Retinoids and Pulmonary Hypertension

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Background—Retinoic acid has antimitogenic effects on smooth muscle cells. Studies on the systemic circulation suggest that it may reduce vascular thickening. Relationships between retinoids and pulmonary hypertension/pulmonary vascular remodeling, however, have not been explored. Thus, the present study examined retinoid levels in plasma of patients with idiopathic pulmonary arterial hypertension and the effects of retinoic acid on human pulmonary artery smooth muscle cell growth.

Methods and Results—We measured retinoid levels by gas chromatograph–mass spectrometer technique in plasma of idiopathic pulmonary arterial hypertension patients and in age- and sex-matched healthy control subjects. Patients had significantly lower levels of all-trans retinoic acid and 13-cis retinoic acid than control subjects but similar 9-cis retinoic acid and retinol levels. In cultured human pulmonary artery smooth muscle cells, all-trans retinoic acid suppressed serotonin-induced cell growth. These cells were found to express the retinoid acid receptors RARα, RARβ, RARγ, RXRα, and RXRβ. Gene array analysis showed that retinoic acid induces the expression of GADD45A, a known cell growth suppressor. Contrary to expectations, plasma from pulmonary hypertension patients suppressed cell growth, likely influenced by factors other than retinoids.

Conclusions—Idiopathic pulmonary arterial hypertension patients have reduced retinoic acid levels, and retinoic acid treatment can elicit growth-inhibitory signals in pulmonary artery smooth muscle cells in vitro. Thus, retinoic acid may influence pulmonary vascular remodeling in humans. (Circulation. 2005;111:782-790.)

Key Words: genes ■ hypertension, pulmonary ■ lung ■ muscle, smooth ■ remodeling

Pulmonary hypertension, defined as a mean pulmonary arterial pressure of >25 mm Hg at rest or 30 mm Hg during exercise, impedes the ejection of blood by the right ventricle and predisposes to right ventricular failure.1 Pulmonary hypertension can develop secondary to various heart and lung diseases and can occur in a primary (or idiopathic) form. Idiopathic pulmonary arterial hypertension (IPAH) is a rare but often fatal condition without a known cure. The estimated annual incidence of IPAH is 1 to 2 cases per million in the general population, with 6% of the cases being familial.1 The median survival is 2.8 years from the time of diagnosis, and current treatment options for IPAH are invasive, expensive, and limited. Although they benefit patients symptomatically and functionally, these treatments usually only partially reverse the pulmonary hypertension and functional impairment.

All-trans retinoic acid (ATRA), an active metabolite of vitamin A, plays important roles in cell differentiation and proliferation during development.2 Recently, the role of retinoids in the development of cardiovascular disease has gained attention because of the ability of ATRA to suppress growth of vascular smooth muscle cells.3,4 In the rat carotid injury model, ATRA decreased neointimal cellularity and extracellular matrix deposition, leading to increased lumen diameter and area.5–7 ATRA also induced favorable remodeling of the injured artery in rabbits.8 In cultured aortic smooth muscle cells, ATRA induced apoptosis9 and inhibited proliferation induced by serotonin,10 platelet-derived growth factor,11 endothelin-1,12 and angiotensin II.13 The suppression of smooth muscle cell proliferation by ATRA depends on retinoic acid receptors.5,11,13,14

ATRA might also have potential for the prevention and/or treatment of pulmonary hypertension. Indeed, ATRA feeding has been shown to inhibit pulmonary hypertension induced by monocrotaline in rats.15 Furthermore, patients with more severe chronic obstructive pulmonary disease, who would be expected to have more pulmonary vascular involvement, have decreased levels of plasma retinol, a precursor of ATRA, compared with patients with mild chronic obstructive pulmonary disease.16 Thus, decreased ATRA levels may predispose to increased pulmonary vascular remodeling, given the evidence that ATRA negatively regulates pulmonary artery smooth muscle cell proliferation. The relationship between retinoids and pulmonary hypertension, however, has not yet been defined.
In the present study, we tested the hypotheses that patients with IPAH have reduced retinoic acid measurements compared with control subjects and that human pulmonary artery smooth muscle cells have functional retinoic acid receptors and ATRA inhibits the growth of these cells.

