Increased Susceptibility to Pulmonary Hypertension in Heterozygous BMPR2-Mutant Mice

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Background—Bone morphogenetic protein receptor-2 (BMPR2)–heterozygous, mutant (BMPR2+/−) mice have a genetic trait similar to that of certain patients with idiopathic pulmonary arterial hypertension (IPAH). To understand the role of BMPR2 in the development of IPAH, we examined the phenotype of BMPR2+/− mice and their response to inflammatory stress.

Methods and Results—BMPR2+/− mice were found to have the same life span, right ventricular systolic pressure (RVSP), and lung histology as those of wild-type mice under unstressed conditions. However, when treated with recombinant adenovirus expressing 5-lipoxygenase (Ad5LO), BMPR2+/− mice exhibited significantly higher RVSP than wild-type mice. The increase of RVSP occurred in the first 2 weeks after Ad5LO delivery. Modest but significant muscularization of distal pulmonary arterioles appeared in BMPR2+/− mice 4 weeks after Ad5LO treatment. Measurement of urinary metabolites of vasoactive molecules showed that cysteinyl leukotrienes, prostacyclin metabolites, and PGE2 were all increased to a similar degree in both BMPR2+/− and wild-type mice during 5LO transgene expression, whereas urinary endothelin-1 remained undetectable. Urinary thromboxane A2 metabolites, in contrast, were significantly higher in BMPR2+/− than in wild-type mice and paralleled the increase in RVSP. Platelet activation markers, serotonin, and soluble P-selectin showed a trend toward higher concentrations in BMPR2+/− than wild-type mice. Cell culture studies found that BMP treatment reduced interleukin-1β–stimulated thromboxane A2 production in the pulmonary epithelial cell line A549.

Conclusions—BMPR2+/− mice do not develop pulmonary hypertension spontaneously; however, under inflammatory stress, they are more susceptible to an increase in RVSP, thromboxane A2 production, and vascular remodeling than wild-type mice. (Circulation. 2005;112:553-562.)

Key Words: inflammation ■ hypertension, pulmonary ■ vasoconstriction ■ bone morphogenetic proteins ■ thromboxane

Bone morphogenetic protein receptor-2 (BMPR2) transduces signals of BMPs, which are a family of peptides originally found to induce ectopic bone formation.1 BMPs were later found to be structurally related to transforming growth factor-βs, activins/inhibins, and mullerian inhibiting substances2–4 and to play critical roles in embryonic development, tissue morphogenesis, and cell differentiation.5

Recent genetic linkage studies found that heterozygous germline mutations in the BMPR2 gene are associated with idiopathic pulmonary arterial hypertension (IPAH)6–8 (formerly denoted as primary pulmonary hypertension6), a disease characterized by a sustained increase of pulmonary artery pressure of unknown cause.10–12 Approximately 55% of cases of the familial form and between 10% and 26% of cases of the sporadic form of IPAH are found to have heterozygous BMPR2 mutations.8,13 Family members with BMPR2 mutations have only a 15% to 20% chance of developing (clinical) pulmonary hypertension.13 The onset of the disease varies widely, both within families and among individuals carrying the same mutation.7,14 It has thus been suggested that additional genetic or environmental factors are required to develop pulmonary hypertension in individuals with a heterozygous BMPR2 mutation. The nature of these additional factors is currently unknown.

Homozygous BMPR2 mutant (BMPR2−/−) mice have been shown to die in utero before mesoderm formation.15 Heterozygous BMPR2 mutant (BMPR2+/−) mice, by contrast, are reported to be morphologically normal and fertile.15 Transgenic mice expressing a dominant-negative BMPR2 gene have been recently examined.16 The study showed that...
postnatal expression of the mutant BMPR2 gene in smooth muscle leads to a significant increase of right ventricular systolic pressure (RVSP) and relatively modest pulmonary vascular remodeling in mice.

