Gene expression profile in flow-associated pulmonary arterial hypertension with neointimal lesions

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van Albada ME, Bartelds B, Wijnberg H, Mohaupt S, Dickinson MG, Schoemaker RG, Kooi K, Gerbens F, Berger RM. Gene expression profile in flow-associated pulmonary arterial hypertension with neointimal lesions. Am J Physiol Lung Cell Mol Physiol 298: L483–L491, 2010. First published December 18, 2009; doi:10.1152/ajplung.00106.2009.—Pulmonary arterial hypertension (PAH) is a pulmonary angio proliferative disease with high morbidity and mortality, characterized by a typical pattern of pulmonary vascular remodeling including neointimal lesions. In congenital heart disease, increased pulmonary blood flow has appeared to be a key mediator in the development of these characteristic lesions, but the molecular mechanisms underlying the pulmonary vascular lesions are largely unknown. We employed a rat model of flow-associated PAH, which induced specific neointimal lesions. We identified gene expression profiles in rats specifically related to the addition of increased pulmonary blood flow to monocrotaline and the associated occurrence of neointimal lesions. Increased pulmonary blood flow induced the expression of the transcription factors activating transcription factor-3 (ATF3) and early growth response factor-1 (EGR-1), for which presence was confirmed in neointimal lesions. Monocrotaline alone induced increased numbers of activated mast cells and their products. We further identified molecular pathways that may be involved in treatment with the prostacyclin analog iloprost, a vasoactive compound with clinically beneficial effects in patients with PAH, which were similar to pathways described in samples from patient studies. These pathways, associated with the development of angio proliferative lesions as well as with the response to therapy in PAH, may provide new therapeutic targets.

PULMONARY ARTERIAL HYPERTENSION (PAH) is a progressive angio proliferative disease with high morbidity and mortality (4), associated with a characteristic pattern of vascular remodeling of the small pulmonary arteries. This vascular remodeling consists of proliferation of endothelial and smooth muscle cells, fibrosis, and inflammation, leading to formation of concentric neointimal lesions and plexiform lesions (37). These latter vascular lesions can be regarded as pathognomonic for PAH (37). Although the histopathology is well described, the pathogenesis of the disease and its typical vascular lesions is largely unknown (28). In patients with congenital heart diseases that develop PAH, increased pulmonary blood flow has been clinically identified as a key mediator in the development of the pathognomonic vascular lesions of PAH (10, 33, 37) and through unknown mechanisms.

Prostacyclin, a vascular endothelium-derived vasoactive compound with vasodilatory and antiproliferative properties, is believed to interfere with the pathogenesis, since prostacyclin analogs have been demonstrated to have beneficial clinical effects in patients with PAH (3, 30). These analogs have been suggested to be able to reverse the pulmonary vascular remodeling process of pulmonary hypertension (PH) in experimental settings (31), although the latter has not yet been demonstrated in patients (2).

To identify pathways that are involved in the pathogenesis of the characteristic neointimal lesions, we used a rat model of monocrotaline combined with increased pulmonary blood flow (35). Monocrotaline in the rat induces pulmonary hypertension characterized by muscularization of the small pulmonary arteries (23), whereas the addition of increased pulmonary blood flow to monocrotaline has been demonstrated to induce the development of neointimal proliferation (5, 26, 35), thereby resembling the histopathology characteristic for patients with PAH. We used this rat model of flow-associated PAH to identify pathways that are specifically induced by increased pulmonary blood flow and to test whether these pathways are affected by prostacyclin analog treatment. For that purpose, we performed a semirandomized crossover design microarray experiment with mRNA isolated from total lung tissue according to the MIAME standards (6). We verified the most relevant results by RT-PCR and immunohistology.

METHODS

Animal model. Twenty-four male Wistar rats (Harlan) weighing 250–300 g were used. Animal care and experiments were conducted according to the Dutch Animal Experimentation Act. The Institutional Animal Care and Use Committees approved the experimental protocols.

