Dysregulated Bone Morphogenetic Protein Signaling in Monocrotaline-Induced Pulmonary Arterial Hypertension

Rory E. Morty, Bozena Nejman, Grazyna Kwapiszewska, Matthias Hecker, Anka Zakrzewicz, Fotini M. Kouri, Dorothea M. Peters, Rio Dumitrescu, Werner Seeger, Petra Knaus, Ralph T. Schermuly, Oliver Eickelberg

Background—Mutations in the bmpr2 gene, encoding the type II bone morphogenetic protein (BMP) receptor, have been identified in patients with pulmonary arterial hypertension (PAH), implicating BMP signaling in PAH. The aim of this study was to assess BMP signaling and its physiological effects in a monocrotaline (MCT) model of PAH.

Methods and Results—Expression of BMP receptors Iib and II, and Smads 4, 5, 6, and 8, was downregulated in lungs but not kidneys of MCT-treated rats. Smad1 phosphorylation and expression of BMP/Smad target genes id1 and id3 was also reduced, although ERK1/2 and p38MAPK phosphorylation remained unaffected. BMP receptor and Smad expression, Smad1 phosphorylation, and induction of the BMP/Smad-responsive element of the id1 promoter were reduced in pulmonary artery smooth muscle cells (PASMCs) from MCT-treated rats. As a consequence of impaired BMP/Smad signaling, PASMCs from MCT-treated rats were resistant to apoptosis induced by BMP-4 and BMP-7, and were also resistant to BMP-4 antagonism of proliferation induced by platelet-derived growth factor.

Conclusion—BMP signaling and BMP-regulated physiological phenomena are perturbed in MCT-treated rats, lending solid support to the proposed roles for BMP signaling in the pathogenesis of human PAH. (Arterioscler Thromb Vasc Biol. 2007;27:1072-1078.)

Key Words: bone morphogenetic proteins ■ monocrotaline ■ pulmonary arterial hypertension ■ Smad ■ vascular remodeling ■ VSMC proliferation

Pulmonary arterial hypertension (PAH) is a rare and often fatal disorder of unknown etiology that occurs either sporadically (idiopathic PAH [IPAH]) or as an inherited form (familial PAH [FPAH]), and is characterized by increased pulmonary vascular resistance, which develops as a consequence of vasoconstriction, thrombosis, and vascular remodeling. A key pathological feature of IPAH is remodeling of pulmonary vascular resistance, which develops as a consequence of impaired BMP/Smad signaling, PASMCs from MCT-treated rats were resistant to apoptosis induced by BMP-4 and BMP-7, and were also resistant to BMP-4 antagonism of proliferation induced by platelet-derived growth factor. As a consequence of impaired BMP/Smad signaling, PASMCs from MCT-treated rats were resistant to apoptosis induced by BMP-4 and BMP-7, and were also resistant to BMP-4 antagonism of proliferation induced by platelet-derived growth factor.

Mutations in the bmpr2 gene have been identified in more than 50% of FPAH patients and in 10% to 25% of IPAH patients.8,9 These mutations disrupt BMP/Smad-mediated signaling,9 and potentiate BMP/MAP kinase signaling.10 Reduced levels of Bmp111 and Bmp212 were observed in the lungs of patients with FPAH and IPAH, and in patients with PAH secondary to thromboembolic disease, ventricular septal defects, or mitral valve regurgitation. These data suggest a role for BMP signaling in PAH and are supported by observations in experimental animals.13-15 Two animal models of IPAH are currently in routine use: one using exposure to hypoxia, and the other using the pyrrolizidine alkaloid monocrotaline (MCT).16,17 In both instances, right ventricular hypertrophy and PAH develop. Despite widespread use of the MCT model, no study to date has addressed a role for BMP signaling in MCT-induced PAH. The data that we present in this article illustrate that the BMP/Smad signaling axis in the lung is perturbed in MCT-induced PAH and that this per-
tured signaling has functional consequences for the maintenance of PASMC cell mass, and hence vascular resistance, associated with MCT-induced PAH. As such, these data lend strong support to the proposed roles for dysregulated BMP signaling in the pathogenesis of IPAH.

