Bone morphogenetic proteins induce apoptosis in human pulmonary vascular smooth muscle cells

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PULMONARY VASOCONSTRICTION, vascular wall remodeling, and in situ thrombosis are major causes for the elevated pulmonary vascular resistance and pulmonary arterial pressure (PAP) found in patients with primary pulmonary hypertension (PPH). The pulmonary vascular hypertrophy in primary pulmonary hypertension (PPH) is mainly caused by increased proliferation and decreased apoptosis in pulmonary artery smooth muscle cells (PASMCs). Mutations of the bone morphogenetic protein (BMP) receptor type II (BMP-RII) gene have been implicated in patients with familial and sporadic PPH. The objective of this study was to elucidate the apoptotic effects of BMPs on normal human PASMCs and to examine whether BMP-induced effects are altered in PASMCs from PPH patients. Using RT-PCR, we detected six isoforms of BMPs on normal human PASMCs and to examine BMP-induced effects are altered in PASMCs from PPH patients. Using RT-PCR, we detected six isoforms of BMPs (BMP-1 through -6) and three subunits of BMP receptors (BMP-RIa, -RIb, and -RII) in PASMCs. Treatment of normal PASMCs with BMP-2 or -7 (100–200 nM, 24–48 h) markedly increased the percentage of cells undergoing apoptosis. The BMP-2-mediated apoptosis in normal PASMCs was associated with a transient activation of phosphorilation of Smad1 and a marked downregulation of the antiproliferative protein Bcl-2. In PASMCs from PPH patients, the BMP-2- or BMP-7-induced apoptosis was significantly inhibited compared with PASMCs from patients with secondary pulmonary hypertension. These results suggest that the antiproliferative effect of BMPs is partially due to induction of PASMC apoptosis, which serves as a critical mechanism to maintain normal cell number in the pulmonary vasculature. Inhibition of BMP-induced PASMC apoptosis in PPH patients may play an important role in the development of pulmonary vascular medial hypertrophy in these patients.

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Mutations of the BMP-RII gene have been demonstrated to be the genetic basis for familial PPH. The BMP-RII gene mutations have also been implicated in 15–25% of patients with sporadic PPH, which suggests a common genetic defect in the BMP-RII gene for the pathogenesis of both familial and sporadic PPH (9, 10, 32, 34, 41). However, how mutations in the BMP-RII gene (located at chromosome 2q33) cause PPH is unclear. To reveal the pathological mechanisms by which a mutant BMP-RII gene mediates vascular abnormalities in PPH patients, it is important to understand the functional role of BMP-mediated activation of BMP receptors in PASMCs from normal subjects. This study was designed to characterize the molecular identities of BMPs and BMP receptors in normal human PASMCs and to examine the effects of BMPs on PASMCs isolated from normal subjects and patients with secondary pulmonary hypertension (SPH) and PPH.

MATERIALS AND METHODS

Demographic, clinical, and hemodynamic characteristics of patients. There were five subjects from whom lung tissue was obtained to prepare PASMCs for the study. The diagnosis of PPH was established clinically in two patients on the basis of the criteria used in the National Registry on Primary Pulmonary Hypertension and was confirmed histopathologically. The mean PAP of the two PPH patients (a 57-yr-old woman and a 31-yr-old man) was 51 and 53 mmHg, respectively. Both of the PPH patients had been treated with Flolan, warfarin, digoxin, and furosemide before lung transplantation. Three subjects had pulmonary hypertension (SPH) resulting from known causes: a 69-yr-old male patient with idiopathic pulmonary fibrosis (mean PAP, 33 mmHg), and a 37-yr-old female patient with lymphangioleiomyomatosis (LAM; mean PAP, 50 mmHg). The three SPH patients were treated with aspirin, citalopram, estrogen, or narcotic pain medicines before transplantation.

Cell preparation and culture. Primary cultured PASMCs from transplant patients were used in this study (63, 64). Lung tissue removed from patients in the operating room was immediately placed in cold (4°C) saline and taken to the laboratory for dissection. Peripheral muscular pulmonary arteries (diameter range, 300–500 μm) isolated from the explanted lung tissues within 5 h after transplantation were incubated in Hanks’ balanced salt solution that contained 2 mg/ml collagenase (Worthington Biochemical) for 20 min. The adventitia was stripped, and endothelium was removed. The remaining smooth muscle was digested with (in mg/ml) 2.25 collagenase, 0.5 elastase, and 1 albumin (Sigma) at 37°C to make a cell suspension of PASMCs. The cells were resuspended, plated onto 25-mm coverslips or 10-cm petri dishes, and incubated in a humidified atmosphere of 5% CO2–95% air at 37°C in the smooth muscle growth medium (SMGM, Clonetics). Human PASMCs from normal subjects (who had no implication of pulmonary hypertension) purchased from Clonetics were also used in some experiments. The cells were seeded in flasks at a density of 2,500–3,500 cells/cm² and were incubated in SMGM. The medium was changed after 24 h and every 48 h thereafter. The SMGM was composed of smooth muscle basal medium supplemented with 5% FBS, 0.5 ng/ml human epidermal growth factor (hEGF), 2 ng/ml human fibroblast growth factor (hFGF), and 5 μg/ml insulin. Cells were subcultured or plated onto 25-mm coverslips using trypsin-EDTA buffer (Clontech) when 70–90% confluence was achieved. The morphology of the cells was examined by using an inverted phase-contrast microscope attached to a digital camera.