In the present study, we examined the phenotype of BMPR2mutant mice with respect to (1) their survival rate during prenatal development, (2) their RVSP and lung histology in the basal state, and (3) their responses to inflammatory stress resulting from adenovirus-mediated pulmonary overexpression of 5-lipoxygenase (5LO). Inflammation has been previously suggested to be one of the mechanisms involved in the development of PAH (for reviews, see Voelkel et al., Tuder and Voelkel, Jeffery and Morrell, and Dorfmuller et al). 5LO catalyzes leukotriene formation and facilitates an inflammatory process. Increased 5LO expression or leukotriene production has been demonstrated in patients with PAH and an animal model of PAH. Thus, using this inflammatory mediator as a stimulus may yield useful information about the role of inflammation in the development of pulmonary hypertension in individuals with BMPR2 haploinsufficiency.

Methods

Animals

BMPR2-mutant mice were established by Beppu and colleagues via targeted gene disruption. Because homozygous BMPR2-mutant mice die in utero, breeding of previously suggested to be one of the mechanisms involved in the resulting from adenovirus-mediated pulmonary overexpression of 5-lipoxygenase (5LO). Inflammation has been previously suggested to be one of the mechanisms involved in the development of PAH (for reviews, see Voelkel et al., Tuder and Voelkel, Jeffery and Morrell, and Dorfmuller et al). 5LO catalyzes leukotriene formation and facilitates an inflammatory process. Increased 5LO expression or leukotriene production has been demonstrated in patients with PAH and an animal model of PAH. Thus, using this inflammatory mediator as a stimulus may yield useful information about the role of inflammation in the development of pulmonary hypertension in individuals with BMPR2 haploinsufficiency.

BMPR2mutant mice were carried out by crossing BMPR2mutant mice with wild-type mice. Genotyping of the offspring was performed after weaning. The mutated BMPR2 allele in the mice was identified by a polymerase chain reaction with primer pairs S-GCTTAA AGGCT ATGCT CGAGA CCAGA TTG-3 and 5AGGTG GGCTG GGAAC CT-3. The amplification conditions were as follows: 1 cycle of 120 seconds at 95°C, 30 cycles of 30 seconds at 95°C, 30 seconds at 63°C, and 90 seconds at 72°C, and 5 minutes at 72°C.

The mice were maintained at the Boston University Laboratory Animal Science Center under stress-free conditions, i.e., unlimited food and water access, regular light cycles (12-hour/12-hour light:dark), no forced exercise, and generally pathogen-free. All animals received humane care. The study was approved by the Institutional Animal Care and Use Committee at Boston University.

Right Heart Catheterization

Mice underwent right heart catheterization at various time points during the study. Before catheterization, mice were anesthetized with 90 mg/kg ketamine and 6 mg/kg xylazine. After dissection to expose the right jugular vein, a 1.4F Millar Micro-Tip pressure catheter (Millar Instruments) was inserted into the vein and advanced to the RV. The catheter was connected to a transducer unit interfaced with a signal amplifier and recorder (Gould Instrument Systems), and RVSP was recorded.

Histology

Mouse lungs were perfused with saline and inflated with 10% phosphate-buffered formalin at a pressure of 20 cm H2O. After fixation for 20 hour at 4°C, the lung tissue was processed and paraffin-embedded with a Hypercenter XP System and Embedding Center (Shandon Inc) and cut into 5μm sections. The tissue sections were then heat-dried on slides at 56°C for 1 hour. Deparaffinization and rehydration were carried out by immersing the slides in xylene (2×5 minutes), 100% ethanol (2×1 minute), 95% ethanol (2×1 minute), and deionized water (5 minutes).

For hematoxylin and eosin staining, tissue sections were incubated in Gill-2 hematoxylin (Fisher) for 2 minutes, rinsed with water for 2 minutes, dipped once in acid alcohol (70% ethanol and 1% concen-

trated HCl), rinsed with water for 1 minute, dipped in 1% NH4OH for 15 seconds, rinsed with water for 1 minute, and dipped twice in 1% eosin Y (Fisher). The stained sections were dehydrated by incubation in 95% ethanol for 2×30 minutes, 100% ethanol for 2×30 seconds, and xylene for 2×2.5 minutes and then mounted with Cytoseal60.