Rats were randomly assigned to the four experimental groups: 1) control (n = 6); 2) monocrotaline-treated rats (PH; n = 6; single dose of monocrotaline 60 mg/kg sc; Sigma Chemical, St. Louis, MO); 3) monocrotaline plus aortocaval shunt [PH+Shunt; n = 6; monocrotaline injection followed by the creation of an abdominal aortocaval shunt 1 wk later (35)]; and 4) PH+Shunt treated with the prostacyclin analog iloprost (PH+Shunt+Ilo; n = 6). Osmotic minipumps (model 2004; Alzet, Palo Alto, CA) with iloprost (72 μg·kg⁻¹·day⁻¹; a generous gift of Schering, Weesp, The Netherlands) were implanted subcutaneously in the same operative session as the creation of the abdominal aortocaval shunt.

Rats were weighed and euthanized when a 15% weight loss occurred. Matched rats in the other experimental groups were euthanized...
nized simultaneously. The mean day of death was 36.5 ± 0.6 days after the administration of monocrotaline. At the time of death, rats were anesthetized with pentobarbital (60 mg/kg ip) and ventilated with room air. Pulmonary and systemic arterial pressures were measured as described by Rabinovitch et al. (29), a technique that is routinely used in our laboratory (35).

RNA isolation, probe construction, and real-time PCR analysis. For a detailed description of RNA isolation, probe construction, and real-time PCR analysis, see the data supplement online at the AJP-Lung Cellular and Molecular Physiology web site.

Microarray. The microarray experiment was designed to meet the MIAME criteria for microarray data (6). For a detailed description of slide design and array analysis, see the online data supplement. To analyze the data, clustering of the significantly regulated genes was performed on the basis of their differences as they appeared in different pairwise comparisons according to a method described by Buermans et al. (7). If a gene was significantly different between the control rats and the PH rats then it was denoted with a 1, and if there was no difference observed then it was a 0. This was also done for PH vs. PH+Shunt and for PH+Shunt vs. control rats. With three pairwise comparisons, seven combinations are possible. A gene with no significant difference between controls and PH rats (0), a significant difference between PH rats and PH+Shunt rats (1), and a significant difference between PH+Shunt rats and control rats (1) would then be assigned to cluster 1: 0-1-1. In the remainder of this article, these clusters will be addressed as clusters I-VII (Table 1).

Pulmonary and cardiac histopathology. The pulmonary histopathology in this rat model has been previously described (34, 35). For a detailed description of pulmonary and cardiac histopathology, see the online data supplement.

Statistical analysis. Data are presented as means ± SE. Group differences were analyzed using one-way ANOVA-testing with Fisher protected least significant differences post hoc testing. Correlation analysis was performed with Pearson correlation test. P < 0.05 was considered significant.

RESULTS

Animal model. Monocrotaline treatment and the creation of an abdominal aortocaval shunt induced PAH at the hemodynamic, pathological, and histological level, as described previously (34). Briefly, rats with PH and PH+Shunt had increased pulmonary arterial pressures and increased right ventricular (RV) hypertrophy (Fig. 1) and showed pulmonary vascular remodeling including increased muscularization and in the PH+Shunt group neointimal lesions in the intraacinar pulmonary vessels. Typical examples of histopathology are shown in Fig. 2. Treatment with iloprost reduced pulmonary arterial pressure and RV hypertrophy (Fig. 1) but not pulmonary vascular remodeling (34).

Microarray analysis. Of the 22,012 genes, 9,559 (43%) passed the criteria for further analysis. Of these 9,559, 383 showed significant changes in the analysis.

Classification in clusters. Seven different clusters were formed based on the difference in expression profiles between the experimental groups (Table 1). Clusters I, III, V, and VII contained genes that were affected (in either way) when pulmonary blood flow was added to monocrotaline. In clusters III, V, and VII, pulmonary blood flow opposed previously induced effects by monocrotaline. Cluster II contained genes that were affected by monocrotaline rather than pulmonary blood flow; in clusters IV and VI, pulmonary blood flow enhanced a