Methods

Patients

Patients attending the outpatient Pulmonary Hypertension Clinic at Rhode Island Hospital (Providence) were recruited for prospective studies from January 2000 to February 2002. They were included if they had a mean pulmonary arterial pressure >25 mm Hg, had a pulmonary artery wedge pressure of <15 mm Hg, and had not received treatment for IPAH such as calcium channel blockers, prostacyclins, or endothelin receptor antagonists. Patients were excluded if they had other forms of pulmonary hypertension such as pulmonary hypertension related to connective tissue disease, portopulmonary hypertension, HIV-related pulmonary hypertension, or pulmonary hypertension secondary to interstitial lung disease, chronic obstructive lung disease, thromboembolic disease, or left heart failure. Patients were also excluded if they consumed ethanol daily, had a body mass index <20 or >30 kg/m², or had a history of liver disease or abnormal liver function tests. Control subjects, matched for age and gender, were healthy, receiving no medication and ATRA inhibits the growth of these cells.

Pulmonary Artery Smooth Muscle Cells

Human pulmonary artery smooth muscle cells (Cell Applications) were used for experiments after 3 to 6 passages in cell culture. Cells were treated with ATRA dissolved in dimethyl sulfoxide, and the same amount of dimethyl sulfoxide (0.02% final) was also added in the untreated controls.

Reverse-Transcription Polymerase Chain Reaction

To determine whether different retinoid receptors are expressed in human pulmonary artery smooth muscle cells, total RNA was extracted, and reverse-transcription polymerase chain reaction (RT-PCR) analyses were performed as previously described.19 PCR primers for human RARα were 5’ primer, 5’-ACC CCC TCT ACC CCG CAT CTA CAA G-3’, and 3’ primer, 5’-CAT GCC CAC TTC AAA GCA CTT CTT C-3’, with expected PCR products of 201 bp.20,21; for RARβ, 5’-AGG AGA CCT CGA AAG AAG-3’ and 5’-GTC AAG GGT TCA TGT CCT TTG GC-3’ with 752 bp; for RARγ, 5’-TTG GAG ATG ATG ATG ATC AGC CCT AGC TTG C-3’ and 5’-CAT GCC CAC TTC AAA GCA CTT CTT C-3’ with 327 bp; for RXRα, 5’-TTC GAT ATG ATG ATG ATC ATG TGT CTA ATG GG-3’ with 93 bp; for RXRβ, 5’-ATT AAC TCA ACA GTG TCA ATG GG-3’ with 232 bp; for RXRγ, 5’-ATT AAC TCA ACA GTG TCA ATG GG-3’ with 452 bp; and for RVRγ, 5’-AGC TAC ACA GAT ACC CCA GT-3’ and 5’-TCA CAT CCT CTT TGT ATG GCC-3’. Denaturing was performed at 94°C for 45 seconds; annealing, for 45 seconds at 55°C; and polymerase reactions, for 2 minutes at 72°C. PCR primers for human GADD45A have sequences: 5’-ACA GAG GTC AGG TCA GGT CTG CCT CCT-3’ and 5’-GCT GAG ATG AGG TCA GGT GTG CC-3’. The annealing temperature of 54°C was used.

Electrophoretic Mobility Shift Assays

To evaluate whether ATRA induces activation of DNA binding activity of different ATRA receptors in human pulmonary artery smooth muscle cells, we prepared nuclear extracts and performed binding reactions as previously described.22 Sequences for double-stranded oligonucleotide probe for RAR and RXR are 5’-TTC GAT ATG ATG ATG ATC ATG TGT CTA ATG GG-3’ and 5’-ATT AAC TCA ACA GTG TCA ATG GG-3’. The annealing temperature of 54°C was used.

Western Blot

Cell lysates were prepared and Western blot was performed as previously described using polyclonal antibodies for phosphospecific ERK1/2 (Cell Signaling Technology), ERK1/2, and GADD45A (SantaCruz Biotechnology).