For smooth muscle α-actin staining, the lung sections were incubated with a mouse monoclonal antibody against smooth muscle cell α-actin (Sigma, 1:5000 dilution in 1% immunohistochemical grade bovine serum albumin [BSA]/phosphate-buffered saline [PBS]) at 4°C overnight. The sections were washed in PBS twice for 5 minutes and incubated with biotinylated goat anti-mouse IgG (Jackson ImmunoResearch) at a dilution of 1:500 in BSA/PBS for 30 minutes at room temperature. The sections were then incubated with avidin DH and biotinylated-alkaline phosphatase H (provided in the Vectastain ABC-AP kit, Vector Laboratories) for 30 minutes and then with alkaline phosphatase substrate (provided in Vector Red Substrate kit, Vector Laboratories) for 20 minutes. Two PBS washes were performed between these incubations, and the final washing was performed under running tap water for 5 minutes. The sections were counterstained with Harris modified hematoxylin for 10 seconds, washed with water, dipped in acid alcohol, and rinsed with water.

Delivery of 5LO Transgene

Recombinant replication-deficient adenovirus expressing human 5LO (Ad5LO) was prepared as previously described and was delivered to the lungs of mice by intratracheal instillation. In this procedure, mice were anesthetized with ketamine (90 mg/kg) and given 1% lidocaine by local injection into the midline neck region. Surgical exposure of the trachea was then performed through a midline incision and subsequent blunt dissection to the level of the trachea. A 29-gauge needle bent at a 60° angle was instilled into the trachea, and 2×106 plaque forming units (PFU) of Ad5LO was slowly instilled into the trachea. After instillation, the neck incision was closed with No. 4 silk surgical suture. Buprenorphine, 0.1 mg/kg sc, was given every 8 to 12 hours after the procedure for 3 doses, and animals were monitored until full recovery from anesthesia before returning them to their regular cages.

RNA Isolation and Northern Blotting

Mouse lungs were perfused with saline and homogenized immediately in 10 mL TRIzol reagent (Invitrogen) followed by chloroform extraction for total RNA. Northern blotting was carried out by electrophoresing 20 μg RNA in 1.2% agarose gels containing 4% formaldehyde. The RNA was transferred onto nitrocellulose membranes and hybridized with an [α-32P]dCTP-labeled 5LO probe that encompasses nucleotides 45 to 290 of human 5LO cDNA (GenBank accession No. J03600). Hybridization was carried out at 68°C overnight in MiracleHyb solution (Stratagene) containing 10 μg/mL sheared salmon sperm DNA. The membrane was washed with 2× saline sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) at room temperature for 15 minutes and then with 0.1× SSC/0.1% SDS at 68°C for 20 minutes before exposure to X-ray film. The membrane was then washed in 0.1× SSC/0.1% SDS at 100°C twice for 15 minutes to strip the bound 5LO probe and hybridized with a [α-32P]dCTP-labeled mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe (Ambion) to estimate total RNA loading.

Urinary Collection and Measurement of Urinary Creatinine

Urine samples were collected from the mice 2 days before Ad5LO treatment (day −2) and on days 3, 7, 12, and 21 after treatment. During the collection, mice were maintained in metabolic cages with water supplied but food withheld for 16 hours. Urine samples were collected during the 16-hour period, and the mice were returned to their regular cages after collection. The urine samples were centrifuged at 20,000g for 5 minutes, and the supernatants were stored in aliquots at −80°C.
Urinary creatinine concentration was determined by the picric acid assay and creatinine assay reagents from Teco Diagnostics. In this assay, 0.01 mL creatinine standards (1.25, 2.5, 5, and 10 mg/mL) or urine samples (diluted 5- to 15-fold in water) were added to a 96-well plate and mixed with 0.2 mL of creatinine picric acid reagent and creatinine buffer reagent (mixed before assay at 1:1, vol/vol). The plate was incubated at 37°C for 40 minutes and read at 490 nm. The absorbance from this reading was registered as the total absorbance. Acetic acid reagent (0.01 mL) was then added to each well, and the plate was read again at 490 nm, and the absorbance from the second reading (nonspecific absorbance) was subtracted from the first reading (total absorbance). The absorbance difference (specific absorbance) was used to construct the standard curve and calculate the sample urinary creatinine concentration.

