Downregulation of type II bone morphogenetic protein receptor in hypoxic pulmonary hypertension

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BONE MORPHOGENETIC PROTEINS (BMPs) are signaling molecules capable of binding with BMPs have been cloned in mammals (28). BMPs are synthesized and secreted from a variety of cell types, including pulmonary vascular smooth muscle cells and endothelial cells. BMPs induce the heteromeric complex formation between type II and type I receptors (11, 17, 27, 28). The constitutively active type II receptor kinase activates the type I receptor, which subsequently activates the downstream signaling pathway through Smad phosphorylation and/or MAPK (16).

Recently, heterozygous germline mutations that involve the gene encoding the BMPR-II have been identified to underlie many cases of familial and sporadic primary pulmonary hypertension (PPH) (3, 12, 15). The precise molecular mechanisms of disease pathogenesis remain to be elucidated but are postulated to be involved in the alteration of BMPR-II function. In addition to these mutations, marked reduction of BMPR-II expression in the lung was observed in patients with PPH in which no mutation was identified in the BMPR-II gene and also among patients with secondary pulmonary hypertension (PH) (1). Furthermore, the expression of BMPR-1A was found to be reduced in pulmonary arteriolar endothelial cells derived from nonfamilial pulmonary hypertension (4). These observations suggest that BMP signaling pathways may be implicated in the molecular pathogenesis of secondary PH as well as familial and sporadic PPH. PPH is a rare disorder, and most cases with PH are due to a variety of causes, including chronic obstructive pulmonary disease (COPD), thromboembolism, left side heart failure, collagen vascular diseases, and exposure to appetite suppressants (4, 9). Secondary PH results from sustained vasoconstriction and structural alterations of the pulmonary vascular bed. The major stimuli responsible for these changes are chronic alveolar hypoxia, chronic inflammation, and excessive shear stress. Of those, hypoxic PH is important because chronic alveolar hypoxia is closely associated with the development of PH in patients with COPD and other chronic lung diseases, which results in increased morbidity and mortality (8).

In the present study, the cellular distribution and temporal changes in the expression of BMP-2 and BMPR-II in the rat pulmonary artery under normal conditions and after exposure to hypoxia were investigated to clarify the role of these molecules in the pathogenesis of hypoxic PH. We also investigated the downstream signaling pathways of BMPR to explore the molecular mechanisms involving pulmonary vascular remodeling in hypoxic PH.
DOWNREGULATION OF BMPR-II IN HYPOXIC PH

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METHODS

Animals. All protocols and surgical procedures were approved by the Institutional Animal Use Committee of Juntendo University School of Medicine in accordance with the National Institutes of Health and American Physiological Society guidelines. The methods utilized to isolate the rat lungs are almost identical to those previously reported except strict precautions were exercised to keep the animals under hypoxic conditions before lung preparation, since expression of several genes has been reported to decrease rapidly in rats exposed to chronic hypoxic PH after a brief exposure to normoxia (23). Briefly, adult male Sprague-Dawley rats (6- to 7-wk old, 230–250 g) were kept for 0.5, 3, 7, 14, or 21 days in a specially designed hypobaric chamber. The chamber was depressurized to 380 mmHg in a room with a 12-h light-dark cycle. To minimize exposure to normoxia, rats raised in the hypobaric chamber were transferred to and kept in a chamber filled with 10% oxygen/90% nitrogen immediately before lung preparation. Age-matched controls were maintained in room air. The lungs were isolated from rats after the intraperitoneal administration of 60 mg of pentobarbital sodium and intracardiac injection of heparin (100 units). Cannulas were inserted into the pulmonary artery and left atrium, and the lungs were perfused with 36 cmH2O with PBS. The right lung was excised and cut into 3- to 5-mm-thick slices, which were frozen in liquid nitrogen, and stored at the pulmonary arterial cannula with PBS. The right lung was excised and cut into 3- to 5-mm-thick slices, which were frozen in liquid nitrogen, and stored at −80°C until analysis and determination of total cellular RNA and tissue protein. The left lung was perfused with 4% paraformaldehyde (PFA), infiltrated by infusion of 4% PFA through the cannula inserted in the trachea, fixed in 4% PFA overnight at 4°C, and then embedded in paraffin. Development of hypoxic pulmonary hypertension was determined by the weight ratio of the right ventricle over the left ventricle plus septum (RV/LV+S), as previously described (21, 23). Each group consisted of six to seven experimental animals.

