Serotonin-Induced Smooth Muscle Hyperplasia in Various Forms of Human Pulmonary Hypertension

Elisabeth Marcos, Elie Fadel, Olivier Sanchez, Marc Humbert, Philippe Dartevelle, Gerald Simonneau, Michel Hamon, Serge Adnot, Saadia Eddahibi

Abstract—Hyperplasia of pulmonary artery smooth muscle cells (PA-SMCs) is a hallmark pathological feature of pulmonary hypertension (PH). Serotonin (5-HT) is involved in the hyperplasia through its interactions with specific receptors and internalization by a specific plasma membrane transporter. We investigated the expression and role of the 5-HT transporter (5-HTT) and 5-HT1B, 5-HT2A, and 5-HT2B receptors in lungs and isolated PA-SMCs from patients with primary PH (n=14), pulmonary veno-occlusive disease (n=4), or secondary PH (SPH, n=8) and nonpulmonary hypertensive control subjects. Whereas strong immunostaining for the three receptor types and 5-HTT was seen in remodeled pulmonary vessels from patients in all PH categories, only 5-HTT expression was increased in lungs and cultured PA-SMCs from patients versus controls. The increased growth response of PA-SMCs from patients with primary PH, pulmonary veno-occlusive disease, or SPH to 5-HT or serum was entirely attributable to 5-HTT overexpression, because 5-HTT inhibitors but not 5-HT receptor antagonists abolished 5-HT mitogenic activity and reduced the serum-induced growth response to similar levels in patients as in controls. The L-allelic variant of the 5-HTT gene promoter, which is associated with 5-HTT overexpression, was present homozygously in 14 of 25 (56%) lung transplantation patients with SPH but in only 27% of controls. Polymorphism of the 5-HTT gene promoter was only partly responsible for the increased 5-HTT expression in PH, because PA-SMCs from patients exhibited higher 5-HTT levels than same-genotype cells from controls and no additional promoter sequence alterations were found. We conclude that 5-HTT overexpression is a common pathogenic mechanism in various forms of PH. (Circ Res. 2004;94:1263-1270.)

Key Words: human pulmonary hypertension ■ serotonin transporter ■ serotonin receptors ■ pulmonary artery smooth muscle cells

Pulmonary hypertension (PH) is characterized by an increase in pulmonary vascular resistance that impedes ejection of blood by the right ventricle and leads to right ventricular failure. Primary PH (PPH) is the clinical term used to describe a rare and fatal condition for which no underlying cause can be found. More commonly, PH occurs in association with various diseases, such as collagen vascular disease, portal hypertension, human immunodeficiency virus infection, respiratory disorders, or chronic thromboembolic lung disease.1,2 The hallmark pathological feature of both PPH and secondary PH (SPH) is vascular cell proliferation and obliteration of small pulmonary arteries. In some cases identified as pulmonary veno-occlusive disease (PVOD), pathological lesions predominate on the venous side of the pulmonary vessels. Whether common pathogenic mechanisms underlie pulmonary vascular remodeling in PPH, SPH, and PVOD remains unknown.

Recent studies have emphasized the major role of serotonin (5-hydroxytryptamine [5-HT]) in the process of pulmonary vascular remodeling. An increased risk of PPH has been reported in patients who used appetite suppressants acting on the 5-HT transporter (5-HTT).3–5 However, the mechanism by which 5-HT affects the pulmonary vasculature is still a matter of debate. Although we previously showed that 5-HTT overexpression was associated with pulmonary vascular smooth muscle hyperplasia in patients with PPH,6 studies in relevant animal models provided evidence that not only the 5-HTT7,8 but also several 5-HT receptors, namely 5-HT1B,9 5-HT2A, and 5-HT2B,10 may contribute to the pulmonary vascular remodeling process. However, the respective contributions of 5-HT receptors and the 5-HTT in human PH have not been specifically examined. Moreover, another key issue is whether alterations in the serotonin signaling pathway may be a common pathogenic mechanism in various forms of PH.
TABLE 1. Clinical and Genotype Characteristics of Patients With PH

