Inflammation, endothelial injury, and persistent pulmonary hypertension in heterozygous BMPR2-mutant mice

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Song Y, Coleman L, Shi J, Beppu H, Sato K, Walsh K, Loscalzo J, Zhang Y-Y. Inflammation, endothelial injury, and persistent pulmonary hypertension in heterozygous BMPR2-mutant mice. Am J Physiol Heart Circ Physiol 295: H677–H690, 2008. First published June 13, 2008; doi:10.1152/ajpheart.91519.2007.—Heterozygous bone morphogenetic protein receptor-II-knockout (BMPR2+/−) mice have a similar genetic trait like that in some idiopathic pulmonary arterial hypertension patients. To examine the effect of pulmonary endothelial injury in BMPR2+/− mice, we challenged the mice with two injections of monocrotaline combined with intratracheal instillation of replication-deficient adenovirus expressing 5-lipoxygenase (MCT+Ad5LO). After the challenge (1 wk), BMPR2+/− mice exhibited a doubling of right ventricular systolic pressure that was greater than that of wild-type mice and remained elevated for 3 wk before heart failure developed. Muscularization and thickening of small pulmonary arterioles was evident in the BMPR2+/− lungs at 2 wk after the challenge and became severe at 3 wk. Marked perivascular infiltration of T cells, B cells, and macrophages was associated with the remodeled vessels. Real-time PCR analysis showed that the expression of six endothelial cell markers in lung tissue was decreased to 20–40% of original levels at 1 wk after the challenge in both BMPR2+/− and wild-type mice and largely recovered in wild-type (50–80%) but not BMPR2+/− lungs (30–50%) at 3 wk after the challenge. Macrophage inflammatory protein-1α and fractalkine receptor expression doubled in BMPR2+/− compared with wild-type lungs. Expression of type I and type II BMP receptors, but not transforming growth factor-β receptors, in the challenged BMPR2+/− and wild-type lungs showed a similar pattern of expression as that of endothelial markers. Apoptotic responses at 1 wk after MCT and Ad5LO challenge were also significantly greater in the BMPR2+/− lungs than the wild-type lungs. These data show that BMPR2+/− mice are more sensitive to MCT+Ad5LO-induced pulmonary hypertension than wild-type mice. Greater endothelial injury and an enhanced inflammatory response could be the underlying causes of the sensitivity and may work in concert with BMPR2 heterozygosity to promote the development of persistent pulmonary hypertension.

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HETEROZYGOUS GERMLINE MUTATIONS of bone morphogenetic protein receptor-2 (BMPR2) have been found in patients with idiopathic pulmonary arterial hypertension (IPAH) (10, 20, 46), a progressive disease characterized by extensive remodeling of the pulmonary arterioles and persistent increase of pulmonary artery pressure of unknown cause. Nearly 70% of familial IPAH and 20% of sporadic IPAH patients have a heterozygous BMPR2 mutation (1), but the chance of developing pulmonary arterial hypertension (PAH) among the BMPR2 mutant family members is only 10–20% (32). Therefore, additional factors are believed to be required for the development of PAH in the BMPR2 mutant carriers.

The BMPR2 gene encodes BMPR-II, which forms a complex with the type I receptors BMPR-IA (Alk-3), BMPR-IB (Alk-6), or ActR-I (Alk-2) in the transforming growth factor (TGF)-β superfamily and transduces signals of BMPs and growth differentiation factors (GDFs) (for review, see Ref. 29). Three BMPR2-deficient mouse lines have been established thus far, which include a BMPR2-knockout (null mutant) (4); a vascular smooth muscle-specific, tetracycline-conditional, dominant-negative BMPR2 transgenic (SM22-tet-dnBMPRII) (51); and a U6-small hairpin RNA-transgenic-BMPR2-knockdown (BMPR2 KD) mouse (23). Studies of these mouse lines have shown that the homozygous BMPR2 deficiency leads to embryonic lethality (4). Heterozygous BMPR2-knockout (BMPR2+/−) mice have a 20% lower birth rate than wild-type mice but otherwise are morphologically normal and have the same life span as wild-type mice. BMPR2+/− mice have normal pulmonary artery pressure [measured as right ventricular systolic pressure (RVSP)] under unstressed conditions (25, 40) but are more susceptible to pulmonary hypertension than wild-type mice when subject to chronic hypoxia combined with serotonin infusion (25), or given replication-deficient adenovirus expressing 5-lipoxygenase (Ad5LO) in the lung (40). The SM22-tet-dnBMPR2 mice have spontaneous pulmonary hypertension when dnBMPRII expression is turned off after birth (51). The BMPR2-KD mice, which have 5–10% of BMPR2 expression compared with wild-type mice, do not develop pulmonary hypertension or pulmonary artery medial hypertrophy spontaneously. Instead, these mice exhibit dilated and thin-walled vessels, severe mucosal hemorrhage in the gastrointestinal tract, and a shortened life span (23). These data indicate that reduced BMPR2 gene dosage alone, even to an extreme extent, is insufficient to cause severe PAH.