TABLE 1. Age and Sex of IPAH Patients Compared With Their Matched Control Subjects

<table>
<thead>
<tr>
<th>Status</th>
<th>Age, y</th>
<th>Gender</th>
<th>NYHA Class</th>
<th>Status</th>
<th>Age, y</th>
<th>Gender</th>
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<tbody>
<tr>
<td>1 IPAH</td>
<td>69</td>
<td>F</td>
<td>III</td>
<td>1 Control</td>
<td>69</td>
<td>F</td>
</tr>
<tr>
<td>2 IPAH</td>
<td>65</td>
<td>M</td>
<td>III</td>
<td>2 Control</td>
<td>64</td>
<td>M</td>
</tr>
<tr>
<td>3 IPAH</td>
<td>66</td>
<td>M</td>
<td>III</td>
<td>3 Control</td>
<td>68</td>
<td>M</td>
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<tr>
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<td>F</td>
<td>II</td>
<td>4 Control</td>
<td>37</td>
<td>F</td>
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<tr>
<td>5 IPAH</td>
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<td>F</td>
<td>III</td>
<td>5 Control</td>
<td>59</td>
<td>F</td>
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<tr>
<td>6 IPAH</td>
<td>57</td>
<td>F</td>
<td>III</td>
<td>6 Control</td>
<td>51</td>
<td>F</td>
</tr>
<tr>
<td>7 IPAH</td>
<td>42</td>
<td>F</td>
<td>III</td>
<td>7 Control</td>
<td>40</td>
<td>F</td>
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<tr>
<td>8 IPAH</td>
<td>32</td>
<td>F</td>
<td>II</td>
<td>8 Control</td>
<td>33</td>
<td>F</td>
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</tbody>
</table>

Mean ± SD 54 ± 16.2 53 ± 14.5
Gene Array Analysis
To identify the effects of ATRA on gene expression, we performed gene array analysis from human pulmonary artery smooth muscle cells treated with ATRA. Cells were treated with ATRA 2 μmol/L for 20 hours, and total RNA was isolated and subjected to mRNA expression analysis using the GE Array Q Series Human Signal Transduction Pathway Finder Array (HS-008, SuperArray Biotechnology Corp). RT labeling was used for synthesizing and labeling cDNA probes, and the chemiluminescent detection method was used. Three separate experiments were performed. The complete gene table can be obtained at www.superarray.com.

Statistical Analysis
Differences between groups were considered statistically significant if P<0.05 as determined by Student t test.

Results
Patients
Eight IPAH patients and 8 age- and gender-matched healthy control subjects were studied. As summarized in Table 1, ages of IPAH patients and control subjects ranged from 32 to 69 years. Patients were mostly female. Their nutritional status was considered normal, as determined by body mass index, physical examination, and baseline laboratory results such as complete blood count, liver enzymes, and a complete metabolic panel (data not shown). Functional classes were NYHA II through IV, and most of the patients had severe pulmonary hypertension, with mean pulmonary artery pressures ranging

Table 2: Hemodynamic Parameters of 8 Patients With IPAH

<table>
<thead>
<tr>
<th>Patient</th>
<th>CVP, mm Hg</th>
<th>mPAP, mm Hg</th>
<th>PAWP, mm Hg</th>
<th>CO, L/min</th>
<th>CI, L·min⁻¹·m⁻²</th>
<th>PVR, dynes/cm²</th>
<th>SVR, dynes/cm²²</th>
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<td>1</td>
<td>6</td>
<td>61</td>
<td>6</td>
<td>4.87</td>
<td>2.55</td>
<td>979</td>
<td>1199</td>
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<td>2</td>
<td>7</td>
<td>47</td>
<td>10</td>
<td>3.0</td>
<td>1.6</td>
<td>987</td>
<td>2090</td>
</tr>
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<td>3</td>
<td>15</td>
<td>77</td>
<td>15</td>
<td>4.01</td>
<td>2.2</td>
<td>1240</td>
<td>1418</td>
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<td>4</td>
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<td>12</td>
<td>3.8</td>
<td>1.91</td>
<td>666</td>
<td>2166</td>
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<td>6</td>
<td>4</td>
<td>52</td>
<td>7</td>
<td>4.76</td>
<td>2.53</td>
<td>722</td>
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<td>70</td>
<td>15</td>
<td>3.72</td>
<td>2.02</td>
<td>1182</td>
<td>1784</td>
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<td>8</td>
<td>9</td>
<td>43</td>
<td>12</td>
<td>3.58</td>
<td>2.02</td>
<td>692</td>
<td>1586</td>
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<tr>
<td>Mean±SD</td>
<td>9±3</td>
<td>57±13</td>
<td>11±3</td>
<td>4±0.6</td>
<td>2.1±0.3</td>
<td>931±219</td>
<td>1661±332</td>
</tr>
</tbody>
</table>

