Elevated levels of activin A in clinical and experimental pulmonary hypertension

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PULMONARY HYPERTENSION (PH) frequently leads to progressive right-sided heart failure (HF) and ultimately transplantation or death (12). The clinical classification of PH divides the disease into groups, including pulmonary arterial hypertension (PAH) and PH due to chronic thrombotic disease [chronic thromboembolic PH (CTEPh)] (26). The group PAH includes idiopathic PAH (IPAH) and PAH related to risk factors or associated conditions (APAH). The pathogenic hallmark of PAH is increased pulmonary vascular resistance due to vasoconstriction, vascular cell proliferation and remodeling, and thrombosis (17). A number of mediators are involved in these processes, including endothelin (ET)-1, inflammatory cytokines, and decreased endothelial release of prostacyclin (10, 17). However, the signaling pathways involved in development of PH are far from clear.

Growing evidence links the transforming growth factor (TGF)-β superfamily to PH (17). This family consists of a number of cytokines, including bone morphogenetic proteins (BMPs) (3, 17), and, notably, 80% of patients with familial PAH and 10–30% of patients with IPAH have mutations in the BMP receptor II gene (9), and these patients also present with PH at a younger age and with more severe hemodynamic compromise (28). Activin A is another member of the TGF-β superfamily and signals, like TGF-β isofoms, primarily via Smad2 and Smad3 (23). Follistatin regulates activin A bioactivity by preventing activin A/receptor interaction, representing a short-loop feedback system (7). Our laboratory has previously reported a potential pathogenic role for activin A in HF and coronary artery disease, linking this cytokine to cardiovascular disorders (27, 31). Based on these findings and its function in inflammation and collagen deposition, we hypothesized a role for activin A also in PH.

In this study, we have examined whether activin A is upregulated in human PH by measuring levels of circulating activin A. To investigate synthesis and localization of pulmonary activin A, we utilized an experimental model of hypoxia-induced PH. In mouse lungs, we also explored signaling pathways that can be activated by activin A, such as phosphorylation of Smads, which are mediators of TGF-β signaling. Possible pathophysiological mechanisms initiated by activin A were explored by exposing pulmonary arterial smooth muscle cells in culture to this cytokine. Elevated levels of activin A and follistatin were found in patients with PH, and activin A levels were significantly related to mortality. Immunohistochemistry of lung autopsies from PH patients and lungs with experimental PH localized activin A primarily to alveolar macrophages and bronchial epithelial cells. Mice with PH exhibited increased pulmonary levels of mRNA for activin A and follistatin in the lungs, and also elevated pulmonary levels of phosphorylated Smad2. Finally, we found that activin A increased proliferation and induced gene expression of endothelin-1 and plasminogen activator inhibitor-1 in pulmonary arterial smooth muscle cells, mediators that could contribute to vascular remodeling. Our findings in both clinical and experimental studies suggest a role for activin A in the development of various types of PH.

METHODS

Patients and controls. Forty-seven patients with PH (mean pulmonary arterial pressure > 25 mmHg at rest, with a normal pulmonary capillary wedge pressure ≤ 12 mmHg), in New York Heart Association functional classes III-IV, referred to our tertiary center for the characterization of suspected chronic precapillary PH, were recruited consecutively in the time period between 2000 and 2006 (Table 1). The study population was divided into three groups, according to type of PH (24, 26): 1) patients with IPAH (n = 15); 2) patients with...
APAH (n = 18) [collagen vascular disease (n = 13), liver cirrhosis (n = 2), and human immunodeficiency virus infection (n = 3)]; and 3) patients with CTEPH (n = 14) verified with pulmonary angiograms. The data presented in Table 1 represent their medication at admission to our center. Following baseline evaluation, all patients were put on anticoagulants; diuretics were added in APAH, employed from autopsy of seven patients with PH (IPAH, n = 20), ET receptor antagonists (n = 10), or sildenafil (n = 3). The patients were followed prospectively for a mean period of 22 mo (0.5–72 mo). Fourteen sex- and age-matched individuals (7 men and 7 women, 44 ± 12 yr) undergoing right-sided heart catheterization during electrophysiological studies of supraventricular arrhythmias, but with otherwise normal hemodynamic function and myocardial structure, served as controls. All patients and controls were Caucasians. Serum samples were obtained from pulmonary and femoral arteries, as described (10). The regional ethical committee approved the study, and informed consent was obtained from each subject.