Pulmonary endothelial injury caused by toxins, reactive oxygen species, autoimmune activity, and shear stress has been known to lead to severe PAH. To examine whether BMPR2+/− mice are more sensitive to pulmonary endothelial injury, the present study challenged BMPR2+/− mice with the combined delivery of monocrotaline (MCT) and Ad5LO. MCT is known to cause pulmonary endothelial injury and pulmonary hyper-

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tension in humans and rats (39) but has little effect in mice (30). 5-LO and its products, leukotrienes, have been previously found to mediate or facilitate the MCT-induced pulmonary hypertension in rats (37, 42). We have previously shown that addition of Ad5LO to MCT challenge in rats significantly exacerbates the pulmonary hypertension caused by MCT (17). To enhance the effect of MCT in the mouse, the present study used combined MCT and Ad5LO treatment. An empirical adjustment of the doses of the reagents was made to discriminate the responses between BMPR2+/− and wild-type mice. In the following study, we examined pulmonary endothelial injury in the challenged mice by measuring the expression levels of a panel of endothelial cell markers in the lung and monitored the development of pulmonary hypertension in the mice by right heart catheterization and histological analysis. The different responses between BMPR2+/− and wild-type mice to the MCT+Ad5LO challenge are analyzed in an effort to identify the role, if any, of endothelial injury and inflammation in developing persistent pulmonary hypertension in the setting of BMPR2 heterozygosity.

METHODS

Animals. The BMPR2+/− mouse line was established by targeted gene disruption (4). Breeding of BMPR2+/− mice was carried out by crossing BMPR2+/− mice with wild-type mice (C57/BL6 mice from Taconic Farms). Genotyping of the offspring was performed after weaning, using a PCR method as described previously (40). The mice were maintained under stress-free conditions, with 12:12-h light cycles, and received humane care. This study was approved by the Institutional Animal Care and Use Committee of Boston University and by the Harvard Medical Area Standing Committee on Animals.

Animal treatment. Age- and sex-matched wild-type and BMPR2+/− mice were injected with MCT intraperitoneally at 500 mg/kg at day 0 and at day 7. At day 10, Ad5LO (2 × 10⁸ plaque-forming units) was administered to the mice by intratracheal instillation (MCT+Ad5LO treatment) (for the detailed procedure, see Ref. 40). After the MCT treatment (1 wk) or 1, 2, or 3 wk after the MCT+Ad5LO treatment, mice were subjected to right heart catheterization and histological analysis. The different responses between BMPR2+/− and wild-type mice to the MCT+Ad5LO challenge are analyzed in an effort to identify the role, if any, of endothelial injury and inflammation in developing persistent pulmonary hypertension in the setting of BMPR2 heterozygosity.

Histology. Mouse lung vessels were perfused with saline through the pulmonary artery and inflated with 10% phosphate-buffered formalin at a pressure of 20 cmH₂O through the trachea. After fixation for 20 h at 4°C, the lung tissue was processed and paraffin-embedded using a Hypercenter XP System and Embedding Center (Shandon, Pittsburgh, PA). The paraffin-embedded lung tissue was cut into 5-μm sections. Hematoxylin and eosin staining was performed according to a previously described method (40). For immunohistochemical staining, the lung sections were incubated with 3% H₂O₂ for 30 min to retrieve (heating the lung sections in 10 mM citric acid, pH 6.0, for 4 min) and received humane care. This study was approved by the Institutional Animal Care and Use Committee of Boston University and by the Harvard Medical Area Standing Committee on Animals.

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Right and left heart catheterization. Mice were anesthetized with 90 mg/kg ketamine and 6 mg/kg xylazine before catheterization. For right heart catheterization, the right jugular vein was dissected and topically treated with 1% lidocaine solution. A 1.4-Fr Millar Mikro-Tip pressure catheter (Millar Instruments) was inserted in the vein and advanced to the right ventricle following the waveforms. The Millar pressure catheter was connected to a Millar pressure transducer unit that was interfaced with a signal amplifier and recorder (Gould Instrument Systems). The data shown in Fig. 1 were collected using this system. To determine cardiac output in the treated mice, left heart catheterization was performed. The right carotid artery was dissected, and a Millar pressure-volume catheter was advanced through the artery in the left ventricle following the waveforms. The pressure-volume catheter was connected to a Millar Pressure-Volume System (MPVS-400), which contains signal conditioning hardware and an integrated PowerLab data acquisition system. The data are processed by PVAN data analysis software. Because of the size of mouse heart, placing two catheters in the heart at the same time interferes with the pressure readings in both chambers. Thus, to compare right ventricular pressure with left ventricular pressure in the same mouse, right heart catheterization using a Millar pressure catheter (a pressure-volume catheter for the murine right heart has yet to be developed) was performed immediately after left heart catheterization.