CVP indicates central venous pressure; mPAP, mean pulmonary artery pressure; PAWP, pulmonary artery wedge pressure; CO, cardiac output; CI, cardiac index; PVR, pulmonary vascular resistance; and SVR, systemic vascular resistance.

Table 3: Levels of Retinoids

<table>
<thead>
<tr>
<th></th>
<th>Total RA, ng/mL</th>
<th>ATRA, ng/mL</th>
<th>13-cis-RA, ng/mL</th>
<th>11-cis-RA, ng/mL</th>
<th>9-cis-RA, ng/mL</th>
<th>Retinol, μmol/L</th>
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</thead>
<tbody>
<tr>
<td>1 IPAH</td>
<td>1.549</td>
<td>0.353</td>
<td>0.479</td>
<td>0.274</td>
<td>0.274</td>
<td>1.891</td>
</tr>
<tr>
<td>2 IPAH</td>
<td>2.320</td>
<td>0.587</td>
<td>0.538</td>
<td>0.237</td>
<td>0.588</td>
<td>1.473</td>
</tr>
<tr>
<td>3 IPAH</td>
<td>2.220</td>
<td>0.665</td>
<td>0.460</td>
<td>0.143</td>
<td>0.529</td>
<td>1.089</td>
</tr>
<tr>
<td>4 IPAH</td>
<td>1.774</td>
<td>0.624</td>
<td>0.391</td>
<td>0.150</td>
<td>0.268</td>
<td>1.156</td>
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<tr>
<td>5 IPAH</td>
<td>3.039</td>
<td>0.734</td>
<td>0.659</td>
<td>0.288</td>
<td>0.659</td>
<td>1.069</td>
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<tr>
<td>6 IPAH</td>
<td>2.019</td>
<td>0.872</td>
<td>0.380</td>
<td>0.138</td>
<td>0.281</td>
<td>2.307</td>
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<tr>
<td>7 IPAH</td>
<td>1.983</td>
<td>0.774</td>
<td>0.539</td>
<td>0.131</td>
<td>0.292</td>
<td>1.873</td>
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<tr>
<td>8 IPAH</td>
<td>2.612</td>
<td>0.794</td>
<td>0.735</td>
<td>0.210</td>
<td>0.372</td>
<td>2.133</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>2.190*</td>
<td>0.675*</td>
<td>0.523*</td>
<td>0.196</td>
<td>0.408</td>
<td>1.624</td>
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<tr>
<td>Control</td>
<td>0.474</td>
<td>0.161</td>
<td>0.124</td>
<td>0.064</td>
<td>0.160</td>
<td>0.492</td>
</tr>
<tr>
<td>1 Control</td>
<td>2.830</td>
<td>0.910</td>
<td>0.890</td>
<td>0.210</td>
<td>0.240</td>
<td>1.546</td>
</tr>
<tr>
<td>2 Control</td>
<td>2.680</td>
<td>0.950</td>
<td>0.850</td>
<td>0.200</td>
<td>0.320</td>
<td>1.494</td>
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<tr>
<td>3 Control</td>
<td>2.640</td>
<td>1.120</td>
<td>0.940</td>
<td>0.190</td>
<td>0.210</td>
<td>1.595</td>
</tr>
<tr>
<td>4 Control</td>
<td>2.340</td>
<td>0.970</td>
<td>0.510</td>
<td>0.000</td>
<td>0.620</td>
<td>1.627</td>
</tr>
<tr>
<td>5 Control</td>
<td>3.390</td>
<td>1.210</td>
<td>0.930</td>
<td>0.130</td>
<td>0.650</td>
<td>1.825</td>
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<tr>
<td>6 Control</td>
<td>3.070</td>
<td>1.180</td>
<td>0.890</td>
<td>0.230</td>
<td>0.370</td>
<td>1.993</td>
</tr>
<tr>
<td>7 Control</td>
<td>3.560</td>
<td>1.500</td>
<td>1.110</td>
<td>0.200</td>
<td>0.370</td>
<td>2.333</td>
</tr>
<tr>
<td>8 Control</td>
<td>3.090</td>
<td>1.240</td>
<td>0.910</td>
<td>0.130</td>
<td>0.400</td>
<td>2.077</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>2.950</td>
<td>1.135</td>
<td>0.879</td>
<td>0.161</td>
<td>0.398</td>
<td>1.811</td>
</tr>
<tr>
<td>SD</td>
<td>0.406</td>
<td>0.194</td>
<td>0.168</td>
<td>0.075</td>
<td>0.161</td>
<td>0.299</td>
</tr>
</tbody>
</table>