Table 1. Gene cluster classification

<table>
<thead>
<tr>
<th>Cluster</th>
<th>CON vs. PH</th>
<th>PH vs. PH+Shunt</th>
<th>CON vs. PH+Shunt</th>
<th>Genes</th>
<th>Up/Down</th>
<th>Regulated by Iloprost Treatment</th>
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<tbody>
<tr>
<td>I</td>
<td>0</td>
<td>1</td>
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<td>9</td>
<td>5/4</td>
<td>5</td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>18</td>
<td>16/2</td>
<td>1</td>
</tr>
<tr>
<td>III</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>69</td>
<td>18/51</td>
<td>21</td>
</tr>
<tr>
<td>IV</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>38</td>
<td>22/16</td>
<td>4</td>
</tr>
<tr>
<td>V</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>75</td>
<td>67/8</td>
<td>3</td>
</tr>
<tr>
<td>VI</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1/0</td>
<td>1</td>
</tr>
<tr>
<td>VII</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>15</td>
<td>13/2</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>225</td>
<td>142/83</td>
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<td>41</td>
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CON, control; PH, monocrotaline-treated rats; PH+Shunt, monocrotaline plus aortocaval shunt [monocrotaline injection followed by the creation of an abdominal aortocaval shunt 1 wk later (35)]. Up/Down, up-/downregulated.
monocrotaline-induced effect. Changes in the different genes per cluster are presented in graphs in Fig. 3.

Cluster I contained those genes that were altered specifically by the addition of increased pulmonary blood to monocrotaline (Table 1), hence they are specific for flow-associated PAH. It included nine genes (for a complete list, see data supplement); five of these genes were also affected by iloprost therapy. Two of these genes are of particular interest since these have been shown to be important transcription factors in systemic vascular disease (16, 25) but have not yet been linked to flow-associated PAH. These genes, activating transcription factor-3 (ATF-3) and early growth response protein-1 (EGR-1), were both upregulated in the PH/Shunt rats (but not in the PH rats), whereas they were downregulated in the PH/Shunt+Ilo rats. Real-time RT-PCR confirmed the changes shown by gene array (Fig. 4A). The changes in expression in EGR-1 were even more pronounced with real-time RT-PCR compared with the gene array. Immunohistochemistry showed the presence of EGR-1 in endothelial layers of pulmonary neointimal lesions (Fig. 4B) but not in the control group. Of the other genes, three are yet unknown, and one is a protein phosphatase.

Cluster II contained those genes that were altered specifically by monocrotaline, of which only one was also affected by iloprost (a protein tyrosin phosphatase). Five of these genes play a role in inflammation (immunoglobulin J chain precursor isoform 2, IgE-Fc receptor high affinity I α-polypeptide, complement component 1, heme oxygenase-1, and the immunoglobulin 4G6 heavy chain variable region), whereas two others are markers of mast cell activation, chymase 1 and tryptase-β1. The monocrotaline-induced genes also included the potassium channel Kcnk1, previously described to be involved in PAH (13).

Cluster III contained genes that may be involved in flow-associated PAH. These gene expression profiles were affected by monocrotaline, but these effects were opposed by the addition of pulmonary blood flow. This cluster contained 69 genes, of which the majority was downregulated by increased flow. Among these were previously described genes such as endothelin-1 and a serotonin receptor, which was downregulated in PH and upregulated in PH/Shunt compared with PH. Thirty-five percent of these genes (n = 21) were also affected by iloprost treatment, mostly in the opposite direction of the effect of the shunt, including a gene encoding for a potassium channel (Shaker). Endothelin was not affected by iloprost.

Cluster IV contained genes that are also possibly involved in flow-associated PAH, but now additional pulmonary blood flow enhanced a (not significant) effect induced by monocrotaline-induced effect. Changes in the different genes per cluster are presented in graphs in Fig. 3.

Fig. 2. Pulmonary histology of intraacinar vessels: A: normal vessel in rat from CON group; B: medial hypertrophy in rat with PH (arrow); C and D: vascular occlusion by neointimal lesion (arrows) in rats with PH+Shunt.
These genes needed both stressors, monocrotaline and increased flow, to be significantly induced. This cluster contained 38 genes, of which only 4 were affected by iloprost. A third of the genes in this cluster were related to cellular proliferation and inflammation. Notably, some genes participating in the Wnt pathway were involved, among which was frizzle-related protein (Frzb).