RT-PCR analysis. mRNA expression of BMP-2 and BMPR-II was evaluated with semiquantitative RT-PCR. RT-PCR was performed as previously described (22). Briefly, reverse transcription was performed using Superscript reverse transcriptase (Invitrogen), oligo(dT)12-18 (Pharmacia Biotec, Milwaukee, WI), and 2 μg of denatured total RNA isolated from the rat lung as a template. To use RT-PCR semiquantitatively, we assessed the relationship between cycle numbers and optical density of the PCR products by varying the PCR conditions: the mixture was denatured at 94°C (30 s), annealed at 80°C until analysis and determination of total cellular RNA and tissue protein. The left lung was perfused with 4% paraformaldehyde (PFA), infiltrated by infusion of 4% PFA through the cannula inserted in the trachea, fixed in 4% PFA overnight at 4°C, and then embedded in paraffin. Development of hypoxic pulmonary hypertension was determined by the weight ratio of the right ventricle over the left ventricle plus septum (RV/LV+S), as previously described (21, 23). Each group consisted of six to seven experimental animals.

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Table 1. Primer sequences and PCR conditions for BMP-2, BMPR-II, and 18S rRNA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Size</th>
<th>AT (°C)</th>
<th>PCR Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-2</td>
<td>Forward 5'-TCCATACGAGAAGAGCACTC-3' Reverse 5'-TTGATGTCTTCTTACGCCTG-3'</td>
<td>489</td>
<td>60</td>
<td>24</td>
</tr>
<tr>
<td>BMPR-II</td>
<td>Forward 5'-GGATTGTGAGAGTCAATC-3' Reverse 5'-CTCTGAACTTCTGCATC-3'</td>
<td>387</td>
<td>55</td>
<td>25</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>Forward 5'-AAAATTTTTAAATTTGGGCCC-3' Reverse 5'-GGTTAATACCCCTTGTTAG-3'</td>
<td>350</td>
<td>55</td>
<td>12</td>
</tr>
</tbody>
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AT, annealing temperature; BMP, bone morphogenetic protein; BMPR, bone morphogenetic protein receptor.

Western blotting. Frozen lung tissues were homogenized in radioimmunoprecipitation assay buffer [150 mM NaCl, 1.5 mM MgCl2, 10 mM NaF, 10% glycerol, 4 mM EDTA, 1% Triton X-100, 0.1% SDS, 50 mM HEPES, pH 7.4, and 1% deoxycholate with 1 mM protease inhibitor cocktail (Calbiochem-Novaviochem, La Jolla, CA)] and centrifuged for 15 min at 12,000 g to remove cellular debris. The protein concentration was estimated using a Micro BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL) by using BSA as the standard. An equal amount of protein (10–50 μg) from each sample was diluted with 2× reducing sample buffer (0.5 M Tris-Cl, 2% 2-mercaptoethanol, 87% glycerol, 10% SDS, and 0.1% bromphenol blue) and boiled for 5 min. The protein suspensions were electrophoretically separated on 6, 10, or 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Mipore). The membranes were blocked for nonspecific binding in a 5% skimmed milk solution at room temperature for 1 h and then incubated with the primary antibody overnight at 4°C. Antibodies against BMP-2 and BMPR-II (Santa Cruz Biotechnologies, Santa Cruz, CA) were used at 1 μg/ml, and antibodies against phosphorylated Smad1/5/8, phosphorylated p38 MAPK, phosphorylated Erk1/2 MAPK, and cleaved caspase-3 (Cell Signaling) were used at 1:1,000 dilution in 1% BSA/0.1% Tween 20/Tris-buffered saline (TBS). Thereafter, the membranes were washed 3 × 10 min with 0.1% Tween 20/TBS, incubated for 1 h with horseradish peroxidase-conjugated anti-goat IgG (for BMP-2, BMPR-II) or anti-rabbit antibodies (for phosphorylated Smad1/5/8, p38 MAPK, Erk1/2 MAPK, caspase-3; Cell Signaling) at 1:10,000 dilution in 1% BSA/0.1% Tween/TBS, reacted with enhanced chemiluminescence substrate (Amersham Pharmacia Biochem), and finally exposed to radiographic films (Amersham). The films were scanned, and relative density of the obtained products was measured using the QuantityOne software (Bio-Rad, Hercules, CA). To confirm equal protein loading, blots for BMP-2 and BMPR-II were stripped with Restore Western (Pierce Biotechnologies) and reprobed using an anti-actin antibody (Santa Cruz). In cases of phosphorylated p38 MAPK, phosphorylated Erk1/2 MAPK, phosphorylated Smad1/5/8, and cleaved caspase-3, the same blots were subsequently stripped with Restore Western and reprobed with anti-total p38 MAPK, anti-total Erk1/2 MAPK, anti-Smad5, or anti-uncleaved caspase-3 antibodies (Cell Signaling), respectively, as internal controls. The results of p38 MAPK, Erk1/2 MAPK, Smad, or caspase-3 were calculated as the ratio of phosphorylated kinase vs. total kinase, phosphorylated Smad1/5/8 vs. Smad5, or cleaved (active) enzyme vs. uncleaved (inactive) enzyme, respectively.

