Ceramide and sphingosine-1 phosphate in COPD lungs

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ABSTRACT

Studies of chronic obstructive pulmonary disease (COPD) using animal models and patient plasma indicate dysregulation of sphingolipid metabolism, but data in COPD lungs are sparse. Mass spectrometric and immunostaining measurements of lungs from 69 COPD, 16 smokers without COPD and 13 subjects with interstitial lung disease identified decoupling of lung ceramide and sphingosine-1 phosphate (S1P) levels and decreased sphingosine kinase-1 (SphK1) activity in COPD. The correlation of ceramide abundance in distal COPD lungs with apoptosis and the inverse correlation between SphK1 activity and presence of emphysema suggest that disruption of ceramide-to-S1P metabolism is an important determinant of emphysema phenotype in COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD), including chronic airflow limitation and emphysema phenotypes, is primarily caused in susceptible individuals by decades of smoking. The pathogenesis of COPD is incompletely elucidated, and therefore few disease-modifying therapies are available. Sphingolipids have been implicated in COPD, especially in the distal lung cell injury and loss of alveolar tissue, the hallmark of emphysema. A proapoptotic second messenger, ceramide, is at the core of the sphingolipid metabolism (figure 1A), serving as precursor of prosurvival metabolites including sphingosine-1 phosphate (S1P). Interventions to rebalance ceramide[1] and S1P levels or signalling reduced lung apoptosis and preserved airspace integrity in murine models of emphysema.[2] Previous studies supported this paradigm, but they included few subjects or relied on plasma and semiquantitative techniques.[3–5] The largest study of COPD plasma, using metabolomics, identified accelerated ceramide metabolism diverted from S1P synthesis,[4] but specific measurements in well-phenotyped COPD lungs have not been reported. We analysed lung ceramide and S1P and explored their potential correlation with phenotypical COPD parameters.

METHODS

All experiments were IRB approved at the University of Illinois Chicago and Indiana University. Lung Tissue Research Consortium provided deidentified clinical data linked to lung specimens collected by their Core Laboratory from lung biopsies, lobectomies, transplant and lung volume reduction surgeries. Lung specimens were >5 cm away from any excised tumours.

Following extraction and lipid phosphorus (Pi) measurements,[1] sphingolipid analyses were performed via combined liquid chromatography–tandem mass spectrometry, using API4000 Q-trap hybrid triple quadrupole linear ion-trap mass spectrometer (Applied Biosystems-Sciex) with turbo ion spray ionisation source and Agilent 1100 series liquid chromatography (Agilent Technologies).[6]

Paraffin-embedded lung tissue sections were immunostained with active caspase-3 (Cell Signaling) or ceramide (Alexis) biotinylated antibodies (Animal Research Kit, Dako). Randomised deidentified images of distal lung parenchyma were acquired and quantified (Metamorph) with a macro from Dr Rubin Tuder (University of Colorado).

Statistical analysis included ANOVA (normality tested with Bartlett's Test) and Pearson linear regression (Prism 6, GraphPad). Multiple regression models were run in R.

RESULTS

We analysed lungs affected from COPD (GOLD 1–4; n=69), lungs of non-diseased smokers at risk for COPD (n=16) and lungs affected from interstitial lung disease (ILD) (n=13), mostly with idiopathic pulmonary fibrosis (IPF) (table 1; online supplemental table 1).

Subjects were well matched for age, gender and race, but the COPD group had lower body mass index. The majority were ex-smokers, exclusive of three current smokers with COPD. One smoker at risk and eight subjects with COPD had lung cancer. Most subjects with COPD (~70%) had a clinical diagnosis of emphysema and severe disease (75% GOLD 3–4).

COPD samples exhibited highly variable lung ceramides, and as a group they were not significantly different from smokers at risk or ILD. We noted a significant pattern of increasing concentrations of lung ceramides from smokers at risk (previously classified as GOLD0) to GOLD1 and GOLD2 (figure 1B). This pattern changed in severe and very severe COPD lungs (GOLD 3–4), in which most ceramide species were decreased (figure 1C). Lungs from smokers at risk and COPD exhibited (similar) extensive ceramide immunostaining in cells comprising alveolar septae and walls (figure 1D), whereas ILD-affected lungs had variable staining patterns (figure 1D). The ceramide abundance in the distal COPD lungs was significantly associated with active caspase-3 staining on adjacent (5 μM) lung sections (figure 1E) and was inversely correlated with alpha-1 antitrypsin plasma levels (figure 1F).