Fig. 1. Right ventricular systolic pressure (RVSP). Wild-type (open bar) and heterozygous bone morphogenetic protein receptor-II-knockout (BMPR2+/−; hatched bar) mice were not challenged (No Tx) or challenged with two injections of monocrotaline (MCT) at day 0 and at day 7 (MCT), or with two injections of MCT followed by intratracheal instillation of adenovirus-expressing 5-lipoxygenase (Ad5LO) at day 10 (MCT+Ad5LO). RVSPs of the mice were measured at 1 or 3 wk after the MCT challenge for the MCT group, or at 1, 2, or 3 wk after Ad5LO delivery for the MCT+Ad5LO group. Data are presented as means ± SE; n = 6–10/group. *P < 0.05 BMPR2+/− vs. wild-type group.
**Quantification of gene expression.** Mouse lungs were perfused with saline and homogenized immediately in TRIzol reagent (Invitrogen, San Diego, CA) followed by chloroform extraction for total RNA isolation. The RNA was further purified using the RNeasy Mini Kit and on-column DNase digestion with the RNase-free DNase set (Qiagen). mRNA was converted to cDNA by the reverse transcriptase reaction using the GeneAmp Gold RNA PCR reagent kit (Applied Biosystems). Real-time PCR was carried out using the TaqMan method in a 7900HT Fast Real-Time PCR System (Applied Biosystems) with Taqman primers obtained from Applied Biosystems. PCR reactions were carried out with the thermal cycling conditions set as follows: 2 min at 50°C to activate the AmpErase UNG, 10 min at 95°C to activate the AmpliTaq gold enzyme, 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. All samples were run in triplicate, and the template-negative control was included in each primer/probe set reaction. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as an endogenous control and run in triplicate for each sample. A standard dilution curve was initially constructed to ensure the amount of cDNA used for the real-time PCR reaction was within the linear dynamic range of detection. The relative amount of target cDNA was calculated by the $2^{-\Delta\Delta CT}$ method (24), which normalizes the target gene expression to an endogenous control (GAPDH) and compares it with a calibrator (a cDNA sample from an untreated wild-type mouse). The data are presented as the relative-fold in expression of the gene of interest compared with that of wild-type, untreated mice.

**Western blot analysis.** Lung tissue was homogenized in 20 mM Tris-HCl, pH 7.5, 2 mM EDTA buffer containing protease inhibitor cocktail set III from Calbiochem. The homogenate was sonicated for 5 min after addition of 1/10 volume of 10× RIPA buffer and centrifuged at 12,000 rpm for 5 min. The supernatant was collected, and proteins were separated by electrophoresis in 4–15% gradient SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Vascular endothelial growth factor receptor (VEGFR)-2 and β-actin were detected by polyclonal anti-VEGFR-2 antibody (2 μg/ml; ZYMED Laboratories) and monoclonal anti-β-actin antibody (1:2,000 dilution; Santa Cruz Biotechnology), respectively.

**Terminal dUTP nick-end labeling staining.** Mouse lung vessels were perfused with saline through the pulmonary artery and inflated with zinc fixative (BD Bioscience) at a pressure of 20 cmH2O through...
the trachea. After fixation for 24 h at 22°C, the lung tissue was processed and paraffin-embedded. Paraffin sections were dewaxed, rehydrated, and incubated in 100% methanol at 20°C for 30 min. Terminal dUTP Nick-End Labeling (TUNEL) staining was subsequently performed using a kit from Roche (catalog no. 11684795910), and 4',6-diamidino-2-phenylindole counterstaining was used with a mounting solution from Vector (catalog no. H-1200). Zinc fixative-prepared lung sections were used for this purpose, since the formalin-fixed tissue sections appeared to mask the DNA nicks and prevent the labeling. Retrieval steps with formalin-fixed tissue using citrate buffer.

Table 1. Hemodynamic parameters of wild-type and BMPR2+/− mice before and 3 wk after MCT+Ad5LO treatment

<table>
<thead>
<tr>
<th></th>
<th>WT NoTx (n = 6)</th>
<th>B NoTx (n = 4)</th>
<th>WT 3-wk (n = 5)</th>
<th>B 3-wk (n = 6)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>29±2</td>
<td>28±2</td>
<td>30±3</td>
<td>28±5</td>
<td>NS</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>448±22</td>
<td>438±7</td>
<td>416±22</td>
<td>379±17</td>
<td>NS</td>
</tr>
<tr>
<td>RVSP, mmHg</td>
<td>23±2</td>
<td>24±2</td>
<td>25±4</td>
<td>38±2</td>
<td>0.013</td>
</tr>
<tr>
<td>LVESP, mmHg</td>
<td>113±2</td>
<td>108±1</td>
<td>111±3</td>
<td>79±5</td>
<td>0.001</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
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<td>6±1</td>
<td>7±1</td>
<td>11±2</td>
<td>0.04</td>
</tr>
<tr>
<td>LV dP/dt max, mmHg/s</td>
<td>6,836±216</td>
<td>6,667±181</td>
<td>6,826±608</td>
<td>4,719±344</td>
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</tr>
<tr>
<td>LV dP/dt min, mmHg/s</td>
<td>(−) 6,325±223</td>
<td>(−) 6,269±206</td>
<td>(−) 6,020±303</td>
<td>(−) 4,558±220</td>
<td>0.01</td>
</tr>
<tr>
<td>CO, (RVU)</td>
<td>2,057±157</td>
<td>1,945±354</td>
<td>1,894±339</td>
<td>8,68±201</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of mice. WT, wild type; B, BMPR2+/− mice; NoTx, untreated; MCT, monocrotaline; Ad5LO, adenovirus expressing 5-lipoxygenase; HR, heart rate; RVSP, right ventricular systolic pressure; LVESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure; LV, left ventricular; CO, cardiac output; RVU, relative volume units; dP/dt max, maximum change in systolic pressure per unit time; dP/dt min, minimum change in diastolic pressure per unit time. P value: 2-way ANOVA.
Fig. 4. Morphometric analysis of muscularized distal vessels. Wild-type or BMPR2+/− mice were challenged with MCT+Ad5LO. Lung tissues were harvested 1, 2, or 3 wk thereafter. The lung sections were stained with anti-smooth muscle α-actin antibody (A). Positively stained cells are shown in red. Blue indicates counterstained cell nuclei. Bars = 50 μm. The muscularized vessels were counted in 20 consecutive microscopic fields (×200) and grouped according to their outer diameters (15–25 or 26–50 μm) (B). The wall thickness was determined by measuring the medial wall thickness (from the abluminal edge to the adluminal edge of adjacent endothelium) and the external diameter of the vessel and calculated as (medial thickness × 2/external diameter) × 100 (C). Data are presented as means ± SE; n = 6 mice. *P < 0.05 BMPR2+/− vs. wild-type groups under the same treatment.
and heating resulted in nonspecific TUNEL staining, possibly due to DNA hydrolysis.