Measurement of cell death. The cells, grown on 25-mm coverslips, were first washed with PBS and then fixed in 95% ethanol (for 20 min at 0°C) and stained with the membrane-permeable nucleic acid stain 4',6'-diamidino-2-phenylindole (DAPI, Sigma). DAPI (5 μM) was dissolved in an antibody buffer that contained 500 mM NaCl, 20 μM Na3VO4, 10 μM MgCl2, and 20 μM Tris-HCl (pH 7.4). The blue fluorescence emitted at 461 nm was used to visualize the cell nuclei. The DAPI-stained cells were examined by using a Nikon TE 300 fluorescence microscope, and the cell (nuclear) images were acquired by using a high-resolution Solamere fluorescence microscope system (Solamere, City, UT). DAPI staining reveals nuclear morphological changes of cells undergoing apoptosis. For each coverslip, seven fields (with 20–25 cells/field) were randomly selected to determine the percentage of apoptotic cells in the total cells based on the morphological characteristics of apoptosis. The cells with clearly defined nuclear breakage, remarkably condensed nuclear fluorescence, and significantly shrunk cell nuclei were defined as apoptotic cells (30). To quantify apoptosis, terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) assay was also performed with an in situ cell death detection kit (Roche; Ref. 30).

The annexin V-FITC Apoptosis Detection Kit (BD Pharmingen) was used for flow cytometry experiments to detect apoptotic cells (40). Human PASMCs cultured in SMGM with or without apoptosis inducers were washed twice with cold PBS and then resuspended in 1× binding buffer at a concentration of 1 × 10⁶ cells/ml. The cell suspension (100 μl, 1–10⁵ cells) was transferred to a 5-ml culture tube and mixed with 5 μl of annexin V-FITC and 10 μl of propidium iodide (PI). The cells were gently vortexed and incubated for 15 min at room temperature (20–25°C) in the dark. Then 400 μl of 1× binding buffer was added to each tube and analyzed by fluorescence-activated cell sorting (with FACSCalibur) by using CellQuest software (Becton Dickinson, Mountain View, CA). 

RT-PCR. Total RNA was extracted from human PASMCs by using the RNAeasy Mini Kit (Qiagen). Total RNA from human brain and lung tissues was purchased from Gibco (2.5 μg/μl). Genomic DNA was removed with RNase-free DNase according to the manufacturer’s instruction. SuperScript reverse transcriptase (Invitrogen) was used to synthesize cDNA. RNA (2 μg) was first incubated with oligo(dT) (1 μl at 0.5 μg/μl) at 70°C for 10 min. Then 8 μl of a solution that contained 10× buffer, 10 mM dNTP, 20 mM MgCl2, 0.1 M DTT, 40 U/μl RNase-OUT, and 50 U/μl SuperScript II reverse transcriptase were added to the samples and incubated for 10 min at 30°C, 60 min at 42°C, and 5 min at 95°C. RNase-H (1 μl at 2 U/μl; Gibco) was added to each reaction, and the samples were incubated for 20 min at 37°C. The sense and antisense primers were specifically designed from the coding regions of each gene (Table 1). Bax and Bel-2 mRNA expression was detected by using specific primers (EZ41 for Bax and EZ42 for Bel-2) purchased from Oxford Biomedical Research (Rochester Hills, MI). The sense and antisense primers are 5'-TTCTGACGGCAACTTCAAC-L741BMP INDUCES APOPTOSIS

AJP-Lung Cell Mol Physiol • VOL 285 • SEPTEMBER 2003 • www.ajplung.org

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

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<th>Standard Name</th>
<th>GenBank Accession No.</th>
<th>Predicted Size, bp</th>
<th>Sense/Antisense</th>
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<td>BMP-1</td>
<td>NM_006131</td>
<td>448</td>
<td>5'-ACCTCCCAATAGCCTCCAAA-3'/5'-CTCAATAACCCGGCAACACTGCA-3'</td>
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<td>BMP-2</td>
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<td>671</td>
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<td>395</td>
<td>5'-CTGTTACTCGCAAGAAACCTGGA-3'/5'-GCGTCTCGCAAGAAACCTGGA-3'</td>
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<td>Smad7</td>
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<td>297</td>
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<td>847–869</td>
<td>2q11–22</td>
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(antisense; 135 bp) for Bax and 5'-TGGGGGAGGAAGAAGACGAGATGCAA-3' (sense) and 5'-CCCGGCGCATTTTGGAAAGAGGTTGAG-3' (antisense; 153 bp) for Bcl-2 (47). The sense and antisense primers for GAPDH are 5'-GAGGGGACGCTGAGATGTTGAG-3' (sense) and 5'-TGGGGGAGGAAGAAGACGAGATGCAA-3' (antisense; 719 bp). The fidelity and specificity of the sense and antisense oligonucleotides were examined by using the BLAST program.

PCR was performed by using the GeneAmp PCR System (Perkin-Elmer, Norwalk, CT) with platinum PCR supermix (GIBCO). The first-strand cDNA reaction mixture (1 μl) was used in a 50-μl PCR reaction that consisted of 1 μl of each primer (10 μM), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM MgCl2, 200 μM of each dNTP, and 2 U Taq DNA polymerase. The cDNA samples were amplified in a DNA thermal cycler under the following conditions: the mixture was annealed at 55°C (30 s), extended at 72°C (30 s), and denatured at 94°C (30 s) for 32 cycles. This was followed by a final extension at 72°C (10 min) to ensure complete product extension. The PCR products were electrophoresed through a 1.5% agarose gel, and amplified cDNA bands were visualized by ethidium bromide staining. To semiquantify the RT-PCR products, an
invariant mRNA of GAPDH was used as an internal control to normalize the mRNA levels of BMP ligands, BMP receptors, and Smad proteins as well as Bax and Bcl-2. The net intensity values of cDNA bands (e.g., for Bax and Bcl-2 transcripts) measured by a Kodak Electrophoresis Documenta- tion System were normalized to the net intensity values of the GAPDH signals; the ratios are expressed as arbitrary units (AU) for quantitative comparison.