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>SPH</th>
<th>PVOD</th>
<th>PPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (n)</td>
<td>12</td>
<td>8</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>Age, years</td>
<td>45±3</td>
<td>46±3</td>
<td>30±6*</td>
<td>40±4</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>6/6</td>
<td>4/4</td>
<td>3/1</td>
<td>6/8</td>
</tr>
<tr>
<td>PAP, mm Hg</td>
<td>…</td>
<td>49.5±3.5</td>
<td>66.3±4.5*</td>
<td>62.6±3.9*</td>
</tr>
<tr>
<td>PVR, U/mm²</td>
<td>…</td>
<td>16±3</td>
<td>32.5±1†</td>
<td>29±3†</td>
</tr>
<tr>
<td>CI, L/min per m²</td>
<td>…</td>
<td>2.7±0.3</td>
<td>2.0±0.2*</td>
<td>2.0±0.1*</td>
</tr>
<tr>
<td>5-HTT genotype</td>
<td>3/6/3</td>
<td>1/3/4</td>
<td>0/1/3</td>
<td>1/5/8</td>
</tr>
<tr>
<td>SS/LS/LL</td>
<td>33 6 3</td>
<td>33 2 3</td>
<td>33 0 3</td>
<td>40 6 4</td>
</tr>
</tbody>
</table>
| PAP indicates pulmonary arterial pressure; PVR, pulmonary vascular resistance; CI, cardiac index.
*P<0.05, †P<0.01 compared with values for patients with SPH.

Accordingly, we investigated the expression of the 5-HTT and the 5-HT₁B, 5-HT₂A, and 5-HT₃A receptors in lungs and isolated pulmonary artery smooth muscle cells (PA-SMCs) from patients with PH and controls. The respective roles of 5-HTT and 5-HT₁B, 5-HT₂A, and 5-HT₃A receptors on PA-SMC proliferation were assessed in cells from both patients with PH and controls using selective 5-HTT inhibitors and 5-HT receptor antagonists. We found that 5-HTT inhibitors, but not 5-HT receptor antagonists, influenced the PA-SMC growth response to 5-HT, and we consequently examined 5-HTT expression and its effects on PA-SMC growth in patients with various types of PH, namely, PPH, SPH, and PVOD. Because 5-HTT expression is genetically controlled, we characterized the genotype of cells from both patients and controls and investigated whether promoter sequence alterations or modifications in signaling pathways underlie 5-HTT overexpression in patients with PH.

Materials and Methods

Study Population

Studies were performed on lung specimens obtained during lung transplantation in 14 patients with sporadic PPH, 4 patients with PVOD, and 25 patients with SPH. In the SPH subgroup there were 11 cases of histiocytosis, 6 of scleroderma, 2 of sarcoidosis, 2 of Eisenmenger syndrome, and 1 each of sickle cell disease, bronchiectasis, cystic fibrosis, and emphysema; the female to male sex ratio was 9 to 16 and the age range was 13 to 55 years (mean±SD, 41±4). The mean pulmonary arterial pressure in this group was 54±19 mm Hg (range, 34 to 118 mm Hg). Lung specimens from 8 of these patients with SPH (3 histiocytosis, 1 sarcoidosis, 1 scleroderma, 1 sickle cell disease, 1 bronchiectasis, and 1 emphysema) were used for immunohistochemical analysis and isolation of PA-SMCs. Control lung specimens were obtained from 15 patients undergoing lobectomy or pneumonectomy for localized lung cancer. Twelve of these patients were selected as controls based on 5-HTT gene polymorphism distribution in a population of healthy subjects.6 The clinical and genotype characteristics of the 26 patients and 12 controls used for immunohistochemical analysis and studies on PA-SMCs are listed in Table 1. All patients satisfied functional class III-IV criteria. Patients with PPH (n=14) or PVOD (n=4) were receiving prostacyclin at the time of transplantation. Preoperative transthoracic echocardiography was performed in the controls to rule out PH.

Lung Immunohistochemical Analysis

Paraffin sections (5 μm thick) of lung specimens were mounted on Superfrost Plus slides (Fisher Scientific) and incubated with the following antibodies: rabbit anti-human 5-HTT, rabbit anti-human 5-HT₁B (Cruz Biotechnology, Santa Cruz, Calif), mouse anti-human 5-HT₂A, or mouse anti-human 5-HT₃A (Pharmingen, San Diego, Calif), diluted 1:1000 in PBS containing 0.02% BSA. Sections were subsequently exposed for 1 hour to biotin-labeled anti-rabbit or anti-mouse secondary antibodies (Sigma) diluted 1:200 in the same buffer. The sections were then stained with H&E. To quantify distal pulmonary artery immunoreactivity for 5-HT₁B, 5-HT₂A, 5-HT₃A, and 5-HTT, images were acquired from 10 randomly selected fields from each lung section and stored as digital image files. Then images were imported into an image-analysis software package (Perfect Image; Clara Vision) for calculation of the pulmonary arterial area and for scoring intensity of immunostaining. The immunostaining index was expressed as the ratio between this count and the pulmonary arterial area.

