Cross Talk Between Endothelial and Smooth Muscle Cells in Pulmonary Hypertension

Critical Role for Serotonin-Induced Smooth Muscle Hyperplasia

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Background—The mechanism of pulmonary artery smooth muscle cell (PA-SMC) hyperplasia in idiopathic pulmonary artery hypertension (iPH) may involve both an inherent characteristic of PA-SMCs and abnormal control by external stimuli. We investigated the role of pulmonary microvascular endothelial cells (P-ECs) in controlling PA-SMC growth.

Methods and Results—Serum-free medium of quiescent P-ECs elicited marked PA-SMC proliferation, and this effect was greater with P-ECs from patients with iPH than from control subjects and greater with PA-SMCs from these patients than from control subjects. Fluoxetine, which inhibits serotonin-induced mitogenesis by blocking the serotonin transporter, and p-chlorophenylalanine, which inhibits serotonin synthesis by blocking tryptophan hydroxylase (TPH), caused a similar 60% reduction in the growth-promoting effect of P-EC media, whereas endothelin receptor blockers had no effect. Assays of TPH activity in P-EC medium based on p-chlorophenylalanine–sensitive 5-hydroxytryptophan accumulation or serotonin determination indicated serotonin synthesis by P-ECs and an increase in this TPH-dependent process in iPH. Expression of the tph1 gene encoding the peripheral form of the TPH enzyme was increased in lungs and P-ECs from patients with iPH. Lung TPH1 immunostaining was confined to the pulmonary vessel intima.

Conclusions—P-ECs produce paracrine factors governing PA-SMC growth. Serotonin, the main P-EC–derived growth factor, is overproduced in iPH and contributes to PA-SMC hyperplasia. (Circulation. 2006;113:1857-1864.)

Key Words: endothelial cells ■ hypertension, pulmonary ■ serotonin ■ tryptophan hydroxylase

Pulmonary arterial hypertension (PH), whether idiopathic (iPH) or associated with underlying disease (aPH), is an unexplained condition that in severe forms in adults or neonates is fatal and for which no satisfactory treatment is available.1 Medial hypertrophy and intimal thickening of pulmonary arteries are hallmark pathological features that ultimately lead to vessel obliteration. Hyperplasia of pulmonary artery smooth muscle cells (PA-SMCs) is the main underlying pathological change. Whether PA-SMC hyperplasia results from a primary cellular defect or from dysregulation of molecular events governing PA-SMC growth remains debated.1,2 Pulmonary endothelial cells (P-ECs) may be involved in pulmonary vascular remodeling not only through their ability to control vascular tone but also through the production and release of growth factors.3,4 Among growth factors implicated in PH progression, serotonin (5-hydroxytryptamine [5-HT]) is thought to play a prominent role.5–8 PA-SMCs from patients with PH proliferate excessively when stimulated by serum6,9,10 but not when stimulated by various growth factors such as platelet-derived growth factor, epidermal growth factor, fibroblast growth factor, insulin-like growth factor, or transforming growth factor β.5 The mitogenic action of 5-HT on PA-SMCs is mediated by the serotonin transporter (5-HTT), which induces internalization of indoleamine.11,12 Increased 5-HTT expression seems closely linked to the abnormal proliferative phenotype of PA-SMCs in patients with iPH and aPH, in part via polymorphism of the 5-HTT gene promoter.5,7 Endothelin (ET-1) also is implicated in the pathogenesis of PH, although its role as a growth factor remains uncertain.13

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serotonin (5-hydroxytryptamine [5-HT]) is thought to play a prominent role.5–8 PA-SMCs from patients with PH proliferate excessively when stimulated by serum6,9,10 but not when stimulated by various growth factors such as platelet-derived growth factor, epidermal growth factor, fibroblast growth factor, insulin-like growth factor, or transforming growth factor β.5 The mitogenic action of 5-HT on PA-SMCs is mediated by the serotonin transporter (5-HTT), which induces internalization of indoleamine.11,12 Increased 5-HTT expression seems closely linked to the abnormal proliferative phenotype of PA-SMCs in patients with iPH and aPH, in part via polymorphism of the 5-HTT gene promoter.5,7 Endothelin (ET-1) also is implicated in the pathogenesis of PH, although its role as a growth factor remains uncertain.13

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In the present study, we investigated whether P-ECs contributed to iPH progression by releasing growth factors acting on PA-SMC proliferation and whether ET-1 and/or 5-HT are involved in this process. To this end, serum-free media derived from cultured P-ECs collected from the lungs of patients with iPH and control subjects were added to cultured PA-SMCs from the same individuals. Because 5-HT was found to account for most of the growth-promoting effect of P-EC media on PA-SMCs, we then investigated whether 5-HT synthesis occurred in the pulmonary vascular endothelium and whether 5-HT production by endothelial cells was altered in iPH.

