Altered Hypoxic–Adenosine Axis and Metabolism in Group III Pulmonary Hypertension

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Abstract

Group III pulmonary hypertension (PH) is a highly prevalent and deadly lung disorder with limited treatment options other than transplantation. Group III PH affects patients with ongoing chronic lung injury, such as idiopathic pulmonary fibrosis (IPF). Between 30 and 40% of patients with IPF are diagnosed with PH. The diagnosis of PH has devastating consequences to these patients, leading to increased morbidity and mortality, yet the molecular mechanisms involved in the development of PH in patients with chronic lung disease remain elusive. Our hypothesis was that the hypoxic–adenosinergic system is enhanced in patients with group III PH compared with patients with IPF with no PH. Explanted lung tissue was analyzed for markers of the hypoxic–adenosine axis, including expression levels of hypoxia-inducible factor (HIF)-1A, adenosine A2B receptor, CD73, and equilibrative nucleotide transporter-1. In addition, we assessed whether altered mitochondrial metabolism was present in these samples. Increased expression of HIF-1A was observed in tissues from patients with group III PH. These changes were consistent with increased evidence of adenosine accumulation in group III PH. A novel observation of our study was of evidence suggesting

altered mitochondrial metabolism in lung tissue from group III PH leading to increased succinate levels that are able to further stabilize HIF-1A. Our data demonstrate that the hypoxic–adenosine axis is up-regulated in group III PH and that subsequent succinate accumulation may play a part in the development of group III PH.

Keywords: group III pulmonary hypertension; adenosine A2B receptor; idiopathic pulmonary fibrosis; succinate; hypoxia-inducible factor-1A

Clinical Relevance

This study shows that alterations in metabolism lead to enhanced activation of the hypoxic-adenosine axis that contribute to the development of pulmonary hypertension associated with idiopathic pulmonary fibrosis. These findings suggest that targeting elements of the hypoxic-adenosine axis could be used therapeutically for group III pulmonary hypertension, for which currently no effective strategies exist.

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Idiopathic pulmonary fibrosis (IPF) is a terminal disease characterized by progressive fibrosis and respiratory insufficiency, with a median survival of 5 years (1, 2). Pulmonary hypertension (PH), defined as mean pulmonary artery pressure (mPAP) 25 mm Hg or greater at rest, is estimated to have a prevalence among patients with chronic lung diseases (CLDs), such as IPF, between 30 and 40% (3, 4), and are classified as having World Health Organization group III PH (5). Patients with group III PH usually present with lower levels of mPAP compared with group I; however, the development of PH has devastating consequences to patients with CLD, and is thus considered the single most significant predictor of mortality in patients with CLD (4, 6, 7). Regardless of the strong association of PH and mortality, there are currently no effective treatments for IPF and its complications (3, 8).

Despite the fact that mediators of inflammation and tissue remodeling are elevated in patients with lung fibrosis (9, 10), not all patients with lung fibrosis develop PH (3, 4). A potential stimulus of disease progression in patients with IPF could be the prevalence of hypoxemic conditions where worsening dyspnea has been associated with IPF progression (11). These observations are relevant, as hypoxic conditions are known to direct the stabilization of hypoxia-inducible factor (HIF)-1 α and subsequent activation of the adenosinergic axis. That is,

characterized by increased expression of enzymes involved in the synthesis of adenosine, a signaling nucleoside that is generated in conditions of cellular injury and stress, and enhanced expression of adenosine receptors, such as adenosine receptor 2B (ADORA2B) (12–14). Previous research has identified elevated expression of ADORA2B and CD73 (an enzyme involved in the generation of adenosine from ATP) in patients with CLD (15). Furthermore, in an animal model of lung fibrosis and PH, we have shown that genetic or pharmacological blockade of ADORA2B inhibited the development of PH (16). These observations suggest alterations of the hypoxic-adenosinergic axis in the pathogenesis of PH secondary to IPF. Stabilization of HIF-1 α has also been shown to occur in the absence of hypoxic environments (17) through metabolic imbalances that have been observed in patients with PH (18). Taken together, these observations prompted us to evaluate whether alterations of the hypoxic-adenosine axis and metabolic imbalances leading to enhanced HIF-1 α stabilization were apparent in patients with PH secondary to IPF.

Materials and Methods

Subjects

The use of human material for this study was reviewed by the University of Texas

Health Science Center at Houston Committee for the Protection of Human Subjects (Institutional Review Board no. HSC-MS-08-0354). The demographic details of the study population are summarized in Table 1. Deidentified lung explant tissue from patients with IPF and corresponding deidentified clinical parameters were obtained from the Methodist Hospital (Houston, TX). IPF was diagnosed by board-certified pulmonologists upon admittance for lung transplantation at the Houston Methodist Hospital using published guidelines (10). Briefly, the criteria for a positive diagnosis of IPF were defined by the exclusion of other known causes of interstitial lung disease, such as domestic and occupational environmental exposures, connective tissue disease, and drug toxicity; the presence of a usual interstitial pneumonia pattern on high-resolution computed tomography scans and lung function tests consistent with restrictive lung disease (19). Healthy control lung tissue was obtained from the International Institute for the Advancement of Medicine (Edison, NJ).

