A Molecular Mechanism for Therapeutic Effects of cGMP-elevating Agents in Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a progressive, usually fatal disease with abnormal vascular remodeling. Pulmonary artery smooth muscle cells (PASMCs) from PAH patients are hyperproliferative and apoptosis-resistant and demonstrate phenotypic changes, with increased contractile gene expression, decreased contractile gene expression, and abnormal vascular remodeling (5). PKGI is one of several cGMP effectors known to stimulate the activity of Smad1/5/8 signaling, and even in patients with nonmutated BMPR2, BMP receptor complexes and Smad1/5/8 signaling are reduced in vascular lesions, emphasizing the importance of BMP/Smad signaling in PAH (1, 8). How-ever, only 10–20% of people with idiopathic PAH and ~20% of “idiopathic” cases have a heterozygous mutation in the BMPR2 gene (1). These mutations decrease Smad signaling, and even in patients with normal BMPR2, BMP receptor complexes and Smad1/5/8 signaling are reduced in vascular lesions, emphasizing the importance of BMP/Smad signaling in PAH (1, 8). However, only 10–20% of people with BMPR2 mutations develop PAH, suggesting that other genetic and environmental factors contribute to disease penetrance (9, 10). We showed previously that PKGI facilitates Smad1/5/8 signaling in C2C12 myoblasts: PKGI binds to BMPR2 and, upon BMP stimulation, detaches

Progressive narrowing of arterial vessels in pulmonary arterial hypertension (PAH) increases pulmonary arterial pressure, causing right ventricular hypertrophy and ultimately heart failure (1, 2). Structural changes occur in all vessel layers, with medial thickening caused by abnormal proliferation of PASMCs (1). PASMCs can switch from a differentiated, quiescent state to a de-differentiated, proliferative state, but the mechanism is not well understood (3, 4). PAH treatment includes modulation of the NO/cGMP signaling pathway to induce vasodilatation (1), with mounting evidence indicating that cGMP-elevating agents may also ameliorate pathological vascular remodeling (5). PKGI is one of several cGMP effectors in smooth muscle cells (SMCs), involved in regulating proliferation, differentiation, and survival, and might contribute to disease penetrance (9, 10). We showed previously that PKGI facilitates Smad1/5/8 signaling in C2C12 myoblasts: PKGI binds to BMPR2 and, upon BMP stimulation, detaches

The abbreviations used are: PAH, pulmonary arterial hypertension; BMP, bone morphogenetic protein; BMPR1, BMP receptor type 1; BMPR2, BMP receptor type 2; PASMC, pulmonary artery smooth muscle cell; Ba-pCPT-cGMP, 8-(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphate; PKG, cGMP-dependent protein kinase; Rp-pCPT-PET-cGMPS, 8-(4-chlorophenylthio)-β-phenyl-1,2-ethenoguanosine-3',5'-cyclic monophosphorothioate; SM, smooth muscle; SMC, smooth muscle cell; TRITC, tetramethylrhodamine isothiocyanate.

Pulmonary arterial hypertension (PAH) is a progressive, usually fatal disease with abnormal vascular remodeling. Pulmonary artery smooth muscle cells (PASMCs) from PAH patients are hyperproliferative and apoptosis-resistant and demonstrate abnormal vascular remodeling. Conversely, cGMP stimulation of PKGI restored defective BMP signaling in rats with hypoxia-induced PAH, consistent with cGMP-elevating agents reversing vascular remodeling in this PAH model. Our results provide a mechanism for the therapeutic effects of cGMP-elevating agents in PAH and suggest that combining them with BMP mimetics may provide a novel, disease-modifying approach to PAH therapy.

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This article contains supplemental Material, additional references, Figs. 1–5, and Table 1. 