Culture of Human PA-SMCs and Measurement of Cell Proliferation

PA-SMCs were cultured from explants of pulmonary arteries (1.5 to 10 mm in diameter), as previously described,6 and used between passages 1 and 3. The cells were subjected to 48 hours of growth arrest in FCS-free medium and then incubated in DMEM with or without FCS (5%), platelet-derived growth factor (PDGF)-BB (10 ng/mL), or 5-HT (10⁻⁶ mol/L). The effect of 5-HT was also examined in the presence of the 5-HTT inhibitor citalopram (Lundbeck) or fluoxetine (Eli Lilly) and of the specific antagonists of 5-HT₁B, 5-HT₂A, and 5-HT₃A receptors GR127935 (Glaxo Smith Kline), ketanserin (Janssen), and RS127445 (Roche), respectively, all at 10⁻⁶ mol/L. Each of these drugs was added 20 minutes before 5-HT. After incubation for 24 hours, the cells were washed twice with PBS, exposed to ice-cold 10% trichloroacetic acid, and dissolved in 0.1 N NaOH (0.5 mL/well). The incorporated radioactivity was counted. [³H]Thymidine incorporation into DNA is reported as counts per minute per well.

5-HTT and 5-HT Receptor mRNA Levels in PA-SMCs

Total RNA was extracted from PA-SMCs according to the method of Chomczynski and Sacchi.13 Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed on 100 ng of total RNA using the following primers: 5-HT₁B (5'-CTCGAGAACGTCGCTGGT-3' and 5'-CCGTCCTGGTTGGGTCGTTG-3'), 5-HT₂A (5'-ATTCCAGA-CTAAAGGCATT-3' and 5'-AGCTAACGGCGCGTTGGC-3'), 5-HT₃A (5'-ACGGTCTCCTTITCTACAACGCA-3' and 5'-CCGGTGA-CGACGTTGGT-3'), 5-HT₅ (5'-TTACACAGATCATCGGC-3' and 5'-GGATCCTCCTGCTACAGG-3'), 5-HT₆ (5'-ATGAAATGGTGACGTCAGCTCCG-3' and 5'-GCTTGTGTA- TCCACATCTGCTG-3'). Each RNA sample was reverse transcribed (45 minutes at 48°C) and amplified using the access RT-PCR kit (Promega) with the primers in the presence of 2.5 mmol/L MgCl₂. Cycle amplifications were performed at 94°C, 60°C, and 72°C (1 minute each, 28 cycles). PCR products were separated by agarose gel electrophoresis, and bands corresponding to the predicted sizes were revealed with ethidium bromide staining. For quantification of PCR products, images were captured, imported, and analyzed using SYNGENE (Genius system). The optical densities of bands from each sample were normalized for the optical density of the corresponding β-actin PCR product.

Competitive RT-PCR Determination of 5-HTT mRNA

5-HTT mRNA was quantitated by competitive RT-PCR, in which RNAs are reverse-transcribed and the synthesized cDNAs are amplified in the presence of an internal standard consisting of the same target mRNA, with a deletion of ~100 bases.6 5-HTT mRNA levels are expressed as attomole per microgram of total RNA.

Measurement of [³H]5-HT Uptake

5-HTT activity was assessed as previously described in PA-SMCs from patients and controls6,7 subjected to growth arrest in FCS-free medium.
Genotyping

Genomic DNA was extracted from human PA-SMCs using the Mini Genomic Kit (Eurobio). The 5-HTT gene promoter region was amplified by PCR using genomic DNA samples from patients with PPH and from controls. PCR products were separated by electrophoresis in 1% agarose gel and purified using the QIAquick PCR purification kit (QIAGEN). PCR products were then sequenced using a dye-terminator cycle-sequencing system (ABI PRISM 377, Perkin-Elmer Applied Biosystem).