Cluster V contained genes that, like clusters III and IV, may be involved in flow-associated PAH. In contrast to cluster III, the changes induced by monocrotaline were partially offset by additional pulmonary blood flow, implying that pulmonary blood flow opposed the induced changes. This cluster contained a total of 75 genes, most of which increase under the influence of monocrotaline. Only 3 of these genes were also influenced by iloprost treatment, of which 2 are yet unknown, and the remaining 1 is annexin, a calcium-binding protein that has also recently been described to be involved in idiopathic pulmonary fibrosis (19).

Fig. 3. Gene expression plots for the 7 separate clusters. Y-axis: log2 gene expression ratios of the 4 groups. The expression in the CON group was set to 0. *P < 0.05 vs. CON, †P < 0.05 vs. PH, ‡P < 0.05 vs. PH+Shunt.
Fig. 4. Transcription factors induced by pulmonary blood flow. 
A: comparison of gene array and PCR data. The expression in the CON group was set to 0. *P < 0.05 vs. CON, †P < 0.05 vs. PH, ‡P < 0.05 vs. PH+Shunt. B: activating transcription factor-3 (ATF3) and early growth response factor-1 (EGR-1) staining (brown) in intraacinar vessels. a: No staining in normal vessel in rat from CON group. b: EGR-1 staining (arrows) in a neointimal lesion in a rat with PH+Shunt. c: EGR-1 staining (arrows) perivascular and in endothelial layer of a partially occluded vessel in a rat with PH+Shunt. d: ATF-3 staining (arrow) in a neointimal lesion in a rat with PH+Shunt.
Cluster VI contained genes that were induced by monocrotaline and reduced by additional pulmonary blood flow. However, this cluster contained only 1 gene, a helicase that plays a role in RNA metabolism.

Cluster VII contained genes like clusters III and V that may be involved in monocrotaline-induced PAH, but in contrast to cluster V, the altered expression of these genes with PH was offset by additional flow. This cluster contained 15 genes. Carboxypeptidase Z (homologous to Wnt-binding proteins) and caspase 6 were upregulated in the monocrotaline model and downregulated to control levels after addition of the shunt. Six of these 15 genes were also upregulated by iloprost treatment.

Of the 225 clustered genes, a third was involved in proliferation and inflammation (Table 2), supporting the notion that PAH is a proliferative and inflammatory disease. Overall, a total of 199 genes were altered due to iloprost treatment. Forty-one of these genes were also regulated by the model (clusters I–VII). Among the genes regulated by iloprost treatment were several involved in Wnt signaling, namely Wnt-inhibitory factor-1, Wnt5a, α1-catenin, axin, secreted frizzled related protein (Sfrp2), and frizzled homolog 5.

**Mast cell count.** The results of the array showed an increase in expression of mast cell markers in monocrotaline-induced PH with or without additional flow. Specifically, tryptase, chymase, mast cell protease 8, IgE-Fc receptor, and IL-9 receptor were all increased in PH. Mast cells have been suggested previously to play a role in the pathogenesis of PAH (12). We confirmed the array findings by real-time RT-PCR (Fig. 5A). Tryptase, a mast cell protease, and IgE-Fc, involved in mast cell activation, were upregulated after monocrotaline. To assess the presence of mast cells in our model, we counted the number of mast cells in the area of interest, that is in the intraalveolar vessels and in the precinar vessels (Fig. 5, B and C). The number of mast cells per field was significantly increased in rats with PAH. Interestingly, the number of mast cells significantly decreased after iloprost treatment, whereas the expression of mast cell products did not.

**DISCUSSION**

In this model of PAH in rats, we identified specific gene expression profiles related to the initiation of PH using monocrotaline, to the addition of increased pulmonary blood flow, and finally to the treatment with iloprost. Specifically, we showed that increased expression of ATF3 and EGR-1 was associated with flow-induced neointimal lesions, whereas monocrotaline-induced pulmonary hypertension was associated with increased numbers of mast cell products and mast cells around small pulmonary arteries. Finally, members of the Wnt pathway, recently suggested to be involved in human PAH, were affected by iloprost therapy in this model. The identification of these pathways, some not previously associated with the development of neointimal lesions in PAH, may provide new insights and therapeutic targets in this progressive pulmonary vascular disease.

In this study, we used a semirandomized crossover design microarray, which provides outcomes according to the MIAME agreements (6). To be absolutely sure of the validity of our data, we verified some key genes by real-time PCR. Indeed, in those genes, all array findings could be confirmed.