Immunohistochemistry. Immunohistochemical analysis was carried out as previously described (21). Briefly, paraffin sections (4 μm) were deparaffinized, rehydrated, and incubated with blocking serum (10% normal rabbit serum or goat serum) for 30 min at room temperature before being incubated with primary antibody to reduce nonspecific binding of secondary antibodies. The serum was removed, and the sections were incubated with goat anti-BMP-2 antibody.

Table 1. Primer sequences and PCR conditions for BMP-2, BMPR-II, and 18S rRNA
the pulmonary vessels were estimated in lung sections from each animal, and the data were calculated for each group. In each animal, at least 20 arteries [60–200 μm external diameter (ED)] associated with terminal bronchioles or lying within the acinus were analyzed.

The immunohistochemistry experiments were controlled by incubation of the tissue with a nonimmune goat or rabbit IgG at the same concentration as the primary antibody. Furthermore, the specificity of BMP-2 and BMPR-II antibodies was demonstrated by incubation of the tissue with the preabsorbed primary antibodies with their respective blocking peptides (Santa Cruz). Little nonspecific staining was ever observed in either case, confirming the specificity of the staining reactions (data not shown). Pulmonary arteries were distinguished from pulmonary veins on the basis of anatomical location and structure (23).

In situ identification of nuclear DNA fragmentation in rat lungs. After being deparaffinized, the tissue sections were stained by using in situ TdT-mediated dUTP nick end labeling (TUNEL) method with Biotin016-dUTP (Roche Diagnostics, Penzberg, Germany) to identify cells demonstrating nuclear DNA fragmentation. The procedure is based on the method described by Gavrieli et al. (6). Residues of digoxigenin nucleotide are catalytically added to the DNA by TdT, an enzyme that catalyzes a template-independent addition of deoxynucleotide triphosphate to the 3'-OH ends of the double- or single-stranded DNA. The anti-digoxigenin antibody fragment carries a peroxidase (Santa Cruz Biotechnology) at 4 μg/ml, goat anti-BMPR-II at 4 μg/ml, rabbit anti-phospho-p38 MAPK at 1:200 dilution, or rabbit anti-cleaved caspase-3 antibody at 1:200 dilution for 12 h at 4°C. In addition, monoclonal antibodies against vascular cell adhesion molecule-1 (5 μg/ml, Santa Cruz) and α-smooth muscle actin (α-SM actin; 0.2 μg/ml; Dako, Glostrup, Denmark) were used as the endothelial marker and smooth muscle marker, respectively. After being washed with PBS-Tween, the sections were incubated with the secondary antibodies: 1:300 dilution of a biotinylated goat anti-rabbit IgG (Dako, for phosphorylated p38 MAPK and cleaved caspase-3) or biotinylated rabbit anti-goat IgG (Dako, for BMP-2 and BMPR-II) for 45 min at room temperature. To block endogenous peroxidase activity, the sections were immersed in 0.3% H2O2 in methanol for 20 min and then incubated with