Western blot analysis. PASMCs were gently washed twice in cold PBS, scraped into 0.3 ml of radioimmunoprecipitation assay buffer (1× PBS, 1% Nonidet P-40 (Amresco), 0.5% sodium deoxycholate, and 0.1% SDS), and incubated on ice for 45 min during which the cell mixture was shaken for 30 s by vortex three times. Resulting cell lysates were sonicated and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was collected, and protein concentrations were determined by Coomassie Plus protein assay reagent (Pierce Biotechnology) by using BSA as a standard. Protein (20 µg) was mixed and boiled in 2× sample buffer (0.25 M Tris-HCl, pH 6.8, 20% glycerol, 8% SDS, and 0.02% bromophenol blue). Protein suspensions were electrophoretically separated on a 10% acrylamide gel, and protein bands were transferred to nitrocellulose membranes by electroblot in a Mini Trans-Blot cell transfer apparatus (Bio-Rad) under conditions recom- mended by the manufacturer. After 1 h of incubation in a blocking buffer (0.1% Tween 20 and 5% nonfat dry milk powder), the membranes were incubated with the affinity-purified rabbit polyclonal antibody against the phosphory- lated Smad1 (Upstate Biotechnology, Lake Placid, NY) and with the mouse monoclonal antibody against Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Finally, the membranes were washed and exposed to anti- rabbit or anti-mouse horseradish peroxidase-conjugated IgG for 90 min at room temperature. The bound antibody was detected with an enhanced chemiluminescence detection sys- tem (Amersham). The monoclonal anti-α-actin antibody (Up- state) was used as a control.

Immunofluorescence labeling. The PASMCs from SPH and PPH patients were first stained with the membrane-permeant nucleic acid stain DAPI (5 µM, Sigma), and the blue fluorescence emitted at 461 nm was used to estimate total cell numbers in the culture. A specific monoclonal antibody raised against smooth muscle α-actin (Upstate) was then used to evaluate cellular purity of culture, and a secondary antibody (Afinipute donkey anti-mouse IgG) conjugated with FITC (Jackson ImmunoResearch) was used to display the fluorescent image (emitted at 529 nm). The cells were mounted in 10% of 1 M Tris-HCl/90% glycerol (pH 8.5) that contained 1 mg/ml p-phenylenediamine. The cell images were processed by the Solamere fluorescence-imaging system (Solamere Technology Group); the FITC fluorescence was colored red and DAPI fluorescence was colored green to display images with red-green overlay. The DAPI-stained cells that also cross-reacted with the smooth muscle cell α-actin antibody were defined as smooth muscle cells.

Histological preparation. The explanted lungs were grossly examined and fixed overnight in 10% neutral buff- ered formalin. The formalin-fixed lungs were serially cut, and representative sections were sampled from each lobe (typically 2–3 samples/lobe) for microscopic examination. The tissues were routinely processed and embedded in paraffin blocks in an automatic tissue processor (Sakura Tissue-Tek VIP, Torrance, CA). The paraffin-embedded tissues were cut into 5-µm-thick sections for standard hematoxylin eosin staining. Selected sections were also stained with trichrome and elastin.

**RESULTS**

**Molecular identity of BMPs and BMP receptors in human PASMCs.** Using RT-PCR analysis, we detected transcripts of BMP-1, -2, -3, -4, -5, and -6 in normal human PASMCs from subjects who had no implication of pulmonary hypertension (Fig. 1A). However, the BMP-7 transcript was not detectable in PASMCs (Fig. 1A, top), although it was highly expressed in human lung tissues (Fig. 1A, bottom). In human PASMCs, mRNA expression levels of all BMP receptors (BMP-RIa, -R Ib, and -RII) were similar to those in human lung tissues (Fig. 1B), which suggests that BMP recep- tors are highly expressed in human PASMCs. These results indicate that BMP-1, -2, -3, -4, -5, and -6 are the endogenously expressed BMPs in human PASMCs, which can be synthesized and secreted to the intercel- lular space and can activate BMP receptors on adjacent PASMCs. Although BMP-7 seems to be an exogenous BMP for PASMCs, it may be synthesized in other types of pulmonary cells such as vascular endothelial cells and alveolar epithelial cells and subsequently secreted to the intercellular space to affect pulmonary vascular smooth muscle cells.

When two types of transmembrane serine-threonine kinase receptors, BMP-R1 and -RII, are activated by binding to ligands (i.e., BMPs), the type II (BMP-RII) and type I (BMP-RI) receptors form a heterotetrameric complex (22, 35–37, 53, 56). The phosphorylated or activated BMP-RI directly phosphorylates the receptor-activated Smads (R-Smad; e.g., Smad1, Smad5, or Smad8), which then form a heteromeric complex with the co-Smad, Smad4, and enter the nucleus for transcrip- tional regulation. The R-Smad/Smad-4 complex binds to the specific Smad binding site (5’-CGCA-3’) in DNA (located in the gene promoter), and activates or represses Smad-responsive genes (22, 35–37, 48, 53, 56). In human PASMCs, we detected seven isoforms of Smad transcripts, Smad1 to Smad7 (Fig. 1C). The mRNA levels of all Smads in human PASMCs were comparable to those in lung tissues, which suggests that the proteins of the Smad family may serve as important downstream-signaling proteins in PASMCs when BMP receptors are activated by their ligands, BMPs.

**Chemicals.** Staurosporine (ST, Sigma) was prepared as a 1-mM stock solution in DMSO; aliquots of the stock solution were then diluted 1:10,000–500,000 times to the culture media or physiological salt solution for experimentation. Hu- man TGF-β1 (R&D Systems) was prepared as a stock solution in sterile HCl (4 mM) that contained 0.1% BSA. Recombinant human BMP-2 and -7 (R&D Systems) and Fas ligand (Sigma) were prepared as stock solutions in sterile PBS that contained 0.1% BSA. Cells were synchronized or growth-arrested in smooth muscle basal medium for 48 h before each experiment.