**Trypan blue staining.** Trypan blue staining was performed according to a modification of the method described by Fischer and colleagues (13). Mice were anesthetized as above, and the right jugular veins were cannulated. After making an incision in the abdominal aorta, 5 ml trypan blue in Hanks’ balanced salt solution (0.05%) was infused at 2 ml/min. The lungs were further perfused with 5 ml PBS and 2.5 ml zinc fixative before inflation with 0.15 ml zinc fixative and then harvested. After fixation at 22°C for 24 h, lung tissues were

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**Fig. 5. Perivascular CD45-positive cells.** Wildtype (A–C) and BMPR2+/− (D–F) mice were challenged with MCT-Ad5LO, and lung tissues were harvested 1, 2, or 3 wk thereafter. Lung tissue sections were stained with anti-CD45 antibody (A). CD45-positive cells are stained dark brown. Green indicates the counterstained cell nuclei. Bars = 50 μm. The number of CD45-positive cells surrounding pulmonary vessels were counted in 10 consecutive microscopic fields (×200/lung section (B). Data are presented as means ± SE; n = 4 mice. *P < 0.05 BMPR2+/− vs. wild-type groups.
Ad5LO treatment caused a doubling of RSVP in MCT/H11001 at 1, 2, and 3 wk after Ad5LO delivery. As shown in Fig. 1, the sels were more distinct in the BMPR2 precipitation and progressive muscularization of small pulmonary ves-

sels, which became more apparent when vessels were severely occluded.

The time course for the increase in RSVP compared with the time course for histological changes suggests that the RVSP increase at week 1 in these mice was mainly due to increased pulmonary vasoconstriction, since histological changes at this time point were modest. The RVSP in the treated BMPR2+/− mice was not higher at 3 wk than at 2 wk, even though pulmonary vascular remodeling was more severe at the latter time point. Because this observation may be a consequence of worsening RV function with progressive increases in pulmonary vascular resistance, we performed a hemodynamic analysis using a pressure-volume catheter in the left heart of mice with and without MCT+Ad5LO treatment. As shown in Fig. 3, the left ventricular systolic pressure in the treated BMPR2+/− mice was significantly lower than that in the untreated groups or in the treated wild-type mice (79 vs. ~110 mmHg). The relative cardiac output [in relative volume units (RVU)] without adjusting for plasma viscosity or conductance] in the treated BMPR2+/− mice was also significantly lower than in the other groups, 868 vs. ~1,900 RVU (Table 1). The maximum and minimum left ventricular dP/dt was significantly lower in the treated BMPR2+/− mice than that of untreated or treated wild-type mice, possibly related to the underfilling of the left ventricle. The increase in left ventricular end-diastolic pressure (LVEDP) was likely a consequence of increased RV pressure with septal recruitment to the right ventricle, the increased stiffness of which can contribute to an increase in mean LVEDP, although reduced left ventricular contractility cannot be excluded.

Pulmonary vascular remodeling. Anti-smooth muscle α-actin staining was carried out in the lung sections obtained from the MCT+Ad5LO-treated mice to perform morphometric anal-

Changes in RVSP and lung histology. Wild-type and BMPR2+/− mice were injected with MCT at day 0 and day 7, followed by intratracheal instillation of Ad5LO at day 10 (MCT+Ad5LO treatment). RVSP in these mice was measured at 1, 2, and 3 wk after Ad5LO delivery. As shown in Fig. 1, the MCT+Ad5LO treatment caused a doubling of RSVP in BMPR2+/− mice at 1 wk after the treatment, which was maintained over the next 2 wk. The RVSP increase in wild-type mice was mild (~33% greater than that of untreated mice), and the change did not reach statistical significance. MCT injections alone did not cause an increase in RVSP in either type of mouse, as measured at 1 and 3 wk after the second injection of MCT. The study was terminated at 3 wk after the MCT+Ad5LO treatment, since the overall condition of the treated BMPR2+/− mice deteriorated quickly beyond this point (labored breathing, and cold limbs).