**Table 1. Baseline characteristics of the study group**

<table>
<thead>
<tr>
<th>Medication</th>
<th>IPAH</th>
<th>APAH</th>
<th>CTEPH</th>
</tr>
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<tbody>
<tr>
<td>Warfarin</td>
<td>61</td>
<td>56</td>
<td>100</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>0</td>
<td>11</td>
<td>45</td>
</tr>
<tr>
<td>Diuretics</td>
<td>31</td>
<td>39</td>
<td>25</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>0</td>
<td>28</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of subjects. IPAH, idiopathic pulmonary arterial hypertension (PAH); APAH, associated PAH; CTEPH, chronic thromboembolic pulmonary hypertension; RAP, right atrial pressure; MPAP, mean pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; CO, cardiac output; PVR, pulmonary vascular resistance; SvO2, mixed-venous oxygen saturation; peak Vo2, maximal oxygen uptake; NT-pro-BNP, N-terminal pro-brain natriuretic peptide. The medication represents therapy at baseline before any interventional therapy was started.

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Spleen (n = 18) diaphragm, stored at 80°C until real-time RT-PCR analysis. The actual concentration of 10 ng/ml. For measurement of proliferation, the cells were stimulated with recombinant activin A (R&D Systems) for 6 or 24 h, and cell pellets were stored at –80°C until real-time RT-PCR analysis. The actual dosage of activin A was based on preliminary dose-response experiments. Although an activin A concentration of 100 ng/ml was used for further experiments, some stimulatory effect was also seen at a concentration of 10 ng/ml.

**Enzyme immunoassays.** Levels of activin A (Serotec Ltd) and follistatin (R&D systems) were analyzed by enzyme immunoassays.

**Statistical analysis.** When more than two groups were compared, the Kruskal-Wallis test was performed, and, if significant, the Mann-Whitney U-test was performed to determine differences between each pair of groups. For comparison of two groups, the Mann-Whitney U-test was used. For comparisons within the same individuals, the
Wilcoxon signed-rank test was used. Coefficients of correlation were calculated by the Spearman rank test. Multivariate Cox regression analysis was applied to identify the independent predictive value of activin A after adjusting for known predictors of mortality in PH (i.e., peak oxygen uptake, heart rate, systolic blood pressure, right atrial pressure, mixed-venous oxygen saturation). Kaplan-Meier curves were generated, and the log rank test was used to compare mortality rates in relation to activin A and follistatin levels. Data are given as means ± SE, unless otherwise stated. Probability values (2-sided) were considered significant at a value of <0.05.

RESULTS

Serum levels of activin A and follistatin in patients with PAH. PH patients [IPAH (n = 15), APAH (n = 18), CTEPH (n = 14)] had significantly raised serum levels of activin A compared with controls (n = 14), as shown both in mixed-venous (i.e., pulmonary artery) and in arterial blood (i.e., femoral artery) (Fig. 1, A and B). Serum levels of follistatin were also elevated in PH patients, but the increase was most prominent and reached statistical significance in all patient groups only in arterial blood (Fig. 1, C and D). There were no significant differences in activin A or follistatin levels between the different PH categories, suggesting that the increased levels are independent of etiology and related to PH per se. While there was no transpulmonary gradient for activin A, PH patients, but not controls, had significantly higher serum follistatin levels in the femoral than in the pulmonary artery (1,832 ± 130 vs. 1,676 ± 132 pg/ml; P < 0.001), with a similar pattern in all PH subgroups, potentially suggesting a net release of follistatin from the lungs during PH. However, the absolute changes were relatively low, and caution is needed when interpreting these data. Moreover, venous activin A levels were inversely correlated with maximal oxygen uptake during exercise testing (r = −0.42, P < 0.01) and arterial oxygen saturation (r = −0.34, P < 0.05). In contrast, we found no relationships between activin A or follistatin and cardiac output, mixed-venous oxygen saturation, right arterial pressure, pulmonary vascular resistance, or NH2-terminal pro-brain natriuretic peptide (data not shown).

There are several reports of increased plasma levels of activin A and follistatin during chronic renal failure (4, 15), potentially at least partly reflecting increased release of activin A into the circulation secondary to the use of heparin during hemodialysis (4). However, in the present study, no patients were taking heparin, and we found no correlation between creatinine levels and serum levels of activin A and follistatin (data not shown).