**Statistical analysis.** Data are expressed as means ± SE. Statistical analysis was performed by using the paired or unpaired Student’s t-test or ANOVA and post hoc tests (Student-Newman-Keuls) as indicated. Differences were consid- ered to be significant when *P* < 0.05.
In addition to BMPs and BMP receptors, human PASMCs also express three isoforms of TGF-β (TGF-β1, -β2, and -β3) and two types of TGF-β receptors (TGF-β-R1 and -RII; Fig. 2). The mRNA expression levels of TGF-β and TGF-β receptors in human PASMCs were also comparable to those in human lung tissues.

It must be emphasized that the mRNA expression of BMPs, BMP receptors, and the downstream signal transduction proteins (Smads) does not directly represent the protein expression of these ligands, receptors, and signaling proteins. Further immunoblot or immunocytochemistry experiments are needed to demonstrate the protein expression and distribution of BMPs, BMP receptors, and Smads in human PASMCs as well as their functional linkages to the effects of BMPs on cell growth and apoptosis.

A apoptotic effects of BMP-2, TGF-β1, and Fas ligand on human PASMCs. It has been demonstrated that low doses (10–100 nM) of BMPs and TGF-β1 inhibited human PASMC proliferation in the presence of low concentrations of serum (38). To test whether the antiproliferative effects of BMPs and TGF-β1 also involve mediating apoptosis in human PASMCs, we determined and compared the percentage of cells undergoing apoptosis in cells cultured in media with or without BMP-2 or TGF-β1.

Under baseline control conditions, the percentage of apoptotic cells (as determined by morphological changes of nuclei by using DAPI staining and TUNEL assay) ranged from 2 to 7% in normal human PASMCs. After 48 h of treatment with 200 nM BMP-2, ~22.9 ± 1.9% of the cells contained apoptotic nuclei as identified by DAPI staining (nuclear condensation and breakage) and TUNEL assay (Fig. 3, A and Ca). Treatment of PASMCs with 50 nM of TGF-β1 or 200 nM of Fas ligand (anti-Fas) also increased the percentage of the cells undergoing apoptosis (Fig. 3, A and Ca). The EC₅₀ for BMP-2-induced apoptosis in human PASMCs was ~75 nM (Fig. 3Cb) and for Fas ligand was ~97 nM (Fig. 3Cc). Furthermore, incubation of human PASMCs in media that contained 20 nM ST increased the percentage of apoptotic cells from 3.0 ± 0.7 to...
The time course of the apoptotic effect of ST (20 nM) on PASMCs indicated that the ST-mediated apoptosis in human PASMCs maximized at 20–24 h after treatment (Fig. 3Bb).

BMP-2 activates Smad1 and downregulates Bcl-2 expression. The basic signaling system of BMP is composed of two BMP receptors and the Smad proteins that are phosphorylated and translocated into the nu-
treatment with BMP-2 for 12, 24, and 48 h; **

protein level of summarized data show the protein level of Bcl-2 (normalized to Cb: Western blot analysis of Bcl-2 and /H9251 and after treatment with 200 nM BMP-2 for 12, 24, and 48 h. /H11002 /H11001 agarose gels for Bcl-2 (153 bp) and Bax (131 bp) in control PASMCs (Cont).

Ba treated with (P/H11002) BMP-2; **

p BMP-2 for 2, 4, 8, 24, and 36 h; *

p Smad1 before (open bar) and after (solid bars) treatment with Smad1) and phosphorylated Smad1 (pSmad1) in human PASMCs. These results demonstrate that BMP-7 induces apoptosis in these cells.

Under baseline conditions, 5.35 ± 1.34% of PASMCs were stained positive for annexin V. After treatment with 200 nM BMP-7 for 48 h, there was a significant increase in annexin V staining to 23.57 ± 4.35% (Fig. 5, A and B). The annexin V-positive cells exhibited the decreased cell size that is indicative of apoptotic cell shrinkage. Under baseline conditions, 7.98 ± 5.03% of the cells were in a size range of 50–260 AU; after treatment of the cells with BMP-7, 25.12 ± 8.79% of the cells were in this size range (Fig. 5C). Based on the dose-response curve shown in Fig. 5D (left), BMP-7 (200 nM for 48 h) induced apoptosis in human PASMCs with an EC50 of ~50 nM. The time course indicated that the minimal time required for BMP-7 (200 nM) to induce apoptosis in human PASMCs was between 12 and 24 h, and the time required to cause 50% of the maximal apoptotic effect was ~35 h (Fig. 5D, right). These results demonstrate that BMP-7, although not expressed in PASMCs, also induces apoptosis in human PASMCs.
BMP- and ST-induced apoptosis is inhibited in PASMCs from PPH patients. Intrinsic abnormalities of pulmonary vascular smooth muscle are present in PPH and may be important in its pathogenesis and etiology (3, 9, 15, 45, 55). In patients with pulmonary hypertension secondary to cardiopulmonary disease such as chronic obstructive pulmonary disease and interstitial pulmonary fibrosis, the elevated PAP may arise from different cellular and molecular mechanisms. To elucidate whether the programmed cell death in PASMCs is altered uniquely in PPH, we compared the apoptotic effects of BMPs and ST between PASMCs isolated from two patients with PPH and three patients with SPH, pulmonary hypertension secondary to idiopathic pulmonary fibrosis, emphysema, and lymphangioleiomyomatosis. The averaged mean PAP of the two PPH patients was 52.0 ± 1.4 mmHg, whereas the mean PAP of the three SPH patients was 36.5 ± 12.3 mmHg. Unlike the SPH patients, the PPH patients were treated with Flolan (prostacyclin) intravenously before lung transplantation.