Methods

Animals and Hemodynamics

The government of the State of Hessen approved all animal procedures. Pulmonary hypertension was induced in adult male Sprague-Dawley rats (15 per experimental group; Charles River Laboratories) by a single intraperitoneal injection of MCT (60 mg/kg; Sigma). Animal handling, measurement of hemodynamic parameters, and lung extraction were conducted as described previously.18,19

RNA Isolation, Semi-Quantitative PCR, and Real-Time PCR

Total RNA was isolated from fresh lung tissue using a Qiagen RNeasy kit (Qiagen). Total RNA was screened for mRNA encoding genes of interest by semi-quantitative RT-PCR30 using the intron-spanning primers indicated in supplemental Table I (available online at http://atvb.ahajournals.org). Cycle numbers lie in the logarithmic phase for each PCR. Quantitative changes in gene expression were also analyzed by quantitative real-time PCR (RNA samples from 5 different animals per group, each sample assessed in duplicate) by the ΔΔCt method,21 using primer pairs indicated in supplemental Table I. The ubiquitously-expressed hydroxy methylbilane synthase (hmbg) gene was used as a reference.

Protein Isolation and Immunoblotting

Protein extraction from rat lungs, kidneys, and cultured PASMCs and gel electrophoresis and immunoblotting were performed as described previously.20,22 Blots were probed with rabbit anti-Smad1, anti-pp44/2 (Thr383/Tyr385), anti–phospho-Smad3 (Ser463/Ser465) and anti-Smad4 (Upstate), and rabbit anti-Acvr2a (R&D Systems), rabbit anti–phospho-p44/42(Thr202/Tyr204) (Cell Signaling Technology), rabbit anti-Smad5 (Zymed), goat anti-Smad8 and mouse anti-Acvr2a (R&D Systems), rabbit anti–phospho-Smad1(Ser463/Ser465) and anti-Smad4 (Upstate), and rabbit anti-Acvr1 (Santa Cruz Biotechnology). Rabbit polyclonal antibodies against Bmpr1a, Bmpr1b, and Bmpr2 have been described previously.23 None of the antibodies used exhibited cross-reaction with related proteins. Mouse anti-β-actin or anti--α-tubulin (1:1000; Cell Signaling Technology) served as a loading control. Peroxidase-conjugated secondary antibodies (1:3000 to 1:3500) were from Pierce. Densitometric analysis of protein bands was performed as described previously.20

Immunohistochemistry

Elastin and H&E staining, and expression of BMP receptors, Smads, smooth muscle actin (SMA), von Willebrand factor (vWF), and proliferating cell nuclear antigen (PCNA) was assessed on 3-μm tissue sections,18,19 with mouse anti-SMA (1:850; clone I4A; Sigma) and anti-vWF (1:800; Dako); rabbit anti-Bmpr1a (1:150), -Bmpr1b (1:150), and -Bmpr2 (1:150); and rabbit anti-Smad1, -Smad4 (both at 1:50), Upstate and -PCNA (1:100; Santa Cruz Biotechnology).

Assessment of Proliferation and Apoptosis of PASMC

Primary rat PASMCs were isolated from the second to the fifth branch of the pulmonary artery30,34 from saline-treated or MCT-treated rats, 4 weeks after treatment. The proliferation of PASMCs in response to platelet-derived growth factor (PDGF; 20 ng/ml) applied alone, or together with BMP-4 (20 ng/ml) was studied in 24-well plates by [3H]thymidine incorporation.24 For assessment of apoptosis, PASMCs were seeded at 1×10⁴ cells per well in chamber slides and grown to ~80% confluence. Quiescent cells (cultured in 0.1% [v/v] FBS for 48 hours) were treated with BMP-4 or -7 (100 ng/ml),24 24 hours, after which cells were fixed and processed using the terminal deoxyribonucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) reaction with dUTP-fluorescein isothiocyanate (FITC; in situ cell death detection kit; Roche).

Luciferase Reporter Assay

PASMCs, seeded in 24-well plates (70% confluent), were transiently transfected with LipofectAMINE (Invitrogen),25 with pld120, a reporter construct containing a BMP-responsive promoter upstream of a firefly luciferase gene.26 Alternatively, cells were transfected with pGL3-Basic (containing a promoterless luciferase gene) or pGL3-Control (containing a constitutively-expressed luciferase gene) both from Promega as negative and positive controls, respectively. Cells were incubated with BMP-4 or BMP-7 (20 ng/ml) for 12 hours. Cells were lysed and processed for determination of firefly luciferase activity as recommended by the manufacturer.