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from the receptor complex and associates with activated Smads; the PKGI-Smad complex translocates to the nucleus, where it recruits the transcriptional co-factor TFII-I to enhance Smad-dependent transcription from a BMP response element-containing reporter gene (11). We now demonstrate functional consequences of cGMP/BMP cross-talk in SMCs: cGMP/PKGI and BMP/Smad cooperatively regulate PASMC growth, survival, and differentiation; defective Smad phosphorylation and Smad downstream signaling are observed in mice with reduced PKG expression, and PKG activation in a rat model of PAH improves Smad signaling.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments with Pharmacological Agents—Primary human PASMCs (Lonza) were maintained according to the manufacturer’s instructions and used at passages 4–8. Spontaneously immortalized rat PASMCs (PAC1, a kind gift from A. Rothman) were cultivated in Dulbecco’s modified Eagle medium (DMEM) plus 10% (v/v) fetal bovine serum (FBS) and antibiotics. SMC precursors (C3H/10T1/2 cells (ATCC), passages 6–16) were cultivated in basic medium Eagle supplemented with 10% (v/v) FBS and 100 units/ml penicillin and 100 mg/ml streptomycin. Cells were serum-starved in 0.1% (v/v) FBS for 4 h (for Id1/2/3 Acta2, Tagln, and Ctn). Total RNA was isolated using TRI Reagent (Molecular Research Center) and used at passages 4–8. mRNA levels were determined with 2-actin and/or β-actin served as internal references to calculate relative mRNA levels using the $2^{-\Delta \Delta C T}$ method.

Quantitative PCR Array was performed with primary human PASMCs, transfected with control or PKGI siRNA, and treated 24 h later with 3 nM BMP2 for an additional 24 h, using primers provided on Human Cell Cycle RT2 Profiler PCR Array plates (Qiagen). Data were analyzed with RT2 Profiler PCR Array Data Analysis version 3.5 (Qiagen). For validation, human Quanti-Tect primers from Qiagen were used. Primer sequences and catalogue numbers are provided in the supplemental Material.

Collagen Matrix Contraction Assay—Collagen contraction assay was performed as described (13), with minor changes. Serum-deprived PASMCs were treated with 3 nM BMP2 for 48 h. Cells were trypsinized, and equal cell numbers were embedded into collagen matrices, which were incubated for another 18–20 h under full serum conditions. Collagen gel sizes were measured with ImageJ (National Institute of Health).

Immunoblotting—Cell extracts were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting with antibodies specific for phospho-Smad1/5/8, phospho-Ser463/465-Smad1/5, phospho-Ser421/422, phospho-Ser407/465-Smad5/8, Smad5, phospho-Ser239-VASP (all from Cell Signaling), PKGI (C-terminal; Abgent), Smad1, β-actin, α-tubulin (all from Santa Cruz Biotechnology), or α2-actin (clone 1A4; Sigma-Aldrich), and Western blots were scanned and analyzed using ImageJ.

Immunofluorescence—Primary human PASMCs and SMC precursors were plated on glass coverslips in 24-well plates, serum-deprived, and stimulated with 3 nM BMP2 for 30 min (Smad1 nuclear translocation) or 24 h (α2-actin expression). Cells were fixed with 3.7% paraformaldehyde and permeabilized in 0.5% Triton X-100. After blocking with 3% BSA, cells were stained using Smad1 primary antibody (Santa Cruz Biotechnology) and Alexa Fluor 555-conjugated goat anti-mouse secondary antibody (Invitrogen), or α2-actin (clone 1A4; Sigma-Aldrich), and FITC-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch). Nuclei were stained using Hoechst 33342. For co-localization studies on lung sections, slides were boiled in 1 mM EDTA, pH 8.0, as described below, and incubated with primary antibodies followed by FITC- and TRITC-labeled secondary antibodies (Jackson Immunoresearch). Coverslips were embedded in Fluoromount-G (Southern Biotech). Pictures were taken from randomly selected fields using a confocal microscope (Olympus FV1000) and 40/0.75 or 10/0.4 objectives. Images were analyzed with Fluoview (Olympus) and Photoshop (Adobe; sizing, contrast adjustment), and identical software settings were used for image acquisition of all samples in a given experiment.