Screening for Mutations in the Gene Encoding Bone Morphogenetic Protein Receptor 2 BMPR2

Mutations in the BMPR2 gene in PA-SMCs from patients with PPH or PVOD were screened as previously described. Briefly, sequences corresponding to exons 1 through 13 of the gene were amplified from genomic DNA samples by PCR with specific primers. PCR products were analyzed as described above.

Statistical Analyses

All data are reported as mean±SEM. ANOVA was used for between-group comparisons. When ANOVA indicated significance, the groups were compared using a Mann-Whitney nonparametric test. \( P < 0.05 \) was considered statistically significant. Genotype frequencies were compared using \( \chi^2 \) tests, and the test for Hardy Weinberg equilibrium was performed in each group.

Results

Clinical and Genetic Characteristics of Patients With Pulmonary Hypertension and Controls

The characteristics of patients whose lung specimens were used for immunohistochemical analysis and studies on cultured PA-SMCs are shown in Table 1. Patients with VOD and PPH did not differ with respect to age or hemodynamic data. Patients with SPH were slightly older than patients with PVOD and PPH and had less-severe PH, as shown by their lower mean pulmonary artery pressure and pulmonary vascular resistance and their higher cardiac index. The control subjects were older than the patients with PPH and PVOD but similar in age to the patients with SPH.

No BMPR2 mutations were identified in patients with PPH or PVOD. The distribution of 5-HTT genotypes in patient subgroups and controls is also shown in Table 1. By selection, the 12 controls reflected the distribution of 5-HTT genotypes in a population of healthy subjects.

Immunolocalization of 5-HTT and 5-HT Receptors in Lungs

In control lungs, 5-HTT, 5-HT1B, and 5-HT2A receptors were predominantly located in the media of pulmonary arteries. Weak staining was also observed in the endothelium. 5-HT2B receptor immunoreactivity was mainly confined to smooth muscle cells of pulmonary arteries in both control subjects and patients with pulmonary hypertension. Bar=100 \( \mu \)M. B, Immunostaining index of the 5-HT transporter and 5-HT1B, 5-HT2A, and 5-HT2B receptors in pulmonary arteries from controls (n=6) and patients with PPH (n=6). Values are mean±SEM. * \( P < 0.05 \), ** \( P < 0.01 \) compared with values for cells from controls. § \( P < 0.05 \) compared with corresponding values from patients with SPH.

Figure 1. A, Immunolocalization of the 5-HT transporter and 5-HT1B, 5-HT2A, and 5-HT2B receptors in lung sections from control subjects and patients with SPH or PPH. 5-HTT and 5-HT2B-like immunoreactivities were located mainly in the smooth muscle cells, but some immunostaining was also associated with pulmonary arterial endothelial cells. 5-HT1B and 5-HT2A-like immunoreactivity was mainly confined to smooth muscle cells of pulmonary arteries in both control subjects and patients with pulmonary hypertension. Bar=100 \( \mu \)M. B, Immunostaining index of the 5-HT transporter and 5-HT1B, 5-HT2A, and 5-HT2B receptors in pulmonary arteries from controls (n=6) and patients with SPH (n=6) or PPH (n=6). Values are mean±SEM. * \( P < 0.05 \), ** \( P < 0.01 \) compared with values for cells from controls. § \( P < 0.05 \) compared with corresponding values from patients with SPH.

5-HTT Promoter and Intron 2 Promoter Sequencing

To look for mutations in the 5-HTT promoter and intron 2, the entire promoter and intron 2 regions were amplified by PCR using genomic DNA samples from patients with PPH and from controls. PCR products were separated by electrophoresis in 1% agarose gel and purified using the QIAquick PCR purification kit (QIAGEN). PCR products were then sequenced using a dye-terminator cycle-sequencing system (ABI PRISM 377, Perkin-Elmer Applied Biosystem).
and SPH cases compared with controls and also a higher staining intensity in PPH than in SPH (Figure 1B). In contrast, the staining intensity for 5-HT receptors in pulmonary arteries did not differ between patients and controls.

5-HTT and 5-HT Receptor mRNA Levels in Cultured PA-SMCs From Patients and Controls

Cultured PA-SMCs from patients with PPH, PVOD, or SPH expressed similar levels of 5-HT1B, 5-HT2A, and 5-HT2B receptor mRNA, as did PA-SMCs from controls (Figure 2). In contrast, 5-HTT mRNA levels were higher in PA-SMCs from patients with PPH and PVOD than in those from controls. PA-SMCs from patients with PPH or PVOD had significantly higher 5-HTT mRNA levels than PA-SMCs from patients with SPH, who did not differ from controls (Figure 2).