Statistical Treatment of Data

Data are presented as mean±SD. Differences between groups were analyzed by ANOVA and the Student–Newman–Keul post-hoc test for multiple comparisons, with a probability value <0.05 regarded as significant.

Results

Induction of PAH and Vascular Remodeling by MCT

Vascular remodeling was evident by thickening of small pulmonary artery vessel walls in rats 4 weeks after MCT administration, together with a significant increase in the right ventricle-to-left ventricle + septum ratio (supplemental Figure IB, ID, and IG), available online at http://atvb.ahajournals.org), which was not observed in saline-treated control animals (supplemental Figure IA, IC, and IG). These changes were accompanied by an increase in right ventricular systolic pressure (supplemental Figure IE) and a decrease in the partial pressure of oxygen in arterial blood (supplemental Figure IF), although no change in systemic arterial pressure was observed (supplemental Figure IF).

Dysregulated Expression of BMP Signaling Machinery in Lungs From MCT-Treated Rats

A moderate to dramatic decrease in the expression of mRNA encoding Bmpr1b, Bmpr2, and Smads 4, 5, 6, and 8 was observed in the lungs, but not the kidneys, of rats 4 weeks after MCT administration, as assessed by semi-quantitative RT-PCR (Figure 1A). Similar data were obtained by real-time RT-PCR (Figure 1B), where a significant reduction in levels of mRNA encoding Bmpr1a, Bmpr2, and Smad4 was observed as early as 2 weeks after MCT administration. Four weeks after MCT administration, levels of mRNA encoding all classical BMP receptors and Smads evaluated, and the alternative BMP receptors Acvr1 and Acvr2a, were significantly decreased, although to varying degrees, relative to saline-treated control rats. Because mast cell chymase (Cma1) levels are elevated in the lungs in IPAH patients,27 and hemeoxygenase-1 (Hmox1) levels are elevated in the lungs in MCT-treated experimental animals that develop PAH,28 we also screened cma1 and hmxox1 mRNA levels as positive controls, and both markers were significantly elevated in the lungs, but not the kidneys, of experimental animals.

Reduced expression of BMP receptors and Smads was confirmed at the protein level by immunoblot (Figure 2A). Quantitation of immunoblot data confirmed a significant decrease in the expression of Bmpr1b and Bmpr2, and Smads
4, 5, and 8 (supplemental Figure II). Bmpr1a exhibited a trend toward reduced expression; however, this trend did not reach statistical significance, whereas Smad1 levels appeared relatively stable.

On histological examination of lungs of MCT-treated rats, inflammatory infiltrates and vessel wall thickening were uniformly observed (Figure 3). Bmpr1a exhibited a moderate decrease in staining intensity of both the alveolar epithelium (supplemental Figure III) and the smooth muscle layer of large vessels from MCT-treated rats (Figure 3B), compared with material from healthy, saline-treated controls (Figure 3A). A strong reduction in staining was observed for Bmpr1b (Figure 3C and 3D) and Bmpr2 (Figure 3E and 3F). Notably, loss of Bmpr2 staining in the vascular endothelium of large vessels was observed (Figure 3F). In the case of Smad proteins, whereas Smad1 staining appeared relatively unaffected in the airways (supplemental Figure III) and vessels from MCT-treated rats (Figure 3G and 3H), Smad4 staining was dramatically reduced in the airway epithelium (supplemental Figure III) and smooth muscle layer of small vessels of MCT-treated rats (Figure 3I and 3J). These changes in BMP receptor and Smad expression paralleled trends observed in our RT-PCR and immunoblot analyses. Together, these data document a dramatic reduction in the expression of the BMP signaling machinery in the lungs of MCT-treated rats.

