# Adenosine A2B Receptor and Hyaluronan Modulate Pulmonary Hypertension Associated with Chronic Obstructive Pulmonary Disease

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**Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death worldwide. The development of pulmonary hypertension (PH) in patients with COPD is strongly associated with increased mortality. Chronic inflammation and changes to the lung extracellular matrix (ECM) have been implicated in the pathogenesis of COPD, yet the mechanisms that lead to PH secondary to COPD remain unknown. Our experiments using human lung tissue show increased expression levels of the adenosine $A_{2B}$ receptor (ADORA2B) and a heightened deposition of hyaluronan (HA; a component of the ECM) in remodeled vessels of patients with PH associated with COPD. We also demonstrate that the expression of HA synthase 2 correlates with mean pulmonary arterial pressures in patients with COPD, with and without a secondary diagnosis of PH. Using an animal model of airspace enlargement and PH, we show that the blockade of ADORA2B is able to attenuate the development of a PH phenotype that correlates with reduced levels of HA deposition in the vessels and the down-regulation of genes involved in the synthesis of HA.**

**Keywords**: adenosine; extracellular matrix; hyaluronic acid; remodeling; vascular

Chronic obstructive pulmonary disease (COPD) is currently the fourth leading cause of death worldwide, and the World Health Organization predicts that it will become the third leading cause of death by 2030 (1). COPD is a heterogeneous disease characterized by airflow obstruction that is not fully reversible. Pathophysiological hallmarks of the disease include remodeling of the small-airway compartment, the loss of elastic recoil by emphysematous destruction of the parenchyma, inflammatory cell infiltration (2), and increased extracellular matrix (ECM) turnover (3). COPD is associated with a wide range of comorbidities, including ischemic heart disease, diabetes, skeletal muscle wasting, osteoporosis, and lung cancer (4). The development of pulmonary hypertension (PH) is a common and fatal complication in patients with COPD (5–7), and is strongly associated with decreased life expectancy (8).

PH is a disorder of the pulmonary vasculature diagnosed by cardiac catheterization. PH is characterized by a mean pulmonary arterial pressure greater than or equal to 25 mm Hg that leads to right ventricular (RV) hypertrophy, followed by right-sided heart failure and death (9). Currently, treatment options are very limited for patients suffering from PH secondary to COPD (10, 11). Thus, to understand the mechanisms that lead to remodeling of the vasculature in COPD is important, with the hope of developing new treatment options for this fatal disorder.

The pathogenesis of PH in COPD is a complex phenomenon characterized by extensive remodeling of the vasculature that results from an increased proliferation of pulmonary artery endothelial and smooth muscle cells, the muscularization of previously nonmuscular arteries, increased vascular tone, and the formation of complex vascular lesions (12). Factors involved in the development of PH in patients with COPD include alveolar hypoxia, inflammation and emphysema that contribute to remodeling, vasoconstriction, and a reduction of the vascular bed (5). Several mediators have been implicated in the development of PH secondary to COPD, such as endothelin-1 (13) and IL-6 (14). In patients with idiopathic pulmonary arterial hypertension, the up-regulation of hyaluronan (HA), a major component of the lung ECM, has been associated with vascular remodeling (15, 16). These observations are of interest, because HA has been shown to play an important role during inflammation and fibrosis (17). However, it remains unknown whether HA plays a role in the pathogenesis of PH secondary to chronic lung diseases such as COPD or idiopathic pulmonary fibrosis (IPF).

Elements of the adenosine signaling system have been closely linked with the production of several mediators, including IL-6 (18, 19), endothelin-1 (18), HA (20, 21), and mediators associated with hypoxia (22, 23). Adenosine is a nucleoside that exerts its actions through G-protein–coupled receptors, including the adenosine 1, 2A,

## CLINICAL RELEVANCE

Our study shows that the adenosine A2B receptor (ADORA2B) and hyaluronan contribute to vascular remodeling and the development of pulmonary hypertension in chronic obstructive pulmonary disease (COPD). The inhibition of ADORA2B was able to attenuate hallmarks of pulmonary hypertension in an animal model of airspace enlargement and vascular remodeling. These findings provide new targets in the development of treatments for pulmonary hypertension in COPD, and contribute to our understanding of how the lung extracellular matrix and adenosine contribute to vascular remodeling in chronic lung diseases.

(*Received in original form February 26, 2013 and in final form June 25, 2013*)

This work was supported by a contract from Gilead Sciences, Inc. (M.R.B.).

*Author Contributions*: H.K.-Q., D.Z., L.B., H.S., and M.R.B. developed the study concept and design. L.J.G.-M., R.B., B.A.B., M.L., and H.S. obtained patient consent forms, collected clinical data, and provided patient samples in a deidentified manner. T.W., N.-Y.C., H.Z., M.P., and H.K.-Q. performed *in vivo* and *in vitro* experiments and analyzed the data. J.G.M. maintained, treated, and genotyped the *Ada*$^{-/-}$ mouse colony. Y.X. and R.A.J. provided expertise on cardiovascular physiology and lung function measurements. H.K.-Q. wrote the manuscript. All authors have read and approved the final manuscript.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

**Am J Respir Cell Mol Biol Vol 49, Iss. 6, pp 1038–1047, Dec 2013**

**Originally Published in Press as DOI: 10.1165/rcmb.2013-0089OC on July 15, 2013**
**Internet address: www.atsjournals.org**

2B, and 3 receptors (ADORA1, ADORA2A, ADORA2B, and ADORA3, respectively) (24). Adenosine is largely generated in response to cell injury, where it exerts both protective and detrimental effects (18, 19, 25). In the context of chronic lung disease, the engagement of ADORA2B via elevated adenosine levels has been implicated in disease progression and tissue remodeling (19, 26). Indeed, increased levels of ADORA2B transcript levels are observed in lung samples from patients with COPD and IPF (27). *In vivo* experiments also demonstrated that the genetic and pharmacological blockade of ADORA2B attenuates the development of fibrosis and PH in an experimental model of fibrosis (18, 19, 25). However, it remains unknown whether adenosine (and in particular, ADORA2B) plays a role in the pathogenesis of PH secondary to COPD.

The goals of this study were to determine whether ADORA2B and HA are increased in PH secondary to COPD, and whether HA production is modulated by ADORA2B. To explore this hypothesis, experiments were performed with human lung explants from patients with COPD, with or without PH, and in an animal model of adenosine-driven chronic lung disease exhibiting airspace enlargement, inflammation, and hallmarks of PH.

## MATERIALS AND METHODS

More detailed methods can be found in the online supplement.

### Subjects

The use of human material for this study was reviewed by the Committee for the Protection of Human Subjects at the University of Texas Health Science Center (Houston, TX). All samples were deidentified. Patients were classified as exhibiting or not exhibiting PH, based on mean pulmonary arterial pressure (mPAP) data collected before transplantation. In total, 13 patients were studied (six without PH, and seven with PH).

### Mice

Adenosine deaminase–deficient ($Ada^{-/-}$) mice were generated and genotyped as described previously (19). Mice homozygous for the *Ada*-null allele were designated $Ada^{-/-}$, whereas control mice, designated $Ada^{+}$, were heterozygous for the *Ada*-null allele. Animal care was conducted in accordance with institutional and National Institutes of Health guidelines. All experiments were reviewed and approved by the Houston Animal Welfare Committee at the University of Texas Health Science Center.

### Experimental Design

$Ada^{-/-}$ mice were identified at birth, and maintained on ADA enzyme therapy from postnatal Day 2 until postnatal Day 30. Chow containing 3-ethyl-1-propyl-8-(1-(3-trifluoromethylbenzyl)-1H-pyrazol-4-yl)-3,7-dihydropurine-2,6-dione (GS-6201) (10 mg/kg/d) or vehicle (Teklad; Harlan Industries, Indianapolis, IN) was supplied starting on Day 21 and for the duration of the experiment. On Day 41, physiological readouts were performed, and the animals were killed for the collection of tissues and fluids for analysis.