streptavidin-biotin-peroxidase complex (diluted 1:300, Dako) for 30 min at room temperature. Subsequently, the immunoperoxidase color reaction was performed by incubation in Tris/HCl containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) and 0.01% H2O2 for 5–10 min at room temperature. The sections were counterstained with hematoxylin. To detect phosphorylated p38 MAPK and cleaved caspase-3, immunohistochemistry was performed in combination with biotinylated tyramide after secondary antibody incubation according to the manufacturer’s instructions (CSA System, Dako). The sections were examined by light microscopy without knowledge of the treatment group, and the intensity of immunostaining was graded semiquantitatively from 0 to 3: grade 0, no staining; grade 1, focal staining or weak staining; grade 2, diffuse moderate staining; and grade 3, strong staining (21, 23). To assess the changes in the expression of BMP-2, BMPR-II, and α-SM actin after exposure to hypoxia, the immunostaining grades of
antibody to the reaction site. For negative controls of DNA fragmentation, sections were stained without TdT. The sections of peroxidase-labeled tissue were visualized by incubation in DAB for 5 min at room temperature. Sections were subsequently counterstained with hematoxylin and were dehydrated and mounted.

Statistical methods. Numerical data are expressed as means ± SE. Comparisons between groups were made with Student’s t-test, ANOVA with Fisher’s post hoc test for multiple comparisons, or repeated-measures ANOVA. Immunohistochemical grading data were analyzed by a Mann-Whitney’s U-test using a proprietary software (Statview; Abacus Concepts, Berkeley, CA). P < 0.05 was considered significant.

RESULTS

Measurement of ventricular weights revealed the RV/LV+S ratios to be 0.26 ± 0.02 in control rats and 0.28 ± 0.01, 0.27 ± 0.03, 0.50 ± 0.02, 0.55 ± 0.02, and 0.55 ± 0.02 in rats exposed to hypoxia for 0.5, 3, 7, 14, and 21 days, respectively (control vs. 7, 14, and 21 days, P < 0.05, n = 6–7). Histological findings with elastic van Gieson staining in rats exposed to hypoxia for 7, 14, and 21 days were also consistent with previous reports of the hypoxic PH model, which shows a gradual increase in medial wall thickness and progressive extension of muscle into smaller arteries (21). The results indicated that a significant degree of right ventricular hypertrophy and vascular remodeling developed after 7–21 days of exposure to hypoxia.

Semiquantitative RT-PCR analysis was utilized to detect changes in mRNA expression of BMP-2 and BMPR-II in rat lung tissues after exposure to hypoxia (Fig. 1). Compared with control rats, the ratio of BMP-2 mRNA to 18S rRNA was approximately three to fourfold higher after 0.5 and 3 days of exposure to hypoxia and then returned to the control levels after 7–21 days. mRNA expression of BMPR-II slightly increased at 0.5 days and then decreased after 3–21 days, but the changes were not statistically significant.