Hematoxylin and eosin-stained lung tissue sections of the treated mice are shown in Fig. 2. Alveolar inflammation was apparent in both wild-type and BMPR2+/− lungs starting at 1 wk after the MCT+Ad5LO treatment. Perivascular inflammation and progressive muscularization of small pulmonary vessels were more distinct in the BMPR2+/− than the wild-type lungs. The thickening of vascular walls appeared at 2 wk after the MCT+Ad5LO treatment and became severe 3 wk later. A loss of lung structure and simplification of the alveolar architecture were apparent in alveoli surrounding remodeled ves-

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Processed and paraffin embedded. Tissue sections were dewaxed in xylene for 2 × 2.5 min, dried, and mounted with cover slides before microscopic examination.

Statistical analysis. Data are presented as means ± SE. Statistical analysis was performed by two-way ANOVA or Student’s t-test. P < 0.05 indicates statistical significance.

RESULTS

Changes in RVSP and lung histology. Wild-type and BMPR2+/− mice were injected with MCT at day 0 and day 7, followed by intratracheal instillation of Ad5LO at day 10 (MCT+Ad5LO treatment). RVSP in these mice was measured at 1, 2, and 3 wk after Ad5LO delivery. As shown in Fig. 1, the MCT+Ad5LO treatment caused a doubling of RSVP in BMPR2+/− mice at 1 wk after the treatment, which was maintained over the next 2 wk. The RVSP increase in wild-type mice was mild (~33% greater than that of untreated mice), and the change did not reach statistical significance. MCT injections alone did not cause an increase in RVSP in either type of mouse, as measured at 1 and 3 wk after the second injection of MCT. The study was terminated at 3 wk after the MCT+Ad5LO treatment, since the overall condition of the treated BMPR2+/− mice deteriorated quickly beyond this point (labored breathing, and cold limbs).

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Fig. 6. Inflammatory cell type in BMPR2+/− lung tissue sections. Lung tissues were harvested from BMPR2+/− mice at 2 wk after MCT+Ad5LO treatment and stained for macrophages (A), B cells (B), T cells (C), and neutrophils (D) using specific antibodies as described in RESULTS. Positively stained cells are dark brown. Green indicates counterstained cell nuclei. Bars = 50 μm.
To confirm that the decreased endothelial cell marker expression was due to cell injury instead of inhibition of mRNA or protein expression, we examined lung tissue for markers of apoptosis and necrosis using a TUNEL assay and trypan blue ex vivo staining, respectively. As shown in Fig. 9, at 1 wk after MCT challenge, the expression of endothelial cell markers, including VE-cadherin, Tie-1, endoglin, and platelet endothelial cell adhesion molecule-1 (CD31), was markedly decreased (to 18–44% of untreated wild-type expression) at 1 wk after MCT + Ad5LO treatment. The expression of these markers had largely recovered (to 51–80% of that in untreated wild-type) in wild-type mice; however, the recovery of these markers in the BMPR2+/- mice was significantly attenuated (30–49% of untreated wild-type expression). To confirm the expression at the protein level, the expression of VEGFR-2, which was examined by Western blot analysis, was also found to be significantly lower in the BMPR2+/- mice than in wild-type mice. The number of muscularized vessels and muscularization of distal pulmonary vessels was significantly more extensive in the treated BMPR2+/- mice than in wild-type mice. The number of muscularized vessels increased markedly at 2 and 3 wk after the MCT + Ad5LO treatment. The number of muscularized vessels with 25–50 μm diameter was 1.5- to 2-fold higher in BMPR2+/- mice than in wild-type mice. The number of muscularized vessels with 25–50 μm diameter was 1.5- to 2-fold higher in BMPR2+/- mice than in wild-type mice (Fig. 4B). An increase in vessel wall thickness was also found in the treated mice, which increased over time, as well (Fig. 4C): the treated BMPR2+/- mice exhibited significantly greater vessel wall thickness than the wild-type mice at 2 and 3 wk after treatment.

Perivascular inflammatory cell identification. To confirm that the perivascular infiltrate of cells seen in MCT + Ad5LO-treated BMPR2+/- mice were inflammatory cells, the lung sections were stained with an anti-mouse CD45 antibody, which recognizes all isoforms of the CD45 leukocyte common antigen. As shown in Fig. 5A, cells in the perivascular infiltrate were, indeed, CD45-positive (leukocytes). The number of perivascular inflammatory cells was significantly higher in the treated BMPR2+/- lungs than in wild-type lungs (Fig. 5B). To identify the cell types in the infiltrate, lung sections from BMPR2+/- mice treated with MCT + Ad5LO and harvested 2 wk after treatment were used for staining. Macrophages, B cells, T cells, and neutrophils were detected by anti-Mac-3, anti-CD45R/B220, anti-CD3 epsilon, and anti-neutrophil antibodies, respectively. As shown in Fig. 6, the perivascular infiltrates were mainly composed of T cells, B cells, and macrophages; few cells in the perivascular infiltrates were neutrophils.

