Functional adaptation and remodeling of pulmonary artery in flow-induced pulmonary hypertension

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Lam, Chen-Fuh, Timothy E. Peterson, Anthony J. Croatt, Karl A. Nath, and Zvonimir S. Katusic. Functional adaptation and remodeling of pulmonary artery in flow-induced pulmonary hypertension. Am J Physiol Heart Circ Physiol 289: H2334–H2341, 2005.—Patients with left-to-right shunt congenital heart disease may develop pulmonary hypertension. Perioperative mortality of these patients is high due to abnormal vasoreactivity of the pulmonary artery (PA). We studied the changes in the PA induced by high pulmonary blood flow in rats with aortocaval fistula. Eight weeks after surgery, morphological changes of the PA were studied and vasomotor function was assessed by isometric force recording. Expression of endothelial nitric oxide synthase (eNOS), VEGF, and cyclooxygenase-2 (COX-2) proteins and levels of cGMP in the PA were analyzed. Rats with high pulmonary blood flow developed pulmonary hypertension, medial thickening, and increasing of internal elastic lamina and basement membrane in the PA. When compared with sham-operated animals, rats with fistula had significantly increased contractions in the PA, whereas relaxations to acetylcholine and NO donor were reduced. Concentrations of cGMP were reduced in the PA of rats with pulmonary hypertension (18.4 ± 3.3 vs. 9.4 ± 1.7 pmol/mg protein; P = 0.04). The altered vasomotor function was normalized by treatment with indomethacin. The PA of rats with fistula expressed higher levels of eNOS, phosphorylated eNOS, and COX-2. Sustained high PA blood flow in rats causes pulmonary hypertension that is morphologically and functionally identical with patients with flow-induced pulmonary hypertension. Abnormal vasomotor function of the PA in these animals appears to be mediated by reduced availability and the biological effect of endogenous NO and the high production of vasoconstrictor prostanoids. Increased eNOS and phosphorylated eNOS are most likely the adaptive changes in response to an increase in PA pressure secondary to high blood flow.

guanosine 3′,5′-cyclic monophosphate; cyclooxygenase-2; endothelium; endothelial nitric oxide synthase

PULMONARY HYPERTENSION, secondary to increased pulmonary blood flow, may develop in patients with left-to-right shunt congenital heart defects or systemic arteriovenous shunt, such as arteriovenous access for hemodialysis (21, 26, 37, 53). The characteristic vascular changes include intimal hyperplasia/fibrosis, medial hypertrophy, extensive extracellular matrix modulation, and, in more severe cases, the formation of plexiform lesion (21). These changes lead to decreased compliance of pulmonary vasculature and changes in vasoreactivity of pulmonary arteries (PAs) (34). Patients with flow-induced pulmonary hypertension have significantly higher perioperative mortality and a lower long-term survival after a cardiac operation (3, 24, 44). During and after an operation, these patients often exhibit fatal pulmonary vasoconstriction (2, 45). Patients with flow-induced pulmonary hypertension also showed significantly impaired endothelium-dependent relaxation in the PAs, whereas endothelium-independent relaxation is relatively preserved (10, 17, 29).

The underlying pathogenesis of flow-induced pulmonary hypertension remains poorly understood (4). The patients with left-to-right congenital heart disease, significant PH, and pulmonary vascular disease were precluded from surgery because of unfavorable peri- and postoperative outcomes (18). Lack of a simple and experimentally validated model presented a major problem in attempts to gain insight into the mechanisms responsible for the development of flow-induced pulmonary hypertension. In the present study, we describe a valid and simple rat model of aortocaval (AC) fistula causing sustained increase of pulmonary blood flow. After pulmonary hypertension was established, we studied the functional, biochemical, and morphological changes in the remodeled PA to gain insight into the mechanisms underlying pathogenesis of flow-induced pulmonary hypertension.

MATERIALS AND METHODS

Surgical procedures of AC fistula. Creation of fistula in the abdominal aorta and inferior vena cava (IVC) in rats has been previously reported as a model for flow-induced pulmonary hypertension (11, 16, 40, 43, 52). The detailed procedure of creating an AC fistula in rats has been described in previous studies from Nath’s and Katusic’s laboratories (30, 38). In brief, Sprague-Dawley rats (~250 g) were anesthetized with an injection of methohexital sodium (50 mg/kg ip). After a midline abdominal incision, IVC and aorta were exposed. The abdominal aorta and IVC were punctured with an 18-gauge needle. The entry point of the needle into the aorta was sealed with a drop of cyanoacrylate glue. Sham-operated rats underwent laparotomy, cross-clamping of the vessels for 30 s without needle puncturing, and the placement of a drop of glue at the abdominal aorta. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic.