### Physiological Measurements

Arterial oxygen saturation, right ventricle systolic pressure (RVSP), heart rate, and RV hypertrophy and lung function measurements were performed as described previously (18).

### Histology and Immunohistochemistry

Lung tissue was processed for histological analysis and stained with Masson trichrome. Immunohistochemistry was performed on 5-μm sections, as described previously (18). To detect HA, sections were incubated with biotinylated HA-binding protein (HABP; Calbiochem, Darmstadt, Germany) overnight at 4°C, and then incubated with the ABC kit (Vector Labs, Burlingame, CA) and developed with Vector Red (Vector Labs). For immunofluorescence, human sections were incubated with α–smooth muscle actin (α-SMA; Sigma-Aldrich, St. Louis, MO) and incubated with Alexa Fluor 555 (donkey anti-mouse; Life Technologies, Carlsbad, CA) or Alexa Fluor 488 (chicken anti-mouse; Life Technologies). Sections were finally counterstained with 4′6-diamidino-2-phenylindole and mounted.

### Morphometry

The methods used to evaluate the extent of vessel muscularization and the number of muscularized vessels in our animal model was based on previously published experiments (18).

### RT-PCR and Protein Expression

Specific transcript levels for mouse ADORA2B, HA synthase (HAS)–1, HAS-2, HAS-3, and $\alpha_1$-procollagen were determined by normalization to 18 s ribosomal RNA (human samples) or apolipoprotein B (mouse samples), and are presented as mean normalized transcript levels using the comparative Ct method ($2\Delta\Delta Ct$). Western blots for ADORA2B (H-40; Santa Cruz Biotechnology, Santa Cruz, CA), ADORA2A (ab115250; antibodies were obtained from Abcam, Cambridge, MA), and HAS-2 (Y-14; Santa Cruz Biotechnology) were performed using lung lysates of human tissue samples (ADORA2B and ADORA2A) or whole mouse lung lysates (HAS-2).

### Expression of HAS-1 in Human Pulmonary Arterial Smooth Muscle Cells

Primary normal human pulmonary arterial smooth muscle cells were obtained from Lonza (Basel, Switzerland) and cultured according to the manufacturer's instructions. Cells were incubated in serum-free basal medium, with or without 5'-(N-ethylcarboxamido) adenosine (NECA) (10 μM) and GS-6201 (100 nM) for 90 minutes. HAS-1 expression was normalized to the average expression levels of housekeeping genes, including hypoxanthine phosphoribosyltransferase 1 (HPRT1), glyceraldehyde 3–phosphate dehydrogenase, and β-actin.

## RESULTS

### Vascular Remodeling in Patients with COPD

PH is associated with vascular remodeling in the lung. These changes include increased vascular smooth muscle mass and the neomuscularization of previously nonmuscular vessels, leading to vessel occlusion (28). In our study, lung sections of patients diagnosed with COPD, with or without a secondary diagnosis of PH, were stained with α-SMA. Our results showed that patients with COPD and an mPAP greater than 25 mm Hg (a hallmark of PH) displayed thickened smooth muscle vascular walls, compared with patients with COPD but an mPAP of less than 25 mm Hg (Figures 1A and 1B). Interestingly, vascular remodeling was observed primarily in fibrotic areas of the lung, where collagen deposition was increased (Figure 1C). These observations suggest a link between vascular remodeling and increased mPAP in patients with COPD.

### Disease Severity Correlates with Increased ADORA2B and Collagen 1A Transcript Levels

Increased transcript levels of ADORA2B and collagen 1A1 (Col1A1) have been previously reported in patients diagnosed with COPD and IPF, compared with patients at Stage 0 COPD and with mild IPF (27). However, whether these mediators were elevated further in patients with PH associated with COPD was not investigated. In the present study, we found that patients with PH secondary to COPD exhibited elevated mRNA and protein levels of ADORA2B, compared with COPD patients without PH (Figure 2A). Increased transcript levels of Col1A1 were also observed in patients with COPD and PH versus patients with COPD but without PH (Figure 2B). To investigate the association between increased mPAP and

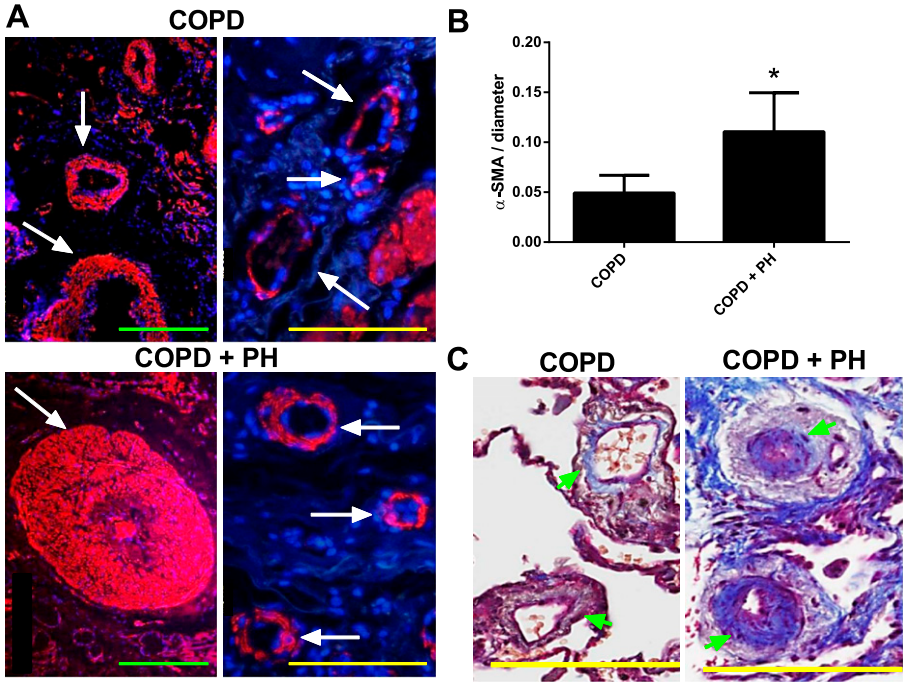


***Figure 1.*** Vascular remodeling in patients with chronic obstructive pulmonary disease (COPD), with or without pulmonary hypertension (PH). (*A*) Immunofluorescently stained lung sections of patients with COPD (*top*) or COPD + PH (*bottom*) for α–smooth muscle actin (α-SMA) (*red*), and counterstained with 4′6-diamidino-2-phenylindole (DAPI; *blue*). *Arrows* indicate α-SMA–positive areas of lung arterioles. *Green scale bars* represent 500 μm, and *yellow scale bars* represent 200 μm. (*B*) Extent of α-SMA present in five vessels for each patient. Results are presented as means ± SEMs ($n = 6$–7 for each group, i.e., COPD or COPD + PH). *$P < 0.05$ refers to comparisons between the COPD and the COPD + PH groups. (*C*) Lung sections of patients with COPD (*left*) or COPD + PH (*right*) were stained with Masson trichrome. Areas positive for collagen stained *blue*, and vessels are indicated by *arrows*. *Scale bars* represent 200 μm.

ADORA2B or Col1A1 transcript levels, associations between mRNA expression levels and mPAP were performed, using linear regression. Both ADORA2B and Col1A1 levels demonstrated a significant correlation with mPAP levels (Figures 2C and 2D). Experiments aimed at elucidating ADORA2A protein expression levels showed no difference between COPD patients with or without PH (*see* Figure E1 in the online supplement). These findings demonstrate that ADORA2B and Col1A1 may be used as molecular markers to track disease severity in patients with COPD as they develop increased mPAPs, as well as suggest that ADORA2B signaling may be involved in this disease.