Fig. 3. Representative photomicrographs of pulmonary arteries from the control and hypoxia-exposed rats stained with anti-BMP-2 (A–F) and anti-BMPR-II (G–L) antibodies. A–C and G–I show conduit arteries, and D–F and J–L show resistance arteries. Minimal endothelial staining for BMP-2 was detected in control (A and D), and a significant increase in BMP-2 expression was identified in the intima in a rat exposed to hypoxia for 3 days (B and E) and decreased 14 days after exposure to hypoxia (C and F). Abundant expression of BMPR-II protein was identified in the intima, media, and adventitia of intrapulmonary resistant artery from the control (G and J) and a rat exposed to hypoxia for 3 days (H and K). BMPR-II expression significantly decreased in a rat exposed to hypoxia for 14 days (I and L). Bar = 50 μm.
Immunoblotting analyses revealed a 17-kDa band for mature BMP-2 protein, and a 110-kDa band for BMPR-II was detected in lung tissue from the control and animals exposed to hypoxia (Fig. 2). Consistent with an elevation in mRNA expression of BMP-2 during the early stages of exposure to hypoxia, expression of BMP-2 protein in lung tissue was also increased by five- to sixfold at 0.5 and 3 days of hypoxia (P < 0.05) and then returned to the control levels after 7–21 days. In contrast, BMPR-II protein levels decreased to one-third to one-fifth of the control values after 7–21 days (P < 0.05).

In control rat lung, faint immunoreactivities for BMP-2 were identified in the intima of conduit arteries and small intrapulmonary arteries (Fig. 3). After exposure of rats to hypoxia for 0.5 and 3 days, there was an increase in immunoreactivities for BMP-2 in the intima and media of pulmonary arteries. The BMP-2 expression in the vascular walls reduced after 7–21 days. The changes in immunoreactivities appeared to be paralleled with that of immunoblotting. Semiquantitative analysis showed BMP-2 increased by ~2.5- to 3-fold in the pulmonary vessels of 60–200 μm ED after 0.5 and 3 days of exposure to hypoxia (Fig. 4A). Abundant immunoreactivities for BMPR-II were demonstrated in the intima, media, and adventitia of pulmonary resistant arteries of the control rats. Although intensity of immunoreactivities in the vascular wall of pulmonary arteries did not change after 0.5 and 3 days, significant decreases in the expression of BMPR-II were detected in rats exposed to hypoxia for 7–21 days (Fig. 3). The immunoreactivity for BMPR-II was reduced to 30–40% of the control animals after exposure to hypoxia for 7–21 days (Fig. 4B). In accordance with the decline of BMPR-II, immunoreactivities for α-SM actin increased in the pulmonary arteries after exposure to hypoxia (Fig. 4C).

Because the Smad pathway and MAPK pathway are known as downstream signal transduction of BMP-2 via BMPR-I and BMPR-II, we identified the expression of p38 MAPK, Erk1/2 MAPK, and Smad1/5/8 in lung tissue with immunoblotting. By using anti-dual phosphorylated p38 MAPK antibody, phosphorylation of p38 MAPK was readily detected in rats under normal conditions (Fig. 5A). Compared with the controls, the ratio of phosphorylated p38 MAPK to total p38 MAPK was increased by 1.5- to 2-fold at 0.5–3 days of exposure to hypoxia and then was significantly reduced to 10–15% of the control levels after 14 and 21 days (P < 0.05). Similarly, the ratio of phosphorylated Erk1/2 MAPK to total Erk1/2 MAPK significantly increased at 3 days, whereas it markedly decreased at 14 and 21 days (P < 0.05; Fig. 5B). These changes in p38 MAPK or Erk1/2 MAPK activity did not appear to be due to upregulation or downregulation of total p38 MAPK or Erk1/2 MAPK, since Western blot analysis revealed comparable levels of total p38 MAPK or Erk1/2 MAPK in the control and hypoxia-exposed rat lungs. Immunoblot analysis for phosphorylated Smad1/5/8 demonstrated a 60-kDa band in the controls and rats exposed to hypoxia. There were no significant changes in the expression of Smad1/5/8 after exposure to hypoxia (Fig. 5C).

Among the p38 MAPK-responsive genes, many encode proteins that are required to arrest cell growth and induce apoptosis. In the present study, apoptosis in the lung vasculature was determined by measuring caspase-3 activity at various points after exposure to hypoxia, since caspase-3 represents a family of cysteine proteases that acts as principal apoptotic mediators (19). Immunoblot analysis by using polyclonal antibodies to uncleaved and cleaved caspase-3 was performed to detect proapoptotic activity in the lungs of the controls and rats exposed to hypoxia. Nineteen- and 17-kDa bands for the active form (cleaved) and a 35-kDa band for the inactive form (uncleaved) of caspase-3 were constitutively expressed in the control rat lungs (Fig. 5D). The active form of caspase-3 significantly increased at 0.5 and 3 days of exposure to hypoxia and then decreased after 14 and 21 days. The ratio of 19- to 35-kDa bands was increased fourfold at 0.5 days and reduced to <30% of the control after 14 and 21 days (P < 0.01 and P < 0.05, respectively).