RA indicates retinoic acid.

*P<0.05 vs controls.
from 40 to 77 mm Hg (see Table 2). No patients or control subjects consumed ethanol regularly, which may deplete hepatic stores of vitamin A.26

Measurements of Retinoid Levels

Different isomers of retinoic acid were measured in plasma samples of normal control subjects and IPAH patients with a sensitive GC/MS technique. As shown in Table 3, mean total retinoic acid level in age- and sex-matched control subjects was 2.95 ng/mL, whereas IPAH patients had significantly reduced levels of 2.19 ng/mL (P<0.05). Total retinoic acid levels in IPAH patients ranged from 1.55 to 3.04 ng/mL, with half having total retinoic acid levels of ≤2 ng/mL. Analysis of various retinoic acid isoforms revealed that the altered total retinoic acid levels in IPAH patients were due to a 40% decrease in ATRA and 13-cis retinoic acid levels (Table 3). Whereas the mean ATRA level of control subjects was 1.14 ng/mL, some IPAH patients had ATRA levels as low as 0.35 ng/mL. No statistically significant differences were noted in levels of 11-cis or 9-cis retinoic acid. Importantly, no significant differences were noted for plasma retinol (vitamin A) levels between IPAH patients and control subjects. Thus, differences in retinoic acid levels are not likely to be due to altered dietary intake of vitamin A.

Levels of Carotenoids and Tocopherols

Table 4 shows the levels of various carotenoids in plasma of IPAH patients and control subjects. Although no differences in lutein, cryptoxanthin, or lycopene were noted, plasma levels of β-carotene, a major provitamin A carotenoid and precursor of retinoic acid, were 50% less in IPAH patients than in control subjects. We also found a 30% reduction in plasma α-tocopherol levels compared with control subjects (Table 4), consistent with the occurrence of oxidative stress in IPAH patients.

Expression of Retinoic Acid Receptors in Human Pulmonary Artery Smooth Muscle Cells

Similar to studies on rat aortic smooth muscle cells,11 we found that human pulmonary artery smooth muscle cells express RARα, RARβ, RARγ, RXRα, and RXRβ, but not RXRγ, as determined by RT-PCR analysis. As shown in Figure 1A, 30 cycles of PCR generated products of 201, 752, and 327 bp, which correspond to RARα, RARβ, and RARγ, respectively. Detection of RXR required 40 cycles of PCR, which generated 93- and 510-bp products, corresponding to RXRα and RXRβ, respectively. However, the RXRγ product that has an expected size of 532 bp was not generated. We performed PCR for up to 50 cycles to confirm that RXRγ was not detectable.

We also found that ATRA increased the expression of RARα and RARβ in human pulmonary artery smooth muscle cells (Figure 1B). Furthermore, ATRA induced the activation of DNA binding activities toward RAR and RXR consensus sequences (Figure 1C). These results indicate that human pulmonary artery smooth muscle cells express retinoic acid receptors and that ATRA can elicit signal transduction in these cells.