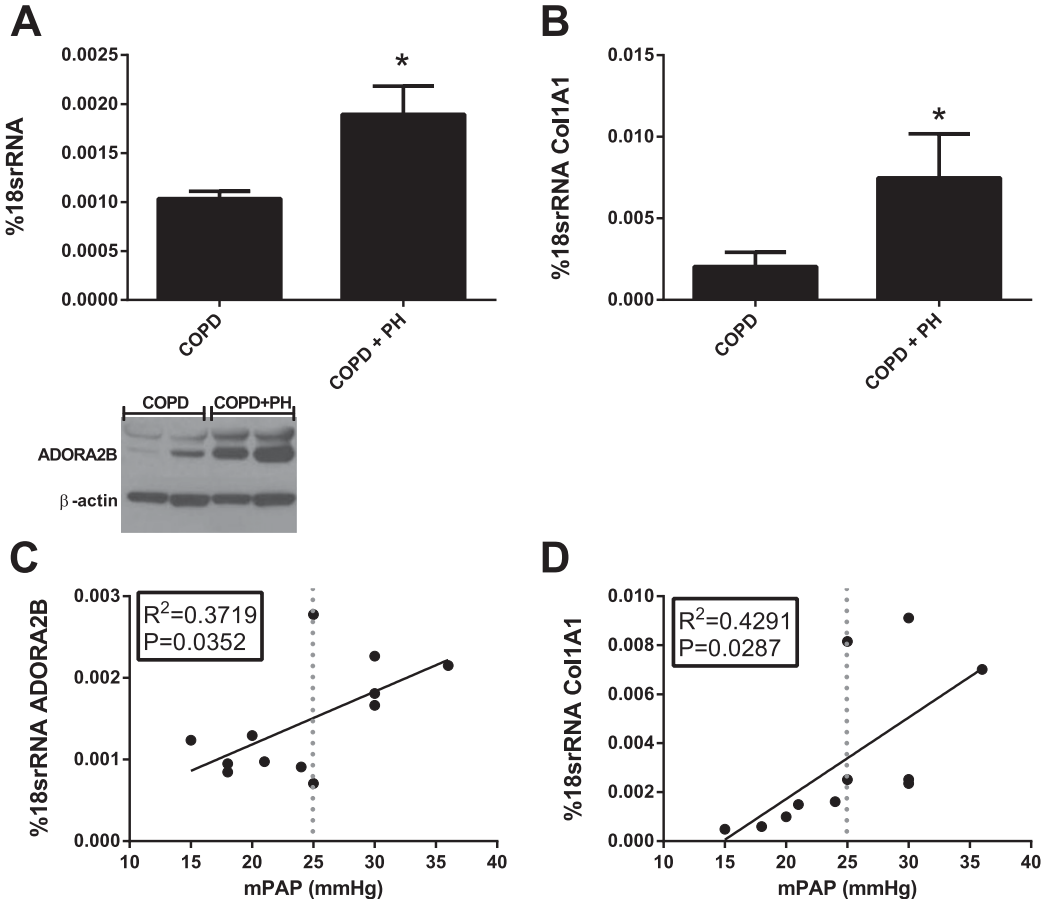


***Figure 2.*** Transcript levels of the adenosine A2B receptor (ADORA2B) and α-procollagen (Col1A1) from patients with COPD or COPD + PH, and associations with mean pulmonary arterial pressure (mPAP). Patients with PH were identified as those with an mPAP less than or equal to 25 mm Hg. Transcript levels for ADORA2B (*A*) and Col1A1 (*B*) were normalized to 18 s rRNA of patients with COPD or COPD + PH. Pearson correlation and linear regression analyses were performed for associations between ADORA2B (*C*) or Col1A1 (*D*) and mPAPs for all patients with COPD (with and without PH). ADORA2B levels correlated strongly with mPAP (*C*; $R^2 = 0.3719$, $P = 0.0352$), as did Col1A1 levels (*D*; $R^2 = 0.4291$, $P = 0.0287$). Data on or right of the *dotted line* represent those with PH (mean PAP ⩽ 25 mm Hg; $n = 6$–7 patients per group). *$P < 0.05$.

### Airspace Enlargement and Vascular Remodeling in a Mouse Model of Airspace Enlargement

To examine the mechanisms that lead to increased vascular remodeling and PH secondary to COPD, we used $Ada^{-/-}$ mice that exhibited airspace enlargement (a feature of emphysematous COPD), vascular remodeling, inflammation, and lung fibrosis (19). Adenosine deaminase (ADA) is an enzyme that metabolizes adenosine into inosine, and reduced ADA activity has been reported in patients with COPD (27). Mice lacking ADA demonstrate increased levels of adenosine in the lung (19) and the phenotype of COPD. In the present study, formalin-fixed and paraffin-embedded (FFPE) lung sections from ADA-competent ($Ada^{+}$), $Ada^{-/-}$, or $Ada^{-/-}$ mice treated with GS-6201 (an ADORA2B antagonist) were stained with hematoxylin-and-eosin or dual immunohistochemistry for α-SMA and the proliferation marker Ki67. These experiments revealed airspace enlargement and thickening of the vascular smooth muscle wall that was accompanied by an increased proliferation of cells within the vascular wall in $Ada^{-/-}$ mice, which was reversed after treatment with the ADORA2B antagonist GS-6201 (Figure 3). These findings highlight the involvement of ADORA2B signaling in modulating remodeling processes in the lung.

### Effects of an ADORA2B Antagonist on Cardiovascular Physiology in the $Ada^{-/-}$ Mouse

We next investigated the effects of elevated adenosine and ADORA2B blockade on the cardiovascular system. RVSP measurements (a common readout to identify high blood pressures in the lung) (29) and the extent of right ventricle hypertrophy (RVH) were analyzed. Our results showed that $Ada^{-/-}$ mice developed an increased RVSP, together with heightened RVH values (Figures 4A and 4B). These increased hallmarks of PH were significantly reduced after treatment with GS-6201 (Figures 4A and 4B). Measurements of heart rate showed no significant changes between different treatment groups (Figure 4C). A morphometric analysis of FFPE sections stained with α-SMA revealed both an increased muscularization of vessels and a greater number of muscularized vessels in $Ada^{-/-}$ compared with $Ada^{+}$ mice. GS-6201 was able to inhibit the extent of α-SMA–positive area in vessels (muscularization), but the number of α-SMA–positive vessels did not change (Figures 4D and 4E). Similarly, mean linear intercept analysis from hematoxylin-and-eosin–stained sections revealed increased airspace enlargement in $Ada^{-/-}$ mice compared with control mice, and this enlargement was attenuated after GS-6201 treatment (Figure 4F). No changes in systemic blood pressure were apparent between treatment groups (data not shown). These findings demonstrate the effects of GS-6201 on attenuating the cardiovascular changes associated with PH in a model of chronic lung injury exhibiting airspace enlargement and vascular remodeling.

### Lung Function in $Ada^{-/-}$ Mice and the Effects of an ADORA2B Antagonist

The effects of ADORA2B blockade on lung function in the $Ada^{-/-}$ mouse model were investigated. In these experiments, we found that resistance and elastance are increased, and that compliance is reduced, in $Ada^{-/-}$ mice compared with control mice (Figures 5A–5C). In addition, $Ada^{-/-}$ mice demonstrated decreased levels of arterial oxygen saturation compared with control mice (Figure 5D), which is consistent with clinical observations of patients with COPD (30). In our experiments, treatment with GS-6201 was able to significantly inhibit both the changes in lung function and the decreased levels of arterial oxygenation in $Ada^{-/-}$ mice (Figures 5A–5D). These results demonstrate the therapeutic potential of ADORA2B blockade in improving lung function and gas exchange in chronic lung disease, where high levels of adenosine are present. We also obtained parameters for tissue elastance and resistance as well as pressure volume (PV)-loop measurements from quasistatic conditions, revealing increased resistance, elastance, and a reduction in compliance and the Salazar-Knowles parameter A in $Ada^{-/-}$ mice (Figures E2A–E2H).