Comparable findings were obtained with immunohistochemical analyses for phosphorylated p38 MAPK and activated caspase-3 (Fig. 6). Immunoreactivities for phosphorylated p38 MAPK were mainly localized in the intima, media, and adventitia of the pulmonary arteries in the control animals. The immunoreactivities increased at 0.5 and 3 days of exposure to hypoxia but significantly decreased at days 14 and 21. Immunoreactivities for cleaved (active) caspase-3 were readily identified in the vascular wall of pulmonary arteries in the control animals. Increased amounts of immunoreactivities were detected at 0.5 and 3 days, with slight immunoreactivities at days 14 and 21. These results suggest that caspase-3 is activated.
Control subjects and patients with secondary PH but failed to initiate signal transduction via BMPR-I and BMPR-II: 1) binding to preformed receptor complexes and inducing a conformational change that activates this complex and results in activation of Smad pathway; and 2) binding to the high-affinity BMPR-I receptors, subsequently recruiting unliganded BMPR-II into the ligand-mediated signaling complex that induces p38 MAPK pathway (16). Although it is unknown which pathway is predominant in the pulmonary vasculature, our results suggest that p38 MAPK may play important roles in the downstream signal transduction of BMP ligands and their receptors for the following reasons. First, our experiments demonstrated that upregulation of BMP-2 occurs during the early periods of exposure to hypoxia and appears to be correlated with increased phosphorylation of p38 MAPK. Second, downregulation of BMPR-II during prolonged periods of exposure to hypoxia appears to be associated with the decline of suppressor cells from patients with PPH who are harboring heterogeneous BMPR-II mutations (13). Furthermore, Zhang et al. (31) revealed that BMP-2 induced apoptosis in human pulmonary smooth muscle cells. BMP-2 is also reported to act as a stimulus of anabolic activity and promoter synthesis of extracellular molecules, such as type II collagen in mesenchymal cells (5). Together, these studies and our findings suggest BMP-2 and its receptors, like other TGF-β family members, may play an important role to maintain normal structure and cellular components of pulmonary vasculature through regulation of apoptosis, synthesis of extracellular molecules, and suppression of cell proliferation.

The ligand BMP-2 has at least two different options to initiate signal transduction via BMPR-I and BMPR-II: 1) binding to preformed receptor complexes and inducing a conformational change that activates this complex and results in activation of Smad pathway; and 2) binding to the high-affinity BMPR-I receptors, subsequently recruiting unliganded BMPR-II into the ligand-mediated signaling complex that induces p38 MAPK pathway (16). Although it is unknown which pathway is predominant in the pulmonary vasculature, our results suggest that p38 MAPK may play important roles in the downstream signal transduction of BMP ligands and their receptors for the following reasons. First, our experiments demonstrated that upregulation of BMP-2 occurs during the early periods of exposure to hypoxia and appears to be correlated with increased phosphorylation of p38 MAPK. Second, downregulation of BMPR-II during prolonged periods of exposure to hypoxia appears to be associated with the decline of suppressor cells from patients with PPH who are harboring heterogeneous BMPR-II mutations (13). Furthermore, Zhang et al. (31) revealed that BMP-2 induced apoptosis in human pulmonary smooth muscle cells. BMP-2 is also reported to act as a stimulus of anabolic activity and promoter synthesis of extracellular molecules, such as type II collagen in mesenchymal cells (5). Together, these studies and our findings suggest BMP-2 and its receptors, like other TGF-β family members, may play an important role to maintain normal structure and cellular components of pulmonary vasculature through regulation of apoptosis, synthesis of extracellular molecules, and suppression of cell proliferation.