PA pressure measurement and tissue preparations. Eight weeks after the operation when significant PA remodeling was established (11, 16, 40, 43, 52), rats were reanesthetized. The trachea was cannulated, and lungs were mechanically ventilated (model 683; small-animal, volume-controlled ventilator; Harvard Apparatus). The thoracic cavity was opened, and the heart and lungs were exposed. A needle connected with a pressure transducer (Kent Scientific) was introduced into the PA trunk, and the mean PA pressure (MPAP) was displayed by a computerized data acquisition system (DASY). MPAP was recorded at least 1 min after the hemodynamics of the animal were stabilized. The rats were then euthanized (pentobarbital sodium,

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250 mg/kg ip), and the trunk and main PAs were harvested for vasomotor reactivity experiments, dihydroethidium assay, Western blot analysis, and cGMP measurement. The left lung and the main PAs of some animals were dissected and fixed in 10% formalin or Trump’s fixative for histology examinations.

**Histology examinations.** Biopsies of formalin-fixed tissues were embedded in paraffin wax and sectioned (5 μm). Sectioned tissues were stained with hemotoxylin and eosin and Verhoeff staining. Ultrastructural changes in the main and intralobular PAs of both groups were examined under a transmission electron microscope. Generation of superoxide anions in the frozen PA was detected by using the dihydroethidium assay under a confocal microscope (14).

**Vasomotor reactivity.** Main PA rings (2 mm long) were mounted in organ chambers containing 25 ml of Krebs solution consisting of (in mmol/l) 118.6 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25.1 NaHCO3, 10.1 glucose, and 0.026 EDTA at 37°C (94% O2-6% CO2). Changes in force were recorded continuously using an isometric force-displacement transducer (Grass FT03; Grass Instrument). Each ring was gradually stretched to 2 g (28). After a 45-min equilibration period, the rings were contracted by the addition of KCl (40 mM) or increasing concentrations of phenylephrine (PE, 10⁻⁹ to 10⁻⁵ M; Sigma). To study the relaxation, isolated PA was first contracted with an EC₅₀ (the concentration required to induce 50% of maximum contraction) of PE, which was determined from the cumulative concentration-response curves for PE (47). Concentration-response curves were then obtained by cumulative addition of acetylcholine (10⁻⁹ to 10⁻⁵ M; Sigma), and a nitric oxide (NO) donor, diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA-NONOate, 10⁻⁹ to 10⁻⁵ M; Sigma), during contraction to EC₅₀ of PE. Some of the preparations were incubated for 15 min before each contraction with a cell permeable superoxide dismutase mimetic Mn(III)-tetrakis(4-benzoic acid)porphyrin (MnTBAP; 10⁻⁵ M; Biomol) (14) or indomethacin (10⁻⁵ M; Sigma). MnTBAP or indomethacin was present in each particular chamber throughout the experiments. Papaverine (3 × 10⁻⁴ M; Sigma) was used to complete relaxations of the vessels. All experiments were performed in vessels with intact endothelium.

**Western blot analysis.** Soluble protein extracts (30–50 μg) of isolated PA were loaded into polyaryclamide gels (9–12%) and transferred onto nitrocellulose membranes. Anti-endothelial NO synthase (eNOS; Transduction), anti-phosphorylated eNOS (eNOS-S1177; Transduction), anti-VEGF (A-20; Santa Cruz Biotechnology), and anti-cyclooxygenase (COX)-2 (Transduction) antibodies were used. After being washed, the membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibodies, and bands were visualized by using enhanced chemiluminescence (Amersham Pharmacia). Equal loading of proteins for each blot was confirmed by the Ponceau S staining (36). Protein levels were quantified by scanning densitometry (Scion Image, Scion).

**Measurement of cGMP.** Isolated PA segments were incubated in Earle’s salts solution containing 0.1% BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin with IBMX (10⁻³ M; Sigma) for 30 min to inhibit the degradation of cyclic nucleotides by phosphodiesterases. PAs were homogenized, and tissue concentrations of cGMP were determined by a radioimmunoassay kit (Amersham Pharmacia) (15).