As shown in Fig. 6A, both SPH and PPH patients exhibited significant pulmonary vascular wall thickening, and the overall thickness of pulmonary arterial wall was similar between SPH and PPH patients (Fig. 6B). The PASMCs prepared from these hypertrophied peripheral pulmonary arteries, which had been cultured and passaged for the same period of time before experimentation, had similar morphologies in SPH and PPH patients (Fig. 7). However, the growth rate (data not shown) and cytoplasmic free Ca++ concentration (63) were significantly increased, whereas K+ channel activity was markedly reduced (63, 64), in PASMCs from PPH patients compared with SPH patients.

To determine whether basal apoptosis and BMP-mediated apoptosis are different in PASMCs from SPH and PPH patients, we isolated PASMCs from each of the patients’ lung tissues and plated the cells on coverslips in six-well petri dishes. In each of the following experiments, the percentage of the cells undergoing apoptosis either under baseline control conditions or after treatment with BMPs (BMP-2 and -7) was measured in at least 12 coverslips of PASMCs from each of the patients. To compare the basal apoptosis rate and BMP-mediated apoptosis between SPH and PPH patients, the percentage of the cells undergoing apoptosis was averaged, respectively, among all the PASMCs from PPH patients compared with SPH patients.

Fig. 5. Apoptotic effect of BMP-7 on human PASMCs. A: representative cell nuclei stained with DAPI and annexin-V in a control cell and a cell treated with 200 nM of BMP-7. Apoptotic cell was stained positively with annexin V (green). B: PASMCs stained with annexin V-FITC and propidium iodide (PI) were analyzed by flow cytometry. A scatter plot of PI (y-axis) vs. annexin V fluorescein (x-axis) indicates the alive cells (bottom-left quadrant), necrotic cells (top-left quadrant), end-stage apoptotic cells (top-right quadrant), and early apoptotic cells (bottom-right quadrant). Number of annexin V-positive apoptotic cells (top- and bottom-right quadrants) was increased from 7.44% in control cells to 23.57% in BMP-7-treated cells. C: treatment with BMP-7 (200 nM for 48 h) displays a shift of the cell population to lower values in the forward scatter scale (M1), which is indicative of cell shrinkage. D: dose-response curve (left) and time course (right) of BMP-7-induced PASMC apoptosis; n = 21 fields of cells from 3 coverslips for each data point; *P < 0.05, **P < 0.001 vs. control cells (0 nM BMP-7 or 0 h in BMP-7 treatment). Baseline control levels of apoptosis in control cells are indicated (horizontal dashed lines).
tested from the two PPH patients (PPH-PASMCs) and all the PASMCs tested from the three SPH patients (SPH-PASMCs).

In PASMCs isolated from these patients, the apoptotic nuclei under baseline conditions were found in 7.2 ± 0.8% (n = 32) of SPH-PASMCs and 4.3 ± 0.5% of PPH-PASMCs (n = 32; P < 0.01). Treatment of the cells with BMP-2 and -7 induced apoptosis in PASMCs from both SPH and PPH patients. In SPH-PASMCs, BMP-2 and -7 increased the percentage of apoptotic cells from 7.35 ± 1.08 to 27.85 ± 2.47% (a 2.79-fold increase) and from 9.11 ± 1.25 to 29.29 ± 2.28% (a 2.21-fold increase), respectively (Fig. 8, A and B, left). In PPH-PASMCs, BMP-2 and -7 increased the percentage of apoptotic cells from 4.73 ± 0.88 to 12.56 ± 1.29% (a 1.65-fold increase) and from 5.52 ± 0.73 to 14.03 ± 1.15% (a 1.54-fold increase), respectively (Fig. 8, A and B, left).

To test whether PASMC apoptosis is inhibited in PPH, we compared the BMP-2- and -7-induced apoptosis between SPH- and PPH-PASMCs by determining the net increase of apoptotic nuclei after treatment (i.e., the difference between the percentage of cells undergoing apoptosis before and after treatment). As shown in Fig. 8, A and B (right), BMP-2-mediated PASMC apoptosis was inhibited by 68% in PPH patients (24.49 ± 2.07 in SPH-PASMCs vs. 7.83 ± 1.51% in PPH-PASMCs; P < 0.001), and BMP-7-mediated apoptosis was inhibited by 58% in PPH-PASMCs (20.18 ± 2.99 in SPH-PASMCs vs. 8.50 ± 2.02% in PPH-PASMCs; P < 0.001). These results suggest that PASMCs isolated from PPH patients are more resistant to BMP-2 and -7 than cells isolated from SPH patients.

Furthermore, the ST-mediated apoptosis was also inhibited in PPH patients by 30.7% compared with cells from SPH patients (Fig. 8C). These results suggest that there are other apoptotic pathways that may be abnormal in PPH-PASMCs in addition to the BMP-mediated apoptotic pathway.

Downregulated BMP-RII in PASMCs from PPH patients. Mutations in the BMP-RII gene have been found in 20–25% patients with sporadic PPH (9, 10, 32, 34, 41). Using immunohistological approaches, Atkinson et al. (4) reported that protein expression of BMP-RII was markedly decreased in the peripheral lung of PPH patients. The downregulation of BMP-RII not only existed in the PPH patients that harbored BMP-RII gene mutations but also was found in the PPH patients without BMP-RII gene mutations (4). Consistent with these results, we also observed that mRNA expression of BMP-RII in PASMCs from PPH patients was much lower than that in PASMCs from SPH patients (Fig. 9, A and B). However, mRNA levels of BMP-RIa and -RIb appeared to be comparable in PASMCs from PPH and SPH patients (Fig. 9, A and B). These results suggest that reduced expression of BMP-RII protein in PPH