Methods

Study Population

We studied lung specimens obtained during lung transplantation in 8 patients with iPH (4 women, 4 men) and during lobectomy or pneumonectomy for localized lung cancer in 9 control subjects (2 women, 7 men). In the lung specimens from control subjects, pulmonary arteries were studied at a distance from tumor areas. Age (mean±SD) was 46±12 years in the patients with iPH and 53±10 years in the control subjects. The mean pulmonary artery pressure in the group with iPH was 63±11 mm Hg (range, 48 to 85 mm Hg), mean pulmonary vascular resistance was 2.45±0.53 mm Hg·L⁻¹·min⁻¹·m⁻² (range, 16 to 37 mm Hg·L⁻¹·min⁻¹·m⁻²), and mean cardiac index was 2.0±0.2 L·min⁻¹·m⁻² (range, 1.6 to 3 L·min⁻¹·m⁻²). Transthoracic echocardiography was performed preoperatively in the control subjects to rule out PH. None of the patients with iPH had been treated with appetite suppressants, and 6 were treated medicinally in the same individuals. Because 5-HT was found to account for most of the growth-promoting effect of P-EC media on PA-SMCs, the cells were subjected to growth arrest in MCDB131 medium (0% FCS) with or without PCPA (10 to 50 μmol/L), NSD1015 (3-hydroxybenzylhydrazine dihydrochloride, 50 μmol/L), or Bosentan (10⁻³ mol/L) for the next 24 hours. P-EC-SMC proliferation was measured with or without 10⁻³ mol/L fluoxetine and with or without Bosentan (10⁻³ mol/L). PA-SMC proliferation was also assessed in response to 5% FCS with or without fluoxetine (10⁻³ mol/L) or PCPA (10⁻⁴ mol/L) added 20 minutes before FCS. The doses of fluoxetine (10⁻³ mol/L) and PCPA (10⁻⁴ mol/L) were chosen according to previous studies showing selective inhibition of 5-HTT⁶⁻¹² and TPH¹⁵⁻¹⁷ activities, respectively. For each condition, [³H]thyomidine 0.6 μCi/mL was added to each well. After incubation for 24 hours, the cells were washed twice with phosphate-buffered saline (PBS), treated with 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.

Determination of TPH Activity

P-ECs in MCDB131 medium supplemented as described above were seeded in 6-well plates at a density of 300 000 cells/mL for 24 hours. The cells were then subjected to growth arrest in MCDB131 medium (0% FCS) with the decarboxylase inhibitor NSD1015 (3-hydroxybenzylhydrazine dihydrochloride, 50 μmol/L), NSD1015+L-tryptophan (10 μmol/L), or NSD1015 (50 μmol/L)+L-tryptophan (10 μmol/L)+PCPA (10 μmol/L) for 2 hours at 37°C. Cells were scraped in their own medium (1 mL), 50 μL of high-performance liquid chromatography (HPLC) solution (2 mol/L perchloric acid, 0.1% EDTA, 0.1% Na₂SO₄, wt/vol) was added, and the mixture was frozen immediately at −80°C. The reaction product, 5-hydroxy-tryptophan (5-HTP), in P-EC homogenates was measured with HPLC coupled with electrochemical detection (HPLC-ECD) as previously described.¹⁸

Measurements of 5-HT and ET-1 Concentrations in P-EC Medium

The P-EC medium used to measure 5-HT and ET-1 levels was obtained from serum-starved cells in MCDB131 medium (0% FCS) for 24 hours with or without PCPA (10 to 50 μmol/L). ET-1 levels in P-EC media were determined by ELISA (R&D Systems, Lille, France), and 5-HT levels were measured by HPLC-ECD. Absolute values were calculated from 5-HT peak areas with reference to authentic serotonin standards and were not corrected for recovery.¹⁸