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This study has demonstrated the cellular distribution of BMP-2 and BMPR-II in the pulmonary vascular tissue from the control rats and animals exposed to hypoxia. Our observations suggest that BMP-2 is predominantly expressed by endothelial cells, whereas a lower level of expression is demonstrated in the vascular smooth muscle of the control rats. BMPR-II is localized in the endothelial cells, smooth muscle cells, and adventitial fibroblasts.

Although BMPs and their receptors play a critical role in embryonic lung morphogenesis, little is known about the functional properties of these proteins in the adult lung under normal conditions (11, 17). Several recent papers revealed the biological effects of BMP-2 on vascular smooth muscle cells. It has been demonstrated that BMP-2 suppressed the proliferation of pulmonary arterial smooth muscle cells derived from control subjects and patients with secondary PH but failed to...
p38 MAPK phosphorylation. Third, we failed to demonstrate any correlation between changes in expression of BMP-2 or BMPR-II and that of phosphorylated Smad1/5/8 after exposure to hypoxia. However, we could not rule out the possibility that the activation of Smad pathway also serves as a subsequent response to hypoxia-induced BMP-2 upregulation, because there are several studies that demonstrated the activation of Smad proteins in the pulmonary vascular cells by BMP ligand stimulation in other types of PH (18, 20, 29).

We demonstrated upregulation of BMP-2 accompanied with activation of p38 MAPK and Erk1/2 MAPK in the vascular wall of intrapulmonary arteries after 0.5 and/or 3 days of exposure to hypoxia. Biological significance of the upregulation of BMP-2 is unknown, but its downstream signaling of p38 MAPK or Erk1/2 MAPK is implicated as a key regulator of cellular growth and proliferation (18, 29). There is considerable evidence in the literature that MAPK can be activated in response to hypoxic stress and is speculated to promote cellular proliferation (16, 20, 25, 29). On the other hand, p38 MAPK is also demonstrated to play a role as an antiproliferative regulator of vascular smooth muscle by inducing apoptosis and/or producing antiproliferative prostaglandins (30, 31). Our experiments revealed that caspase-3 was activated in the vascular wall of pulmonary arteries after 0.5 and 3 days of exposure to hypoxia. Furthermore, we also demonstrated that TUNEL-positive apoptotic cells were increased in the vascular wall of pulmonary arteries during the early periods of exposure to hypoxia. These results suggested that apoptotic activity was increased during the early periods of exposure to hypoxia, a time point that precedes the development of PH. Because BMP-2 and p38 MAPK are known to induce apoptosis, upregulation of BMP-2 may result in activation of apoptosis through the p38 MAPK pathway (7). A recent report demonstrating induction of apoptosis in human pulmonary vascular smooth muscle cells by BMP-2 also supports our hypothesis (31). Although apoptosis may likely serve as a critical mechanism to maintain normal cell number and antiproliferative effect, biological roles for the activation of apoptosis in the development of hypoxic PH is under investigation. Some investigators suggest that apoptosis precedes vascular remod-

Fig. 6. Representative photomicrographs of intrapulmonary arteries from the control and hypoxia-exposed rats stained with anti-phosphorylated p38 MAPK (A–C) and anti-cleaved (activated) caspase-3 (D–F) antibodies. Immunoreactivities for phosphorylated p38 MAPK were mainly localized in the intima, media, and adventitia of the vessel from a control rat (A). The expression of phosphorylated p38 MAPK significantly increased 3 days after exposure to hypoxia (B), but markedly decreased at 14 days (C). Minimal immunoreactivity for caspase-3 was identified in the vascular wall of the pulmonary artery from a control rat (D). Activation of caspase-3 was demonstrated in the pulmonary arteries from rats exposed to hypoxia for 0.5 days (E), and then slight immunoreactivity was detected in the vessel from animals exposed to hypoxia for 21 days (F). Bar = 50 μm.
eling in hypoxic PH, and inhibition of apoptotic cell death by a broad inhibitor of caspase prevented the development of intravascular pulmonary endothelial cell growth and severe pulmonary hypertension in this model (24). Therefore, like other TGF-β signaling, the net results of BMP-2 signaling on vascular growth and structure are complex. Whether upregulation of BMP-2 during the early periods of exposure to hypoxia inhibits or promotes cell proliferation and vascular remodeling may be highly context specific, depending on receptor type, cell type, downstream signals, transcriptional program, and other environmental factors. Furthermore, additional factors possibly affect the expression of phosphorylated p38 MAPK and Erk1/2 MAPK and activation of caspase-3, because there are many molecules capable of controlling both MAPK and apoptotic pathways.