Our array analysis yielded several genes that have previously been suggested to be involved in the pathogenesis of PAH in humans (11, 13). Examples are the involvement of endothelin-1, potassium channel expression, serotonin signaling, and proinflammatory and proapoptotic activation (13). On the other hand, this gene array also yielded several pathways that have not been connected yet with the development of angioproliferative lesions in PAH. Finally, we identified genes specifically induced by increased pulmonary blood flow.

**ATF3 and EGR-1.** We identified two genes that were specifically induced by increased pulmonary blood flow and have never been identified in neointimal lesions in PAH: ATF3 and EGR-1. We confirmed the presence of the protein products in neointimal lesions in the rats (and absence of the protein products in control rats), suggesting that both transcription factors are involved in the neointimal proliferation phase of the disease. These transcription factors are well-suited candidates as inducers of the proliferative phase, since they both have been shown to be present in systemic vascular endothelium (16, 25) and are both inducible by shear stress, inflammation, and hypoxia (17, 22, 25, 38). Furthermore, both transcription factors induce inflammation and proliferation, two hallmarks of the characteristic pulmonary vascular remodeling seen in PAH patients (13). Although both genes are present in vascular endothelium, they are not exclusive endothelial cell products, as they are also present in fibroblasts and vascular smooth muscle cells (16, 22). Indeed, EGR-1 has been shown to be upregulated in isolated fibroblasts after hypoxia-induced pulmonary hypertension (1). Yet, both genes were not activated in monocrotaline-induced PH, suggesting that vascular smooth muscle cell proliferation, the characteristic vascular profile in monocrotaline models, was not induced by these transcription factors. It is still unknown which cell types contribute to the development of the characteristic neointimal lesions (32). Interestingly, both genes are known as early response genes, whereas in our model they appear to play a role also in the end-stage of the disease (39). Further studies to the temporal expression and effects of inhibition of these genes are necessary to delineate their role in the development of pulmonary vascular remodeling in flow-associated PAH.

**Mast cell involvement.** Genes that were selectively regulated by monocrotaline were mainly associated with inflammation (e.g., heme oxygenase-1). This confirms that inflammation is important in the vascular remodeling that occurs as a consequence of monocrotaline administration. Interestingly, several genes associated with the proliferation of mast cells (IL-9) or presence of mast cells (IgE-Fc receptor, tryptase-β1, and chymase 1) were induced by monocrotaline, both with and without flow. These results were confirmed with RT-PCR. Moreover, we showed an increased presence of mast cells in the small

Table 2. Functional classification of genes

<table>
<thead>
<tr>
<th>Function</th>
<th>Genes, n</th>
<th>Clusters</th>
<th>Iloprost</th>
</tr>
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<tbody>
<tr>
<td>Proliferation: cell cycle, apoptosis, and oncogenes</td>
<td>41  42</td>
<td>41  42</td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td>36  16</td>
<td>36  20</td>
<td></td>
</tr>
<tr>
<td>Structural/membrane protein/receptors</td>
<td>19  32</td>
<td>19  32</td>
<td></td>
</tr>
<tr>
<td>Signal transduction</td>
<td>38  32</td>
<td>38  32</td>
<td></td>
</tr>
<tr>
<td>Enzymes involved in other processes</td>
<td>55  57</td>
<td>55  57</td>
<td></td>
</tr>
<tr>
<td>Ribosomal proteins and unknown</td>
<td>225 199</td>
<td>225 199</td>
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</tr>
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</table>
intraacinar pulmonary vessels that are known to be particularly involved in PAH. Also, their presence decreased after iloprost therapy. Indeed, increased mast cell counts have been described in lung biopsies of patients with PAH associated with congenital heart disease (12). We believe the increased mast cell count in this model is likely to reflect a mechanism in the pulmonary vascular remodeling rather than a direct effect of monocrotaline itself since Vaszar et al. (36) showed that mast cells and their related protein expression levels started to rise significantly as late as 21 days after monocrotaline injection and not earlier. The involvement of mast cells in the development of PAH has received little attention, although they may be of specific interest since mast cells are known as potent angiogenic stimulators (8, 24). Mast cells may play a role in angiotensin II-mediated vasoconstriction (18), supposedly via specific serine proteases from their granules (14, 21). Chymase, one of the serine proteases excreted by mast cells, is involved in the conversion of angiotensin I to angiotensin II (18) and of big endothelin to endothelin-1 (9), two powerful vasoconstrictors with mitogenic properties. Together, these data suggest that the presence of excessive mast cells in pulmonary arteries of human and experimental PAH contributes to the development of the disease. Interestingly, iloprost treatment reduced mast cell presence but not their gene products (Fig. 5). Further studies are necessary to delineate the role of mast cell products in pulmonary vascular remodeling in PAH and to investigate their potential in therapeutic strategies.