Hyperplasia of Cultured PA-SMCs From Patients With PPH, PVOD, or SPH

In cells cultured in serum-free medium, 5-HT produced an increase in [3H]thymidine incorporation that was more marked in PA-SMCs from patients with PPH, PVOD, or SPH than in those from controls (Table 2). The PA-SMC proliferation response to 5-HT was significantly stronger in patients with PPH or PVOD than in those with SPH. Pretreatment of the cells with the 5-HTT inhibitors fluoxetine or citalopram (10⁻⁶ mol/L)^7,10 completely abolished the 5-HT-induced increase in [3H]thymidine incorporation, causing DNA synthesis to return to a similar basal level in PA-SMCs from patients and controls (Table 2 and Figure 3A). In contrast, the mitogenic response to 5-HT was not affected by incubation of the cells with 10⁻⁶ mol/L of the 5-HT2A, 5-HT2B/C, and 5-HT1B/D receptor antagonists ketanserin, RS-127445, and GR127935, respectively (Table 2). We found that doses of citalopram as low as 10⁻⁷ mol/L completely abolished the PA-SMC growth response to 5-HT, whereas doses of ketanserin, RS-127445, or GR127935 as high as 10⁻⁶ mol/L failed to affect this response. The PA-SMC growth response to FCS (containing ≈350 nmol/L of 5-HT⁷^8) was also significantly stronger in cells from patients than in those from controls (Figure 3B), with a greater increase in DNA synthesis in the PPH and PVOD groups than in the SPH group. In the presence of citalopram (10⁻⁶ mol/L), the growth response to FCS was reduced and the [3H]thymidine incorporation differences among patient groups and between patients and controls were abolished (Figure 3B).

As illustrated in Figure 3C, PDGF also markedly increased [3H]thymidine incorporation in PA-SMCs, but this effect did not differ between patients with PPH, PVOD, or SPH and controls. Furthermore, the stimulating effect of PDGF on

**Table 2.** [3H]Thymidine Incorporation in Pulmonary Artery Smooth Muscle Cells

<table>
<thead>
<tr>
<th>Additives</th>
<th>Controls</th>
<th>SPH</th>
<th>PVOD</th>
<th>PPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>315±101</td>
<td>379±99</td>
<td>345±122</td>
<td>384±135</td>
</tr>
<tr>
<td>5-HT (10⁻⁶ mol/L)</td>
<td>1990±205</td>
<td>3807±282*</td>
<td>4508±151†</td>
<td>4477±139†</td>
</tr>
<tr>
<td>5-HT + GR127445</td>
<td>1732±177</td>
<td>3371±234*</td>
<td>4197±186†</td>
<td>4341±212†</td>
</tr>
<tr>
<td>5-HT + ketanserin</td>
<td>1933±224</td>
<td>3534±189*</td>
<td>4214±200†</td>
<td>4476±196†</td>
</tr>
<tr>
<td>5-HT + RS127445</td>
<td>1875±231</td>
<td>3421±167*</td>
<td>4307±168†</td>
<td>4557±217†</td>
</tr>
<tr>
<td>5-HT + citalopram</td>
<td>414±53</td>
<td>445±33</td>
<td>477±60</td>
<td>512±38</td>
</tr>
<tr>
<td>5-HT + fluoxetine</td>
<td>390±48</td>
<td>411±59</td>
<td>462±73</td>
<td>476±64</td>
</tr>
</tbody>
</table>

Incorporation of [3H]thymidine in cells from controls or from patients with SPH, PVOD, or PPH after exposure to 5-HT alone or in the presence of GR127935, ketanserin, RS127445, citalopram or fluoxetine (10⁻⁶ mol/L).

*P<0.05, †P<0.01 compared with values for cells from controls.

‡P<0.05 compared with values for cells from SPH.
DNA synthesis was not modified by citalopram (10−6 mol/L) (Figure 3C).