Fig. 1. Serum levels of activin A (A and B) and follistatin (C and D) in patients with pulmonary hypertension (PH) classified as idiopathic (IPAH, n = 15) and associated pulmonary arterial hypertension (APAH, n = 18) and chronic thromboembolic PH (CTEPH, n = 14), and in sex- and age-matched control subjects (n = 14). Blood was collected from the pulmonary artery (A and C) or femoral artery (B and D). Values are means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control subjects. Kaplan-Meier curves show the cumulative incidence of death during the observation period, according to dichotomized levels of activin A (above or below median levels) at baseline in venous blood (pulmonary artery) (E) and arterial blood (femoral artery) (F).
Activin A levels in relation to mortality in PH patients. During a mean follow-up of 22 mo, 11 patients died (3 patients with IPAH, 6 with APAH, and 2 with CTEPH), all cardiopulmonary deaths. When generating Kaplan-Meier curves showing the cumulative incidence of death during the observation period, according to dichotomized levels of activin A or follistatin (above or below median levels), we found a higher mortality rate in those with high (i.e., above median 796 pg/ml) compared with those patients with low activin A levels, as shown in both mixed-venous and in arterial blood (Fig. 1, E and F). No such association was found between follistatin levels and mortality (data not shown). However, the present study was not designed or powered to investigate the role of activin A as a prognostic marker in PH, and these observations must be regarded as preliminary. Accordingly, only univariate data are presented, and larger studies are needed to evaluate activin A as a prognostic marker in PH.

Activin A and follistatin in experimental PH. An experimental model of hypoxia-induced PH was utilized to investigate synthesis and localization of pulmonary activin A. The ratio between the weight of the right ventricle and left ventricle plus septum was increased at 1 wk in the hypoxia group compared with controls (0.23 ± 0.01 vs. 0.17 ± 0.01; P < 0.05, n = 8/8) and at 2 wk of hypoxia (0.25 ± 0.01 vs. 0.17 ± 0.01; P < 0.001, n = 8/8), indicating right ventricular hypertrophy due to PH. At both time points, the left ventricular weight-to-body weight ratio was unaltered in the hypoxia group. Moreover, pulmonary artery acceleration time measured by Doppler echocardiography was reduced after hypoxia for 1 wk (19.1 ± 0.5 vs. 23.9 ± 0.8 ms) and 2 wk (18.0 ± 0.9 vs. 24.9 ± 1.1 ms, hypoxia group vs. control group; P < 0.05 for both comparisons), indicating PH.

Activin A is a homodimer of activin βA-subunits, and at the mRNA levels, we found significantly elevated pulmonary expression of activin βA after 2 wk of hypoxia, whereas the increase did not reach statistical significance at 1 wk of hypoxia (n = 8/8, Fig. 2A). The protein level of pulmonary activin A was significantly higher in the hypoxia group compared with control at 1 wk and unchanged at 2 wk of hypoxia (n = 8/8, Fig. 2B). A similar pattern was also seen in serum with elevated serum levels of activin A after 1 wk of hypoxia, but not after 2 and 4 wk, although the difference did not reach statistical significance (P = 0.07 vs. control mice). As depicted in Fig. 2C, activin A levels in the lungs increased over time in control animals. The reason for this pattern is at present unclear, but, as activin A levels have been reported to increase with age in humans (20), it is possible that this is what is reflected in our measurements in the mouse model. We found an increase in the pulmonary mRNA levels of follistatin throughout the study period during hypoxia-induced PH (n = 8/8, Fig. 2C), but with no significant changes in protein levels (data not shown). Finally, we examined the gene expression of activin receptor II, the activin A-binding part of the activin A receptor complex, and found significantly increased gene expression after both 1 and 2 wk of hypoxia (n = 8/8, Fig. 2D). This finding may suggest increased activin A receptor function and thus increased activin A responses during PH.

Smad2 phosphorylation in experimental PH. Activin A primarily signals via activation of the Smad2/3 pathway. We found that mice with hypoxia-induced PH had a marked increase in the ratio of phosphorylated Smad2 to total Smad2/3 at 1 and 2 wk of hypoxia, demonstrating activation of this pathway (n = 8/8, Fig. 3, A and B). The total levels of Smad2/3 were not changed in mice with PH (n = 8/8, Fig. 3, A and C). It may seem contradictory that Smad2/3 signaling is still increased, whereas pulmonary activin A levels have dropped. However, as TGF-β also signals via this pathway, our findings may at least partly reflect that, in addition to upregulation of activin A, as shown in the present study, hypoxia-induced PH.
control mice. is also characterized by persistently increased TGF-β activity, as also suggested by others (6).