Effects of ATRA on Serotonin-Induced Cell Growth

To determine whether ATRA has inhibitory effects on human pulmonary artery smooth muscle cell growth, cells were treated with serotonin (5-HT), a known inducer of
pulmonary artery smooth muscle cell proliferation. Treatment of quiescent cells with serotonin (1 μmol/L) caused an 80% increase in cell number after 6 days, consistent with proliferation of these cells. ATRA (2 μmol/L) completely abrogated the serotonin-induced increase in cell number (Figure 2). ATRA also induced a small number of apoptotic cells (10% to 15%) as monitored by comet assay. ATRA did not cause necrotic cell death as determined by Trypan blue staining.

**Effects of ATRA on Serotonin-Induced ERK Activation**

Phosphorylation and activation of p44/42 mitogen-activated protein kinases (ERK) have been shown to play an essential role in the growth of bovine pulmonary artery smooth muscle cells induced by serotonin. In human pulmonary artery smooth muscle cells, serotonin rapidly and transiently phosphorylated ERK within 5 minutes, as determined by Western blot using a phospho-specific ERK antibody. ATRA caused partial inhibition of serotonin-induced ERK phosphorylation, whereas the specific MEK inhibitor PD98059 caused complete inhibition (Figure 3).

**Effects of ATRA on Gene Expression**

To further identify which genes might be targeted by ATRA in inhibition of cell growth, gene array analyses were performed with the GE Array Signal Transduction Pathway Finder. This array consists of 96 genes, some of which are constitutively expressed in human pulmonary artery smooth muscle cells (Figure 4). In 3 parallel analyses, 11 genes were found to be consistently upregulated in response to ATRA. These include cyclin-dependent kinase inhibitor 2A (gene 19), engrailed homolog 1 (gene 31), GADD45A (growth arrest and DNA damaging; gene 36), hepatocyte nuclear factor 3α (gene 40), heat shock protein 90 (gene 45), intracellular adhesion molecule (gene 46), insulin-like growth factor binding protein 3 (gene 47), mdm2 (gene 59), quinone oxidoreductase homolog (gene 70), MCP1 (gene 79), and E-selectin (gene 80). Among them, GADD45A may be important in the growth-inhibitory actions of ATRA, because it promotes growth arrest and induces apoptosis of smooth muscle cells.

To confirm the enhancement of GADD45A expression (gene 36) by ATRA in human pulmonary artery smooth muscle cells, RT-PCR and Western blot experiments were performed. As shown in Figure 5A, RT-PCR using human GADD45A primers confirmed that treatment of cells with ATRA for 20 hours enhanced GADD45A mRNA expression. We also found that ATRA enhances GADD45A protein expression as determined by Western blot (Figure 5B).
Effects of Plasma From IPAH Patients and Control Subjects on Cultured Human Pulmonary Smooth Muscle Cell Growth

The experiments described above demonstrated that retinoic acid can modulate growth signaling events in human pulmonary artery smooth muscle cells. Accordingly, we tested the hypothesis that plasma from IPAH patients would influence the growth of these cells. We performed experiments to examine the effects of plasma samples on quiescent human pulmonary artery smooth muscle cell growth. These experiments revealed that plasma from control subjects can serve as a potent growth factor of human pulmonary artery smooth muscle cells (Figure 6). Such growth-promoting ability was dramatically higher than that of FBS, which often is used to maintain these cells. On the other hand, plasma from IPAH patients exhibited limited ability to induce cell growth. Trypan blue staining did not show increases in necrotic cell death by any of these samples (data not shown).

Discussion

We found that IPAH patients have significantly diminished circulating levels of ATRA and 13-cis-retinoic acid compared with matched control subjects, consistent with the hypothesis that insufficient retinoic acid metabolites contribute to the development of IPAH. We have supported our hypothesis with studies demonstrating that human pulmonary artery smooth muscle cells constitutively express genes for the retinoic acid receptors RARα, RARβ, RARγ, RXRα, and RXRβ and, when stimulated by ATRA, increased expression of RARα and RARβ, as well as DNA binding activities for RAR and RXR consensus sequences. We have also shown that ATRA suppresses serotonin-induced proliferation of human pulmonary artery smooth muscle cells, possibly via
cantly different from plasma from control subjects at 10% for 4 days. Values represent mean ± SEM of viable cells as determined by Trypan blue exclusion on a hematocytometer. *Significantly different from plasma from control subjects at P<0.05.