Immunohistochemical Staining and Histomorphometry—Lung tissue paraffin sections were deparaffinized and hydrated, and antigen retrieval was done by boiling (using a 1200-W microwave, full power for 3 × 15 s) in 1 mM EDTA, pH 8.0, for phospho-Smad1/5 staining, or 10 mM citrate, pH 6.0, for α2-actin staining. After blocking in TBS/0.1% Tween 20/5% goat serum, primary antibodies were incubated overnight at 4 °C in antibody diluent (Cell Signaling). After washing, slides were treated with species-specific SignalStain® Boost IHC Detection Reagent (Cell Signaling), developed with a 3,3-diaminobenzidine peroxidase substrate kit (Vector Laboratories), and dehy
RESULTS

PKG1 Facilitates BMP/Smad Signaling in SMCs—We studied interactions between BMP and cGMP signaling in primary human PASMCs, immortalized rat PASMCs, and immortalized SMC precursors (C3H/10T1/2 cells). We found that treating cells with 8-pCPT-cGMP significantly increased BMP-induced Smad phosphorylation (Fig. 1, A and B for primary human PASMCs and murine SMC precursors). Conversely, inhibiting PKG with Rp-pCPT-PET-cGMPS largely blocked BMP-mediated Smad activation (Fig. 1, C and D, and supplemental Fig. 1, A–C, for rat PASMCs). Because pharmacological inhibition of PKG could have off-target effects, we also used an siRNA approach (12) with subsequent viral reconstitution of siRNA-resistant PKG1α. We found that BMP-induced Smad phosphorylation was reduced in PKG1-depleted cells, but could be restored by re-expressing PKG1α (Fig. 1, E and F, and supplemental Fig. 1D). Moreover, inhibiting PKG blocked BMP-induced nuclear Smad1 translocation in primary human PASMCs (Fig. 1G). We previously reported similar findings in C2C12 myoblasts, where shRNA-mediated transient knockdown of PKG1 diminished BMP-induced Smad phosphorylation and nuclear translocation (11).

Smads assemble transcriptional complexes at promoter regions of specific genes, including the inhibitor of differentiation (Id) gene family (7, 11). We found that inhibiting PKG decreased, although activating PKG increased Id1/2/3 mRNA expression in BMP-treated cells (Fig. 1H and supplemental Fig. 1E). Basal Id expression was not significantly affected by the PKG inhibitor. Thus, PKG1 is necessary for BMP-induced Smad1/5 activation and nuclear translocation and BMP-induced Id expression in PASMCs.

cGMP and BMP Cooperate to Control PASMC Proliferation and Apoptosis—Medial hypertrophy in the vascular lesions of PAH is due mainly to abnormal PASMC proliferation and resistance to apoptosis (1), and PASMCs from PAH patients respond less to the anti-proliferative and pro-apoptotic effects of BMP compared with cells from normal subjects (15, 16). Similar to BMP, cGMP/PKG1 signaling represses SMC growth (3, 17). Therefore, we examined whether PKG1 mediated the anti-proliferative effects of BMP in PASMCs and found that inhibiting PKG pharmacologically or by siRNAs (12) largely prevented growth inhibition by BMP (Fig. 2, A and C, and supplemental Fig. 2, A–C). In the latter case, reconstituting siRNA-resistant PKG1α restored BMP-induced growth inhibition (Fig. 2C). As reported by others (16, 18), BMP increased cell death in PASMCs, but PKG inhibition abolished this effect (Fig. 2B and supplemental Fig. 2, D and E). In the absence of BMP, cGMP activation of PKG reduced PASMC growth and survival (Fig. 2D and supplemental Fig. 2F). Smad1/5-depleted cells were relatively insensitive to cGMP-induced growth suppression, but reconstituting siRNA-resistant Smad1 fully restored cGMP anti-proliferative effects (Fig. 2D; knockdown efficiencies of Smad1 and 5 siRNAs are shown in supplemental Fig. 2G). Consistent with these results, the BMPRI inhibitor dorsomorphin (19) blocked basal Smad phosphorylation and reduced cGMP anti-proliferative effects (supplemental Fig. 2, H and I). Furthermore, growth suppression by cGMP required...
Id1/3 (supplemental Fig. 2); knockdown efficiencies of Id1 and 3 siRNAs are shown in supplemental Fig. 2K), similar to the obligatory role of Id1 in BMP-mediated inhibition of SMC proliferation (20, 21). These data indicate that basal PKG activity is necessary for BMP-induced growth suppression and apoptosis in PASMCs and that cGMP/PKG1 and BMP/Smad cooperate to suppress PASMC growth. Their cooperative effect may be partly explained by their convergence on Id1/2/3 (Fig. 1H).