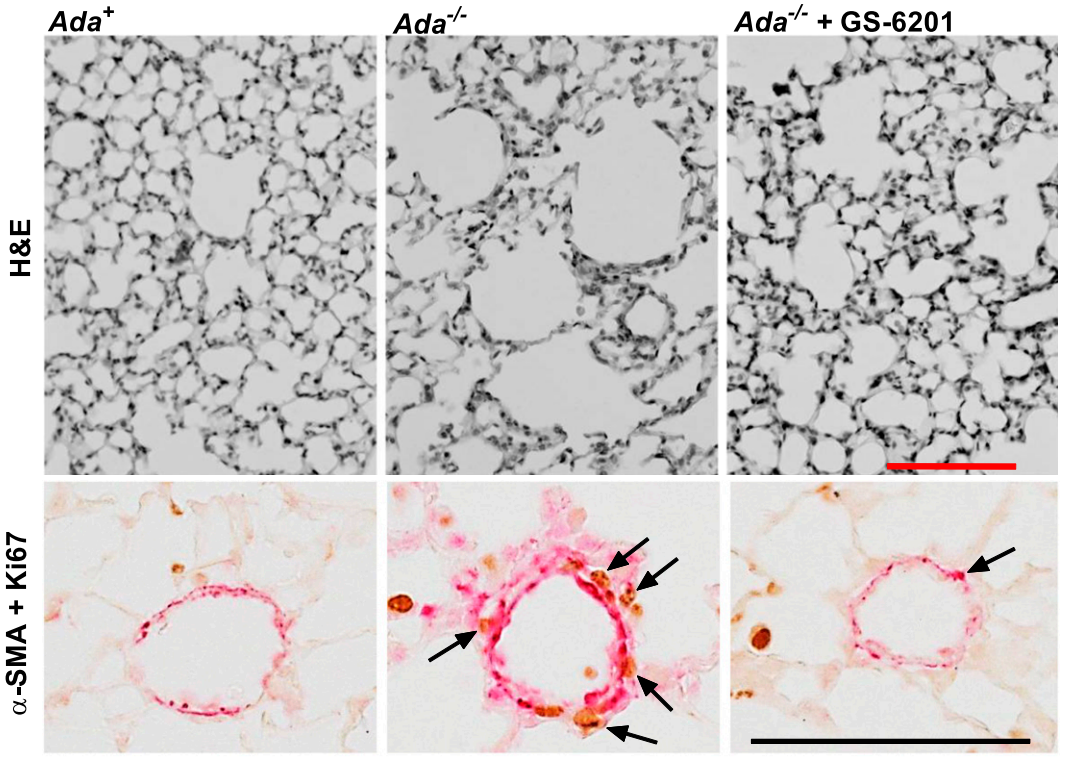


***Figure 3.*** Airspace enlargement and vascular remodeling in the adenosine deaminase–deficient ($Ada^{-/-}$) mouse model. (*Top*) Hematoxylin-and-eosin–stained (H&E) or (*bottom*) dual immunohistochemistry for α-SMA (*red*) and Ki67 (*brown*) lung sections from adenosine deaminase competent ($Ada^{+}$, *left*), $Ada^{-/-}$ (*center*), and $Ada^{-/-}$ mice treated with 3-ethyl-1-propyl-8-(1-(3-trifluoromethylbenzyl)-1H-pyrazol-4-yl)-3,7-dihydropurine-2,6-dione (GS-6201) (*right*). *Arrows* indicate nuclei positive for Ki67 (a marker for cell proliferation) in lung sections from $Ada^{+}$, $Ada^{-/-}$, and $Ada^{-/-}$ mice treated with GS-6201. The *red* and *black scale bars* represent 200 μm.

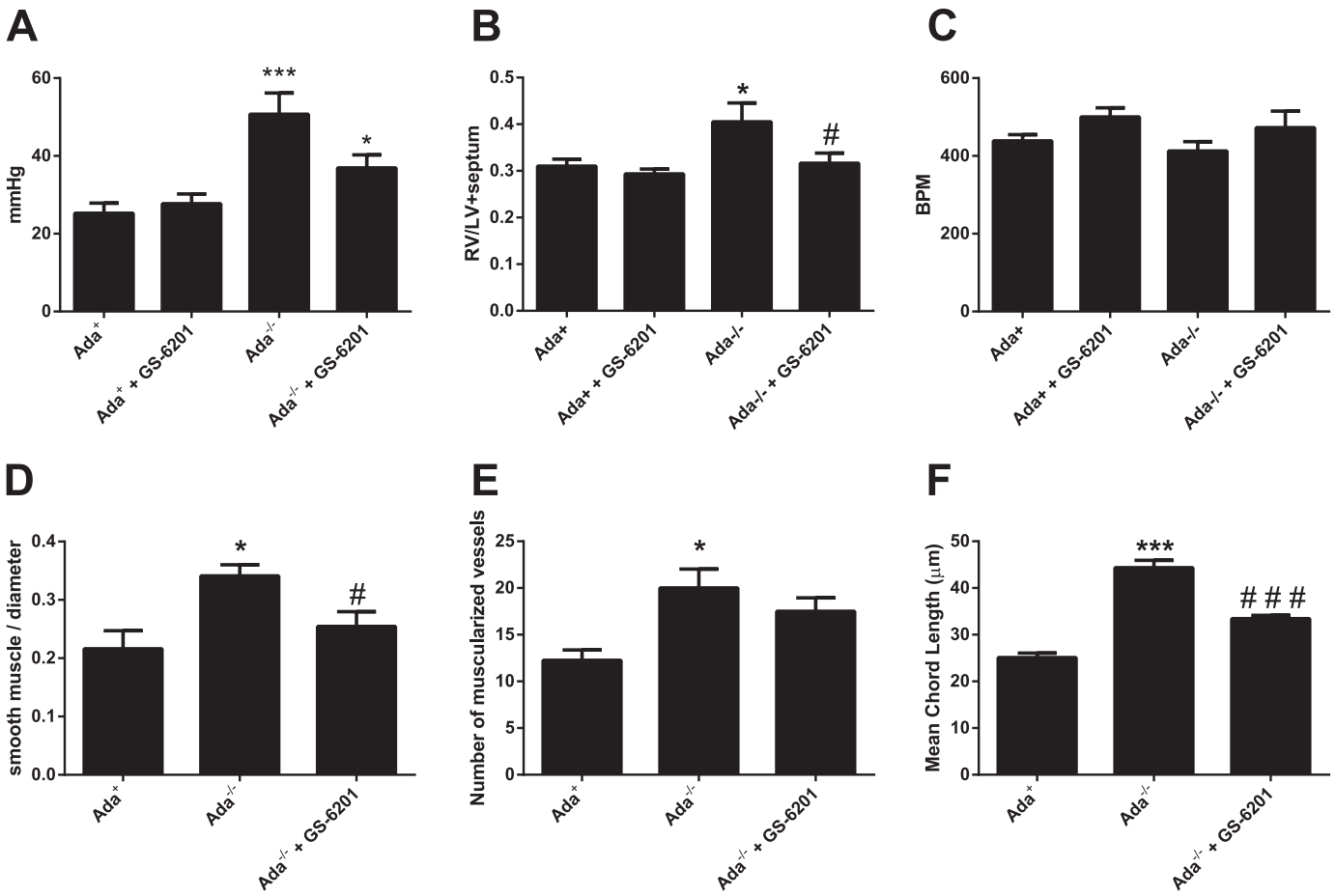


***Figure 4.*** Cardiovascular physiology and lung remodeling in ADA-deficient mice ($Ada^{-/-}$), and the effects of ADORA2B blockade. (*A*) Right ventricle systolic pressure (RVSP) was measured in fully anesthetized mice. (*B*) Right ventricular (RV) hypertrophy was determined by using the Fulton index (measuring the dry weight of the RV relative to the left ventricle [LV] and the septum). (*C*) Mouse heart rates were determined during RVSP measurements. BPM, beats per minute. A morphometric analysis was performed to determine (*D*) the extent of muscularization present in five vessels for each mouse, and (*E*) the number of α-SMA–positive vessels in five random sections of the lung parenchyma. (*F*) Mean chord lengths were used to determine airspace enlargement in $Ada^{-/-}$ mice and the effects of GS-6201 treatment. Results are presented as means ± SEMs ($n = 6$–9 for all treatment groups, i.e., $Ada^{+}$, $Ada^{+}$ + GS-6201, $Ada^{-/-}$, and $Ada^{-/-}$ + GS-6201). ***$P < 0.001$ and *$P < 0.05$ refer to comparisons between $Ada^{+}$ and $Ada^{-/-}$ treatment groups. ###$P < 0.001$ and #$P < 0.05$ refer to comparisons between $Ada^{-/-}$ and $Ada^{-/-}$ + GS-6201 treatment groups. One-way ANOVA with the Newman-Keuls multiple comparisons test was used for statistical analyses.