Total RNA Isolation

Total RNA was isolated from lung, P-ECs, and PA-SMCs with the Trizol reagent (Invitrogen, Cergy-Pontoise, France) and estimated from optical density measurements (260- to 280-nm absorbance ratio). To avoid inappropriate amplification of residual genomic DNA, total RNA was systematically treated by DNase. Total RNA was extracted from P-ECs and PA-SMCs with the Qiagen RNeasy Mini kit (QIAGEN SA, Courtaboeuf, France) according to the manufacturer’s instructions. RNA concentration was determined by standard spectrophotometric techniques, and RNA integrity was assessed by visual inspection of ethidium bromide-stained denaturing agarose gels. RNAs from human intestine, liver, and cerebral tissues were purchased from Clontech, BD-Biosciences (Mountain View, Calif).

cDNA Preparation and Real-Time Quantitative Polymerase Chain Reaction

First-strand cDNA synthesis was carried out with the SuperScript II Reverse Transcriptase System (Life Technologies, Inc, Gaithersburg, Md). A mixture containing 1 μg total RNA, 2 μL deoxynucleotide

Isolation and Culture of Human P-ECs

Human P-ECs were obtained by Dispase I digestion (Roche Diagnostics, Penzberg, Germany) of a 5-cm³ lung tissue fragment left at 37°C overnight. The suspension was filtered, plated onto 0.1% gelatin-coated wells, and grown in MCDB131 medium (Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal calf serum (FCS), 50 μU/mL of penicillin/streptomycin, 4 mmol/L L-glutamine, 25 mmol/L HEPES, 10 U/mL heparin, 1 μg/mL human endothelial cell growth supplement, and 10 ng/mL vascular endothelial growth factor (Promocell, Heidelberg, Germany). Immunomagnetic purification of P-ECs was then performed with anti-PECAM-1 (CD31) monoclonal antibody—labeled Dynabeads® (Dynal, Biotech, Coupiègne, France). To characterize the endothelial cell phenotype, cells were labeled with acetylated low-density lipoprotein (LDL) coupled to a fluorescent carbocyanine dye (DiI-Ac-LDL, Tebu, Le Perray en Yvelines, France) and stained with antibodies against the endothelial cell–specific lectin Ulex europaeus agglutinin-1 (UEA-1, Sigma, Lyon, France).⁶ Experiments also were performed using monoclonal antibodies against desmin and vimentin (Dako, Trappes, France). Cells positive for DiI-Ac-LDL and UEA-1 and negative for desmin and vimentin were labeled as endothelial cells and constituted >95% of our culture cells. Cells were used for the study between passages 3 and 6.

Culture of PA-SMCs

PA-SMCs were cultured from explants as previously described.⁶ To determine the phenotypic characteristics of cultured PA-SMCs, the cells from each culture were assessed for expression of muscle-specific contractile and cytoskeletal proteins, including smooth muscle cell α-actin, desmin, and vinculin.⁶

Measurements of PA-SMC Proliferation

PA-SMCs in Dulbecco’s modified Eagle’s medium supplemented with 15% FCS were seeded in 24-well plates at a density of 5×10⁴ cells per well and allowed to adhere. The cells were subjected to 48 hours of growth arrest in medium containing 0% FCS and then treated with 1 mL conditioned medium from P-ECs with or without fluoxetine (10⁻³ mol/L). P-EC serum-free medium was obtained as follows: At the time of initiating PA-SMC growth arrest, P-ECs were seeded in 24-well plates at a density of 5×10⁴ cells per well and allowed to adhere and grow in the same medium as described above (but without heparin) for 24 hours. The P-ECs were then serum starved in MCDB131 medium with or without the tryptophan hydroxylase (TPH) inhibitor p-chlorophenylalanine (PCPA, 10 mmol/L) for the next 24 hours. PA-SMC proliferation was assessed with or without 10⁻³ mol/L fluoxetine and with or without Bosentan (10⁻³ mol/L). PA-SMC proliferation was also assessed in response to 5% FCS with or without fluoxetine (10⁻³ mol/L) or PCPA (10⁻⁴ mol/L) added 20 minutes before FCS. The doses of fluoxetine (10⁻³ mol/L) and PCPA (10⁻⁴ mol/L) were chosen according to previous studies showing selective inhibition of 5-HTT⁶⁻¹² and TPH¹⁵⁻¹⁷ activities, respectively. For each condition, [³H]thyomidine 0.6 μCi/mL was added to each well. After incubation for 24 hours, the cells were washed twice with phosphate-buffered saline (PBS), treated with 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.