Dysregulated BMP Signaling in Lungs From MCT-Treated Rats

The reduced expression of BMP receptor and Smad proteins observed in the lungs of MCT-treated rats suggested that BMP signaling was most likely also perturbed. Indeed, a dramatic decrease in baseline Smad1 phosphorylation in lung homogenates was observed after MCT-administration (Figure 4A). In contrast, baseline levels of phosphorylated ERK1/2 were only slightly reduced, and levels of phosphorylated p38MAPK were unaffected (Figure 4A). Genes encoding the Id family of helix-loop-helix proteins, in particular, Id1, are direct targets of Smad1-dependent BMP signaling. We therefore evaluated expression of the id1, id2, and id3 genes as a read-out of BMP/Smad signaling. In line with our earlier observations, the expression of both id1 and id3 genes was reduced, as assessed by semi-quantitative RT-PCR (Figure 4B). Further analysis by real-time RT-PCR indicated that id1 mRNA levels were reduced 2 weeks after MCT-administration, whereas mRNA levels of both id1 and id3 were reduced 4 weeks after MCT administration (ΔΔCt of −4.8 and −2.1, respectively; supplemental Figure IV). Taken together, these data demonstrate...
that BMP signaling is impaired in the lungs of MCT-treated rats. This decreased signaling could not be attributed to Smad6, which antagonizes BMP signaling, because levels of mRNA encoding Smad6 were reduced in lungs of MCT-treated rats (Figure 1A and 1B). Given the important and opposing roles of Smad and MAP kinase pathways in regulating cell proliferation, we examined cell proliferation in the lungs of MCT-treated rats using PCNA staining as an indicator of proliferating cells. An increased frequency of proliferating cells was observed in the lungs (Figure 4C, panels a and b), specifically, in the smooth muscle layer of small resistance arteries (Figure 4C, panels c and d) of MCT-treated rats.

**Dysregulated BMP Signaling in PAMSC From MCT-Treated Rats**

The PAMSC is a key player in the pathogenesis of PAH,1–3 and growth and proliferation of PAMSCs are regulated by BMP ligands.30,31 Because we observed increased proliferation of PAMSCs in the lungs of MCT-treated rats (Figure 4C), we examined whether PAMSCs from MCT-treated rats exhibited altered BMP signaling. Indeed, levels of mRNA encoding Bmpr1b and Bmpr2 and Smad4 were significantly lower in PAMSCs from MCT-treated rats, whereas levels of mRNA encoding Bmpr1a and Smad1 levels were not significantly altered between control and MCT-treated groups (ΔΔCT values between −1.8 and −2; Figure 5A).

Whereas Smad1 protein levels were unchanged between PAMSCs from saline- and MCT-treated rats, baseline Smad1 phosphorylation was not evident at all in PAMSCs from MCT-treated rats, although it was evident in PAMSCs from saline-treated rats (Figure 5B). In contrast, ERK1/2 levels and ERK1/2 phosphorylation appeared similar in PAMSCs from both saline- and MCT-treated rats (Figure 5B).

We then transfected a BMP-responsive promoter placed upstream of a firefly luciferase gene on the pId120 reporter plasmid26 into PAMSCs from saline- and MCT-treated rats. Although this promoter was responsive to stimulation by BMP-4 and BMP-7 in PAMSCs from saline-treated rats, the response was significantly (2- to 4-fold) abrogated in PAMSCs from MCT-treated rats (Figure 5C). Together, these data indicate that signaling by the BMP/Smad axis in PAMSCs from MCT-treated rats is impaired.

Proliferation of PAMSCs isolated from both saline- and MCT-treated rats was stimulated by PDGF (Figure 5D). In PAMSCs from saline-treated rats, this effect was antagonized by BMP-4. However, consistent with impaired BMP/Smad signaling in PAMSCs from MCT-treated rats, BMP-4 did not antagonize PDGF-induced proliferation in PAMSCs from MCT-treated rats (Figure 5D). The induction of apoptosis in PAMSCs by BMP ligands is well documented.32,33 We therefore assessed the induction of apoptosis in PAMSCs from saline- and MCT-treated rats by BMP ligands. Consistent with the abrogated BMP/Smad signaling we observed in PAMSCs from MCT-treated rats, PAMSCs from MCT-
treated rats exhibited a 3-fold reduction in apoptosis induced by either BMP-4 or BMP-7 (Figure 6A and 6B). Together, these proliferation and apoptosis data indicate that perturbed signaling in MCT-treated rats does have functional consequences for the maintenance of PASMCs cell mass in MCT-treated rats.