![Fig. 6. Histological examination of pulmonary arteries in lung tissues isolated from patients with secondary and primary pulmonary hypertension (SPH and PPH, respectively). A: muscular arteries seen in two SPH (left) and two PPH (right) patients demonstrate a similar degree of medial thickening as shown (hematoxylin eosin stain, original magnification ×200). B: summarized data show the averaged thickness of pulmonary artery (PA) wall determined by arbitrary measurement of PA wall thickness on the histological slices in SPH and PPH patients.](http://ajplung.physiology.org/)

![Fig. 7. Morphology and purity of human PASMCs in culture. A: phase-contrast photomicrographs show cultured PASMCs prepared from SPH and PPH patients. B: cultures that were stained with the smooth muscle a-actin antibody (red) and the nucleic acid dye, DAPI (green) show that all DAPI-positive cells cross-react with the a-actin antibody in both SPH- and PPH-PASMCs. Horizontal bars denote 20 μm.](http://ajplung.physiology.org/)

![Fig. 8. A and B (right), BMP-2-mediated PASMC apoptosis was inhibited by 68% in PPH patients (24.49 ± 2.07 in SPH-PASMCs vs. 7.83 ± 1.51% in PPH-PASMCs; P < 0.001), and BMP-7-mediated apoptosis was inhibited by 58% in PPH-PASMCs (20.18 ± 2.99 in SPH-PASMCs vs. 8.50 ± 2.02% in PPH-PASMCs; P < 0.001). These results suggest that PASMCs isolated from PPH patients are more resistant to BMP-2 and -7 than cells isolated from SPH patients.](http://ajplung.physiology.org/)

![Fig. 9. A and B: summarized data show the averaged thickness of pulmonary artery (PA) wall determined by arbitrary measurement of PA wall thickness on the histological slices in SPH and PPH patients.](http://ajplung.physiology.org/)
patients (4) may be due to inhibited transcription of the BMP-RII gene in PASMCs, and that the downregulated or mutated BMP-RII may lead to dysfunction of BMP signaling (9, 46). Indeed, BMP-2-mediated activation or phosphorylation of Smad1 was significantly reduced in PASMCs from PPH patients (22.82 ± 9.05 AU) compared with cells from SPH patients (37.53 ± 6.51 AU; n = 5 experiments; P < 0.01 by paired t-test; Fig. 9C). The 39% reduction of BMP-2-mediated phosphorylation or activation of Smad1 in PPH-PASMCs suggests an inhibited BMP-RII function in PPH-PASMCs. It is unclear, however, whether the inhibited BMP-RII function is due to mutation or downregulation of BMP-RII in these cells (4, 9, 38, 46).

DISCUSSION

Apoptosis is crucial for normal development and homeostasis of multicellular organisms. The programmed cell death plays an important role in cell number control in various tissues and organs by balancing cell growth and multiplication and by eliminating unnecessary cells (14, 20, 26, 54). Therefore, the precise control of apoptosis and proliferation of PASMCs plays a critical role in maintaining normal pulmonary vascular structure and function (3, 43, 45, 50, 55). In patients with PPH, hypertrophy of the pulmonary vascular wall and obliteration of small pulmonary arteries are the major causes for the elevated pulmonary vascular resistance and PAP. The pulmonary vascular remodeling characterized by medial hypertrophy due to smooth muscle cell growth is one of the important factors contributing to narrow the vascular lumen and increase the pulmonary vascular resistance.
sistance (3, 43, 45, 50, 55). It has been demonstrated that increased PASMC proliferation and/or inhibited PASMC apoptosis both contribute to induce pulmonary vascular medial hypertrophy (3, 7, 8, 43, 50, 55). However, the precise mechanisms involved in the regulation of PASMC proliferation and apoptosis in PPH are still incompletely understood.

PASMC proliferation and apoptosis are regulated by many vasoactive agonists, growth factors, and cytokines (3, 9, 43, 50, 55). The TGF-β family includes multifunctional peptides that are involved in the regulation of embryonic development and tissue homeostasis. The TGF-β family members, which include TGF-βs, activins, and BMPs, are secreted intercellular signaling molecules that regulate cell proliferation, differentiation, migration, and apoptosis (9, 22, 35–39, 41, 53, 56–61). Because mutations of the BMP-RII gene have been found in familial and sporadic PPH patients (10, 32, 34, 41), it is important to elucidate the physiological role of the BMP signaling system in the regulation of normal PASMC growth and apoptosis, and to define the difference of BMP-mediated effects on PASMCs from SPH and PPH patients.

**Molecular identification of BMP signaling system in human PASMCs.** BMPs, which belong to the large superfamily of TGF-β, initiate several distinct signaling cascades by binding to two types of transmembrane serine-threonin kinase receptors, BMP-RI and -RII (29, 35–37, 44). The BMP signaling cascades play an important role in embryonal development and adult tissue homeostasis (5, 27). The well-recognized roles of TGF-β superfamily members in regulating cell proliferation, differentiation, and apoptosis suggest that BMPs must be involved in regulating vascular smooth muscle cell proliferation and apoptosis under both physiological and pathological conditions (9, 22, 35–39, 41, 53, 56–61).

The results from this study show that 1) six types of BMP transcripts (BMP-1 through -6), three types of BMP receptor transcripts (BMP-R1a, -R1b, and -R1I), and seven types of Smad transcripts (Smad1 through -7) were detected by using RT-PCR analysis in PASMCs from normal subjects; 2) BMP-7 was not detectable in PASMCs (prepared from either normal subjects or patients with SPH and PPH) but was highly expressed in lung tissues; and 3) three isoforms of TGF-β transcripts (TGF-β1, -β2, and -β3) and two types of TGF-β receptor transcripts (TGF-β-R1I and -R1II) were found in PASMCs. These results suggest that the BMP/TGF-β signaling system may play an important role in the regulation of human PASMC growth and apoptosis.