Polymerase Chain Reaction

First-strand cDNA synthesis was carried out with the SuperScript II Reverse Transcriptase System (Life Technologies, Inc, Gaithersburg, Md). A mixture containing 1 μg total RNA, 2 μL deoxynucleotide
triprophosphate mix (10 mmol/L), and 100 ng random primers in a total volume of 12 μL was incubated for 5 minutes at 65°C and chilled on ice. Then, 4 μL of 1st Strand Buffer, 2 μL of DTT (0.1 mmol/L), and 40 U of ribonuclease inhibitor (RNase-Out, Invitrogen, Carlsbad, Calif) were added to the samples, which were then heated at 95°C for 2 minutes. After 1 μL SuperScript reverse transcriptase II (200 U/μL) was added, the mixture was incubated for 10 minutes at 25°C, 50 minutes at 42°C, and 15 minutes at 70°C. The cDNA was diluted 1:20 for use in real-time quantitative polymerase chain reaction (PCR). Amplification was performed in triplicate with an ABI Prism 7000 (Applied Biosystems, Foster City, Calif). Predeveloped sequence detection reagents specific for human tph1, tph2, and 5-HTT (Assays-on-Demand Gene Expression Products, Applied Biosystems), including forward and reverse primers, and a TaqMan MGB probe (fluorophores dye-labeled) were purchased as mixtures and used at 1.25 μL/25 μL PCR. Each 25 μL PCR reaction mix also included 1× TaqMan universal PCR master mix (P/N4304437, Applied Biosystems). The thermal cycle conditions were 2 minutes at 50°C for optimal AmpErase UNG activity, 10 minutes at 95°C to activate the AmpliTaq polymerase, and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The fluorescent signal at each cycle was generated by the release of fluorophores from the quencher caused by the 5′-exonuclease activity of AmpliTaq polymerase and was plotted against the cycle number. The threshold cycle Ct, defined as the cycle number at which an increase above background fluorescence could be reliably detected, was determined for each sample with a GeneAmp software. Relative quantification was performed with the comparative ΔΔCt method by normalization with 18s ribosomal RNA.

Lung Immunohistochemical Labeling of TPH and 5-HTT

Paraffin sections (5 mm thick) of lung specimens were mounted on Superfrost Plus slides (Fisher Scientific, France). For 5-HTT immuno staining, the slides were dewaxed in 100% toluene, and the sections were then rehydrated by immersion in decreasing ethanol concentrations (100%, 95%, and 70%) and then in distilled water. Endogenous peroxidase activity was blocked with H2O2 in methanol (0.3% vol/vol) for 10 minutes. After 3 washes with PBS, the sections were preincubated in PBS supplemented with 3% (wt/vol) bovine serum albumin for 30 minutes and then incubated overnight at 4°C with polyclonal rabbit anti-TPH or goat anti–5-HTT antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif) diluted 1:1000 in PBS containing 0.02% bovine serum albumin. The sections were exposed for 1 hour to biotin-labeled anti-goat secondary antibodies (Dako, France) diluted 1:200 in the same buffer and then to streptavidin biotin horseradish peroxidase solution. Peroxidase staining was carried out with 3,3′-diaminobenzidine tetrahydrochloride dihydrate (DAB, Sigma, St Louis, Mo) and hydrogen peroxide. Finally, the sections were stained with hematoxylin and eosin.

Statistical Analyses

All data are reported as mean±SEM. To assess the effects of treatment with the medium derived from P-ECs of patients and control subjects on the proliferation of PA-SMCs from patients and control subjects, ANOVA was used for between-group comparisons. When ANOVA indicated significance and an interaction, the groups were further compared by use of a nonparametric test. To assess the effects of various treatments on smooth muscle cell growth induced by EC media, the nonparametric Kruskal-Wallis test was performed. When the Kruskal-Wallis test showed a significant difference, the groups were further compared using a nonparametric Student-Newman-Keuls test. The nonparametric Mann-Whitney test was used to compare patients and control subjects in the other parts of the study. Values of P<0.05 were considered statistically significant.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Growth of PA-SMCs in Response to Serum-Free Media Derived From Cultured P-ECs