Expression of endothelial cell markers in lung tissue. To assess the pulmonary endothelial injury caused by MCT + Ad5LO challenge, the expression of six endothelial cell markers in lung tissue of wild-type and BMPR2+/- mice was examined. These endothelial cell markers are as follows: VEGFR-1 (or Flt-1), VEGFR-2 (Flk-1 or Kdr), VE-cadherin, Tie-1, endoglin (or CD105), and platelet endothelial cell adhesion molecule-1 (or CD31). Real-time PCR was carried out to quantify the expression levels of these genes in lung tissue homogenates. As shown in Fig. 7, the expression of all markers was markedly decreased (to 18–44% of untreated wild-type expression) at 1 wk after MCT + Ad5LO treatment. No difference was found between BMPR2+/- and wild-type mice, suggesting that substantial endothelial cell injury had occurred in both types of mice to a similar extent. At 3 wk after the MCT + Ad5LO treatment, the expression of these markers had largely recovered (to 51–80% of that in untreated wild-type) in wild-type mice; however, the recovery of these markers in the BMPR2+/- mice was significantly attenuated (30–49% of untreated wild-type expression). To confirm the expression at the protein level, the expression of endothelial cell markers had largely recovered (to 51–80% of that in untreated wild-type) in wild-type mice; however, the recovery of these markers in the BMPR2+/- mice was significantly attenuated (30–49% of untreated wild-type expression). To confirm the expression at the protein level, the expression of VEGFR-2, which was examined by Western blot analysis, was also found to be significantly lower in the BMPR2+/- mice than in wild-type mice. The number of muscularized vessels and muscularization of distal pulmonary vessels was significantly more extensive in the treated BMPR2+/- mice than in wild-type mice. The number of muscularized vessels increased markedly at 2 and 3 wk after the MCT + Ad5LO treatment. The number of muscularized vessels with 25–50 μm diameter was 1.5- to 2-fold higher in BMPR2+/- mice than in wild-type mice. The number of muscularized vessels with 25–50 μm diameter was 1.5- to 2-fold higher in BMPR2+/- mice than in wild-type mice (Fig. 4B). An increase in vessel wall thickness was also found in the treated mice, which increased over time, as well (Fig. 4C): the treated BMPR2+/- mice exhibited significantly greater vessel wall thickness than the wild-type mice at 2 and 3 wk after treatment.

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Fig. 9. Terminal dUTP nick-end labeling (TUNEL) staining. Mice were challenged with MCT + Ad5LO, and the lung tissues were harvested 1 wk thereafter. Tissue sections were stained with TUNEL reagents and counterstained with 4′,6-diamidino-2-phenylindole. Representative images of the stained sections are shown in A. TUNEL-positive stained cells are shown in green (right), nucleated cells are shown in blue (middle), and the corresponding lung structures are shown in gray (left). Ctrl-W and Ctrl-B indicate untreated wild-type and untreated BMPR2−/− lung sections, respectively; D7-W and D7-B indicate MCT + Ad5LO-challenged wild-type and BMPR2−/− lungs at day 7, respectively. B: percentage of TUNEL-stained cells among all nucleated cells in each section. Data are presented as means ± SE; n = 4 mice. *P < 0.05 BMPR2−/− vs. wild-type groups.
MCT+Ad5LO challenge, <3% of the cells in the wild-type lungs were TUNEL positive, whereas ~10% of the cells in the BMPR2 lungs were apoptotic. Trypan blue staining was carried out by perfusing the lungs with trypan blue in Hanks’ balanced salt solution, followed by PBS washing and Zn fixation. The staining was carried out at 4 days after the MCT+Ad5LO challenge, since severely injured cells could undergo complete lysis and limit the nuclear staining by trypan blue. The numbers of the trypan blue-positive cells appeared to be similar between the challenged wild-type and BMPR2+/− lungs (Fig. 10). Distal areas of lung tissue had more stained cells than the proximal areas (data not shown), indicating that injury occurred in smaller vessels.

Expression of chemokines and receptors of BMPs and TGF-β. The RNA samples collected in the above study were also examined for the expression levels of chemokines/chemokine receptors, including monocyte chemotactic protein-1 (MCP-1 or CCL-2), macrophage inflammatory protein-1α (MIP-1α or CCL-3), RANTES, fractalkine (CX3CL-1), and the fractalkine receptor (CX3CR-1). As shown in Fig. 11, MCP-1 expression increased greatly after challenge (8-fold in wild-type and 12-fold in BMPR2+/− mice), which declined at 3 wk after treatment (to 3-fold in wild-type and 5-fold in BMPR2+/− mice). The trend toward higher expression of MCP-1 in the BMPR2+/− than the wild-type lungs, however, did not reach statistical significance. This pattern appeared to reflect overall airway inflammation in the treated BMPR2+/− and wild-type lungs. MIP-1α and fractalkine receptor expression was approximately two times as great in the challenged BMPR2+/− lungs as in the wild-type lungs. The increased expression appeared at 1 wk and persisted to 3 wk after the challenge.