5-HTT Activity in Cultured PA-SMCs From Patients With PPH, PVOD, or SPH

Compared with [3H]5-HT uptake in PA-SMCs from controls, [3H]5-HT uptake measured in cells from patients with PH was markedly increased. This increase was significantly larger in cells from patients with PPH or PVOD than in those from patients with SPH (Figure 4). At 10−6 mol/L, fluoxetine or citalopram decreased [3H]5-HT uptake to the same level in PA-SMCs from the three patient groups and the control group (Figure 4).

5-HTT Expression in Cultured PA-SMCs From Patients and Controls as a Function of 5-HTT Gene Polymorphism

The influence of 5-HTT gene polymorphism on 5-HTT expression and activity was evaluated in controls and patients with PH. Twelve controls were selected in these studies according to their 5-HTT genotype; 3 were LL, 6 LS, and 3 SS. The 14 patients with PPH were classified according to their 5-HTT genotype (Table 1). Because only one patient with PPH was homozygous for the S allele, data from this patient were not used for the statistical analysis but are shown in Figure 5. In the controls, 5-HTT mRNA levels and [3H]5-HT uptake were found to be significantly greater in cells homozygous for the L variant than in cells having one or two copies of the S variant (Figure 5). Similar differences were noted between genotypes in patients with PPH, but for each genotype, PA-SMCs from patients with PPH had markedly greater 5-HTT mRNA levels and [3H]5-HT uptake activity than PA-SMCs from controls (Figure 5).

5-HTT Promoter and Intron 2 Sequence in Patients With Primary Pulmonary Hypertension

Because 5-HTT overexpression in patients with PPH might be attributable to mutations in the regulatory regions of the 5-HTT gene, including the promoter region and intron 2, we sequenced these regions in both the patients and controls. Compared with the sequence obtained in controls, none of the patients had deletion, missense, or nonsense mutations in the 5-HTT promoter or intron 2 region (not shown).

Distribution of 5-HTT Genotypes in Patients With Secondary Pulmonary Hypertension and Controls

In the overall population of lung transplantation patients with SPH, the SS/LS/LL genotypes were distributed according to the Hardy-Weinberg equilibrium, 3/8/14, respectively. We used as controls a previously reported population of 84 healthy white subjects (mean age, 46±11 years) whose SS/LS/LL genotype distribution was found to be 16/45/23, respectively. We found that the homozygous form of the long variant (LL) was higher in the group of lung transplantation patients with SPH (56%) than in controls (27%) (P<0.05).

Discussion

The main objective of this study was to examine the expression and functional roles of the 5-HTT and three 5-HT receptors in PA-SMCs from patients with PH and to test whether common patterns of alteration are present in different types of human PH.

In the first part of this study, we used immunohistochemistry to examine remodeled pulmonary arteries from patients with severe PH. We found that medial thickening of pulmonary arteries was associated with strong immunostaining of 5-HTT and of the 5-HT receptors 5-HT1B, 5-HT2A, and 5-HT2B. Comparison of the staining intensity index between patients and controls revealed that 5-HTT, but not receptor protein, was increased in pulmonary arteries from patients with PPH, PVOD, or SPH. Additional studies revealed a marked increase in 5-HTT expression in PA-SMCs cultured from the lungs of patients with PPH, PVOD, or SPH compared with those from controls. In contrast, the expressions of 5-HT1B, 5-HT2B, and 5-HT2A receptors did not differ between PA-SMCs from patients with PH and controls. Functional studies showed that antagonists for the 5-HT1B, 5-HT2A, and 5-HT2B receptors did not alter the mitogenic effect of 5-HT on PA-SMCs from either patients or controls, whereas 5-HTT inhibitors completely abolished this effect. This confirms our initial observation that 5-HT–induced proliferation of human PA-SMCs is mediated mainly through internalization of indoleamine via its transporter. These results also suggest that 5-HT receptors previously shown to affect PH in experimental animals may exert their action through mechanisms distinct from putative direct effects on SMC hyperplasia. Indeed, 5-HT1B receptors have been shown to mediate 5-HT–induced contraction of human pulmonary arteries, and enhancement of 5-HT1B–mediated and 5-HT2A–mediated contraction has been found in pulmonary arteries from hypoxic rats. Therefore, the previous findings that rats treated with selective 5-HT1B or 5-HT2A receptor antagonists and knock-out mice for the 5-HT1B receptor (5-HT1B−/−) develop less-severe PH and a milder degree of vascular remodeling than relevant paired controls probably reflect the loss of 5-HT–mediated pulmonary vasoconstriction under such conditions. Mice with genetically or pharmacologically inactivated 5-HT2B receptors have also been shown to be protected against hypoxia-induced PH. However, 5-HT2B receptors have been shown to not be involved in pulmonary vasoconstriction, and the present results show that they do not mediate 5-HT–induced PA-SMC proliferation. It is therefore likely that these receptors act on the pulmonary vasculature through more complex and indirect mechanisms, potentially involving endothelial cells, fibroblasts, or extracellular matrix remodeling. The present observation that 5-HT1B, 5-HT2A, and 5-HT2A receptors are not upregulated in remodeled pulmonary arteries and cultured PA-SMCs from patients with PH suggests that they play a minor role in the process of pulmonary vascular remodeling associated with PH. In contrast, the increased 5-HTT expression observed in SMCs of remodeled pulmonary arteries in lung sections (Figure 1) and cultures may be an early event responsible for PA-SMC hyperplasia and subsequent medial hypertrophy of pulmonary vessels.