Urinary Vasooactive Metabolites

The production of cysteinyI leukotrienes, prostacyclin, prostaglandin (PG) E2, endothelin-1, and thromboxane (Tx) A2 in mice was determined by analyzing the urinary concentration of the products or their metabolites by ELISA. For cysteinyI leukotriene measurements, an ELISA kit detecting leukotrienes C4, D4, and E4 from Rocky Mountain Diagnostics was used. All samples were analyzed in duplicate and repeated at least twice.

Results

BMPR2+/− Mice Breeding

BMPR2+/− mice were bred by crossing BMPR2+/− with wild-type mice (C57/BL6 mice). The genotypes of the offspring were determined after weaning. The Table shows the summary of the numbers of BMPR2+/− and wild-type mice obtained from breeding and the distribution of male and female mice in each population. As demonstrated, the numbers of BMPR2+/− and wild-type mice in the offspring were significantly different, 498 and 618, respectively (χ² = 12.9, P<0.001). The ratio of BMPR2+/− to wild-type mice was 81:100, indicating that ∼20% of BMPR2+/− mice were lost in early development. The majority of the missing BMPR2+/− mice died in utero, although some may have died in the immediate postnatal stage, because the number of pups in each litter was not counted immediately after birth. The female:male distribution in either BMPR2+/− or wild-type offspring was not statistically different. The surviving BMPR2+/− mice were morphologically normal and had no apparent problems in the unstressed state. These mice also had a similar life span (∼18 months) to wild-type mice.

Lung Structure and RVSP in BMPR2+/− Mice

To assess lung development in the BMPR2+/− mice, lung tissue sections from BMPR2+/− and wild-type mice were prepared and stained with hematoxylin and eosin. As shown in Figure 1, no apparent gross abnormalities were observed in the sections from either mouse type, although a minor difference in adhesion of leukocytes to the vessel wall was noted in the BMPR2+/− mice (Figure 1B).

The pulmonary artery pressure in the BMPR2+/− mice was assessed by measuring RVSP via right heart catheterization. In the absence of pulmonary valve disease, RVSP is equivalent to the pulmonary artery systolic pressure. As shown in Figure 2, the RVSP in BMPR2+/− mice was similar to that of wild-type mice from 2 to 18 months of age, indicating that BMPR2+/− mice did not develop pulmonary hypertension spontaneously under unstressed conditions.

Expression of 5LO in Lungs of Mice

To examine the responses of BMPR2+/− mice to pulmonary inflammation, recombinant human 5LO was expressed in the lungs of mice via adenovirus-mediated gene transfer. Ap-
proximately $2 \times 10^6$ PFU of replication-deficient adenovirus expressing recombinant human 5LO (Ad5LO) was administered to the mice via intratracheal instillation. The time course of transgene expression was examined by Northern blotting. As shown in Figure 3, expression of 5LO peaked at day 7 after Ad5LO delivery and decreased significantly by day 21. No difference in transgene expression was found between wild-type and BMPR2$^{+/+}$ mice.