**Localization of activin A and follistatin in pulmonary tissue.** Immunohistochemical analysis revealed the presence of activin A and follistatin immunoreactivities in pulmonary tissue from both humans (Fig. 4) and mice with PH (Fig. 5). First, the strongest expression of activin A and follistatin in both human and murine lungs was seen in alveolar macrophages (Fig. 4, D and G; Fig. 5, D and G). While the staining pattern of activin A was similar within alveolar macrophages in all patients, the amount of alveolar macrophages showed variations, but with no significant differences between the various diagnostic subgroups of PH. Second, while weak expression of activin A was observed in other interstitial cells and in arterial and venous SMC in lungs from PH patients (Fig. 4C), fairly strong activin A immunoreactivity was seen in vascular SMC in murine lungs (Fig. 5B). Thus both increased number of alveolar macrophages and increased expression in vascular SMCs may be responsible for the increase in activin A levels in PH lungs. Third, follistatin immunoreactivity was fairly strong in SMC and epithelial cells, i.e., ciliated cells in the conductive airway, in pulmonary tissue from both humans and mice with PH (Fig. 4F and 5F).

**Activin A induces ET-1 and PAI-1 gene expression and proliferation in pulmonary artery SMC and ET-1 gene expression in endothelial cells.** To further elucidate a potential pathogenic role in PH, we investigated the effect of activin A on pulmonary arterial SMCs. Several significant findings were revealed (Fig. 6). First, activin A significantly increased the proliferation of these cells. Second, PAI-1 has been shown to be essentially involved in vascular remodeling processes, possibly involving effect on SMC (5), and, notably, activin A induced a marked increase in gene expression of PAI-1 in pulmonary arterial SMCs (Fig. 6B). Third, ET-1 is also implicated in the pathogenesis of PH (13), and activin A promoted an increase in mRNA levels of pre-pro-ET-1 in pulmonary arterial SMCs, as well as in HUVECs (Fig. 6, C and D). Finally, in PH patients, serum levels of activin A were significantly correlated with serum levels of ET-1 (Fig. 7), and, although caution is needed when interpreting correlation analyses, these findings may potentially suggest an in vivo interaction between these two mediators.

**DISCUSSION**

The discovery that a majority of patients with familial PAH have mutations in the BMPR2 gene has drawn attention to the pathogenic role of TGF-β superfamily in PH (17). Herein we demonstrate markedly elevated serum levels of the TGF-β superfamily member activin A in different groups of PH patients, including patients with IPAH, APAH, and CTEPH. The relationship between high activin A levels and mortality in PH patients further suggests a role for activin A in PH. However, this study was not designed to investigate the role of activin A as a prognostic marker in PH, and the survival data should be interpreted with caution.

Strong expression of activin A in alveolar macrophages has been reported in pulmonary fibrosis, contributing to cell proliferation and fibrosis (21). We extend these findings by showing strong activin A immunostaining in lungs during both clinical and experimental PH, and its relation to alveolar macrophages further suggests a role for inflammatory cells in PH. Moreover, the increase in mRNA levels of both activin A and follistatin in lung tissue in the mouse model indicates local synthesis of these peptides during the development of PH. Our findings of an increased ratio of phosphorylated Smad2 to total Smad2/3 within the lungs in experimental PH further support increased pulmonary activin A activity during PH. Richter et al. (25) have previously reported increased phosphorylated Smad2 in the endothelial cells from the lungs in PH patients, and our findings in the present study may suggest that increased activin A activity could contribute to the increased Smad2/3 signaling during PH.

The present study also illustrates that the regulation of activin A during experimental PH is rather complex. Thus, while there was an early increase in protein levels of activin A within lung tissue, this was followed by a decrease at the end of the observation period. At present, we have no firm explanation for this pattern, but it is tempting to hypothesize that this may reflect that chronic as opposed to acute hypoxia could downregulate the expression of this cytokine, at least in this
An early increase followed by a subsequent normalization or decrease has also been reported during brain and fetal hypoxia (29). In contrast to the protein level, the mRNA levels of activin A were significantly elevated in pulmonary tissue after 2 wk of hypoxia, suggesting that activin A may be subjected to posttranscriptional regulation in this model.