Tissue Collection

Tissue was collected at the time of lung explantation and processed on site within 20 minutes. The lobes were identified from each lung explant and, for each lobe, the center was identified, taking the airway as a reference point. A 1-cm transverse center cut was performed to obtain a section from the center of each lobe. Consequently, this tissue was divided into three sections to separate external (closest to pleura), center, and internal (closest to bronchi) portion according to the airway. A piece from each portion was then cut for histology and an adjacent section was flash frozen in liquid nitrogen. For studies involving lung tissue, and to account for the heterogeneity of fibrotic deposition in patients with IPF, lower lobes were selected from patients with IPF (blinded to a diagnosis of PH) where similar fibrotic deposition was present, based on Masson's trichrome-stained sections and Ashcroft scores (20) performed by an observer blinded to diagnosis.

Quantitative RT-PCR, Immunoblots and ELISA, Histology, and Immunohistochemistry

Please refer to the online supplement for experimental details. Briefly, RT-PCR

Table 1. Demographic Description of Study Population

Characteristics	IPF (n = 19)	IPF + PH (n = 12)	P Value
Female sex	6/19	6/12	0.3051
Male sex	13/19	6/12	0.3051
Age, years	65.47 $\pm$ 1.9	56.83 $\pm$ 3.67	0.029
mPAP, mm Hg	19.06 $\pm$ 0.89	33.08 $\pm$ 1.99	<0.0001
Systolic PAP, mm Hg	31 $\pm$ 1.47	51.17 $\pm$ 2.46	<0.0001
Diastolic PAP, mm Hg	12.17 $\pm$ 0.68	22.58 $\pm$ 1.92	<0.0001
6MWD, m	233.8 $\pm$ 28.20	145.1 $\pm$ 25.82	0.0415
BMI	27.45 $\pm$ 1.04	30.94 $\pm$ 1.74	0.0773
FVC, %	55.95 $\pm$ 4.87	45.29 $\pm$ 6.55	0.1953
DL _{CO} , %	25.41 $\pm$ 2.84	20.53 $\pm$ 3.68	0.3414
TLC, %	56.06 $\pm$ 4.03	54.34 $\pm$ 6.29	0.8106
FEV ₁ , %	55.47 $\pm$ 5.82	45.99 $\pm$ 7.13	0.3143
PaO ₂ , mm Hg	60.43 $\pm$ 3.30	55.44 $\pm$ 4.32	0.365
PaCO ₂ , mm Hg	41.47 $\pm$ 1.16	39.32 $\pm$ 1.21	0.233
Bicarb, mEq/L	26.86 $\pm$ 1.08	25.69 $\pm$ 0.94	0.4589

Definition of abbreviations: 6MWD, 6-minute walk distance; Bicarb, bicarbonate; BMI, body mass index; DL_{CO}, diffusing capacity of the lung for carbon monoxide; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; IPF, idiopathic pulmonary fibrosis; mPAP, mean pulmonary artery pressure; PaCO₂, partial pressure of carbon dioxide in arterial blood (mm Hg); PaO₂, partial pressure of oxygen in arterial blood (mm Hg); PAP, pulmonary artery pressure; PH, pulmonary hypertension; TLC, total lung capacity.

experiments were performed using SYBR Green I master mix (Mannheim, Germany). For immunoblots, protein from lung tissue lysates was extracted with RIPA buffer. Histology and immunohistochemistry (IHC) was performed on formalin-fixed paraffin embedded (FFPE) samples using standard protocols.

Statistical Analysis

A Student's *t* test (unpaired) was performed for comparisons between two distinct groups. One-way ANOVAs with the Newman-Keuls correction were performed for multiple group analysis. Associations between transcript levels of two genes or clinical data were established by linear regression and Pearson's correlation analysis. A Chi-square calculation was made for categorical data. A *P* value of 0.05 or less was considered to be significant. All statistical analyses were performed with GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

Results

Vascular Remodeling Is Apparent in Group III PH Independent of the Level of Fibrosis

We first assessed histologically the presence of fibrotic deposition in the lungs of patients with IPF alone and IPF with a secondary diagnosis of PH. For this experiment, we performed Ashcroft scoring on lung sections obtained for each lobe (upper and lower) from our patient cohort (Table 1). We report no significant differences in the deposition of fibrosis assessed histologically (Figures 1A and 1B), consistent with expression levels of collagen 1A1 (Figure 1C). However, evidence of vascular remodeling was seen histologically in patients with IPF plus PH compared with those with IPF alone (Figures 1D and 1E), correlative with elevated mPAP (Figure 1F).