Serum-free media from cultured P-ECs obtained from the lungs of patients with iPHT and control subjects were added to PA-SMCs cultured in 0% serum. This produced a marked increase in [3H]thymidine incorporation (Figure 1A). The increase was larger when PA-SMCs were stimulated with media of P-ECs from patients with iPHT compared with P-ECs from control subjects. Moreover, PA-SMCs from patients with iPHT grew faster than those from control subjects in response to serum-free medium from homologous P-ECs. The highest growth rate was observed for PA-SMCs from patients with iPHT treated with serum-free medium from homologous P-ECs (Figure 1A). To determine the contribution of ET-1 to P-EC media-stimulated PA-SMC growth, we first tested the endothelin receptor antagonist Bosentan (Figure 1B), which did not alter the total growth response. Treatment with Bosentan also did not affect PA-SMC growth (data not shown). To evaluate the contribution of 5-HT, we added fluoxetine (10−5 mol/L) to inhibit 5-HTT in PA-SMCs. This diminished the growth response to P-EC medium by ∼60%. With fluoxetine, growth rates of PA-SMCs from control subjects were identical with media of P-ECs from control subjects and from patients with iPHT (Figure 1C). Fluoxetine also reduced the growth-stimulating action of serum, which contains serotonin (Figure 1D). Levels of both ET-1 and 5-HT were markedly increased in the media of P-ECs from patients with iPHT compared with those from control subjects (Figure 2A).

Evidence for Serotonin Synthesis by P-ECs

To investigate whether 5-HT measured in P-EC serum-free media was released from an indoleamine pool stored in P-ECs or was synthesized de novo by these cells, experiments were performed after P-EC treatment with PCPA,15 a selective inhibitor of TPH, the rate-limiting enzyme of 5-HT biosynthesis. P-EC pretreatment with PCPA (10−5 mol/L) reduced 5-HT to undetectable levels in the medium and reduced PA-SMC growth to an extent similar to that obtained by PA-SMC pretreatment with fluoxetine (as previously reported) but was not affected by PCPA, demonstrating that PCPA did not induce nonspecific effects on PA-SMC growth.

We then measured TPH activity in P-EC homogenates by quantifying 5-HT accumulation after addition of NSD1015, an inhibitor of aromatic L-amino acid decarboxylase19 (Figure 2B and 2C). Only after the substrate L-tryptophan was added to the incubation mixture did 5-HT become detectable. Accumulation of 5-HTP was 3 times greater in P-ECs from patients with iPHT than from control subjects. Addition of 10 μmol/L of the TPH inhibitor PCPA nearly abolished 5-HTP accumulation in P-ECs from control subjects but induced only 50% inhibition in P-ECs from patients with iPHT. Neither 5-HTP accumulation nor 5-HT release was detected using PA-SMCs from patients with iPHT or control subjects.
Expression of TPH in Lungs and Cultured Cells From Patients With iPH

TPH is encoded by 2 genes, the well-characterized tph1 gene expressed in enterochromaffin cells20 and the recently identified tph2 gene expressed in the central nervous system.21 Using real-time quantitative PCR to measure tph1 and tph2 mRNA, we found that tph2 mRNA was detected selectively in cerebral tissues, whereas tph1 mRNA was detected predominantly in the gut and, to a lesser extent, in whole-lung homogenates and cerebral tissues from humans. No significant amounts of tph1 mRNA were detected in the liver (Figure 3A). Levels of tph1 mRNAs in whole-lung homogenates from patients with iPH were increased ~3-fold compared with control subjects (Figure 3B). Expression of tph1 in quiescent cultured P-ECs also was markedly increased in patients with iPH compared with control subjects (Figure 3B). Staining with toluidine blue was negative, ruling out contamination of P-EC cultures by mast cells. No tph1 mRNA was detected in PA-SMCs from patients with iPH or control subjects. In contrast, 5-HTT expression predominated in PA-SMCs and was greater in PA-SMCs from patients with iPH than from control subjects (Figure 3B).