Regarding PH, activin A may affect the major cell types and processes involved in the pathogenesis. First, proliferation and migration of vascular SMC are central pathogenic events in PH (17). Activin A has previously been suggested to play a role in airway remodeling by promoting proliferation of SMC in lungs during asthma (8), and herein we show that activin A significantly enhances proliferation of human pulmonary artery SMC, further indicating a role for activin A in vascular remodeling. Second, Hoeper et al. (16) have previously reported increased plasma levels of PAI-1 in PAH patients, and we demonstrate that activin A increases gene expression of this serine protease inhibitor in pulmonary artery SMC. PAI-1 has...
Fig. 5. Representative photomicrographs of pulmonary tissue sections from a control mouse (A, B, and E) and a mouse with PH (C, D, F, and G), demonstrating activin A (B–D) and follistatin (E–G) immunoreactivities. A: pulmonary section stained with omission of the primary antibody as control, demonstrating no immunostaining of any of the cellular elements of the pulmonary tissue. Activin A and follistatin immunoreactivities were seen in bronchial epithelial cells (B and C), in vascular smooth muscle cells (E and F), and in alveolar macrophages (D and G, arrows), and in cells in the interalveolar septa. Scale bars in A, B, C, E, and F = 100 μm; in D and G = 25 μm.
been shown to be essentially involved in vascular remodeling processes, possibly by facilitating migration and/or proliferation of SMC (5, 11). If such PAI-1-inducing effects also occur in SMC within the pulmonary arterioles in PAH patients, it would link activin A not only to vascular remodeling, but also to thrombus formation during PAH. Third, activin A has been shown to stimulate proliferation and differentiation of lung fibroblasts and to promote bleomycin-induced pulmonary fibrosis (2, 22), and similar activin A-mediated effects on fibroblasts could contribute to the collagen deposition observed during PH (2). Finally, in the present study, we demonstrate that activin A induces gene expression of ET-1, linking activin A not only to pulmonary vascular remodeling, but also to the vasoconstriction that characterizes PH (13). However, our in vitro data should be interpreted with some caution. Although it is conceivable that activin A levels may be much higher within a fibrotic and inflammatory environment, as in the lung from patients with PH, than in serum, the actual dosage of activin A that was used in the present study was rather high.

Thus, further studies are needed to clarify if activin A is an important mediator and not only a marker in PH.

Follistatin is the main biological inhibitor of activin A bioactivity, binding it with high affinity, thereby inhibiting receptor interaction (7). Thus, while raised activin A levels during PH may reflect pathogenic or causative mechanisms, the rise in follistatin may be protective. In line with this, activin A is a main inducer of follistatin expression, and the raised levels of follistatin during PH may, therefore, represent a counterregulatory mechanism to the raised activin A levels (7). Interestingly, during experimental PH, there was no increase in protein levels of follistatin, as opposed to the early increase in protein level of activin A, potentially resulting in less inhibition of activin A activity. However, follistatin may also bind other TGF-β superfamily members such as myostatin (1), potentially resulting in inhibition of other responses, and further studies are needed to delineate the interaction between activin A and follistatin and possibly other mediators during PH.

In conclusion, our findings may suggest that the involvement of the TGF-β superfamily in the pathogenesis of PH should include activin A, potentially contributing to vascular remodeling, fibrosis, thrombus formation, and vasoconstriction during PH, representing a hitherto unrecognized pathogenic mediator in this disorder.

ACKNOWLEDGMENTS

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GRANTS

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Fig. 6. A: effect of activin A on pulmonary artery smooth muscle cell (PASMC) proliferation, as determined by incorporation of [3H]thymidine. Gene expression, quantified using real-time quantitative RT-PCR and normalized against β-actin, of plasminogen activator inhibitor (PAI)-1 (B) and endotelin (ET)-1 (C) after stimulation of PASMC with activin A (100 ng/ml) for 6 and 24 h is shown. D: gene expression of ET-1 in human umbilical vein endothelial cells stimulated with activin A (100 ng/ml) for 6 and 24 h. Values are means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 vs. unstimulated.

Fig. 7. Correlation between serum levels of activin A and ET-1 in patients with PH.

Fig. 7. Correlation between serum levels of activin A and ET-1 in patients with PH.
REFERENCES