**Statistical analysis.** Results are presented as the means ± SE; n represents the number of animals studied in each experimental group. Sham and AC fistula rats were compared by an unpaired t-test or ANOVA for repeated measures followed by multiple group comparisons using Tukey’s test, where appropriate. Statistical significance was accepted at a level of P < 0.05.

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**Fig. 1.** Representative histological sections of main pulmonary artery (PA) of sham-operated rats (A) and rats with fistula (B) (×40) and intralobular PA of sham-operated rats (C) and rats with fistula (D) (×200). Significant medial thickening is shown in PA of fistula rats. Experiments were performed on three different rats in each group.
RESULTS

PA pressure and morphological analysis. Eight weeks after creation of a fistula, MPAP was significantly elevated in the rats with the fistula when compared with sham-operated animals (19.8 ± 1.2 vs. 10 ± 0.7 mmHg, respectively; *P* < 0.001; *n* = 4–5 rats). Medial thickening was observed in the main and intralobular PAs of rats with the AC fistula (Fig. 1, B and D), compared with sham-operated rats (Fig. 1, A and C). Verhoeff stain showed no evidence of intimal thickening in the remod-

Fig. 2. Representative ultrastructural sections of PA as seen under electron microscopy for sham-operated rats (A and C) and rats with aortocaval (AC) fistula (B and D). Irregular thickening of internal elastic lamina (IEL) and basement membrane (BM) with widened subendothelial space is shown in PA of rats with fistula (B). Fragmented IEL and deposition of collagen (closed arrows) in subendothelial space of PA of rats with AC fistula (D). Closed arrows indicate endothelial cells. Open arrowheads indicate IEL. Magnifications are ×2500 (A), ×3000 (B), ×7500 (C and D). Experiments were performed in main and intralobular PAs on three different animals for each group.

Fig. 3. Dihydroethidium assay was performed in frozen PA to measure generation of superoxide anion in sham-operated rats (A) and rats with flow-induced pulmonary hypertension (B). Similar dihydroethidium fluorescent densities were detected in blood vessels of both groups. Experiments were performed in four to six different animals for each group.
eled PAs (data not shown). Examination of PA ultrastructure showed thickening of the internal elastic lamina (IEL) and basement membrane, with discrete widening of subendothelial space in the PA of rats with fistula (Fig. 2B). Analysis with higher magnifications (×7,500) further demonstrated the fragmentation of IEL and deposition of collagen fibers in the widened subendothelial space (Fig. 2D). Similar fluorescent densities of dihydroethidium were detected in the PA wall of the two groups (n = 4–6 rats; Fig. 3, A and B).

Vascular reactivity. Contractions to KCl (40 mM) and PE (10−9–10−5 M) were significantly increased in the PA of rats with the AC fistula (Table 1 and Fig. 4A, I). EC50 of PE was lower in the fistula group (Table 1). Endothelium-dependent relaxations to acetylcholine were significantly impaired in rats with pulmonary hypertension, and there was a significant reduction in the maximal relaxation (96.9 ± 0.9 vs. 81.5 ± 3.3%; P < 0.001, n = 8–10 rats; Fig. 4B, I). Relaxations induced by DEA-NONOate were also significantly right-shifted in PA of rats with fistula, but the maximal relaxation was similar with sham-operated animals (Fig. 4C, I). In rats with pulmonary hypertension, MnTBAP, a superoxide dismutase mimetic, did not affect the vasomotor function of PA (Fig. 4, A, II–C, II). However, treatment with indomethacin suppressed the contraction and potentiated the relaxations of the PAs in animals with pulmonary hypertension (Fig. 4, A, III–C, III).

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Table 1. Responses of pulmonary artery to KCl and phenylephrine

<table>
<thead>
<tr>
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<th>Contraction to KCl (40 mM), g</th>
<th>Max Contraction, g</th>
<th>EC50, (×10−5 M)</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
<td>0.93±0.09</td>
<td>0.95±0.08</td>
<td>6.30±3.21</td>
</tr>
<tr>
<td>Fistula</td>
<td>1.55±0.04*</td>
<td>1.61±0.09*</td>
<td>3.94±2.25†</td>
</tr>
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Values are means ± SE; n = 8–10 animals. PE, phenylephrine; EC50, concentrations of PE that cause 50% of maximal contraction. *P < 0.001 and †P = 0.08 (power of statistics is 30%) compared with sham-operated animals analyzed by unpaired t-test.