### HA Synthesis Is Modulated by ADORA2B and Is Increased in Remodeled Vessels of Patients with PH Linked to COPD

Altered turnover of the lung ECM is a feature of COPD, IPF, and other pulmonary diseases (3). Our results showed that remodeling of the vasculature in patients with PH secondary to COPD was observed in regions rich in collagen deposition (Figure 1C), and that Col1A1 expression correlated strongly with mPAP (Figure 2D). Collagen is one of many components of the lung ECM that is also composed of fibronectin, vitronectin, proteoglycans, and glycosaminoglycans (GAGs) (31). Of the GAGs, HA has been associated with idiopathic PH (16), and has also been reported to promote angiogenesis in models of lung fibrosis (32). To investigate whether HA was up-regulated in our mouse model of airspace enlargement and PH, transcript levels for HAS enzymes were evaluated according to RT-PCR. The results from these experiments showed a significant increase in the levels of HAS-1 and HAS-2 in $Ada^{-/-}$ mice (Figures 6A and 6B). Furthermore, $Ada^{-/-}$ mice treated with GS-6201 demonstrated attenuated levels of HAS-1 and HAS-2 (Figures 6A and 6B). Consistent with these changes, HAS-2 protein levels were increased in $Ada^{-/-}$ mice compared with $Ada^{+}$ mice. Treatment with GS-6201 was able to attenuate the increase in HAS-2 protein levels in $Ada^{-/-}$ mice (Figure 6C). In line with elevated HAS-2 levels, an increased presence of HA was observed histologically in $Ada^{-/-}$ mice compared with $Ada^{+}$ mice (Figure 6C), and $Ada^{-/-}$ mice treated with GS-6201 exhibited reduced HA levels. Interestingly, the colocalization of HA and α-SMA in the vasculature was observed in $Ada^{-/-}$ mice, but not in the $Ada^{+}$ or $Ada^{-/-}$ + GS-6201 groups (Figure 6D). To examine the role of ADORA2B further in modulating the expression of HAS enzymes, human smooth muscle cells were cultured under control (vehicle) conditions, exposed to the adenosine receptor agonist NECA, or treated with both NECA and GS-6201. The results of these studies revealed that smooth muscle cells exposed to NECA showed a significant increase in HAS-1 mRNA expression, compared with both control cells and cells exposed to NECA + GS-6201 (Figure 6E). These results demonstrate that ADORA2B signaling can directly up-regulate the components of HA synthesis in pulmonary vascular smooth muscle cells.

To determine whether these changes were mirrored in human subjects, we examined whether increased HA was present in remodeled vessels of patients with PH associated with COPD.

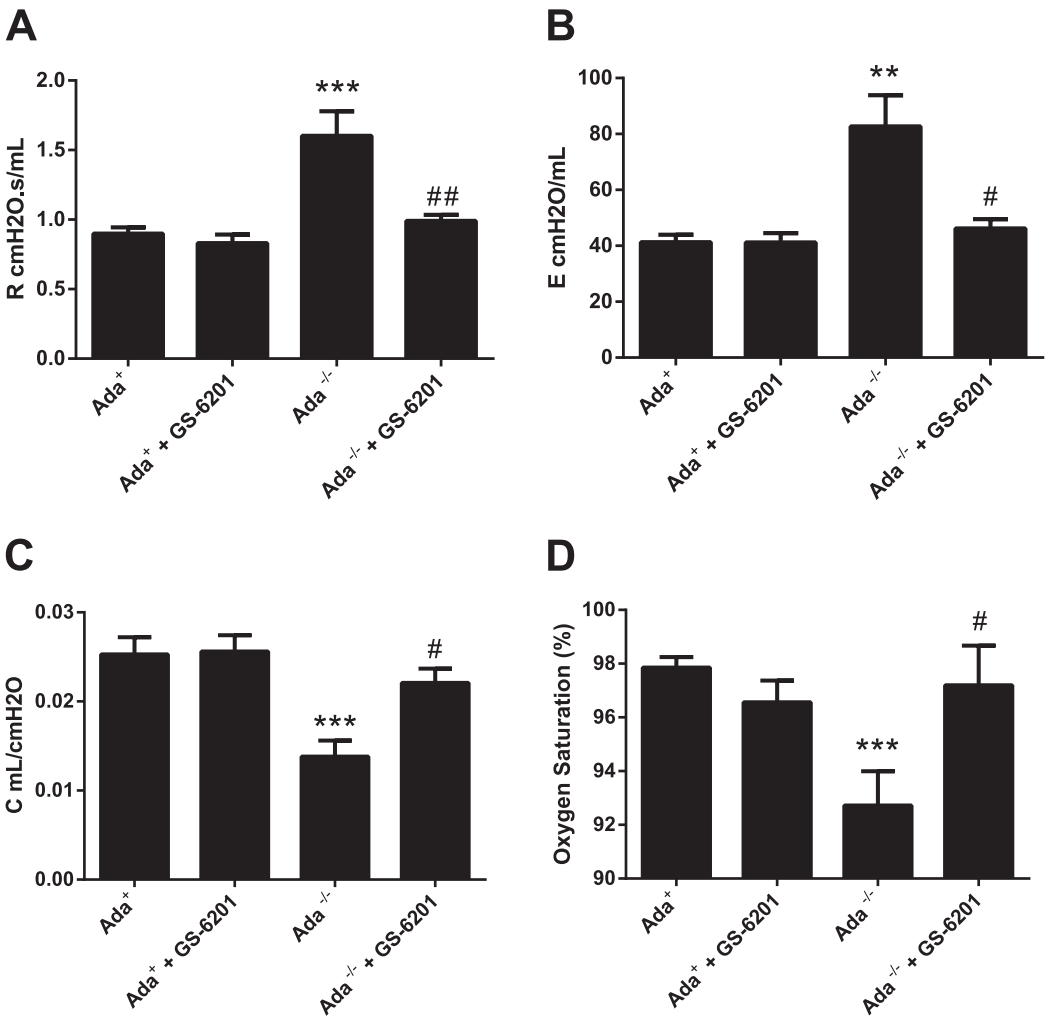


***Figure 5.*** Lung function measurements in $Ada^{-/-}$ mice, and the effects of GS-6201. Dynamic resistance (*A*), elastance (*B*), and compliance (*C*) of the lungs were measured (R, dynamic resistance; E, elastance; C, compliance). These measurements were performed using a FlexiVent system (Scireq, Montreal, PQ, Canada) in tracheotomized and anesthetized mice. (*D*) Arterial oxygenation levels were determined in awake mice by pulse oximetry, using the MouseOx system (Starr Lifesciences, Oakmont, PA). Results are presented as means ± SEMs ($n$ = 6-9 for all treatment groups, i.e., $Ada^{+}$, $Ada^{+}$ + GS-6201, $Ada^{-/-}$, and $Ada^{-/-}$ + GS-6201). ***$P < 0.001$ and **$0.001 < P < 0.01$ refer to comparisons between $Ada^{+}$ and $Ada^{-/-}$ treatment groups. ##$0.001 < P < 0.01$ and #$P < 0.05$ refer to comparisons between $Ada^{-/-}$ and $Ada^{-/-}$ + GS-6201 treatment groups. One-way ANOVA with the Newman-Keuls multiple comparisons test was used for statistical analyses.