Wnt signaling. In this study, we found that certain components of the Wnt pathway were affected by iloprost therapy (i.e., Wnt inhibitory factor-1, Wnt5a, Sfrp2, Fzd5, α-1-catenin, axin, and calmodulin-3). Recently, the Wnt pathway has been shown to be an important pathway in pulmonary development as well as in pulmonary inflammation and oncology (27) but had not been linked to PAH before. While this manuscript was under preparation, a study in lung explants of patients with idiopathic PAH was published that also showed involvement of the Wnt pathway (20). Indeed, we found similar members of subfamilies of gene pathways activated in our rat model (e.g., Rac1 and Wnt11). The Wnt family controls a variety of processes including proliferation and migration (27). Target genes of the Wnt pathways include matrix metalloproteinases, cyclooxygenase-2, and the VEGF receptor. All of these substances are believed to play a role in the pathogenesis of pulmonary hypertension. The factors of the Wnt system that were affected by therapy in our model were both activators as well as inhibitors of the Wnt pathway, hence the net effect of iloprost intervention of the Wnt pathway remains difficult to predict (15). Of note is that most of the Wnt genes in our model were significantly affected by iloprost therapy rather than by the disease itself. Our animal model allows for separation of gene products regulated by the disease process vs. those regulated by the therapeutic regimen and is therefore an important addendum to interpret data derived from patients with end-stage PAH who in the current era are likely to be treated.

Fig. 5. Mast cell involvement. A: comparison of gene array and PCR data. The expression in the CON group was set to 0. *P < 0.05 vs. CON. B: mast cell counts in the lungs of the different rat groups. *P < 0.05 vs. CON, ‡P < 0.05 vs. PH+Shunt. C: typical example of mast cell staining in pulmonary vasculature of rats with PH.
with antipulmonary hypertensive drugs, including prostacyclin analogs. Whether the Wnt pathway is involved in the development of the pulmonary vascular lesions or in the effect of therapeutic strategies remains to be determined.

Effects of therapy. When comparing our gene profiles with those obtained from patient studies (11, 20), it became apparent that most of the genes that have been described previously in humans were, in our model, affected by iloprost rather than by the disease itself. For example, several oncogenes and anion channels described by Geraci et al. (11) were found to be affected by iloprost (DEAD-box protein, Shaker, chloride channel, and cytochrome c oxidase), whereas the array in our model showed that they were not significantly induced by monocrotaline or pulmonary blood flow. Similarly, most of the genes involved in the Wnt pathway (20) were only significantly affected by iloprost. Hence, the question arises whether gene profiles found in patients with end-stage PAH are also influenced by therapeutic agents. It is yet unknown whether the beneficial clinical effects of iloprost in patients with PAH are reached through vasodilatory properties or through actual reversal of pulmonary vascular remodeling (34). This study illustrates that linking changes in gene and protein profiles to pulmonary vascular remodeling in vivo are necessary to address these issues.

In conclusion, in this rat model of flow-associated PAH, we showed that increased expression of ATF3 and EGR-1 was associated with flow-induced neointimal lesions, whereas monocrotaline-induced pulmonary hypertension was associated with increased numbers of activated mast cells around small pulmonary arteries. Finally, members of the Wnt pathway, recently suggested to be involved in human PAH, were affected by iloprost therapy in this model. The identification of these pathways, some not previously associated with the development of neointimal lesions in PAH, may provide new therapeutic targets in this progressive pulmonary vascular disease.

GRANTS

This work was supported by the Sebald Foundation.

DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