**RVSP of Mice After Receiving Ad5LO**

The RVSP of wild-type and BMPR2$^{+/+}$ mice was examined initially at day 12 after administration of Ad5LO and compared with that of mice that received adenovirus-expressing green fluorescence protein (AdGFP). The choice of time point was based on a previous study, which showed that rats receiving Ad5LO and monocrotaline treatment developed pulmonary hypertension 10 days later. As shown in Figure 4, RVSP was significantly increased in Ad5LO-treated BMPR2$^{+/+}$ mice compared with those with no treatment (23±1 versus 12±1 mm Hg, P<0.05). The RVSP response of BMPR2$^{+/+}$ mice was not absolutely specific for exogenous 5LO expression, because the mice that received AdGFP had an elevated RVSP as well, though to a significantly milder degree (Figure 4). Adenovirus-mediated gene transfer is known to cause inflammation. Thus, the increase in RVSP found in BMPR2$^{+/+}$ mice likely reflects a general response to inflammation, which is intensified by 5LO overexpression. Wild-type mice exhibited little increase in RVSP under either Ad5LO or AdGFP treatment, suggesting that BMPR2$^{+/+}$ mice are more sensitive to an inflammation-mediated RVSP increase than are wild-type mice.

To investigate the time course of change of RVSP in the mice, BMPR2$^{+/+}$ or wild-type mice were treated with Ad5LO and examined by right heart catheterization at days 0, 3, 7, 10, 12, 14, or 21 after Ad5LO delivery. As shown in Figure 5, the increase of RVSP in BMPR2$^{+/+}$ mice peaked at day 7 (33±3 mm Hg) and returned to near normal by day 21 (14±2 mm Hg, compared with day 0, 12±1 mm Hg). The RVSP of wild-type mice increased mildly at day 7 (18±2 mm Hg) and became normal at day 12 (13±1 mm Hg). The increase of RVSP in the BMPR2$^{+/+}$ mice was consistent with the time course of transgene expression (shown in Figure 3) and was significantly higher than that of wild-type mice.

To determine whether the change in RVSP was associated with pulmonary vascular remodeling, immunohistochemical staining for smooth muscle $\alpha$-actin was carried out in lung tissue sections prepared from wild-type and BMPR2$^{+/+}$ mice after Ad5LO delivery. As shown in Figure 6, lung sections obtained at day 7 after Ad5LO delivery showed no sign of vascular remodeling in either type of mouse, although significant inflammatory cell infiltration was present. Increased muscularization of distal pulmonary arterioles was found in lung sections obtained at day 28 after Ad5LO treatment. Counting the $\alpha$-actin–stained distal pulmonary arterioles (vessels that are located distal to terminal bronchioles, adjacent to the alveolar duct, noncollapsed, and with diameters <40 μm) showed that BMPR2$^{+/+}$ mouse lungs had a significantly greater number of muscularized vessels than lungs from wild-type mice (36±3 versus 7±1) (Figure 6G). The muscularized distal arterioles accounted for $\approx$10% of total distal arterioles in the BMPR2$^{+/+}$ lungs. The degree of muscularization (thickness of the muscle layer), however, was mild. Because this increase in muscularization occurred after the maximal increase in RVSP, the increased RVSP observed in BMPR2$^{+/+}$ mice in the first 2 weeks after Ad5LO delivery was mild. Because this increase in muscularization occurred after the maximal increase in RVSP, the increased RVSP observed in BMPR2$^{+/+}$ mice in the first 2 weeks after Ad5LO delivery was mild.
delivery was not due to pulmonary vascular remodeling but rather to enhanced pulmonary vasoconstriction.

**Generation of Vasoactive Molecules During 5LO Overexpression**

To understand the molecular basis of the enhanced pulmonary vasoconstriction in BMPR2+/−/− mice during 5LO expression, urine samples were collected from the mice 2 days before (day 2) and 3, 7, 12, and 21 days after Ad5LO delivery. Urinary levels of several vasoactive molecules, including cysteinyl leukotrienes, prostacyclin metabolites, PGE2, TxB2, and endothelin-1, were determined. As shown in Figure 7, the concentration of cysteinyl leukotrienes in urine was undetectable before Ad5LO delivery (day 2) but was markedly increased after 5LO expression. The production of cysteinyl leukotrienes peaked at day 7 (≈20 000 pg/mg cysteinyl leukotrienes/urinary creatinine) and diminished by day 21. There was no difference between wild-type and BMPR2+/−/− mice in cysteinyl leukotriene production.