Lung sections incubated with HABP showed increased HA adjacent to remodeled vessels in patients with PH and COPD, compared with vessels of patients with COPD but without PH (Figures 7A and 7B). In addition, transcript levels of HAS-2 were increased in patients with COPD + PH, compared with patients with COPD but without PH (Figure 7C). Pearson correlation and linear regression analyses revealed a close correlation between HAS-2 expression and mPAP (Figure 7D), implicating HAS-2 in the progression of PH in patients with COPD. Remarkably, HAS-2 transcript levels were correlated with ADORA2B mRNA levels (Figure 7E), demonstrating a strong link between ADORA2B upregulation and heightened HAS-2 expression. Taken together, these observations indicate a potential role of HA and ADORA2B in promoting vascular remodeling in patients with COPD. Our data show an association between the heightened expression of certain ECM components that modulate the remodeling response and are attenuated after treatment with an ADORA2B antagonist, and postulate a novel therapeutic target for the treatment of PH secondary to COPD.

## DISCUSSION

PH is a fatal complication of COPD, and several studies have documented its devastating effects on mortality, including an approximately 50% decline in survival (7). Currently, no effective treatments are available for PH in COPD (10, 11), and clinical trials using presently approved therapies for pulmonary arterial hypertension (PAH) in patients with COPD and PH have proven disappointing (10, 11, 33, 34). Thus, the need exists to elucidate the mechanisms that lead to PH in COPD, with the aim of developing specific treatments for this disorder.

The pathogenesis of PH in COPD is heterogeneous, and is thought to involve chronic inflammation, emphysema, irregular ECM turnover, and hypoxia (5). However, the molecular mechanisms involved are not fully understood. One molecule that is generated under these conditions of chronic inflammation, emphysema, and hypoxia is the signaling molecule adenosine (22, 35). Elements of the adenosinergic signaling system are known to be elevated in chronic lung diseases, including COPD and IPF (26, 27). However, little is known of how adenosine is able to orchestrate the remodeling of the vasculature leading to PH in COPD. In this study, we demonstrate that patients with COPD and PH present with remodeled vessels, characterized by increased smooth muscle and collagen deposition. In addition, we show that elevated ADORA2B and Col1A1 transcript levels correlate with increased pulmonary arterial pressures. Using an experimental model of adenosine-mediated airspace enlargement, we show that these mice exhibit the phenotype of PH, including elevated RVSP, RVH, and vascular remodeling. Moreover, the treatment of these mice with GS-6201, an ADORA2B antagonist, was able to halt damage to the lungs, including the development of PH. Our data suggest that the mechanisms leading to vascular remodeling involve ADORA2B-mediated increases in HAS-2 and subsequent elevations of HA, a component of the ECM that is able to promote tissue remodeling (17, 36) and is elevated in patients with PAH (15, 16). These observations are consistent with our human

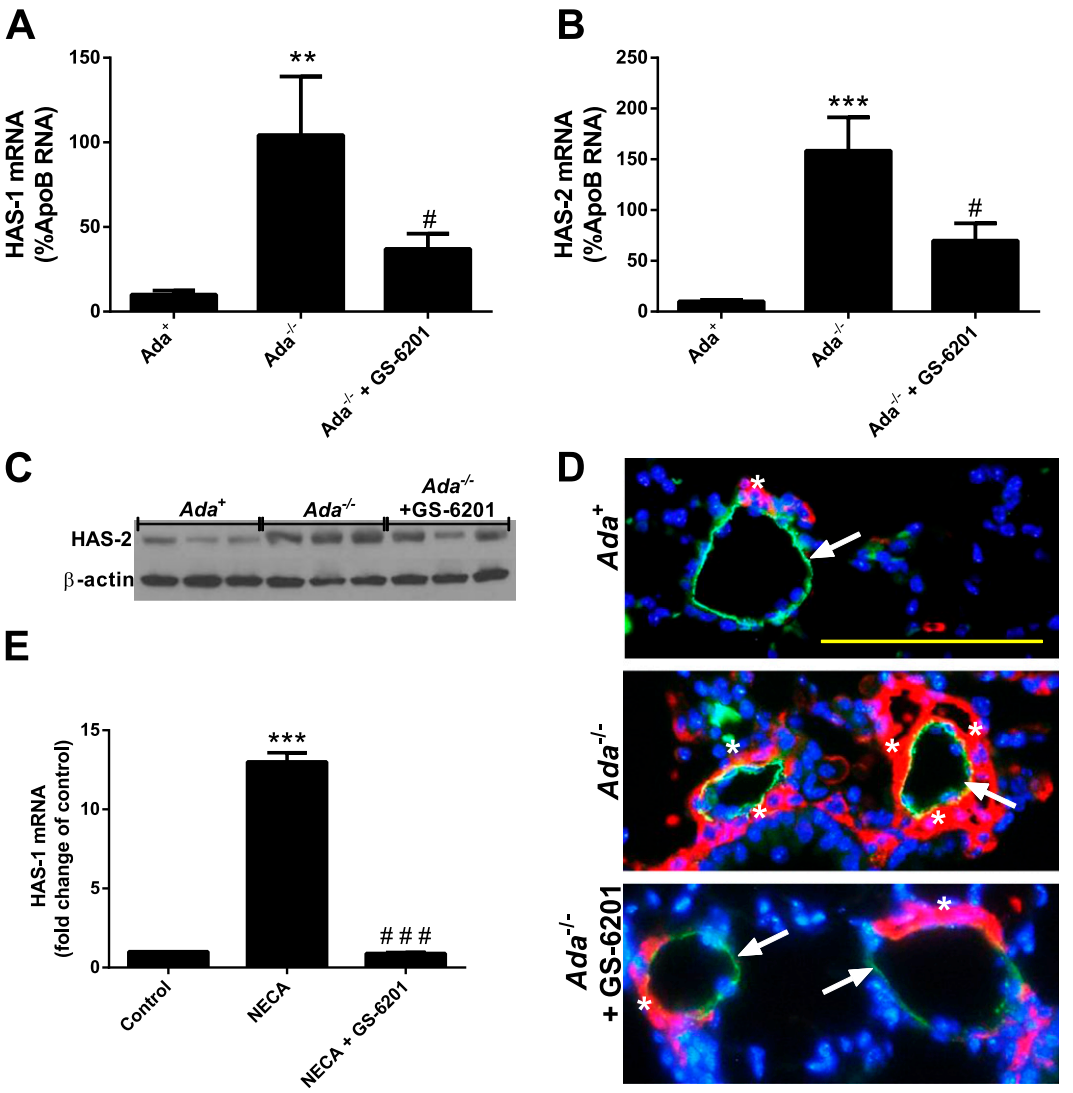


*Figure 6.* ADORA2B modulates the expression of hyaluronan synthase (HAS) genes. The mRNA expression levels of HAS-1 (*A*) and HAS-2 (*B*) were determined relative to apolipoprotein B (ApoB) RNA. Results are presented as means ± SEMs ($n = 4$–6 for all treatment groups, i.e., $Ada^{+}$, $Ada^{-/-}$, and $Ada^{-/-}$ + GS-6201). $^{***}P <$ 0.001 and $^{**}0.001 < P <$ 0.01 refer to comparisons between $Ada^{+}$ and $Ada^{-/-}$ treatment groups. $^{\#}P < 0.05$ refers to comparisons between the $Ada^{-/-}$ and $Ada^{-/-}$ + GS-6201 treatment groups. One-way ANOVA with the Newman-Keuls multiple comparisons test was used for statistical analyses. (*C*) Western blotting for HAS-2 and β-actin (internal control) from whole-lung lysates of $Ada^{+}$, $Ada^{-/-}$, and $Ada^{-/-}$ + GS-6201 mice. (*D*) Immunohistochemical staining in lung sections from $Ada^{+}$, $Ada^{-/-}$, and $Ada^{-/-}$ + GS-6201 treatment groups for hyaluronan (HA), using biotinylated HA-binding protein (HABP; *red*), α-SMA (*green*), and nuclear DAPI stain (*blue*). *Asterisks* highlight areas with high HA presence, and *arrows* indicate α-SMA–positive sections. Colocalization of α-SMA and HA appears *yellow* (*scale bar* represents 200 μm). (*E*) HAS-1 expression levels of human smooth muscle cells exposed to control samples, 5′-(N-ethylcarboxamido) adenosine (NECA), or NECA + GS-6201. $^{***}P < 0.001$ refers to comparisons between control and NECA-treated groups. $^{\#\#\#}P < 0.001$ refers to comparisons between NECA-exposed and NECA + GS-6201–exposed cells ($n = 6$–7 patients per group).

data showing increased HA staining and HAS-2 expression. Furthermore, HAS-2 transcript levels correlate with increasing mPAP levels in patients with COPD. In an effort to link ADORA2B and HA production, we show that ADORA2B levels correlate significantly with HAS-2 transcript levels. Taken together, our data indicate a pivotal role for ADORA2B signaling in the pathogenesis of COPD and in orchestrating changes in the ECM composition that lead to vascular remodeling and PH.