Immunolocalization of TPH and 5-HTT in Lungs From Patients With iPH

Immunohistochemical analyses revealed that TPH immunostaining was confined to the intima of pulmonary vessels; no staining was seen in the media (Figure 4). Even in remodeled vessels with marked muscular hypertrophy from iPH patients, tph1-like immunoreactivity was present in the intima, whereas it was less marked in obstructed vessels in which endothelium was absent or replaced by a neointima. In contrast, 5-HTT immunostaining was located predominantly in the media in lung specimens from patients with iPH and from control subjects, producing uniform staining in the media of vessels with marked muscular hypertrophy (Figure 4).
Discussion

The present results show that cultured P-ECs constitutively produce and release growth factors that act on PA-SMCs. The stimulating effect of P-EC media on PA-SMC growth was greater with P-ECs from patients with iPH than from control subjects and with PA-SMCs from patients than from control subjects, indicating that alterations in cross talk between these 2 cell types are critical to pulmonary vessel remodeling. Furthermore, 5-HT accounted for 60% of the growth-promoting activity of P-EC media. Human P-ECs expressed tph1 and produced 5-HT, and both tph1 expression and 5-HT production were increased in P-ECs from patients with iPH compared with control subjects. Finally, we provided evidence that increases in both tph1 expression by P-ECs and 5-HTT expression by PA-SMCs contributed to PA-SMC hyperplasia in iPH.

These findings are of major interest for several reasons. First, the pulmonary endothelium is usually thought to affect the underlying vascular smooth muscle chiefly by releasing vasoactive factors that affect PA-SMC contractility. The present results provide new insight into the potential role of P-ECs in producing and releasing paracrine factors that influence the growth of PA-SMCs. Endothelial cells from distal microvessels instead of proximal pulmonary arteries were used in these experiments because they may be more representative of the mechanism of pulmonary vascular remodeling, which begins in distal vessels. Previously, P-EC dysfunction in iPH has been shown to be associated with decreased production of nitric oxide22 and prostacycline23 and increased production of ET-1.24 In the present study, we show that a critical aspect of P-EC dysfunction in iPH is excessive release of growth factors that induce PA-SMC hyperplasia. During development, factors derived from endothelial cells are known to participate in blood vessel formation and maturation by recruiting and stabilizing vascular wall cells.4,25 Previous in vitro evidence that serum-free media derived from angiopoietin-1–treated P-ECs induced PA-SMC growth is consistent with such a process in the pulmonary circulation.26 The present results, obtained in nonstimulated quiescent cultured cells from adult patients, suggest that P-ECs may constitutively produce and release growth factors that act on PA-SMCs and whose physiological function may consist of recruiting PA-SMCs and maintaining a smooth muscle coat around the pulmonary vessels.3,4 Interestingly, PA-SMC proliferation induced by serum-free medium of quiescent P-ECs was greater with P-ECs from patients with iPH than from control subjects, indicating dysregulation of this process in iPH. We cannot exclude that these findings might be related to the severity of PH in our patients rather than to the pathogenesis of the disease. However, because P-ECs were studied outside their in vivo environment, the results suggest that excessive release of growth factors by P-ECs is an intrinsic abnormality closely linked to the pathogenesis of iPH.

Second, the main factor involved in endothelium–smooth muscle interaction is serotonin. Fluoxetine, which blocks the growth-promoting effect of 5-HT on PA-SMCs by inhibiting 5-HTT, diminished the growth response to P-EC medium by \(\approx 60\%\), whereas endothelin receptor antagonists had no effect. These data indicate that 5-HT was the main growth