To search for additional genes mediating anti-proliferative effects of cGMP and BMP, we examined a panel of 72 cell cycle-relevant genes in primary human PASMCs cultured with and without BMP and/or PKGI siRNA (Fig. 2E; Fig. 3D shows PKGI knockdown). BMP decreased expression of 42% of the genes (including Cdk2 and Bcl2; significance cut-off 1.3-fold; supplemental Table 1). Of the BMP2-affected genes, 29% were sensitive to PKGI depletion. Quantitative RT-PCR analysis confirmed that four genes were co-regulated by BMP and cGMP (Fig. 2, F and G): (i) Anapc2 encodes a catalytic subunit of the anaphase-promoting complex (22); (ii) Mre11A is part of a DNA protection-repair complex (23); (iii) Rad1 is a component of a cell cycle checkpoint complex activated by DNA damage (24); and (iv) Bcl2 is an anti-apoptotic protein (16, 18). All four genes are essential for regulation of cell growth (16, 18, 22–24).

Thus, BMP/cGMP co-regulate several genes involved in growth and survival of PASMCs.

PKGI Is Essential for BMP-induced SM-specific Contractile Gene Expression—In response to environmental stimuli, SMCs undergo reversible switching between a differentiated, contractile state with high SM-specific gene expression, and a de-differentiated, proliferative state characterized by low SM-specific gene expression (3, 4). De-differentiation and proliferation of PASMCs contribute to the progressive narrowing of small pulmonary arteries in PAH due to thickening of the muscle layer (1, 2). BMP induces SM-specific gene transcription in a Smad-dependent manner, thereby maintaining a differentiated PASMC phenotype (25). We hypothesized that cGMP/PKG1 could be involved in BMP regulation of the PASMC phenotype, because PKGI sustains a differentiated state in aortic SMCs (3, 12). We found that pharmacological inhibition or siRNA depletion of PKGI in primary human PASMCs and SMC precursors blocked BMP-induced Acta2 (α2-actin or SM-α-actin), Tagln (transgelin or SM22), and Cnn (calponin) expression (Fig. 3, A and B, shows immunofluorescence staining of α2-actin, with a longer excitation time shown in supplemental Fig. 3A and Western blot analysis of α2-actin expression in supplemental Fig. 3B;Fig. 3, C and D,
shows RT-PCR quantification of all three mRNAs, and PKGI knockdown efficiency.

Increased contractile gene expression in SMCs induces shrinkage of collagen lattices (13), and we found that inhibiting PKG activity reduced BMP-induced collagen gel contraction (Fig. 3E). Thus, cGMP and BMP signaling cooperate to enhance contractile gene expression, thereby maintaining PASMC differentiation.

PKGI Deficiency Correlates with Impaired Smad Phosphorylation and Smad-dependent Gene Expression in Vivo, Causing Vascular Remodeling—To explore cGMP/PKGI interaction with BMP/Smad signaling in vivo, we examined Smad phosphorylation in lungs from homo- or heterozygous Prkg1 (PKGI) knock-out mice. We found reduced Smad1/5/8 phosphorylation in lungs of 6-week-old homo- and heterozygous Prkg1 knock-out mice compared with wild type littermates (Fig. 4A).
Prkg1 is a critical mediator of BMP-induced contractile gene expression. A, human PASMCs received BMP2 and/or Rp-pCPT-PET-cGMPS (Rp) for 24 h and were analyzed for α2-actin by immunofluorescence staining (green, with nuclei counterstained in blue). Scale bar, 5 μm. B, SMC precursors were stimulated and stained as in A, and the number of α2-actin-positive cells was determined (relative to cells receiving only BMP2; median ± S.E., n = 6, *, p < 0.05, two-tailed Wilcoxon Rank test). C, human PASMCs were treated with BMP2 and/or Rp for 24 h and examined for Acta2, Tagln, and Cnn mRNA expression by quantitative RT-PCR (normalized to Gapdh and to unstimulated cells; n = 4, **, p < 0.01; ***, p < 0.001; one-way ANOVA). D, Acta2, Tagln, Cnn, and Prkg1 mRNA expression was measured by real-time RT-PCR in human PASMCs transfected with siRNAs targeting GFP or Prkg1 and treated with BMP2 for 24 h. mRNA levels were normalized to Gapdh and to control siRNA-treated, unstimulated cells (*, p < 0.05; **, p < 0.01; ****, p < 0.001; one-way ANOVA). E, after treatment with BMP2 and/or Rp for 48 h, equal numbers of PASMCs were embedded into collagen matrices; gel contraction was measured relative to untreated controls (n = 6, **, p < 0.01; ***, p < 0.001, one-way ANOVA).