The mRNA expression of BMP receptors (BMPR-II, BMPR-IA, and BMPR-IB) and TGF-β receptors (TGFβR-II and TGFβR-I) is shown in Fig. 12. BMPR-II expression in the untreated BMPR2+/− mice was 50% that of wild-type mice, consistent with the genotypes of these mice. After the MCT+Ad5LO treatment (1 wk), the BMPR-II expression in the wild-type and BMPR2+/− lungs decreased to 22 and 11%, respectively, of that in untreated wild-type lungs. The decreased expression partly recovered at 3 wk after the treatment, to 50 and 19% in the wild-type and BMPR2+/− lungs, respectively. The expression of BMPR-IA and BMPR-IB also decreased to 35 and 19%, respectively, of that in untreated wild-type lungs at 1 wk after the MCT+Ad5LO treatment.

Fig. 10. Trypan blue-stained lung section. Mice were challenged with MCT+Ad5LO, and lung tissues were perfused with 5 ml trypan blue solution 4 days later. After perfusing an additional 5 ml PBS and 2.5 ml zinc fixative, lung tissues were harvested and embedded in paraffin. Paraffin sections were dewaxed in xylene and examined under the microscope. Trypan blue-stained nuclei of injured cells are shown in black. NoTx-W and NoTx-B indicate untreated wild-type and BMPR2+/− lung sections, respectively; D4-W and D4-B indicate MCT+Ad5LO-challenged wild-type and BMPR2+/− lungs at day 4, respectively. Bars = 50 μm.

Fig. 11. Chemokines/chemokine receptor expression in MCT+Ad5LO-challenged wild-type and BMPR2+/− lungs. Analysis was performed using the same RNA samples and the same method as described in Fig. 7.
Although the decrease occurred to a similar extent in both wild-type and BMPR2\(^{+/−}\) lungs, the recovery of expression of these receptors was significantly attenuated in the BMPR2\(^{+/−}\) compared with the wild-type lungs (55 vs. 77\% for BMPR-IA, \(P<0.05\), and 28 vs. 43\% for BMPR-IB, \(P<0.05\)). The effect of MCT\(^{+/−}\) Ad5LO challenge was noticeably less severe for TGF-\(\beta\) receptor expression: the expression of TGF\(\beta\)R-I and TGF\(\beta\)R-II in both wild-type and BMPR2\(^{+/−}\) mice had nearly completely recovered by 3 wk after treatment.

**DISCUSSION**

This study showed that BMPR2\(^{+/−}\) mice are more sensitive to MCT\(^{+/−}\) Ad5LO challenge than wild-type mice. Under this challenge, BMPR2\(^{+/−}\) mice developed sustained increases in RVSP, progressive thickening of small pulmonary arterioles, and marked perivascular inflammation of the remodeled vessels. Pulmonary endothelial injury occurred in both types of mice to a similar degree initially but recovered to a significantly lesser degree in the BMPR2\(^{+/−}\) than the wild-type lungs. The chemokine MIP-1\(\alpha\) and fractalkine receptor expression were significantly higher in the challenged BMPR2\(^{+/−}\) than the wild-type lungs, an effect that persisted throughout the 3 wk of experimental protocol. Increased apoptosis was also observed at 1 wk after challenge in the BMPR2\(^{+/−}\) compared with the wild-type lungs. Thus endothelial injury coupled with an enhanced inflammatory response in the BMPR2\(^{+/−}\) mice may account for the increased susceptibility to pulmonary hypertension in these mice.

BMP signaling has been known to play important roles in vasculogenesis and angiogenesis. In vasculogenesis, the mesoderm cells that form yolk sac blood islands are induced to express VEGFR-2 (Flk-1, Kdr) initially and give rise to the first vascular structures later (12). In vitro studies of embryonic stem cell differentiation models have shown that the formation of VEGFR2\(^+\) (Flk-1\(^+\), Kdr\(^+\)) cells from mouse and human embryoid bodies requires BMP-4 signaling (18, 34). An in vivo study of mouse vasculogenesis has also demonstrated that the process requires the activity of hedgehog proteins, forkhead transcription factor Foxf1, and BMP-4 (2). BMP signaling-stimulated angiogenesis has been shown in various in vivo and in vitro models, including tumor development (21, 35, 38), bone formation (9), chick chorioallantoic membrane assay.

**Fig. 12.** Expression of bone morphogenetic protein (BMP) and transforming growth factor (TGF)-\(\beta\) receptors in MCT\(^{+/−}\) Ad5LO-challenged wild-type and BMPR2\(^{+/−}\) lungs. Analysis was performed using the same RNA samples and the same method as described in Fig. 7.

**Fig. 13.** BMP signaling pathways and potential effects in endothelial cells.
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(36), and cultured endothelial cell migration and tube formation. These studies have shown that BMPs activate endothelial cells by induction of expression of inhibitor of differentiation (Id-1; see Ref. 45 and 50) and by stimulating VEGF production from surrounding nonendothelial cells (9). Id1 and Id3 double-knockout (Id1–/– Id3–/–) mice have been shown to be angiogenically defective and tumor resistant (28), and this defect is found to be a consequence of impaired VEGF-driven mobilization of VEGFR2+ circulating endothelial progenitors and impaired proliferation and incorporation of VEGFR1+ cells (27). Thus decreased BMP signaling could affect endothelial repair when angiogenesis/neovascularogenesis is in demand.