In contrast to the upregulation of BMP-2 signaling during the early periods, expression of BMPR-II and activity of p38 MAPK and Erk1/2 MAPK were significantly decreased in the pulmonary artery after 14–21 days of exposure to hypoxia. The reduced expression of BMPR-II protein in the hypoxic lung may be due to downregulation in posttranscriptional processing rather than decreased transcription or mRNA instability because mRNA expression of BMPR-II did not significantly change during exposure to hypoxia for 21 days. Our results were comparable with the clinical observations that expression of BMPR-II is markedly reduced in the lungs of patients with PPH in which no mutation was identified in the coding sequence of BMPR-II and those with secondary PH (1, 13). In addition, West et al. (26) elegantly demonstrated that the loss of BMPR-II signaling in smooth muscle cells is sufficient to produce the pulmonary hypertensive phenotype by using a smooth muscle-specific transgenic mouse expressing a dominant negative mutation under the control of tetracycline gene switch. When the mutation was activated after birth, mice developed PH with no increase in systemic arterial pressure. Furthermore, Beppu et al. (2) reported that BMPR-II heterozygous mice that had reduced lung BMPR-II expression developed PH accompanied with wall thickness of muscularized pulmonary arteries. Thus the downregulation of BMPR-II appears to be associated with vascular remodeling and development of hypoxic PH, but how defects in BMPR-II signaling contribute to these changes is unknown. BMP-2 inhibits vascular smooth muscle cell proliferation after balloon injury in rats (14). BMP-2 has also been demonstrated to inhibit growth of pulmonary artery smooth muscle cells derived from control subjects and patients with secondary PH (13). These reports suggest inhibition of BMP signaling pathways by downregulation of BMPR-II results in smooth muscle cell proliferation. Furthermore, BMP-2 was reported to induce apoptosis in human pulmonary vascular smooth muscle cells derived from control subjects and patients with secondary PH (31). Consistent with this report, we demonstrated a significant reduction of apoptotic activity accompanied with downregulation of BMPR-II in pulmonary arteries after prolonged exposure to hypoxia. These observations suggest that failure of BMP-induced growth suppression and inhibition of apoptosis of vascular smooth muscle cells can concurrently mediate thickening of the pulmonary vasculature, which subsequently reduces the inner lumen diameter of pulmonary arteries, increases pulmonary vascular resistance, and increases pulmonary arterial pressure. Thus disruption of BMP signaling pathway by downregulation of BMPR-II may result in pulmonary vascular remodeling due to the failure of critical antiproliferative programs.

In summary, we have demonstrated a temporal profile of BMP signaling in control rats and animals exposed to hypoxia for 21 days. During the early period of hypoxia, expression of BMP-2 and its downstream signal molecule p38 and Erk1/2 MAPK was upregulated in the intrapulmonary arteries. Although BMPR-II protein was abundantly expressed in the vascular wall of pulmonary arteries in the control rats, prolonged exposure to hypoxia was associated with a marked downregulation of BMPR-II and inactivation of p38 and Erk1/2 MAPK. These results suggest abrogation of BMP
signaling may be a common molecular pathogenesis in the development of PH in various etiologies, including hypoxic PH and PPH. However, at this stage, we do not know how much the alteration of BMP signaling is involved in the pathogenesis of hypoxic PH since the relatively low disease penetration of familial PPH-bearing mutations in BMPR-II gene suggests that its development probably requires additional environmental and/or genetic events.

REFERENCES