Molecular Markers of Hypoxia Are Enhanced in Patients with IPF and PH

In an effort to understand the mechanisms leading to enhanced vascular remodeling in PH secondary to lung fibrosis, we hypothesized that increased stabilization of HIF-1A (Figure 2A) may be present in the lungs of patients with group III PH. Immunoblots for HIF-1A reveal increased stabilization in IPF plus PH compared

with IPF alone (Figure 2B). Consistent with this, IHC for HIF-1A showed increased signals in remodeled vessels of patients with IPF plus PH compared with patients with IPF alone (Figure 2C). An ELISA for HIF-1A revealed increased levels in IPF lung samples versus control and a significant increase in HIF-1A in patients with IPF plus PH relative to patients with IPF with no PH (Figure 2D). In addition, we report increased expression from lung tissue lysates of the hypoxia-inducible genes CD73, GLUT1, NOS3, PAI1, and PDK1 (Figures 2E–2I). These findings suggest that HIF-1A stabilization is present in patients with IPF and PH compared with IPF alone. Interestingly, correlation analysis between mPAP in patients with and those without a secondary diagnosis of PH (see Figure E1 in the online supplement) only revealed a significant association between elevated mPAP and reduced diffusing capacity of carbon monoxide (DL_{CO}) or reduced 6-minute walk distance (6MWD) (Figures E1A and E1B). These observations further support the notion of augmented hypoxemic conditions as a stimulus for the development of PH in patients with IPF. DL_{CO} is a measure of gas exchange capacity of the lungs, and is considered an important clinical marker of hypoxemia in patients. Transcript levels for HIF-1A and HIF-2A did not show significant changes in expression between IPF and IPF plus PH datasets (Figures E2A and E2B). Independent channels for our staining for HIF-1A are shown in the online supplement (Figure E2C).

Stabilization of HIF-1A has also been reported in conditions of normoxia, due to alterations in succinate metabolism (21–23). To determine the extent of succinate metabolism, we assessed levels of succinate dehydrogenase (SDH) complex, subunit A (SDHA), a key enzyme in the degradation of succinate to fumarate (21–23), from patients with IPF and an mPAP of 25 mm Hg or less (no PH) and patients with IPF with a mPAP 25 mm Hg or greater (IPF + PH). We found reduced levels of SDHA in patients with IPF plus PH (Figure 3A), consistent with reduced succinate metabolism (SDH activity, Figure 3B), and an enhanced accumulation of succinate in patients with IPF plus PH compared with healthy control subjects and patients with IPF alone (Figure 3C). Interestingly, our data

show a significant correlation between HIF-1A and succinate levels in patients with IPF with or without PH (Figure 3D). Experiments measuring complex IV and pyruvate dehydrogenase did not reveal significant differences between the groups (Figure E3). Elevated succinate levels have been implicated as a mechanism for HIF-1A stabilization devoid of hypoxia that is mediated through reduced ability of prolyl-hydroxylases to bind to HIF-1A (24). In support of this, our results show reduced levels of PHD1 in patients with IPF plus PH compared with normal control subjects (Figure 3E). No changes in PHD2/3 levels were observed (data not shown). Expression of genes associated with mitochondrial regulation (25) revealed a marked reduction in peroxisome proliferator-activated receptor γ , coactivator 1 α (PPARG1A) and mitofusin 2 (MFN2) expression levels between normal control subjects and patients with IPF or patients with IPF plus PH; however, no differences were seen between IPF and IPF plus PH samples (Figure 4). These results suggest alterations in mitochondrial biology in diseased tissue that may account for alterations in mitochondrial metabolism in these samples.

Adenosine Metabolism Is Reduced in Patients with IPF and PH

HIF-1A is known to induce expression of CD73, an ectopic enzyme involved in the generation of adenosine (26) (Figure 2D). Therefore, we examined the extent of adenosine metabolism in patients with IPF alone and IPF plus PH. After cellular injury, ATP is released extracellularly and then catabolized to AMP by CD39; CD73 is then involved in the catabolism of AMP to adenosine, which is then broken down by adenosine deaminase (ADA) to inosine (Figure 5A). We report a correlation between increased transcripts for CD39 and mPAP (Figure 5B); enzyme kinetic studies from lung homogenates for CD73 and ADA revealed heightened activity of CD73 and reduced activity for ADA that correlated with elevated mPAP values (Figures 5C and 5D). IHC for CD73 revealed increased signals for CD73 in IPF sections and a further increase in IPF plus PH sections in areas surrounding remodeled vessels (Figure 5E). We also report a significant correlation between

Figure 1. Fibrosis and vascular remodeling in idiopathic pulmonary fibrosis (IPF) and pulmonary hypertension (PH). (A) Masson's trichrome–stained lung sections showing parenchymal areas from three distinct patients with IPF (*top three panels*) or IPF plus PH (*bottom three panels*). Scale bar represents 100 μ m. (B) Ashcroft scores from upper and lower lobes from patients with IPF or IPF plus PH. (C) Collagen 1A1 (COL1A1) expression levels relative to 18s ribosomal RNA (18sRNA) expression levels from patients with IPF or IPF plus PH. (D) Masson's trichrome–stained lung sections showing representative vessels from three distinct patients with IPF (*top three panels*) or IPF plus PH (*bottom three panels*). Scale bar represents 100 μ m. (E) Vessel remodeling assessed by measuring the distance between the adventitia and the lumen. (F) Mean pulmonary arterial pressure (mPAP) levels from patients with IPF or IPF plus PH. Results are presented as means $\pm$ SEM. Significance level: *** $P < 0.001$ refers to t -test comparisons between IPF and IPF plus PH groups.