Figure 2. Synthesis of 5-HT and ET-1 in P-ECs from patients with iPH and control subjects. A, 5-HT and ET-1 levels measured in conditioned media derived from cultured P-ECs from patients with iPH (n=8) or control subjects (n=9) after a 24-hour incubation. Each bar is mean±SEM. *P<0.05, **P<0.01 vs control subjects. B, TPH activity in P-ECs from patients with iPH and control subjects. Experiments were performed in the presence of the decarboxylase inhibitor NSD1015 (50 \(\mu\)mol/L). *P<0.01 vs control subjects. C, Pathway for serotonin biosynthesis, indicating the target enzymes PCPA and NSD1015.
factor released by P-ECs and acting on PA-SMCs. Serotonin was also found to be the main growth factor released by P-ECs on stimulation by angiopoietin-1.26 We therefore investigated whether 5-HT in P-EC medium was produced by de novo synthesis of 5-HT by P-EC or was released from an indoleamine pool stored in P-ECs. The first step in the biosynthesis of 5-HT is catalyzed by TPH, the rate-limiting enzyme of the pathway.27 TPH is therefore a marker for 5-HT synthesis. We found that P-EC treatment with PCPA,15 a selective TPH inhibitor,16,17 reduced the levels of 5-HT and 5-HTP to undetectable values in the medium and reduced PA-SMC growth to an extent similar to that obtained by PA-SMC pretreatment with fluoxetine. A strong argument that the effects of PCPA and fluoxetine were mediated specifically through 5-HT-dependent mechanisms is that no further effects were produced when the 2 drugs were combined. Thus, when optimal depletion of intracellular 5-HT was already achieved by 5-HT synthesis inhibition (with PCPA), 5-HTT blockade by fluoxetine did not further inhibit PA-SMC proliferation. Moreover, strong TPH immunoreactivity was seen in the endothelium of normal pulmonary vessels, indicating that TPH expression occurred in vivo in the normal lung. TPH is encoded by 2 genes, the well-characterized tph1 gene expressed in enterochromaffin cells20 and the recently identified tph2 gene expressed in the central nervous system.21 We found that only the tph1 gene was expressed in whole-lung homogenates and in quiescent cultured human P-ECs. Thus, serotonin is synthesized by P-ECs in the normal lung as a result of TPH1 enzyme activity and appears to be the main growth factor produced by P-ECs and acting on PA-SMCs in a paracrine fashion. Such cross talk between ECs and SMCs mediated by 5-HT appears unique to the pulmonary circulation, because 5-HT does not seem to act as a potent mitogenic factor on SMCs in systemic vessels.5,28

Third, dysregulation of lung 5-HT synthesis by P-ECs appears to make a major contribution to smooth muscle hyperplasia in iPH. We found that TPH expression and 5-HT synthesis were increased in P-ECs from patients with iPH compared with control subjects. Moreover, increased 5-HT production by P-ECs from patients with iPH was responsible for most of the increased PA-SMC proliferation that occurred on stimulation with P-EC media from patients with iPH compared with control subjects. However, neither PCPA nor fluoxetine completely abolished the growth-promoting effect of the P-EC media, indicating that pathways other than those mediated by serotonin were involved in the proliferative response. Because ET receptor antagonists had no effects and because only small amounts of
factors such as platelet-derived growth factor and epidermal growth factor were detected in P-EC medium (data not shown), it is unlikely that these factors were involved. Further support for the hypothesis that 5-HT was the main growth factor in P-EC media came from the finding that PA-SMCs from patients with iPH grew faster than those from control subjects when stimulated with a given P-EC medium. This is consistent with our previous observation that PA-SMCs from patients with iPH overexpress 5-HTT, which mediates the mitogenic action of indoleamine.6,7 Therefore, iPH is characterized by increased expression of TPH1 by P-ECs and of 5-HTT by PA-SMCs. The present results constitute evidence that combined excesses of 5-HT synthesis by P-ECs and of 5-HT internalization by PA-SMCs interact directly to induce PA-SMC hyperplasia in iPH.

The current concept is that 5-HT production outside the central nervous system occurs chiefly in the enterochromaffin cells,29 which release 5-HT into the bloodstream, where this indoleamine is taken up by platelets. Under normal conditions, the lung vascular bed is not exposed to excessive circulating 5-HT levels because platelets can store large amounts of 5-HT. Although a high risk for PH has been shown in a few patients with abnormal 5-HT platelet storage30 and elevated circulating 5-HT levels, as well as in individuals or experimental animals treated with the serotonin-releasing drug dexfenfluramine8,31 and in Fawn-hooded rats32 (which have a platelet storage disease), platelet alterations are usually mild or absent in patients with iPH or aPH. The present results showing that 5-HT production outside the central nervous system occurs chiefly in the enterochromaffin cells,29 which release 5-HT into the bloodstream, where this indoleamine is taken up by platelets. Under normal conditions, the lung vascular bed is not exposed to excessive circulating 5-HT levels because platelets can store large amounts of 5-HT. Although a high risk for PH has been shown in a few patients with abnormal 5-HT platelet storage30 and elevated circulating 5-HT levels, as well as in individuals or experimental animals treated with the serotonin-releasing drug dexfenfluramine8,31 and in Fawn-hooded rats32 (which have a platelet storage disease), platelet alterations are usually mild or absent in patients with iPH or aPH. The present results showing that 5-HT is produced locally, within the pulmonary vessel wall, at the site of the remodeling process, provide new insight into the role for 5-HT in the pathogenesis of PH. Hyperplasia of PA-SMCs in iPH appears to result from both dysregulation of serotonin production by P-ECs caused by tph1 overexpression and an increased PA-SMC response to serotonin caused by overexpression of the selective serotonin transporter 5-HTT.