**Antiproliferative effect of BMPs involves induction of apoptosis.** In human aortic smooth muscle cells, BMP-2 and -7 inhibit cell proliferation (11, 57) and increase cell differentiation (11), whereas BMP-2 inhibits rat vascular smooth muscle cell proliferation (39). Recently, Morrell and colleagues (38) demonstrated that TGF-β1 and BMP-2, -4, and -7 at doses of 1–100 ng/ml inhibited [3H]thymidine incorporation in PASMCs from normotensive and SPH patients cultured in medium that contained FBS. The antiproliferative effect of TGF-β1 and BMP-2, -4, and -7 (which appeared to be independent of the doses ranging from 0.1 to 100 ng/ml) was significantly attenuated and even reversed to be proliferative in PASMCs from PPH patients (38).

Furthermore, compared with normotensive and SPH patients, the protein expression of BMP-RII in lung tissues (e.g., in pulmonary vascular endothelium and arterial smooth muscle) was markedly reduced in PPH patients with and without mutations of the BMP-RII gene (4). These results provide compelling evidence that BMPs and their receptors and downstream signal transduction are required for preventing normal PASMCs from overgrowth (i.e., hypertrophy and hyperplasia), which is important in maintaining the thin pulmonary vascular wall and low pulmonary vascular resistance under normal conditions. Mutation and/or downregulation of the BMP ligands and receptors as well as defects in the downstream signaling pathway would therefore enhance pulmonary vascular remodeling and increase pulmonary vascular resistance and PAP in patients with PPH (4, 9, 10, 32, 34, 41, 46).

**Role of inhibited PASMC apoptosis in development of pulmonary vascular remodeling.** The results from this study demonstrate that, in PASMCs isolated from normal subjects and SPH patients, 1) BMP-2 (which is highly expressed in PASMCs) and BMP-7 (which is not expressed or the expression level is extremely low in PASMCs) both significantly increased the percentage of the cells undergoing apoptosis at doses ranging from 100 to 200 nM; and 2) the BMP-2-mediated apoptosis in PASMCs was associated with activation or phosphorylation of Smad1 and with a marked inhibition of Bel-2 expression. In PASMCs isolated from PPH patients, the BMP-2- and -7-mediated apoptosis as well as the ST-induced apoptosis were all significantly inhibited (by 30–68%) compared with PASMCs isolated from normal subjects and SPH patients. Furthermore, the mRNA expression of BMP-RII and the BMP-2-mediated phosphorylation or activation of Smad1 were markedly inhibited in PASMCs from PPH patients compared with cells from SPH patients. These results suggest that 1) an additional mechanism involved in the antiproliferative effect of BMPs is to induce apoptosis in normal human PASMCs when the cells are exposed to high doses of BMPs, and 2) the inhibited apoptosis in PASMCs from PPH patients may contribute to the initiation and/or progression of pulmonary vascular medial hypertrophy in these patients. It is also important to note that the SPH-PASMCs and PPH-PASMCs were derived from similarly sized vessels (the peripheral resistance pulmonary arteries with the diameter ranges from 300 to 500 μm) and yet responded differently to the apoptotic inducers (BMP-2, -7, and ST).

Apoptosis is a highly regulated process that eliminates unnecessary cells (14, 20, 26, 54) such as cells migrated into the vascular lumen and hypertrophied cells accumulated in the pulmonary vasculature (8, 43). Thus timing and location of cell death as well as cell growth and division must be precisely controlled to...
maintain the normal pulmonary vascular structure. Compared with PASMCs from normal subjects and patients with SPH, PASMCs from PPH patients exhibited a significant resistance to apoptotic inducers such as BMP-2, -7, and ST. The inhibited apoptosis in PPH-PASMCs would thus at least in part contribute to the thickening of the pulmonary vascular wall that is observed in patients with PPH. Our histological data indicate that SPH and PPH patients both exhibited significant pulmonary vascular remodeling characterized by medial hypertrophy or vascular wall thickening in small pulmonary vessels. However, the BMP- and ST-mediated apoptosis was only inhibited in PPH-PASMCs but not in SPH-PASMCs. These results suggest that pulmonary arterial medial hypertrophy is caused by different mechanisms in patients with SPH and PPH. A unique mechanism involving the BMP signaling pathway responsible for normal apoptosis in PASMCs is inhibited in PPH patients but not in SPH patients.

The PPH patients from whom we isolated PASMCs for this study were treated with Flolan (prostacyclin) before lung transplantation, whereas the SPH patients were not treated with Flolan but were treated with other drugs such as aspirin, citalopram, estrogen, or narcotic pain medicines. We cannot exclude the possibility that the difference of BMP-mediated apoptosis between SPH-PASMCs and PPH-PASMCs was related to different treatment for SPH and PPH patients. It is, however, unlikely that the inhibited apoptosis in PPH-PASMCs was due to continuous treatment of the PPH patients with Flolan, since prostacyclin has been demonstrated to inhibit PASMC proliferation and cause regression of pulmonary arterial medial hypertrophy. If the decreased apoptotic effect of BMPs on PPH-PASMCs compared with SPH-PASMCs was due to the Flolan treatment, one would have to assume that prostacyclin inhibits PASMC apoptosis and/or attenuates BMP-mediated apoptosis. To our knowledge, prostacyclin has not been demonstrated to have antiapoptotic effects on vascular smooth muscle cells and to interfere with the BMP signaling system.

Whether the apoptosis rate is inhibited in vivo in pulmonary arteries from the PPH patients is unknown. Rabinovitch and colleagues have reported that the induction of PASMC apoptosis by suppression of metalloprotease and tenascin-C expression results in regression of pulmonary medial hypertrophy and reduction of PAP (7, 8, 43). Taken together with our results shown in this study, we speculate that inhibition of apoptosis in PASMCs is involved in the development and progression of pulmonary arterial medial hypertrophy, whereas induction or enhancement of PASMC apoptosis may be targeted to develop therapeutic approaches for pulmonary vascular remodeling in patients with PPH. As shown in Fig. 8, an apoptotic response to BMP-2 or -7 (~15% or two- to threefold above the baseline) still existed in PPH-PASMCs. It is unclear whether this “residual” apoptosis induced by BMP-2 or -7 in PPH-PASMCs would be sufficient to remove unnecessary or “misguided” PASMCs in vivo and to prevent pulmonary medial hypertrophy in these patients.