Figure 5. Effects of ATRA on GADD45A expression. A, Quiescent human pulmonary artery smooth muscle cells were treated with ATRA (2 μmol/L) for 20 hours. Total RNA was subjected to RT-PCR to monitor GADD45A and G3DPH mRNA levels. Bar graph represents mean ± SEM (n=3) of ratio of GADD45A to G3DPH mRNA as determined by densitometry. B, Cells were treated with ATRA (2 μmol/L) for durations indicated. Cell lysates were prepared and subjected to Western blot using GADD45A and tubulin antibodies.

mechanisms involving inhibition of ERK activation and upregulating expression of genes, including GADD45A. Although we have not established a mechanism for the reduction in circulating ATRA and 13-cis-RA levels, our findings identify possible molecular targets for ATRA inhibition of pulmonary vascular remodeling.

Possible mechanisms for the reduced ATRA and 13-cis-RA levels in IPAH patients include reduced production or release related to decreased dietary intake of vitamin A, vascular changes secondary to the pulmonary hypertension, enhanced clearance or metabolism, or a combination of these factors. The decreased levels of α-tocopherol and β-carotene in the IPAH patients compared with normal subjects suggest the occurrence of oxidative stress, which could interfere with ATRA synthesis. Previous studies have demonstrated that patients with IPAH have evidence of increased lipid peroxidation and increased 8-hydroxy guanosine and nitrotyrosine expression associated with reduced superoxide dismutase activity.28 Thus, oxidative injury to synthetic metabolic pathways could lead to reduced accumulation of ATRA and 13-cis-retinoic acid in the IPAH patients.

The lower circulating levels of the retinoic acid precursor β-carotene that we observed in the IPAH patients raise the possibility that a reduced dietary intake of β-carotene contributed to the reductions in ATRA and 13-cis-retinoic acid levels. However, other carotenoids that we measured, including lutein, cryptoxanthin, and lycopene, were not lower in the IPAH patients. Retinoid acid levels also depend on dietary intake of vitamin A; however, the circulating level of this vitamin was not different between control subjects and IPAH patients. Furthermore, the lack of alteration in 9-cis-retinoic acid levels suggests more strongly that dysregulated metabolism of ATRA and 13-cis-RA rather than reduced dietary intake of vitamin A or β-carotene is responsible for the reduced ATRA levels.

If ATRA and 13-cis-RA are important in regulating pulmonary vascular remodeling, then molecular targets must be present to convey regulatory signals. Retinoic acid receptors are required for ATRA and 13-cis-RA signal transduction. Retinoids interact with nuclear receptors to translocate into the nucleus and activate transcription of target genes. The retinoic acid receptors bind both 9-cis-RA and ATRA, whereas the retinoid X receptors bind 9-cis-RA only. Although 13-cis-RA is not a direct receptor ligand, it probably isomerizes to ATRA or 9-cis-RA to interact with different receptors. Our results indicate that, similar to findings from cultured systemic vascular smooth muscle cells, all RA and retinoid X receptors, except for RXRγ, are constitutively expressed in human pulmonary artery smooth muscle cells. Furthermore, we observed that DNA binding activity for RARs and RXRs was increased with ATRA treatment, suggesting a direct effect of ATRA (or possibly one of its isomers) on transcription of target genes. This indicates that the retinoic acid receptors are functional and stimulate signal transduction in human pulmonary artery smooth muscle cells and demonstrates the feasibility of human pulmonary artery smooth muscle cell regulation by retinoids.