These findings are of potential clinical interest, although data obtained in cell culture may not reflect in vivo events. Inhibition of 5-HT synthesis by PCPA or 5-HT transport by fluoxetine caused similar reductions in PA-SMC growth in vitro. Only 5-HTT inhibition could be used to treat PH in humans. According to a preliminary report on a case-control study conducted in Europe in 1996, antidepressant medication use was significantly lower in patients with iPH than in control subjects, suggesting that these medications might lower the risk of iPH development.33 Clearly, the potential consequences of these drugs on human PH deserve to be investigated.

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Disclosures
None.

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Figure 4. Immunolocalization of TPH and 5-HTT in lung sections from control subjects and patients with iPH. In a lung section from a control subject (A) and in lung sections from patients with iPH (B through D), TPH immunostaining was confined to pulmonary endothelial cells and was absent in obstructed vessels lacking an endothelium (D). E, A lung section from a control subject with 5-HTT–like immunoreactivity located mainly in smooth muscle cells. F through H, In lung sections from patients with iPH, 5-HTT–like immunoreactivity is still prominent in the medial layer of pulmonary arteries with marked muscular hypertrophy. Scale bar=100 μm in all sections.
Enodothelial cell dysfunction is the main target of current therapeutic strategies for pulmonary arterial hypertension (PH). Loss of nitric oxide–mediated vasodilation, diminished prostacyclin synthesis, and excessive endothelin synthesis and release by pulmonary microvascular cells (P-ECs) during PH have led to the development of the 3 therapeutic options currently available: endothelin receptor antagonists, prostacyclin or prostacyclin derivatives, and type 5 phosphodiesterase inhibitors. That endothelial cells can participate in pulmonary vascular remodeling by producing and releasing growth factors is of therapeutic relevance. The finding that serotonin is one of those endothelial cell–derived growth factors argues for a therapeutic role of selective inhibitors of the serotonin transporter, the overexpression of which leads to smooth muscle hyperplasia in PH. Because endothelial cell–derived growth factors act in concert, combination drugs that act selectively on endothelial cell-derived mediators such as endothelin-1 and serotonin might have additive effects. Moreover, at least 50% of endothelial cell–derived growth factors released by P-ECs remain to be identified and represent potential molecular targets for future therapies. Investigation of the cross talk between endothelial and smooth muscle cells may therefore help to define how therapeutic strategies for PH can be combined and to identify new molecular targets for future treatments.

**CLINICAL PERSPECTIVE**

Endothelial cell dysfunction is the main target of current therapeutic strategies for pulmonary arterial hypertension (PH). Loss of nitric oxide–mediated vasodilation, diminished prostacyclin synthesis, and excessive endothelin synthesis and release by pulmonary microvascular cells (P-ECs) during PH have led to the development of the 3 therapeutic options currently available: endothelin receptor antagonists, prostacyclin or prostacyclin derivatives, and type 5 phosphodiesterase inhibitors. That endothelial cells can participate in pulmonary vascular remodeling by producing and releasing growth factors is of therapeutic relevance. The finding that serotonin is one of those endothelial cell–derived growth factors argues for a therapeutic role of selective inhibitors of the serotonin transporter, the overexpression of which leads to smooth muscle hyperplasia in PH. Because endothelial cell–derived growth factors act in concert, combining drugs that act selectively on endothelial cell-derived mediators such as endothelin-1 and serotonin might have additive effects. Moreover, at least 50% of endothelial cell–derived growth factors released by P-ECs remain to be identified and represent potential molecular targets for future therapies. Investigation of the cross talk between endothelial and smooth muscle cells may therefore help to define how therapeutic strategies for PH can be combined and to identify new molecular targets for future treatments.
Cross Talk Between Endothelial and Smooth Muscle Cells in Pulmonary Hypertension: Critical Role for Serotonin-Induced Smooth Muscle Hyperplasia
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