HIF-1A levels and CD73 activity in patients with IPF with and without PH (Figure 5F). Additional statistical analysis revealed significant differences in CD73, but not ADA activity, between patients with IPF and patients with IPF plus PH (Figures E4A and E4B). These results suggest that the capacity for adenosine accumulation is heightened in patients with PH secondary to IPF. Although adenosine levels would be ideal, adenosine is rapidly metabolized by ADA and adenosine kinase, and thus it is not possible for us to accurately measure levels in explanted tissue; in addition, it would be impossible to discern

between intra- and extracellular levels of adenosine.

Altered Equilibrative Nucleotide Transporter Expression Is Observed in Patients with IPF and PH

An important mechanism involved in reducing extracellular levels of adenosine is the cellular uptake of adenosine by equilibrative nucleotide transporters (ENTs) (27). Immunoblots for ENT1 revealed a reduction in ENT1 protein expression in patients with IPF plus PH compared with patients with IPF alone (Figure 6A). These observations were consistent with IHC for ENT1 showing decreased ENT1 staining in

patients with IPF plus PH compared with patients with IPF alone (Figure 6B). These findings further support the accumulation of extracellular adenosine as a mechanism of disease progression in IPF.

ADORA2B Expression Correlates with Elevated mPAP Values

Adenosine can activate several G protein–coupled receptors that include ADORA1, ADORA2A, ADORA2B, and ADORA3 (28). We examined transcript levels of each receptor by RT-PCR from RNA extracted from lung homogenates and performed a Pearson's correlation and linear regression against mPAP. No

Figure 2. Representations of the molecular markers of hypoxia in PH secondary to IPF. (A) Diagram of the hypoxia pathway. (B) Immunoblot for hypoxia-inducible factor (HIF)-1A and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from lung lysates from IPF and IPF plus PH lung tissue. (C) Immunofluorescence for α-smooth muscle actin (α-SMA; green signals), HIF-1A (red signals), and counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue signals). Scale bar represents 50 μm. Stabilization and nuclear localization of HIF-1A is shown in purple (white arrowheads). HIF-1A levels determined from ELISA from normal controls, IPF, and IPF plus PH lung tissue (D). Expression levels normalized to control lung of CD73 (E), GLUT1 (F), NOS3 (G), PAI1 (H), and PDK1 (I) from patients with IPF and patients with IPF plus PH. Results are presented as means ± SEM. (D) Significance levels: ***P < 0.001 and *P < 0.05 refers to ANOVA comparisons between control and IPF or IPF plus PH groups. #P < 0.05 refers to ANOVA comparisons between IPF and IPF plus PH groups. (E–I) Significance level: *P < 0.05 refers to t-test comparisons between IPF and IPF plus PH groups.

Figure 3. Succinate metabolism is altered in IPF plus PH. (A) Immunoblot from lung lysates comparing control against IPF and patients with IPF plus PH for succinate dehydrogenase (SDHA; succinate dehydrogenase complex, subunit A) and GAPDH as a control. (B) SDH activity in isolated mitochondrial extracts and (C) succinate levels determined from lung lysates from control subjects, patients with IPF, and patients with IPF plus PH. (D) Simple linear regression with 95% confidence interval (CI) showing the relationship between HIF-1A and succinate levels ($r^2 = 0.1524$ and $P = 0.0329$). (E) Immunoblot from lung lysates comparing control subjects against patients with IPF and patients with IPF plus PH for prolyl-hydroxylase-1 (PHD1) and GAPDH as a control. Results are presented as means $\pm$ SEM. Significance levels: *** $P < 0.001$ and * $P < 0.05$ refers to ANOVA comparisons between control and IPF or IPF plus PH groups. ## $0.001 < P < 0.01$ and # $P < 0.05$ refers to ANOVA comparisons between IPF and IPF plus PH groups.

significant correlation was observed between ADORA1, ADORA2A (albeit a downward trend), or ADORA3 and mPAP levels (Figures 6A–6C). These observations are also seen in supplementary statistical analysis, where significant changes between IPF and IPF plus PH cohorts are only reported for ADORA2B, but not for the other adenosine receptors (Figures E5A–E5D). However, increased ADORA2B transcripts correlated with elevated mPAP values and reduced 6MWD (Figures 7D and 7E). These findings are supported by immunoblots from lung homogenates for ADORA2B showing increased ADORA2B protein deposition in patients with IPF plus PH compared with those with IPF alone (Figure 7F). Our results implicate a role for ADORA2B in the pathogenesis of PH secondary to lung fibrosis, as first suggested in an experimental model of PH secondary to lung fibrosis (29).