An important finding from this study is that PA-SMCs isolated from patients with various patterns of PH grew faster than PA-SMCs from controls when stimulated by 5-HT or by serum but not by other growth factors such as PDGF. We
examined cells obtained from 14 patients with PPH, 4 patients with PVOD, and 8 patients with SPH related to various diseases. To our knowledge, this is the first study that quantified the functional response of isolated PA-SMCs in such a large series of patients with PH. It is noteworthy that a large difference in the PA-SMC growth responses to serum and 5-HT was observed between patients and controls, with no overlap of values between the two groups. The explanation

Figure 3. Stimulatory effect of 5-HT (A), FCS (B) (5%), and PDGF (C) on [3H]thymidine incorporation (in cpm/well) in cultured PA-SMCs from controls (n=12) and from patients with SPH (n=8), PVOD (n=4), or PPH (n=14). Cells were incubated with either 5-HT (10^{-6} mol/L) alone, FCS (5%) alone, or PDGF (10 ng/mL) alone (left) or in the presence of citalopram (10^{-6} mol/L) added 20 minutes before 5-HT, FCS, or PDGF (right). Values are mean±SEM. *P<0.01 compared with values for PA-SMCs from controls. §P<0.05 compared with corresponding values for PA-SMCs from patients with SPH.

Figure 4. [3H]5-HT uptake in PA-SMCs from controls and patients with SPH (n=8), PVOD (n=4), or PPH (n=14). The concentration of [3H]5-HT in the uptake buffer was 10 nmol/L. *P<0.05, **P<0.01 compared with values for cells from controls. §P<0.05 compared with corresponding values for cells from patients with SPH. In presence of citalopram, residual [3H]5-HT accumulation was very low and was similar in patients and controls.
for the slightly lower levels of 5-HTT expression and 5-HT–induced PA-SMC proliferation in the patients with SPH than in those with PPH or PVOD. It is not clear whether chronic exposure to PPH and PVOD patients to prostacyclin treatment before isolation of tissue could have altered the observed differences in their PA-SMC responses compared with SPH. It is also possible that plasma levels of 5-HT could directly interact with 5-HTT expression. Interestingly, the level of pulmonary artery pressure was also lower in the patients with SPH than in those with PPH or PVOD (Table 1). However, no relationship was found between PA-SMC proliferation and the LL genotype.

The difference in 5-HT–induced PA-SMC proliferation between patients and controls was abolished by the 5-HTT inhibitors fluoxetine and citalopram, implying that it was related to 5-HTT overexpression. This finding supports the concept that increased 5-HTT expression contributes to the pathogenesis not only of PPH but also of other forms of severe PH. Until now, few common mechanisms shared by individuals with PH attributable to different causes had been identified. The present results suggest that a final common molecular pathway may involve increased 5-HT signaling through increased expression of its transporter.