Further evidence for the functionality of the retinoic acid signaling system in human pulmonary artery smooth muscle cells and its possible relevance to pulmonary hypertension derives from our experiments demonstrating inhibition of human pulmonary artery smooth muscle cell proliferation by ATRA. Using serotonin to induce proliferation in human pulmonary artery smooth muscle cells,27 we showed that ATRA completely blocked the increase in cell number. However, we cannot be certain that this finding has physiological relevance, because the concentration used approaches circulating levels seen in patients...
with acute promyelocytic leukemia who are undergoing therapy with ATRA and is 10^7 times higher than normal serum levels. Nevertheless, our observations using pharmacological concentrations of ATRA suggest a potential use of ATRA as a therapeutic agent to reduce pulmonary vascular remodeling.

In addition, although we have demonstrated that ATRA stimulates signal transduction, we have not completely identified the signal transduction pathway involved. Serotonin-induced smooth muscle cell proliferation has been shown to occur via ERK, and we showed that ATRA partially inhibited phosphorylation of ERK induced by serotonin in human pulmonary artery smooth muscle cells. This indicates that ATRA, at least in part, exerts its growth-inhibitory effect via influencing the ERK-dependent pathway.

To gain further insight into the possible pulmonary vascular effects of ATRA, we performed gene array analysis after exposing human pulmonary artery smooth muscle cells for 20 hours to the same concentration of ATRA that completely inhibited proliferation. Among the 11 genes that were consistently upregulated by ATRA, GADD45A appears to be a rational candidate for involvement in the smooth muscle cell growth-inhibitory effect, because it has previously been shown to promote growth arrest and apoptosis in vascular smooth muscle cells.

We further confirmed the upregulation of GADD45A gene expression by ATRA using RT-PCR, as well as protein expression using Western blot. These experiments demonstrate that ATRA serves to regulate gene expression in human pulmonary artery smooth muscle cells and that some of its effects may be mediated via GADD45A expression, although we readily acknowledge that we have not yet conclusively established this connection and that other pathways may be involved.

We further acknowledge a number of other limitations of our design. Although we examined blood samples from patients consecutively undergoing right heart catheterization and made efforts to examine samples only from those meeting criteria for IPAH, we cannot be certain that ours is a representative sample of all such patients. Furthermore, our patient samples were obtained from central veins (via the introducing catheter), whereas the samples from normal control subjects were taken from peripheral veins. However, Craft et al have reported no differences in retinol levels between venous and capillary serum samples. Also, short of experiments demonstrating that manipulations of ATRA or GADD45A in experimental models or in clinical trials affect the severity or outcomes of pulmonary hypertension, we acknowledge that we have not established pathophysiological roles for these agents. Rather, we hope that our results will serve to generate further hypotheses and to stimulate further experimentation.

Experiments on the effects of plasma from IPAH patients and control subjects on human pulmonary artery smooth muscle cell growth surprisingly showed that plasma samples from control subjects are potent growth promoters, whereas those from IPAH patients have markedly reduced ability to promote cell growth. These observations are contrary to our central hypothesis that increased retinoic acid levels suppress cell growth, because our results show that plasma from control subjects have higher levels of retinoids than IPAH patients. However, these observations do not refute our hypothesis, because these plasma samples were taken from patients who had already developed significant degree of pulmonary hypertension. It might be revealing to perform similar experiments on cell growth using plasma from patients who are in the process of developing pulmonary vascular remodeling. Furthermore, it is intriguing to hypothesize that plasma from IPAH patients might contain other cell growth suppressive or death factors that serve as defense mechanisms to reduce the degree of pulmonary vascular thickness. Identifications of such endogenous factors might help in the development of therapeutic agents to reduce pulmonary vascular remodeling. Our HPLC results demonstrate increased oxidative stress in IPAH patients, and it is possible that oxidants might serve as such growth suppressive mediators.

In conclusion, we have shown that ATRA levels are reduced in patients with IPAH and have suggested that this reduction may predispose to the development of pulmonary hypertension by permitting pulmonary vascular remodeling via proliferation of smooth muscle cells. Furthermore, we have identified possible pathways by which retinoic acids may act to inhibit human smooth muscle cell proliferation in pulmonary arteries. Further studies are necessary to delineate the pathophysiological and possible therapeutic roles of retinoic acids in pulmonary hypertension.

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