Discussion

A major finding of our study was that the presence of PH secondary to IPF correlated

with physiological and molecular markers of hypoxia that were independent of the degree of fibrosis. These markers included an enhanced evidence of HIF-1A stabilization in patients with IPF plus PH and a significant correlation between mPAP, DL_{CO} , and 6MWD. Our data also indicate an up-regulation of the adenosinergic axis in PH secondary to IPF, leading to enhanced accumulation of adenosine and expression of its receptor, ADORA2B, a receptor involved in aberrant repair processes in the lung (13, 29, 30). A novel finding of our study was decreased mitochondrial metabolism in patients with IPF plus PH, as evidenced by reduced expression levels of PPARG1A and MFN2, diminished activity of SDH, dampened expression of SDHA, and the subsequent accumulation of succinate, a protein that has been shown to inhibit PHDs, leading to further stabilization of HIF-1A (21–23). Interestingly, our data show a strong correlation between increased HIF-1A protein levels and elevated succinate levels that, in turn, are linked with reduced PHD1 levels in patients with IPF plus PH. Taken

together, our results suggest that impaired succinate metabolism is an additional mechanism that stabilizes HIF-1A in addition to hypoxic conditions. This heightened stabilization of HIF-1A leads to enhanced activation of the hypoxic-adenosinergic axis, including augmented adenosine accumulation through changes in enzymatic activity favoring production of adenosine that leads to engagement of ADORA2B, a receptor that has been shown to modulate vascular remodeling and PH (16, 29).

The development of PH is considered to be one of the most significant markers of increased morbidity and mortality in patients with CLDs (3, 31). Despite the increased mortality due to PH, there are no effective clinical or molecular markers that can be used to predict or evaluate its development or progression (5, 7, 32, 33). In line with this, we report no significant correlations between mPAP values and pulmonary function tests or blood gas values, a phenomenon that has been reported previously and has been ascribed to a lower sensitivity of these tests to detect

Figure 4. Markers of mitochondrial stability. Transcript expression levels of peroxisome proliferator-activated receptor γ , coactivator 1 α (PPARGC1A; A) and mitofusin 2 (MFN2; B) from normal, IPF, and IPF plus PH lung samples. Results are presented as means $\pm$ SEM. Significance levels: *** P < 0.001 refers to ANOVA comparisons between control and IPF or IPF plus PH groups.

changes in pulmonary vascular tone (34). However, in the present study, we have shown that elevated mPAP in patients with IPF inversely correlates with DL_{CO} and 6MWD, two clinical readouts that directly and indirectly assess the gas exchange capacity of the lungs. Reduced gas exchange (reduced DL_{CO}) is inexorably linked to compromised oxygen delivery; therefore, these results suggest hypoxemia as a potential stimulus leading

to the development of secondary PH in CLDs.

Although increased levels of HIF-1A have been previously documented in patients with IPF compared with normal control subjects (35), our study takes this observation further and reports enhanced stabilization of HIF-1A in patients with PH with underlying IPF. Importantly, we report increased HIF-1A signals in remodeled vessels of patients with IPF plus

PH compared with those with IPF alone. In addition, we report increased HIF-1A levels by ELISA between patients with IPF and normal individuals that are further enhanced in patients with IPF plus PH compared with individuals with IPF, but no PH. These observations point to a pathological role for enhanced HIF-1A stabilization in vascular remodeling, due to persistent hypoxemic conditions or increased succinate accumulation that leads to enhanced HIF-1A stabilization. In line with this, stabilization of HIF-1A resulting from altered mitochondrial metabolism has been reported to lead to resistance to apoptosis and cellular proliferation in cancer biology (36) through altered succinate metabolism and subsequent PHD inhibition (21–23). Similar alterations in metabolism have also been documented in PH (18), where several parallels between PH and cancer have been discussed (37). Consistent with these observations, we report a decrease in SDHA levels between IPF and IPF plus PH, a decline in SDH activity between control subjects and patients with IPF that is further

Figure 5. Adenosine metabolism deaminases and their correlation to mPAP. (A) Schematic of extracellular adenosine metabolism and the sequential activity of CD39 and CD73, along with adenosine deaminase (ADA), to degrade ATP to the final product of inosine. (B) Simple linear regression with 95% CI showing relationship between mPAP and cellular expression of CD39 ($r^2=0.2208$ and $P=0.057$). (C) Simple linear regression with 95% CI showing relationship between mPAP and CD73 ($r^2=0.2593$ and $P=0.044$). (D) Simple linear regression with 95% CI showing relationship between mPAP and ADA ($r^2=0.2503$ and $P=0.041$). (E) Immunohistochemistry for CD73 (blue signals) and α SMA; red signals from representative lung sections of a control subject (left), a patient with IPF (middle), or a patient with IPF plus PH (right). The scale bar represents 75 μ m. The solid arrowheads point to areas rich in CD73 signals. Simple linear regression with 95% CI showing the relationship between HIF-1A levels and CD73 activity ($r^2=0.4031$ and $P=0.0082$) (F). Ado, adenosine; Ino, inosine.