Fig. 4. Measurements of isometric force of PA in sham- and AC fistula-operated animals. A.I: contraction responses of PA to cumulative addition of phenylephrine (PE, 10−9 to 10−5 M). B.I: relaxation responses to cumulative addition of acetylcholine (ACh). C.I: relaxations to cumulative addition of diethylenemonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA/NO). Relaxations were obtained during contraction to EC50 (the concentration required to achieve 50% of maximum contraction) of PE. A.II–C.II: preincubation of PAs with Mn(III)-tetrakis(4-benzoic acid)porphyrin (MnTBAP) (10−5 M) in rats with AC fistula before each experimental protocol. A.III–C.III: preincubation of PAs with indomethacin (10−5 M) in rats with AC fistula before each experimental protocol. Vascular reactivity of PA in sham-operated animals was not affected by treatment with MnTBAP or indomethacin (data not shown). *P < 0.05 for AC vs. sham-operated rats; †P < 0.05 for AC + MnTBAP vs. sham-operated rats. Data were analyzed by ANOVA and are means ± SE; n = 5–10 rats.
DISCUSSION

In the present study we report several new findings. First, creation of an AC fistula in rats induces changes in PA vasomotor function that are very similar to changes described in the PA of patients with flow-induced pulmonary hypertension. Second, the morphology of hypertensive PAs is consistent with previously described morphological alterations in the blood vessel wall exposed to high blood flow and a subsequent increase in pressure. Third, altered reactivity of the PA exposed to high blood pressure is not affected by superoxide scavenging but is normalized by the inhibition of COX, indicating a significant involvement of endogenous vasoconstrictor prostanoids. Fourth, increased regional blood flow enhances the expression of eNOS protein and its phosphorylation (eNOS-S1177) in the PA. Finally, flow-induced pulmonary hypertension reduces biological activity of NO and production of cGMP in the PA wall.

Consistent with previous reports (11, 43, 52), we found that rats developed significantly higher PA pressure 8 wk after exposure to high pulmonary blood flow. We also showed that there was obvious medial thickening in the PA and arterioles of these animals. In the present study, we are the first to characterize the vasomotor function of rat PA exposed to high blood flow. We provide evidence that contractions of remodeled PA to both KCl and α1-adrenergic receptor agonist are significantly enhanced. Because maximal contractions to depolarization of smooth muscle cells induced by KCl and activation of α1-adrenergic receptors by PE were similar, the augmentation of PA contractions was therefore most likely due to remodeling of blood vessel media. The reduced EC50 of PE also indicates that the remodeled PAs are more sensitive to α1-adrenergic stimulation. These results are consistent with morphological changes detected in media of PA with hypertension. Reported increase in catecholamines-mediated trophic effect (19) and activation of Rho kinase (42) could be the mechanisms responsible for flow-induced vascular smooth muscle hypertrophy, proliferation, and increased reactivity to vasoconstrictors.

Endothelium-dependent relaxation to acetylcholine was significantly impaired in the PA of rats with fistula, whereas...
endothelium-independent relaxation was less affected. Incubation with a cell-permeable superoxide scavenger MnTBAP did not affect the endothelium-dependent relaxation. This finding is in line with our observation that fluorescent densities of dihydroethidium in the PA were not different between the two groups, indicating that impaired PA endothelial function 8 wk after elevated blood flow is less likely due to an increased generation of superoxide anions. However, we are not able to rule out increased production of reactive oxygen species at the early stage of flow-induced pulmonary hypertension, because scavenging of superoxide anions has been shown to potentiate the impaired endothelial function of remodeled PA 9 days and 4 wk after increased PA blood flow in the lamb model (9, 47).

In contrast, augmented contractions to PE and impaired relaxations to acetylcholine of the remodeled PA in the present study were normalized by treatment with indomethacin. These results suggest that increased production of endogenous vasoconstrictor prostanoids may significantly contribute to the flow-induced vascular dysfunction in the PA. Our data also demonstrate that the functional changes in the PA of rats with flow-induced pulmonary hypertension are consistent with changes observed in patients with flow-induced pulmonary hypertension; namely, severe pulmonary vasoconstriction impaired endothelium-dependent relaxations, but relatively normal endothelium-independent relaxations (10, 17, 29, 45).