Because Prkg1−/− mice die within 8 weeks of birth from severe gastrointestinal dysfunction (14), we concentrated subsequently on adult (16-week-old) Prkg1+/− mice, which appear phenotypically normal. We found that basal Smad1/5/8 phosphorylation in lung tissue was down-regulated in 7 of 26 heterozygotes compared with matched wild type littermates (significance cut-off 1.3-fold; Fig. 4C–E). Similarly, expression of Spp1 (osteopontin) and Thbs1 (thrombospondin) mRNA was unaffected by low PKGI; Col1a1 (collagen 1α1) mRNA expression showed a trend toward higher levels in lungs of Prkg1−/− mice, but this did not reach statistical significance (supplemental Fig. 4F).

The correlation among Prkg1 gene expression, Smad phosphorylation, and Smad-dependent gene expression indicates that residual PKGI activity in some heterozygotes was insufficient to maintain normal Smad activity, and reduced Smad signaling was associated with vascular remodeling.

PKG Activation Restores Impaired Smad Phosphorylation in PAH Rats—Similar to findings in PAH patients, Smad1/5/8 phosphorylation is decreased in PASMCs from rats with hypoxia- or monocrotaline-induced PAH (8, 20, 21, 27). However, animals exposed to monocrotaline or hypoxia do not
develop vascular lesions characteristic of human PAH (5, 28). In contrast, rodents kept under hypoxic conditions and treated with the VEGF receptor antagonist SU5416 better mirror the pathological vascular changes seen in PAH patients, and these changes are progressive even after cessation of hypoxia and SU5416 treatment (5, 29). We hypothesized that treating PAH rats with a cGMP-elevating agent may improve defective BMP/Smad signaling in this model. Rats were kept under hypoxic conditions and were treated with SU5416 for 21 days; then, animals were randomized to receive either vehicle or the soluble guanylate cyclase stimulator riociguat for 14 days (5). We now demonstrate that riociguat significantly increased Smad1/5/8 phosphorylation in lungs of hypoxic rats treated with SU5416, without changing Smad1 and Smad5 phosphorylation (Fig. 5B). Consistent with these results, we showed previously that PKGI overexpression in C2C12 cells enhances BMP-induced transcription of a BMP-responsive reporter gene, even in cells co-transfected with BMPR2 constructs containing loss-of-function mutations found in PAH patients (11).

The anti-proliferative and pro-apoptotic effects of BMP are attenuated in PASMCs from PAH patients (15, 16). Decreased BMP2 expression and deficient Smad1/5/8 phosphorylation are observed in small pulmonary arteries of PAH patients and in several animal models of PAH, suggesting that impaired BMP signaling plays a central role in the pathophysiology of PAH (15, 27, 31). Microarray gene expression analysis comparing BMP-treated PASMCs derived from normal subjects and PAH patients also supports a proliferative, apoptosis-resistant phenotype in PAH (15). Id proteins are essential mediators of the anti-proliferative and pro-apoptotic effects of BMP (32). The Id1 protein is upregulated in the nuclei of PASMCs from PAH patients (20, 21). We found that the anti-proliferative effect of BMP was prevented when PKG was inhibited; conversely, the anti-proliferative effect of cGMP in PASMCs required Smad1/5 and Id1/3. Thus, both BMP and cGMP converge to induce Id proteins to inhibit PASMC proliferation. Furthermore, we identified several pro-proliferative and anti-apoptotic genes that were down-regulated by BMP in a PKGI-dependent fashion, namely Anapc2, Mre11A, Rad1, and Bcl2.