A recent study showed that BMP signaling also reduces apoptosis of cultured pulmonary artery endothelial cells under serum-deprived conditions and supports the survival of cultured circulating endothelial progenitors from normal individuals but not from IPAH patients (44). Several studies on animal models of pulmonary hypertension have also shown that increasing expression of VEGF or angiopoietin-1 via cell-mediated gene transfer can markedly attenuate the MCT-induced pulmonary hypertension in rats (6, 53), whereas inhibition of VEGFR-2 leads to severe pulmonary hypertension in hypoxia-treated rats (43). These studies indicate that abnormal angiogenesis and endothelial cell apoptosis following injury are key elements in the pathogenesis of PAH.

The expression of BMP receptors II, IA, and IB was found to be markedly decreased in the MCT + Ad5LO-challenged mice at 1 wk and recovered to a lesser extent in the BMPR2+/– than the wild-type lungs. This expression pattern is similar to that observed in the expression of endothelial cell markers and thus suggests that BMP receptors may be expressed more abundantly in pulmonary endothelial cells than other cells in the lung. Aktinson and colleagues (3) have previously reported that BMPR2 expression in normal human lung tissue is prominent on the endothelium but minimal in airway and arterial smooth muscle. A recent study of the rat MCT model showed that MCT challenge causes decreased expression of BMP receptors II, IA, and IB in the rat lung but not kidney. Strikingly, this study showed that the protein levels of these receptors in the rat lungs remain largely diminished at 4 wk after the MCT challenge (31). Inability to recover the expression of BMP receptors, or potentially to expand the pool of endothelial (progenitor) cells, in lung tissue could contribute to the MCT-induced pulmonary hypertension in this model.

Marked perivascular inflammation was observed in the MCT + Ad5LO-challenged BMPR2+/– mice. The majority of cells in the perivascular infiltrates were T cells, B cells, and macrophages, but not neutrophils. This pattern is similar to that found in human PAH plexiform lesions (for review, see Ref. 47). Chemokine analysis showed that the expression of MIP-1α and the fractalkine receptor (CX3CR-1) was two times as high in the challenged BMPR2+/– lungs as in the wild-type lungs, and this pattern of differential expression persisted throughout the 3-wk protocol. MIP-1α is a potent lymphocyte and monocyte chemoattractant and is involved in type 1 T helper cell (Th1) immune response (11). The fractalkine receptor is specifically expressed on cytotoxic effector lymphocytes and macrophages and has been implicated in the pathogenesis of several diseases, including coronary artery disease (for review, see Ref. 48). Increased expression of these molecules in the BMPR2+/– lungs suggests that the mice had an enhanced Th1 immune response to the MCT + Ad5LO challenge. BMP signaling is involved in hematopoiesis (22) and has been shown to inhibit T cell maturation (7) and B cell development (19); however, the specific role of BMP signaling in specific immune responses has not been reported. Further study is required to understand the potential role of Th-1 response in pulmonary vascular remodeling, and its relationship to the endothelial injury and apoptosis in the BMPR2+/– mice.

MCT and 5LO have been used in this study to induce pulmonary hypertension. The doses of these agents were chosen to differentiate optimally the responses between BMPR2+/– and wild-type mice toward the challenge. Although these agents were effective for the purpose of this study, they are far from perfect for establishing a mouse model of PAH that mimics the human syndrome. We have noticed that the metabolic rate of MCT varies with mouse strains and ages, e.g., mice with 129 background are significantly more sensitive to MCT, and one-half the dose of MCT (300 mg/kg) causes acute liver and lung injury and early death in these mice. Older mice (> 6 mo) also metabolize MCT more efficiently. Thus closely matched groups are required for this protocol, and the doses need to be adjusted empirically by age if other strains of mice are used.

Although BMP signaling is necessary for maintaining normal vascular structure, studies have also found that BMPs can cause a proinflammatory endothelial phenotype (8) and stimulate an inflammatory response (41). The expression of BMP2 and/or BMP4 in endothelial cells is significantly increased under various endothelial injurious conditions, including high flow shear stress (16), oscillatory shear stress (41), hyperhomocysteinemia (49), or treatment with H2O2 (41), tumor necrosis factor-α (41), or hypoxia (5). The increased expression of BMPs under some of these conditions was found to mediate an inflammatory response. This effect of BMPs could possibly be explained by a different signaling pathway of BMP in which the X-chromosome-linked inhibitor of apoptosis protein (XIAP) plays a critical role in competing with BMP signaling (19); however, the specific role of BMP signaling has been shown to inhibit T cell maturation (7) and B cell development (19); however, the specific role of BMP signaling in specific immune responses has not been reported. Further study is required to understand the potential role of Th-1 response in pulmonary vascular remodeling, and its relationship to the endothelial injury and apoptosis in the BMPR2+/– mice.

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