Why cultured PA-SMCs from patients with PPH, PVOD, or SPH remain capable of overexpressing 5-HTT in the absence of additional stimuli remains incompletely understood. Compelling evidence has been obtained that 5-HTT expression is genetically controlled and that a polymorphism in the promoter region of the human 5-HTT gene affects transcriptional activity. The polymorphism consists of two common alleles with a 44-bp insertion or deletion, designated L and S, respectively. The L allele drives a 2- to 3-fold higher expression of the 5-HTT transcript than the S allele. In previous studies, we found that the LL genotype was significantly more frequent in patients with PPH than in a control population.6 Moreover, we recently reported that the LL genotype determined the severity of PH in familial PPH patients with the SS, LS, or LL genotype, in controls, each bar is the mean ± SEM of data obtained from 6 individuals in each group. In patients with PPH, the LS and LL bars represent the mean ± SEM of data obtained in 5 and 8 individuals, respectively. SS bar represents values obtained in 1 individual. *P < 0.05, **P < 0.01 compared with respective SS values; §P < 0.05 compared with respective LS values; #P < 0.05 compared with respective values in controls.

Figure 5. 5-HTT mRNA levels (A) and [3H]5-HT uptake (B) in PA-SMCs from controls and from PPH patients with the SS, LS, or LL genotype. In controls, each bar is the mean ± SEM of data obtained from 6 individuals in each group. In patients with PPH, the LS and LL bars represent the mean ± SEM of data obtained in 5 and 8 individuals, respectively. SS bar represents values obtained in 1 individual. *P < 0.05, **P < 0.01 compared with respective SS values; §P < 0.05 compared with respective LS values; #P < 0.05 compared with respective values in controls.

The difference in 5-HTT expression among patients with PH compared with controls. Thus, 5-HTT gene overexpression in PA-SMCs from patients with PH may not result from alterations of the regulatory sequences of the 5-HTT gene. It is likely that complex mechanisms are involved, such as alterations of related genes or of signaling pathways involved in the regulation of 5-HTT expression.

Mutations in the gene encoding bone morphogenetic protein receptor 2 (BMPR2) are now considered to be the genetic basis for familial PPH and less than 10% of cases of sporadic PPH. The exact link between the BMPR2 mutant genotype and the pathogenesis of PH remains to be elucidated but probably involves altered BMPR2 function. Recent studies have found markedly reduced BMPR2 immunostaining in patients with PPH even when no mutation was identified. Interestingly, a smaller but significant reduction in BMPR2 immunostaining was also observed in patients with secondary PH. Thus, BMPR2 and 5-HTT expression indicate that BMPR2 protein expression or function are depressed in isolated cells from patients with PH.
Another hypothesis is that PPH may result from several genetic predisposing factors and that altered genetic control of BMPR2 and 5-HTT may play independent roles in the pathogenesis of the vascular lesion characteristic of PPH. Additional studies will be necessary to investigate the potential link between expression and function of BMPR2 and 5-HTT.

An important finding from this study is that exposure of cells to the selective 5-HTT inhibitors fluoxetine and citalopram diminished PA-SMC proliferation and abolished the difference between patients and controls, whether the cells were stimulated by serotonin or by serum. These results are reminiscent of our previous findings that mice deficient in 5-HTT were protected against hypoxic PH or pulmonary vascular remodeling and that selective 5-HTT inhibitors, but not 5-HT receptor antagonists, protect against development of hypoxic PH in mice. Interestingly, 5-HTT–deficient mice and mice treated with 5-HTT inhibitors demonstrated enhanced hypoxic vasoconstriction. Because 5-HT uptake inhibitors are known to increase the plasma levels of 5-HT and because the vasoconstrictor action of 5-HT is mediated by 5-HT receptors, it may be questioned whether 5-HTT inhibitors may have deleterious or beneficial effects in patients with PH. Before these drugs may be used for clinical purpose in patients with PH, careful studies of risks and benefits will need to be performed.

Acknowledgments

This research was supported by grants from INSERM, the Ministère de la Recherche, the Délégation à la Recherche Clinique de l’AP-HP, and the Fondation de France. We are grateful to the pharmaceutical companies Lilly, SmithKline Beecham, Janssen, Lundbeck, and Roche for generously donating the drugs used in this study.

References


Serotonin-Induced Smooth Muscle Hyperplasia in Various Forms of Human Pulmonary Hypertension

Elisabeth Marcos, Elie Fadel, Olivier Sanchez, Marc Humbert, Philippe Dartevelle, Gerald Simonneau, Michel Hamon, Serge Adnot and Saadia Eddahibi

_Circ Res._ 2004;94:1263-1270; originally published online April 1, 2004; doi: 10.1161/01.RES.0000126847.27660.69

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/94/9/1263

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/