Figure 6. Equilibrative nucleotide transporter (ENT) 1 involvement in patients with IPF and patients with IPF plus PH. (A) Immunoblot for ENT1 and GAPDH from normal (control), IPF, and IPF plus PH lung lysates. (B) Immunohistochemistry for ENT1 staining (brown signals) showing its presence around vessels in control, IPF, and IPF plus PH lung sections. The scale bar represents 100 μm . The arrows point to the localization of ENT1 signals; “V” denotes a vessel.

down-regulated in PH associated with IPF, and subsequent succinate accumulation in lung homogenates. In support of these observations, we demonstrate a significant correlation between elevated HIF-1A and increased succinate levels measured by ELISA that are in line with reduced PHD1 expression in patients with IPF plus PH, as shown by immunoblots. Taken together, these results suggest altered metabolism and accumulation of succinate as an

additional mechanism in IPF that, together with hypoxia, lead to enhanced HIF-1A stabilization. Further support for altered mitochondrial function comes from transcript expression levels of PPARG1A and MFN2 showing a marked reduction of these genes that have been shown to contribute to mitochondrial fragmentation in animal models of PH and in patients with PAH (25). PPARG1A is a transcriptional coactivator that plays a

central role in mitochondrial biogenesis and cellular stability (25, 38). It has also been shown to protect mitochondria from reactive oxygen species generated during respiration; thus, depletion of PPARG1A could lead to mitochondrial dysfunction by preventing mitochondrial biogenesis and though reduced reactive oxygen species detoxification (38). Although no significant differences were observed for PPARG1A and MFN2 between IPF and IPF plus PH, we believe that prolonged depletion of PPARG1A levels and subsequent mitochondrial dysfunction in IPF may lead to reduced SDH activity and, ultimately, to the development of PH in patients with IPF. Taken together, these results suggest that, in patients with CLD, such as IPF, increased mitochondrial fragmentation may lead to dysfunctional metabolism, contributing further to HIF-1A stabilization through the accumulation of succinate, leading to vascular remodeling and the development of PH.

Stabilization of HIF-1A is known to lead to the activation of the adenosinergic axis that is characterized by increased CD39 and CD73 expression and reduced expression of ENTs that, together, drive the extracellular accumulation of adenosine (26, 27). In acute conditions,

Figure 7. Simple linear regression with 95% CI to evaluate relationship between different adenosine receptors and mPAP. (A) Relationship between adenosine receptor (ADORA) 1 and mPAP ($r^2 = 0.0485$ and $P = 0.38$). (B) Association between ADORA2A and mPAP ($r^2 = 0.1220$ and $P = 0.155$). (C) Correlation between ADORA3 and mPAP ($r^2 = 0.0554$ and $P = 0.38$). (D) Correlation between ADORA2B and mPAP ($r^2 = 0.1685$ and $P = 0.037$). (E) Correlation between ADORA2B and 6-minute walk distance (6MWD) ($r^2 = 0.2422$ and $P = 0.023$). (F) Immunoblot of patients with IPF and patients with IPF plus PH evaluating ADORA2B, with β -actin as a control.

stimulation of this pathway is protective and leads to antiinflammatory and tissue repair processes (13). However, sustained activation of the adenosinergic axis has been observed in many chronic diseases, including IPF and COPD (13, 15, 39, 40). Both the acute and chronic effects of adenosine are mediated by increased ADORA2B expression, which is itself mediated by hypoxic conditions and HIF-1A stabilization (13, 16, 30). Consistent with these studies, we report a heightened capacity for adenosine accumulation and ADORA2B expression in patients with IPF plus PH compared with those with IPF alone, exemplified by increased CD39 expression that has also been recently reported in group I PH (41), elevated CD73 activity, and reduced

ADA activity. These changes are also accompanied by reduced ENT1 expression observed in immunoblots. Remarkably, IHC for ENT1 did not show positive signals for ENT1 in remodeled vessels from patients with IPF plus PH compared with control subjects, and showed dampened ENT1 signals in IPF vessels. Further support comes from our staining for CD73, showing markedly elevated signals in the parenchymal areas of the lung surrounding remodeled vessels in IPF plus PH. These observations are also in line with data showing a strong correlation between elevated HIF-1A levels and increased CD73 enzymatic activity. These findings provide further evidence for stabilization and subsequent accumulation of adenosine in the surrounding

remodeled vessel as a pathological process in PH associated with IPF.