Urinary levels of prostacyclin metabolites, 6-keto-PGF1α and 2,3-dinor-6-keto-PGF1α, were next measured. As shown in Figure 8A, the basal level of urinary prostacyclin metabolites in BMPR2+/−/− and wild-type mice was similar, ≈10 000 pg/mg (prostacyclin metabolites/urinary creatinine). The prostacyclin concentration was increased by 3- to 3.5-fold at days 3 and 7 after Ad5LO treatment and returned to the basal level by day 12. The increase was similar in BMPR2+/−/− and wild-type mice.

Urinary levels of PGE2, a pulmonary vasodilator, in BMPR2+/−/− and wild-type mice are shown in Figure 8B. The basal level of the compound in the 2 types of mice was 2200 to 4500 pg/mg (PGE2/urinary creatinine). During 5LO overexpression in the mice, the concentration increased to 16 000 to 20 000 pg/mg at days 3 and 7 and returned to basal level by day 12. There was no significant difference in PGE2 production between the wild-type and BMPR2+/−/− mice. Interestingly, the pattern of PGE2 production in the mice was comparable to that of prostacyclin, which suggests that the increased production of these compounds was due to activa-
ulation of an upstream enzyme(s) in PG synthesis, such as cyclooxygenase-2.27

To determine whether the increased RVSP in the BMPR2−/− mice was due to overproduction of vasoconstrictive molecules, the generation of endothelin-1 and TxA2 in mice during 5LO expression was examined. Results showed that urinary endothelin-1 was undetectable in these mice either before or after Ad5LO delivery, indicating no marked increase in the production of this vasoactive peptide in these mice.

TxA2 production in the mice was assessed by measuring urinary concentrations of TxB2 and 11-dehydro-TxB2, metabolites of TxA2. As shown in Figure 9A, the basal level of urinary TxB2 in both wild-type and BMPR2−/− mice was ≈30 000 pg/mg (TxB2/creatinine). During 5LO expression, TxB2 concentration in the BMPR2−/− mice increased to 71 000, 116 000, 91 000, and 59 000 pg/mg at days 3, 7, 12, and 21, respectively. The change in Tx production in wild-type mice, by contrast, was less striking and did not reach statistical significance compared with pretreatment controls, with values of 47 000, 60 000, 39 000, and 24 000 pg/mg on days 3, 7, 12, and 21, respectively. Urinary 11-dehydro-TxB2, in the Ad5LO-treated mice showed a similar pattern of change as TxB2 (Figure 9B). Thus, among the vasoactive molecules examined in this study, a difference between wild-type and BMPR2−/− mice was found only in TxA2 production. The time course of the change in TxB2 level was comparable to that of the RVSP change in the mice.

**Platelet Activation**

Platelet activation is the major source of Tx production in vivo, and urinary Tx metabolites have been used as markers for platelet activation. To determine whether the increased TxA2 production in BMPR2−/− mice under inflammatory stress was due to augmented platelet activation, we examined 2 other platelet activation markers, plasma sP-selectin and urinary serotonin, in the mice. As shown in Figure 10A, untreated wild-type and BMPR2−/− mice had similar plasma sP-selectin levels, 725 ± 26 and 751 ± 99 ng/mL, respectively. Ad5LO treatment increased the plasma sP-selectin concentration to 1326 ± 100 and 1791 ± 187 ng/mL in wild-type and BMPR2−/− mice, respectively. BMPR2−/− mice tended toward a higher sP-selectin level than did wild-type mice, but the difference did not reach statistical significance. A similar trend was found in urinary serotonin concentrations in the 2 types of mice. As shown in Figure 10B, before Ad5LO delivery...
treatment, the urinary serotonin concentrations in wild-type and BMPR2/+/+/ mice were similar, 73%9 and 73%8 pg/mg urinary creatinine, respectively. Seven days after Ad5LO
treatment, urinary serotonin concentration increased to 173%13 and 215%25 pg/mg in wild-type and BMPR2/+/+/ mice, respectively. The difference between wild-type and BMPR2/+/+/ mice did not reach statistical significance. The relatively small difference in sP-selectin and serotonin levels found between wild-type and BMPR2/+/+/ mice after Ad5LO treatment suggested that platelet activation was not the primary source of the increased TxA 2 production in the Ad5LO-treated BMPR2/+/+/ mice.