**DISCUSSION**

Using pharmacological and genetic approaches, we found that BMP/Smad and cGMP/PKGI signaling pathways cooperate in PASMCs to regulate expression of multiple genes involved in proliferation, apoptosis, and maintenance of a differentiated, contractile phenotype (Fig. 5D). Consistent with these results, we showed previously that PKGI overexpression in C2C12 cells enhances BMP-induced transcription of a BMP-responsive reporter gene, even in cells co-transfected with BMPR2 constructs containing loss-of-function mutations found in PAH patients (11).
Studies in different cell types have demonstrated that each of these proteins is essential for sustaining proliferation (16, 18, 22–24). Others have shown that BMP suppresses Bcl2 expression in PASMCs (16, 18), and Bcl2 mRNA is up-regulated in lung tissue from patients with heritable or idiopathic PAH, likely contributing to the apoptosis resistance of vascular cells (33).

Heterozygous BMPR2-deficient mice display either normal hemodynamics or mild PAH at base line but show enhanced susceptibility to inflammation-induced PAH (9, 34). Mice expressing an inducible dominant negative form of BMPR2 in smooth muscle develop PAH when the transgene is activated (16, 18, 22–24). Others have shown that BMP suppresses Bcl2 expression in PASMCs (16, 18), and Bcl2 mRNA is up-regulated in lung tissue from patients with heritable or idiopathic PAH, likely contributing to the apoptosis resistance of vascular cells (33).

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We found that 27% of the \( Prkg1^{+/-} \) mice had defective Smad phosphorylation and that these mice had lower mean \( Prkg1 \) mRNA expression compared with heterozygotes with normal Smad signaling. Expression of the wild type \( Prkg1 \) allele may be modified by epigenetic mechanisms; phenotypic heterogeneity has been observed previously in inbred mouse models with heterozygous targeted disruption of other genes (40, 41). Similarly, in families with a heterozygous \( Bmpr2 \) mutation leading to an unstable transcript, unaffected mutation carriers express significantly more wild type \( Bmpr2 \) mRNA than affected PAH patients (42). This heterogeneity, together with other genetic and environmental modifiers, likely explains incomplete penetrance of the disease. Because some patients with heritable or idiopathic PAH express less \( Prkg1 \) mRNA in lung than normal subjects (33), we propose that PKGI expression below a critical threshold contributes to reduced BMP/Smad signaling, leading to abnormal PASMC gene expression, proliferation, and survival.

Multiple studies have demonstrated that cGMP-elevating agents, including NO, phosphodiesterase inhibitors, and guanylate cyclase stimulators, can induce pulmonary vasodilation and reverse vascular remodeling and right heart hypertrophy in rodent models of PAH (43–46). We used a rat model of PAH induced by hypoxia and SU5416 that closely mimics the vascular changes seen in human PAH patients and found that riociguat not only improved pulmonary hemodynamics and vascular remodeling (5), but also increased Smad phosphorylation. Riociguat, the first guanylate stimulator to enter clinical development, has yielded promising results in phase II and III clinical trials in PAH patients (47, 48).

In conclusion, we found that cooperation between the cGMP/PKG and BMP/Smad pathways is essential for maintaining a low proliferative, differentiated PASMC phenotype (Fig. 5D) and that a critical threshold of basal PKGI activity is required for normal Smad activation and Smad-dependent gene expression. PKGI activity in PASMCs may modify disease penetrance in \( Bmpr2 \) mutation carriers. This work provides a molecular basis for reversal of vascular remodeling by PKG activation in PAH animal models and at least partly explains the beneficial effects of cGMP-elevating agents in PAH patients (1, 47). Combining PKG activators and BMP agonists may lead to novel, personalized medical strategies for PAH patients.

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cGMP and BMP Signaling in Pulmonary Hypertension

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