In conclusion, our results point to altered metabolism and protracted exposure to hypoxic conditions as potential stimuli for the development of PH in underlying IPF, a process that appears to be modulated by chronic activation of the hypoxic-adenosinergic axis and subsequent engagement of ADORA2B. These findings are significant, as altered succinate metabolism could be used to track disease progression and to identify patients who could benefit from adenosine-based therapeutics. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

- Bjoraker JA, Ryu JH, Edwin MK, Myers JL, Tazelaar HD, Schroeder DR, Offord KP. Prognostic significance of histopathologic subsets in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 1998;157:199–203.
- Latsi PI, du Bois RM, Nicholson AG, Colby TV, Bisirtzoglou D, Nikolakopoulou A, Veeraraghavan S, Hansell DM, Wells AU. Fibrotic idiopathic interstitial pneumonia: the prognostic value of longitudinal functional trends. *Am J Respir Crit Care Med* 2003;168:531–537.
- Behr J, Ryu JH. Pulmonary hypertension in interstitial lung disease. *Eur Respir J* 2008;31:1357–1367.
- Nathan SD, Noble PW, Tuder RM. Idiopathic pulmonary fibrosis and pulmonary hypertension: connecting the dots. *Am J Respir Crit Care Med* 2007;175:875–880.
- Poor HD, Girgis R, Studer SM. World health organization group III pulmonary hypertension. *Prog Cardiovasc Dis* 2012;55:119–127.
- Lettieri CJ, Nathan SD, Barnett SD, Ahmad S, Shorr AF. Prevalence and outcomes of pulmonary arterial hypertension in advanced idiopathic pulmonary fibrosis. *Chest* 2006;129:746–752.
- Patel NM, Lederer DJ, Borczuk AC, Kawut SM. Pulmonary hypertension in idiopathic pulmonary fibrosis. *Chest* 2007;132:998–1006.
- Blackwell TS, Tager AM, Borok Z, Moore BB, Schwartz DA, Anstrom KJ, Bar-Joseph Z, Bitterman P, Blackburn MR, Bradford W, et al. Future directions in idiopathic pulmonary fibrosis research: an NHLBI workshop report. *Am J Respir Crit Care Med* 2014;189:214–222.
- King TE Jr, Pardo A, Selman M. Idiopathic pulmonary fibrosis. *Lancet* 2011;378:1949–1961.
- Raghu G, Collard HR, Egan JJ, Martinez FJ, Behr J, Brown KK, Colby TV, Cordier JF, Flaherty KR, Lasky JA, et al.; ATS/ERS/JRS/ALAT Committee on Idiopathic Pulmonary Fibrosis. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. *Am J Respir Crit Care Med* 2011;183:788–824.
- Ley B, Collard HR, King TE Jr. Clinical course and prediction of survival in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2011;183:431–440.
- Eckle T, Kewley EM, Brodsky KS, Tak E, Bonney S, Gobel M, Anderson D, Glover LE, Riegel AK, Colgan SP, et al. Identification of hypoxia-inducible factor HIF-1A as transcriptional regulator of the A2B adenosine receptor during acute lung injury. *J Immunol* 2014;192:1249–1256.
- Karmouty-Quintana H, Xia Y, Blackburn MR. Adenosine signaling during acute and chronic disease states. *J Mol Med (Berl)* 2013;91:173–181.
- Kong T, Westerman KA, Faigle M, Eltzschig HK, Colgan SP. HIF-dependent induction of adenosine A2B receptor in hypoxia. *FASEB J* 2006;20:2242–2250.
- Zhou Y, Murthy JN, Zeng D, Belardinelli L, Blackburn MR. Alterations in adenosine metabolism and signaling in patients with chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis. *PLoS One* 2010;5:e9224.
- Karmouty-Quintana H, Zhong H, Acero L, Weng T, Melicoff E, West JD, Hemnes A, Grenz A, Eltzschig HK, Blackwell TS, et al. The A2B adenosine receptor modulates pulmonary hypertension associated with interstitial lung disease. *FASEB J* 2012;26:2546–2557.
- Eckle T, Brodsky K, Bonney M, Packard T, Han J, Borchers CH, Mariani TJ, Kominsky DJ, Mittelbronn M, Eltzschig HK. HIF1A reduces acute lung injury by optimizing carbohydrate metabolism in the alveolar epithelium. *PLoS Biol* 2013;11:e1001665.
- Zhao Y, Peng J, Lu C, Hsin M, Mura M, Wu L, Chu L, Zamel R, Machuca T, Waddell T, et al. Metabolomic heterogeneity of pulmonary arterial hypertension. *PLoS One* 2014;9:e88727.
- Pellegrino R, Viegi G, Brusasco V, Crapo RO, Burgos F, Casaburi R, Coates A, van der Grinten CPM, Gustafsson P, Hankinson J, et al. Interpretative strategies for lung function tests. *Eur Respir J* 2005;26:948–968.
- Ashcroft T, Simpson JM, Timbrell V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *J Clin Pathol* 1988;41:467–470.
- Koivunen P, Hirsilä M, Remes AM, Hassinen IE, Kivirikko KI, Myllyharju J. Inhibition of hypoxia-inducible factor (HIF) hydroxylases by citric acid cycle intermediates: possible links between cell metabolism and stabilization of HIF. *J Biol Chem* 2007;282:4524–4532.
- Koivunen P, Lee S, Duncan CG, Lopez G, Lu G, Ramkissoon S, Losman JA, Joensuu P, Bergmann U, Gross S, et al. Transformation by the (R)-enantiomer of 2-hydroxyglutarate linked to EGLN activation. *Nature* 2012;483:484–488.
- Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson DG, Mansfield KD, Pan Y, Simon MC, Thompson CB, Gottlieb E. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF- α prolyl hydroxylase. *Cancer Cell* 2005;7:77–85.
- Mills E, O'Neill LA. Succinate: a metabolic signal in inflammation. *Trends Cell Biol* 2014;24:313–320.
- Ryan JJ, Marsboom G, Fang YH, Toth PT, Morrow E, Luo N, Piao L, Hong Z, Ericson K, Zhang HJ, et al. PGC1 α -mediated mitofusin-2 deficiency in female rats and humans with pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2013;187:865–878.
- Synnestvedt K, Furuta GT, Comerford KM, Louis N, Karhausen J, Eltzschig HK, Hansen KR, Thompson LF, Colgan SP. Ecto-5'-nucleotidase (CD73) regulation by hypoxia-inducible factor-1