Regulation of gene transcription by BMP signaling system. Binding of BMP ligands (e.g., BMP-2 and -7) to either of the receptors (BMP-RI or -RII) encourages the two types of receptors to dimerize with one another and to form a ligand-receptor complex. The activated BMP-RI phosphorylates the R-Smad proteins (e.g., Smad3, -5, and -8), which then dimerize with co-Smad (e.g., Smad4). The R-Smad and co-Smad complex translocates into the nucleus and controls transcription of the target genes that contain the Smad binding sequence (5′-CAGAC-3′ and 5′-GTCTG-3′) in their promoter (22, 35–37, 44, 48, 56). In addition to the R-Smads and co-Smads, vertebrates and humans also express the antagonistic Smads including Smad6 and -7 (18, 19, 23–25), which mediate negative feedback within TGF-β/BMP signaling pathways and regulatory inputs from other pathways. The antagonistic Smads serve as an R-Smad decoy to compete for the activated tyrosine kinase and therefore to inhibit activation of R-Smads (35). Smad6 is an antagonistic Smad that preferentially inhibits BMP signaling by blocking activation of Smad1, -5, or -8 (18, 23, 24).

In addition to activating gene transcription by binding onto the Smad-binding sequence in the promoter, increased Smads in the nucleus can form heterogeneous polymers with corepressors such as the homeodomain protein TGIF (59) and the two related proteins c-Ski and Snon (2, 33, 51) to induce repression of target gene transcription (35, 48, 56). An additional inhibitor of Smads is the Smad-interacting protein-1 (35), a zinc-finger/homeodomain protein that interacts with Smad1 and -5 in mammalian cells and inhibits BMP-mediated effects.

Increased Smads in the nucleus can also form heterogeneous polymers with other transcription factors (e.g., activator protein-1) to mediate apoptosis (60). Furthermore, Smad proteins have been reported to interact with calmodulin (65), a Ca2+-sensitive protein in the cytosol. Overexpression of calmodulin inhibits Smad activation and attenuates the response of TGF-β/BMP signal transduction (65), which suggests that an increase in cytoplasmic Ca2+ concentration may exert an inhibitory effect on the TGF-β/BMP signaling pathway by activating calmodulin (13) and thereby attenuates TGF-β/BMP-mediated apoptosis in human PASMCs.

Cellular mechanisms involved in BMP-induced apoptosis. It has been demonstrated that BMPs inhibit proliferation in rat (39) and human (11, 57) aortic smooth muscle cells and human PASMCs (38) and induce apoptosis in human PASMCs (this study), normal human lung epithelial cells (61), and HepB3 cell lines (60). The precise mechanisms by which BMP-2 and -7 induce apoptosis in human PASMCs are still unknown. Our study indicates that treatment of PASMCs from normal subjects with BMP-2 decreased Bcl-2 mRNA and protein expression. It is unknown whether and which of the nuclear corepressors are
involved in the BMP-mediated downregulation of Bcl-2 gene transcription in PASMCs. Bcl-2 is an antiapoptotic protein that attenuates apoptosis by 1) inhibiting cytochrome c and apoptotic protease release (28, 52, 62); 2) blocking K⁺/H⁺11001 channels (12); 3) maintaining Ca²⁺/H⁺11001 in the sarcoplasmic and endoplasmic reticulum (21, 31); and 4) regulating proton flux in mitochondria (49). In lung tissues from sporadic and familial PPH patients, Geraci et al. (16) showed that the mRNA expression of Bcl-2 was upregulated. The upregulated Bcl-2 gene transcription may be related to the mutations in the BMP-RII gene and/or dysfunction of BMP signaling. These results imply that modulation of Bcl-2 gene expression is a critical mechanism in directing human PASMCs to undergo proliferation or apoptosis (Fig. 10).

Taken together with the data from this study and from other investigators, we speculate that BMP-mediated apoptosis in PASMCs from normal subjects is at least in part due to downregulation of Bcl-2 expression, whereas decreased BMP-receptor signaling (e.g., in PPH patients who have BMP-RII mutations) may lead to the upregulation of Bcl-2 (16) and inhibition of cytochrome c- or caspase-9-dependent apoptosis in PASMCs. The subsequent decrease in the ratio of cell apoptosis to proliferation favors PASMC growth, thereby mediating pulmonary vascular medial hypertrophy (Fig. 10). It is unknown whether the PPH patients from whom we obtained PASMCs contain any mutations of BMP-RII as reported by Machado et al. (34). The resistance to BMP-induced apoptosis in PASMCs from the PPH patients suggests that reduced BMP-RII signaling and/or BMP-RII/Smad-mediated gene transcription play an important role in the pathogenesis of PPH in patients with or without mutations of the BMP-RII gene (4, 9, 10, 32, 34, 41).

Summary. In normal PASMCs, the antiproliferative effects of BMPs (e.g., BMP-2 and -7) involve the induction of apoptosis. The cellular mechanisms by which BMPs induce PASMC apoptosis involve phosphorylation of Smad1 and downregulation of Bcl-2 expression. In PASMCs from PPH patients, the basal apoptosis and the BMP-mediated apoptosis rates are both inhibited compared with PASMCs from SPH patients. Although they need to be verified by in vivo study, the results from these in vitro experiments suggest that inhibition of apoptosis in PASMCs may play a role in
the development of pulmonary vascular remodeling in patients with PPH.

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DISCLOSURES

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BMP INDUCES APOPTOSIS