**Discussion**

Members of the TGF-β superfamily of growth factors are emerging as important mediators of vascular disorders, including atherosclerosis, restenosis, hereditary hemorrhagic telangiectasia, and PAH.34 The BMPs and their receptors constitute an important subgroup within this superfamily, having been accredited with roles in the pathogenesis of familial and idiopathic forms of PAH.2,4 We assessed the expression and function of the BMP/Smad axis in MCT-induced PAH in rats, a popularly used (albeit poorly understood) model of IPAH.16,17 Our data demonstrated pronounced dysregulation of BMP/Smad signaling in MCT-induced PAH in rats, and that this perturbed signaling has functional consequences for the maintenance of PASMC cell mass in MCT-treated rats.

In a hypoxia-induced PAH model in rats, Takahashi et al15 demonstrated a downregulation of Bmpr2 in the pulmonary arteries in response to chronic hypoxia. In whole-lung extracts from these rats, phosphorylation of p38MAPK was also reduced; however, no change in phosphorylated Smad1/5/8 was observed. These authors concluded that the BMP/MAP kinase axis, but not the BMP/Smad axis, was perturbed in hypoxia-induced PAH. Our data contrast sharply with those results, because we observed a dramatic reduction in both phosphorylated Smad1 and transcription of the BMP/Smad1-responsive genes Id1 and Id3. In contrast, only a slight reduction in ERK1/2 phosphorylation, and no change in p38MAPK phosphorylation, was observed in whole-lung homogenates from MCT-treated rats with severe PAH. In PASMCs isolated from these rats, BMP signaling was functionally impaired (as assessed by a luciferase reporter assay), in the extracellular matrix composition and remodeling.16,17 However, we demonstrate here that MCT also affects the airways, and PASMCs from MCT-treated rats remain atypical after serial passage, suggesting that persistent changes are induced in that cell type. It could be suggested that MCT induces a transdifferentiation of PASMCs into cells with an altered phenotype; however, these ideas remain entirely speculative.
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sufficient to generate a hypertensive condition, because
observation that loss of functional Bmpr2 alone is not
particularly with respect to Bmpr2, implicating both receptors
similar pattern is observed in MCT-induced PAH in rats,
have been observed in lungs from patients with IPAH, and a
TurboNet: IPAH and FPAH. Total Smad1 levels and baseline ERK1/2 phosphor-
MCT-induced rat model of PAH with human IPAH and
TurboNet: IPAH and FPAH. Total Smad1 levels and baseline ERK1/2 phosphor-
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the proposed roles for BMP signaling in the pathogenesis of human PAH.

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Disclosures

None.

References


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This online supplement consists of one table (Supplementary Table I) and four figures (Supplementary Figure I - Supplementary Figure IV).
Supplementary Table I. Primers employed for RT-PCR. Forward and reverse primers are indicated for real-time (rt) and semi-quantitative (sq) PCR reactions.

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<th>Reverse Primer</th>
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<th>Annealing temperature (ºC)</th>
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Supplementary Figure I

Monocrotaline induces pulmonary arterial hypertension and vascular remodeling in rats. Elastin staining (A and B) and dual-staining for von Willebrand factor (brown) and smooth muscle actin (purple) (C and D) in small pulmonary arteries (indicated by arrows) in 3 µm sections prepared from the lungs of rats four weeks after administration of vehicle alone (A and C), or monocrotaline (B and D). (E) Right ventricular systolic pressure (RVSP), (F) systemic arterial pressure (SAP), (G) right ventricle-to-left ventricle+septum ratio (RV/LV+S), and (I) partial pressure of oxygen in arterial blood (paO2) were determined in control rats, four weeks after saline administration, and in monocrotaline-treated rats, two and four weeks after monocrotaline administration. *, p < 0.05, relative to controls.
Supplementary Figure II. Expression of BMP signaling machinery in MCT-treated rats assessed by immunoblot. These data represent the quantitation of immunoblot data is illustrated in Figure 1. *, p < 0.05, relative to controls. CTRL, control.
Supplementary Figure III. Localization of BMP receptors and Smad proteins in the airways of MCT-treated rats. BMP receptors and Smads were localized (red color) in the airways (a) and vessels (v) of saline- and MCT-treated rats.
Supplementary Figure IV. Expression of BMP target genes encoding Id1-Id3 was assessed in lungs from MCT-treated rats by real-time RT-PCR (black bars, two weeks; gray bars, four weeks, relative to saline-treated controls). wk, weeks. #, p < 0.05, relative to saline treated controls; *, p < 0.05, between indicated groups.