- mediates permeability changes in intestinal epithelia. *J Clin Invest* 2002;110:993–1002.
27. Eltzschig HK, Abdulla P, Hoffman E, Hamilton KE, Daniels D, Schönfeld C, Löffler M, Reyes G, Duszenko M, Karhausen J, *et al.* HIF-1-dependent repression of equilibrative nucleoside transporter (ENT) in hypoxia. *J Exp Med* 2005;202:1493–1505.
 28. Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV: nomenclature and classification of adenosine receptors. *Pharmacol Rev* 2001;53: 527–552.
 29. Karmouty-Quintana H, Weng T, Garcia-Morales LJ, Chen NY, Pedroza M, Zhong H, Molina JG, Bunge R, Bruckner BA, Xia Y, *et al.* Adenosine A2B receptor and hyaluronan modulate pulmonary hypertension associated with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 2013;49:1038–1047.
 30. Sun CX, Zhong H, Mohsenin A, Morschl E, Chunn JL, Molina JG, Belardinelli L, Zeng D, Blackburn MR. Role of A2B adenosine receptor signaling in adenosine-dependent pulmonary inflammation and injury. *J Clin Invest* 2006;116:2173–2182.
 31. Chaouat A, Naeije R, Weitzenblum E. Pulmonary hypertension in COPD. *Eur Respir J* 2008;32:1371–1385.
 32. Nadrous HF, Pellikka PA, Krowka MJ, Swanson KL, Chaowalit N, Decker PA, Ryu JH. Pulmonary hypertension in patients with idiopathic pulmonary fibrosis. *Chest* 2005;128:2393–2399.
 33. Pitsiou G, Papakosta D, Bouros D. Pulmonary hypertension in idiopathic pulmonary fibrosis: a review. *Respiration* 2011;82: 294–304.
 34. Nathan SD, Shlobin OA, Ahmad S, Urbanek S, Barnett SD. Pulmonary hypertension and pulmonary function testing in idiopathic pulmonary fibrosis. *Chest* 2007;131:657–663.
 35. Tzouveleakis A, Harokopos V, Paparountas T, Oikonomou N, Chatziioannou A, Vilaras G, Tsiambas E, Karameris A, Bouros D, Aidinis V. Comparative expression profiling in pulmonary fibrosis suggests a role of hypoxia-inducible factor-1alpha in disease pathogenesis. *Am J Respir Crit Care Med* 2007;176:1108–1119.
 36. Semenza GL. HIF-1 mediates metabolic responses to intratumoral hypoxia and oncogenic mutations. *J Clin Invest* 2013;123: 3664–3671.
 37. Guignabert C, Tu L, Le Hire M, Ricard N, Sattler C, Seferian A, Huertas A, Humbert M, Montani D. Pathogenesis of pulmonary arterial hypertension: lessons from cancer. *Eur Respir Rev* 2013;22: 543–551.
 38. Austin S, St-Pierre J. PGC1 α and mitochondrial metabolism—emerging concepts and relevance in ageing and neurodegenerative disorders. *J Cell Sci* 2012;125:4963–4971.
 39. Esther CR Jr, Lazaar AL, Bordonali E, Qaqish B, Boucher RC. Elevated airway purines in COPD. *Chest* 2011;140:954–960.
 40. Goodarzi MT, Abdi M, Tavilani H, Nadi E, Rashidi M. Adenosine deaminase activity in COPD patients and healthy subjects. *Iran J Allergy Asthma Immunol* 2010;9:7–12.
 41. Visovatti SH, Hyman MC, Bouis D, Neubig R, McLaughlin VV, Pinsky DJ. Increased CD39 nucleotidase activity on microparticles from patients with idiopathic pulmonary arterial hypertension. *PLoS One* 2012;7:e40829.