The exact prevalence of PH in the general population of patients with COPD is not fully known, because patients with stable COPD do not typically undergo cardiac catheterization (8). Furthermore, the symptoms of PH are similar to those of COPD, including dyspnea, fatigue, and exercise limitation (2), adding further to the complexity of diagnosing PH in these patients. As a result, data pertaining to the prevalence of PH have typically focused on patients with Stage II or Stage III COPD awaiting lung transplantation, for whom hemodynamic data from heart catheterization form part of the normal transplant evaluation (5, 8). In these studies, the prevalence of PH in COPD accounted for 30–50% of the number of patients (5, 6). Alarmingly, a recent study by Sertogullarindan and colleagues (37) focusing on clinically stable COPD patients with a history of tobacco smoking or biomass exposure demonstrated a prevalence of PH ranging from 37.5–60%. These findings provide further impetus to determine the prevalence of PH in stable COPD and to elucidate the mechanisms that result in the development of PH.

The severity of COPD is normally determined by forced expiratory volume in 1 second ($FEV_1$). However, consensus has been established that $FEV_1$ alone cannot adequately describe the complexity of this disease (38). Interestingly, lung function data, including the percentage-diffusing capacity of the lung for carbon monoxide, predicted forced vital capacity, and total lung capacity, do not correlate with the presence of PH in chronic lung diseases such as IPF (39) or COPD (14). These observations are in line with the results of our study, as summarized in Table 1. Thus, the need exists to uncover biological markers of disease that correlate with mPAP, and that can be used as diagnostic markers to better classify the severity of COPD. An important finding of our study involves the demonstration that transcript levels of ADORA2B and Col1A1, which are typically increased in patients with COPD or IPF (27), are further enhanced in patients with PH, and correlate with increasing mPAPs, suggesting their possible use as biomarkers for disease progression and the development of PH.

Because of the complex nature of PH associated with chronic lung disease, most research has focused on studying the mechanisms that lead to idiopathic or familial PAH (11, 18). PAH is

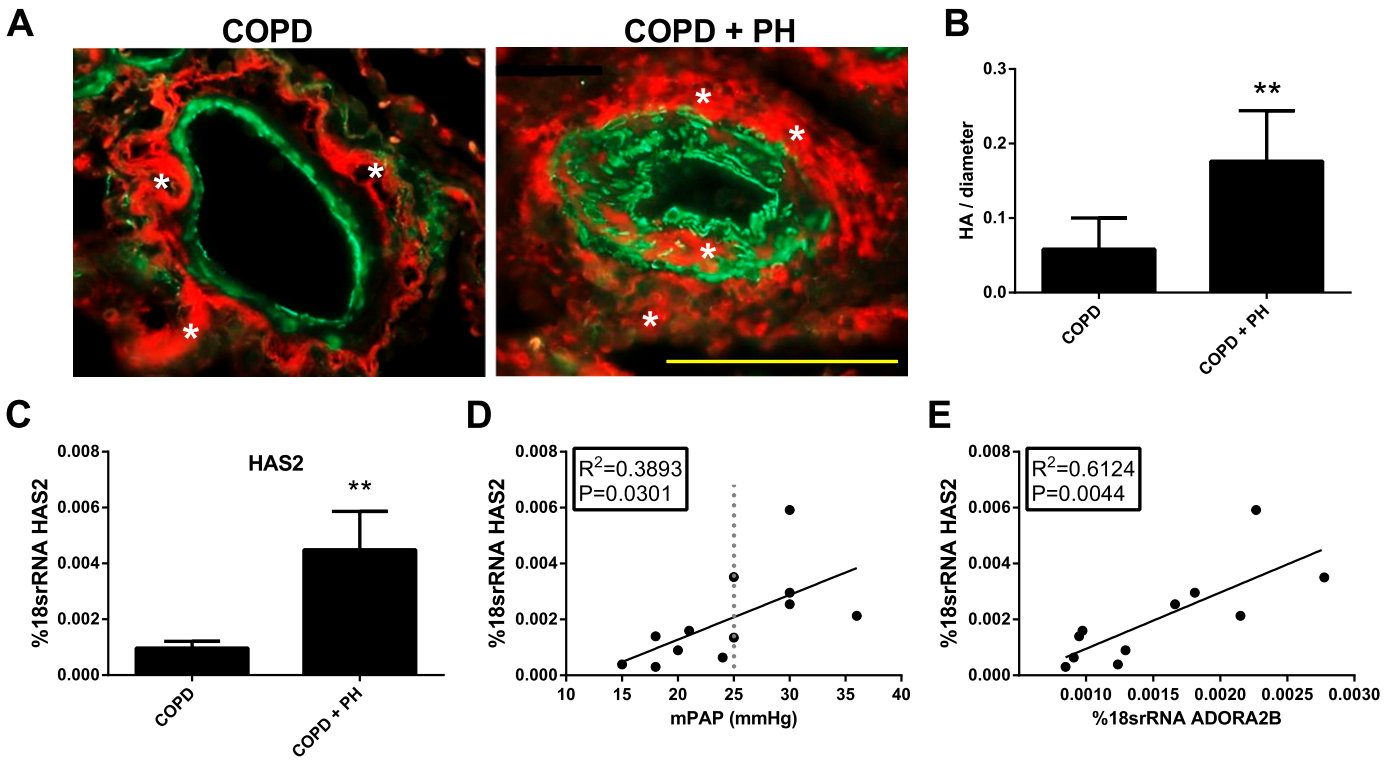


***Figure 7.*** Increased presence of HA and HAS in human patients with COPD + PH, compared with patients with COPD but without PH. (*A*) Dual immunohistochemical staining for α-SMA (*green*) and HA, using HABP (*red*) from lung sections of a patient with COPD but without PH (*left*) or a patient with PH secondary to COPD (*right*). The *asterisks* mark areas with high HA deposition. *Scale bar* represents 200 μm. (*B*) Extent of HA present in or adjacent to five vessels for each patient. Results are presented as means ± SEMs ($n = 6$–7 for each group, i.e., COPD or COPD + PH). **$0.001 < P < 0.01$ refers to comparisons between the COPD and the COPD + PH groups. (*C*) Transcript levels for HAS-2. Data are presented as means ± SEMs. **$0.001 < P < 0.01$ refers to Student *t* test comparisons between different patient cohorts. Pearson correlation and linear regression analyses were undertaken for associations between (*D*) HAS-2 and mPAP and (*E*) HAS-2 and ADORA2B for all patients with COPD (with and without PH). HAS-2 levels correlated strongly with mPAP (*C*; $R^2 = 0.3893$ and $P = 0.0301$), as did HAS-2 with ADORA2B levels (*D*; $R^2 = 0.6124$, and $P = 0.0044$). Data on or right of the *dotted line* (*C*) represent those with PH (mean PAP ⩽ 25 mm Hg; $n = 6$–7 patients per group).

characterized by pulmonary vascular remodeling that is not associated with ongoing chronic lung injury (40). As a result, numerous animal models can be used to study PAH (28). Unfortunately, fundamental pathophysiological differences exist between PAH and COPD-associated PH (11), and these differences preclude using animal models of PAH to study PH in COPD. Currently, limited models are available that can be used to study the development of PH in chronic lung disease. To study the potential mechanisms that lead to PH in COPD, as well as the central role that adenosine may play in orchestrating the process of vascular remodeling, the $Ada^{-/-}$ model of chronic lung injury was chosen. $Ada^{-/-}$ mice are characterized by elevated levels of adenosine, emphysematous-like destruction of the parenchyma, chronic inflammation, and lung fibrosis (19). However, whether hallmarks of PH were present was not previously investigated. In this study, we report that $Ada^{-/-}$ mice present with increased RVSP, RVH, and vascular remodeling, characterized by the increased muscularization of pulmonary arterioles and the neovascularization of previously nonvascular vessels that are characteristic of PH (41). These changes were accompanied by alveolar destruction and impairments in lung function. Interestingly, our experiments in lung function reveal that contrary to previously reported data (42), increased airway resistance and elastance were observed. These observations included both dynamic and PV-loop data under quasistatic conditions. These differences can be explained by pointing out that in the $Ada^{-/-}$ model, elevated levels of adenosine promote the destruction of the airway, but may also contribute to the heightened airway resistance. Indeed, several studies showed elevated levels of adenosine in the exhale condensate of patients with asthma that were thought to be responsible for the increased airway hyperresponsiveness reported in these patients (26). In addition, $Ada^{-/-}$ mice also present with lung fibrosis (19), which can also contribute to the increases in resistance and elastance and the reduction in compliance (18) reported here.