To further determine the molecular mechanisms underlying functional abnormalities in the PA, we investigated the protein expression and functional changes of the signaling molecules in the remodeled vascular wall. Because sustained increased PA blood flow has been previously shown after the creation of AC fistula in rats (11), we first studied changes in the key flow-regulated enzymes. We found that protein expression and phosphorylation of eNOS were significantly enhanced in the high-flow PA. With regard to expression of eNOS, our data are consistent with the previous reports in rats (11, 16, 43) and lambs (6), as well as patients with flow-induced pulmonary hypertension (4). We also detected increased expression of VEGF in the PA of rats with high pulmonary flow. Upregulated VEGF suggests the ongoing process of tissue remodeling in the PA exposed to high flow and pressure (33). Furthermore, in addition to increased shear stress secondary to high pulmonary blood flow, enhanced VEGF may also cause phosphorylation of eNOS in the PA of rats with fistula (13, 22, 41, 49). Activation of eNOS releases NO in the vessel wall to maintain the normal vascular tone (vasodilation) and to inhibit vascular smooth muscle cell proliferation (8). Therefore, the upregulation of eNOS and NO appears to be a particularly important adaptive response given a progressive increase in PA pressure and development of pulmonary hypertension (4).

Our results demonstrated that basal levels of cGMP were actually reduced in the high-flow PA, indicating that despite increased expression of eNOS and phosphorylated eNOS, biological activity of NO is decreased. This finding is at variance with previous observations (5–7) obtained in fetal lambs with cardiopulmonary shunt, in which higher concentrations of cGMP were detected in lung tissue. In the condition of chronic high pulmonary blood flow (also known as models of volume-overload heart failure) (25), increased CGMP in the lung tissue can be caused by activation of heme oxygenase-1 (30). However, in the present study cGMP was measured in the PA rather than lung tissue, thereby reflecting changes of cGMP in the vascular wall. Furthermore, our findings are consistent with changes in eNOS and cGMP reported in the arterial wall of experimental animals with systemic hypertension. The expression and activity of eNOS are increased in the hypertensive animals (39, 51), whereas endothelial function is impaired and cGMP levels are reduced or not affected (39, 46).

We demonstrated characteristic ultrastructural changes in the subendothelial regions of the intralobular PA of rats with fistula, which were compatible with those observed in arteries exposed to high blood flow (27, 31, 32). Remodeled PA exhibited widened subendothelial spaces with irregular thickening of IEL and basement membrane. Fragmented IEL, an important characteristic change of flow-induced arterial remodeling (32), was seen in the PA of these animals. Within the widening subendothelial spaces, we also found abnormal deposition of collagen fibers, which was also reported in the omental artery of women with preeclampsia (48). The importance of these structural changes for vascular function is unclear and remains to be determined.

Numerous studies have shown that increased formation of COX-dependent vasoconstricting factors, especially thromboxane A2, is involved in abnormal vasomotor function in pulmonary hypertension (35). Therefore, we examined the expression and function of COX-2, the inducible isof orm of COX, in the rat PA. Pulmonary hypertension was associated with significant elevation of COX-2. Treatment with a COX inhibitor, indomethacin, normalized the vasomotor function of PA. These functional changes suggest that an increased formation of vasoconstrictor prostanoids is responsible for the abnormal vasomotor function of PA. Indeed, our findings are consistent with previously reported studies on patients and animals with pulmonary hypertension. In patients with left-to-right shunt congenital heart defects, plasma and urine levels of thromboxane B2 (a stable metabolite of thromboxane A2) were increased (1, 23), whereas urine levels of 2,3-dinor-6-ketoprostaglandin F1α (a stable metabolite of prostacyclin) were reduced (1). Similar results were also reported in patients with other forms of pulmonary hypertension (12, 50) and hypoxic-induced pulmonary hypertension in pigs (20).

Using a rat model of increased PA blood flow by creating an AC fistula, we observed the development of pulmonary hypertension and characteristic morphological and functional changes in the PA that are consistent with changes described in patients with flow-induced pulmonary hypertension. The propensity of hypertensive PA toward vasoconstriction appears to be caused by physiological antagonism between NO and vasoconstrictor prostanoids.

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