**Effect of BMP on TxA 2 Production in A549 Cells**

Pulmonary epithelial cells are another source of TxA 2 production in vivo.28,29 We therefore examined the effect of BMPs on TxA 2 production in a human pulmonary epithelial cell line, A549. As shown in Figure 11, untreated A549 cells released a small amount of TxB 2 (207±19 pg/mg total cell protein) in the basal state, and IL-1β stimulated TxB 2 production by >10-fold (2822±41 pg/mg). BMP-2 did not affect basal level of TxB 2 production (208±11 pg/mg) but reduced IL-1β–stimulated production by ≈20% (2215±186 pg/mg). BMP-6 had an effect (2339±41 pg/mg) similar to BMP-2 in the assay. These data indicated that BMP signaling did not directly affect TxA 2 production in A549 cells but reduced the stimulating effect of IL-1β on the synthesis of this prostanoid.

**Discussion**

To understand the relation between BMPR2 haploinsufficiency and pulmonary hypertension, this study examined BMPR2/+/+/ mice and explored 3 issues: (1) whether BMPR2 haploinsufficiency affects the development of the mice; (2) whether haploinsufficiency alone leads to pulmonary hypertension; and (3) whether haploinsufficiency increases the susceptibility to pulmonary hypertension in the setting of inflammation. The results showed that BMPR2 heterozygosity caused ≈20% fatality in mice during embryonic development but did not lead to pulmonary hypertension under unstressed conditions. The study also showed that BMPR2/+/+/ mice were more sensitive to inflammation-induced pulmonary hypertension than wild-type mice.

BMP signaling plays an important role in embryonic development and is involved in gastrulation, mesoderm formation, neural patterning, skeletal development, and organogenesis (for reviews, see Hogan5 and Zhao30). Homozy-
opment.30 By contrast, animals with heterozygous mutations in these genes are viable and have been reported to be grossly phenotypically normal. In this study, we found that ~20% of BMPR2\textsuperscript{+/-} mice died in utero and/or during weaning, which suggests that haploinsufficiency of BMPR2 affects early development. Although we observed no apparent abnormality in the surviving BMPR2\textsuperscript{+/-} mice under unstrained conditions in this study, some of these mice may have had minor developmental defects, and these defects could affect the phenotype of the mice under stressed conditions.

Inflammation has been suggested to be an important mechanism in the development of PAH. Evidence supporting the hypothesis includes the following: (1) Inflammatory cells are found in the vicinity of remodeled pulmonary vessels with plexiform lesion; (2) proinflammatory cytokines and chemokines are increased in patients with PAH; (3) PAH is a common complication of autoimmune diseases involving systemic inflammation, such as scleroderma and systemic lupus erythematosus; and (4) in a rat model of PAH, monocrotaline administration causes pulmonary endothelial injury and inflammation, which is followed by pulmonary vascular remodeling (for reviews, see Voelkel et al,\textsuperscript{17} Tuder and Voelkel,\textsuperscript{18} Jeffery and Morrell,\textsuperscript{19} and Dorfmuller et al\textsuperscript{20}). In the present study, we examined the effect of inflammation on the development of pulmonary hypertension in BMPR2\textsuperscript{+/-} mice. Adenovirus-mediated overexpression of 5LO in the lung was used as the inflammatory stress because 5LO expression is increased in the human disease. The results showed that BMPR2\textsuperscript{+/-} mice responded to the inflammation with an immediate marked increase in pulmonary hypertension (peaking at day 7) and delayed muscularization of distal pulmonary arterioles (28 days after Ad5LO treatment). The early increase of RVSP is related to enhanced pulmonary vasoconstriction, due, at least in part, to increased TxA2 production caused by the inflammation. Further study is required to identify the specific mechanism. The overall degree of muscularization found in the Ad5LO-treated BMPR2\textsuperscript{+/-} mice is mild. This could be due to the transgene expression, as well as that inflammation is transient. Further studies are required to demonstrate whether increased pulmonary vascular injury by sustained inflammation leads to extensive pulmonary vascular remodeling and persistent pulmonary hypertension in BMPR2\textsuperscript{+/-} mice.