In this experimental model of chronic lung injury, we report that after treatment with the ADORA2B antagonist GS-6201, destruction of the parenchyma can be prevented, consistent with previous observations (19). More importantly, treatment with GS-6201 was able to inhibit the hallmarks of PH, including elevated RVSP, RVH, and vascular remodeling. These observations are consistent with previous observations in which this same drug was able to significantly inhibit increased RVSP and vascular remodeling in a mouse model of chronic bleomycin exposure (18). Similarly, ADORA2B blockade restored lung function parameters to normality, including arterial oxygenation levels, consistent with previous experiments by our group (18). Taken together, these findings indicate a central role of ADORA2B in promoting lung damage and in the development of PH.

We focused our attention on mediators that were not only able to regulate inflammation, but that also contributed directly to the vascular remodeling process. One such mediator is HA, a multifaceted GAG that can promote or inhibit lung pathology, depending on its molecular weight and its accessibility to HABPs (17). In experimental models of disease, heightened levels of HA have been shown to modulate inflammation and fibrosis (17), where an accumulation of HA and its subsequent degradation to low

**TABLE 1. CHARACTERISTICS OF PATIENTS USED IN THE STUDY**

| | COPD | COPD + PH |
|---|---|---|
| Age, yr | 62.4 ± 4.0 | 67 ± 1.6 |
| mPAP, mm Hg | 18 ± 1.4 | 28 ± 1.8* |
| PAP, Sys/Dias mm Hg | 30 ± 2.1/13 ± 1.4 | 40 ± 1.8/22 ± 2.3* |
| 6MWD, meters | 807 ± 152 | 736 ± 205 |
| BMI | 23.3 ± 1.9 | 26.8 ± 2.0 |
| FVC% | 58.3 ± 4.1 | 51.0 ± 4.9 |
| $D_{LCO}$% | 26 ± 6.7 | 11.8 ± 2.3 |
| TLC% | 118 ± 8.6 | 116 ± 6.7 |
| $FEV_1$% | 25.4 ± 2.8 | 21.6 ± 1.8 |
| $Pa_{O_2}$, mm Hg | 64.6 ± 5.2 | 61.32 ± 6.4 |
| $Pa_{CO_2}$, mm Hg | 48.1 ± 2.9 | 51.3 ± 3.6 |
| BICARB, mEq/L | 29.4 ± 1.8 | 32.2 ± 2.1 |

*Definition of abbreviations*: BICARB, bicarbonate level in the lungs; BMI, body mass index; COPD, chronic obstructive pulmonary disease; Dias, diastolic; $D_{LCO}$%, percentage diffusing capacity of the lung for carbon monoxide; $FEV_1$%, percentage forced expiratory volume in 1 second; FVC%, percentage forced vital capacity; mPAP, mean pulmonary arterial pressure; PAP, pulmonary arterial pressure; PH, pulmonary hypertension; 6MWD, 6-minute walking-distance; Sys, systolic; TLC%, percentage total lung capacity.

Fifty percent of patients were male, and 50% were female, in both the COPD and COPD + PH cohorts. All data are presented as means ± SEMs from 6–7 patients for each cohort (COPD vs. COPD + PH).

* $P < 0.001$ refers to Student *t* test comparisons between COPD versus COPD + PH cohorts.

molecular weight (LMW) fragments contribute to the inflammatory and tissue remodeling process (17). Furthermore, increased HA has been observed in the monocrotaline model of PH (43), further implicating its role in vascular remodeling. Adenosine via ADORA2A was recently demonstrated to modulate the expression of HA in bleomycin-induced lung injury (20, 21). In our model of chronic PH associated with chronic lung injury, we demonstrate increased levels of HAS-1 and HAS-2, as well as increased HA-staining adjacent to small pulmonary vessels. These changes are accompanied by an increased deposition of α-SMA, consistent with vessel remodeling. Close inspection of immunofluorescently stained sections reveals the coexpression of HA and α-SMA, suggesting that HA may play a role in regulating vascular remodeling during PH. Indeed, several studies have demonstrated that HA promotes the migration and proliferation of arterial and vascular smooth muscle cells (44, 45), as well as inducing angiogenesis (46), possibly via interactions with CD44 (47). These findings, together with our observations, suggest an important role for HA in vascular remodeling leading to PH in COPD. Interestingly, we report that treatment with GS-6201 inhibited the increased expression of HAS-1 and HAS-2 mRNA levels that were accompanied by a reduction in HA deposition. Using human arterial smooth muscle cells, we confirmed these observations *in vitro* by showing that the activation of ADORA2B promotes the expression of HAS-1. The results of these studies are in line with previous observations, where adenosine was shown to regulate HA levels (20, 21). However, contrary to these previous observations, we show that ADORA2B but not ADORA2A is responsible for the up-regulation of HA. Our study involved a major difference in that we used a chronic model of lung injury as opposed to an acute model of lung damage. In our $Ada^{-/-}$ mice, the activation of ADORA2B was associated with disease progression (19). It is also important to consider that although the blockade of ADORA2B prevents increased HA in our model, Collins and colleagues (21) and Scheibner and colleagues (20) showed that the activation of ADORA2A down-regulated HA. This phenomenon is consistent with the anti-inflammatory and proinflammatory dualities of adenosine action in the context of acute and chronic injury (25), and point out the need to further dissect the acute and chronic functions of adenosine and its signaling system during various stages of disease progression.

Elevated levels of HA have been reported in patients with COPD (48), pulmonary fibrosis (49), and PAH (15, 16). However, whether increased levels of HA are present in PH during COPD remains unknown. In this study, we found an increase in the deposition of HA adjacent to and within areas of remodeled vessels of patients with COPD and PH, compared with nonremodeled vessels of patients with COPD but without PH. We also show increased levels of HAS-2 transcripts that significantly correlated with rising mPAP levels, suggesting a direct role of this molecule in the pathogenesis of PH during COPD. Moreover, we found a significant correlation between HAS-2 and ADORA2B transcript level expression, suggesting a direct role of this adenosine receptor subtype in the regulation of HA levels in chronic lung disease.

In conclusion, our data suggest that in patients with COPD, prolonged hypoxia as a result of chronic inflammation, airway obstruction, and airway remodeling may result in the up-regulation and heightened activation of ADORA2B in pulmonary artery smooth muscle cells (PASMCs), leading to enhanced HA production through increased HAS expression and the HA-induced remodeling of the vasculature. This study provides important clinical, mechanistic, and potential diagnostic data that suggest a role for ADORA2B antagonism as treatment for PH secondary to COPD. In support of this observation, theophylline, a phosphodiesterase inhibitor and nonselective adenosine antagonist, has been known to improve cardiac function, including lowering mPAP in patients with COPD (50). Interestingly, in our patient cohort, only one patient was receiving theophylline treatment, and did not present with a secondary diagnosis of PH.

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

***Acknowledgments***: The authors thank Kelly Volcik, Ph.D., for critical review of the manuscript.

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