positive cells (red) in the vessel wall stain positively for Id1 (green), as shown in the merged images. In the monocrotaline-exposed rat, the hyperplastic media is shown to be positive for α-SMA but lacking in Id1 expression. Following sildenafil treatment, there is increased immunostaining for Id1 in α-SMA positive cells.