BMPR2\textsuperscript{+/-} mice were found to produce significantly higher amounts of TxA\textsubscript{2} than wild-type mice during 5LO overexpression. This observation is consistent with previous reports that TxA\textsubscript{2} production is significantly enhanced in patients with PAH.\textsuperscript{31-33} Mechanistically, however, this finding raises the question of how BMPR2 deficiency leads to increased TxA\textsubscript{2} production.
production. No report in the literature has linked BMP signaling to TxA₂ production. Because the TxA₂ levels in the wild-type and BMPR²/⁻ mice were the same before AdSLO delivery, the difference in TxA₂ production in these mice resides in the different responses to SLO overexpression or inflammation. We therefore examined platelet activation in vivo and pulmonary epithelial cell activation in cell culture, because TxA₂ syntheses is most abundantly expressed in platelets (2187 ng/mg protein),³⁴ and the lung has the highest content of the enzyme, 765 ng/mg, among solid-organ tissues.³⁴ The cells in lung that express TxA₂ synthesize are mainly bronchial epithelial cells and alveolar macrophages in humans and also small pulmonary artery smooth muscle cells in rats.²⁹ Examining plasma sP-selectin and urinary serotonin, 2 platelet activation markers, showed that both of the markers tended to be higher in BMPR²/⁻ than wild-type mice, but neither difference reached statistical significance. Thus, platelet activation may not be the primary or the only source of the enhanced TxA₂ production in BMPR²/⁻ mice during SLO expression. The pulmonary epithelial cell line AS49 was found to produce a very small amount of TxA₂ under basal conditions, but the production of this prostanoid was stimulated markedly by IL-1β. BMP did not affect the basal level of TxA₂ production but reduced IL-1β stimulation significantly by 20%. These effects suggest that BMP signaling does not regulate TxA₂ production directly but interferes with IL-1β signaling.

IL-1β signaling activates 2 pathways, one leading to nuclear factor (NF)-κB activation and the other, mitogen-activated protein kinase, Janus NH₂ terminal kinase (JNK), and subsequently, activator protein (AP)-1. A previous study has shown that BMP-7 inhibits IL-1β-induced JNK and AP-1 activation but does not affect IL-1β-induced NF-κB activation in human mesangial cells.³⁵ If a similar selective inhibition occurs in pulmonary epithelial cells, it could explain the partial inhibitory effect of BMPs on the IL-1β-stimulated TxA₂ production in AS49 cells. Further studies are required to understand the specific interaction between BMP and IL-1 signaling in these cells.

TxA₂ is a potent vasoconstrictor and platelet activator.³⁶,³⁷ It also inhibits voltage-gated potassium channels³⁸ and has synergistic effects with serotonin in causing vascular smooth muscle cell proliferation.³⁹ These effects, when persistently produced by multiple inflammatory insults or stresses, could contribute to pulmonary vascular remodeling and sustained pulmonary hypertension. Understanding the relation between BMPR² haploinsufficiency and TxA₂ production under inflammatory stress could shed light on the mechanism of heterozygous BMPR² mutation–mediated IPAH.

Acknowledgment
This study was supported by NIH grants HL58976, HL55993, and HL61795 (to Dr Loscalzo) and grants from the American Heart Association (0256282N) and Pfizer (Atorvastatin Research Award) (to Dr Zhang).

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_Circulation._ 2005;112:553-562; originally published online July 18, 2005;
doi: 10.1161/CIRCULATIONAHA.104.492488
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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