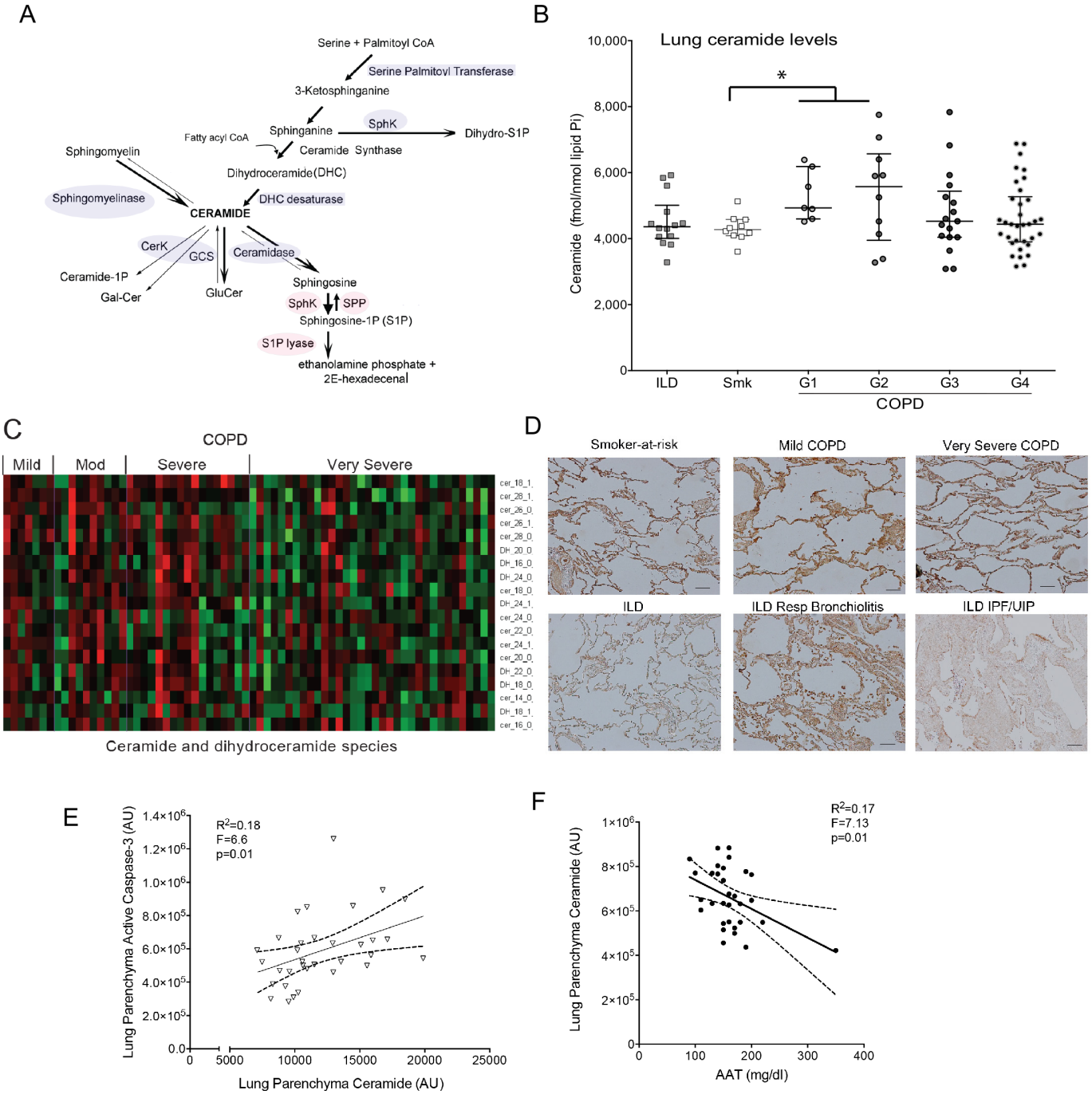

Figure 1 Lung ceramides. (A) Schematic of sphingolipid metabolism. (B) Total ceramide levels in lung homogenates from subjects in the indicated groups (median and IQR, each data point representing an individual subject; ANOVA, Kruskal-Wallis; overall p=0.09; *p<0.05 Smk versus GOLD1 (G1) and G2; post-test for linear trend Smk, G1, and G3 p=0.03). (C) Heat map of lung levels ceramide (cer) and dihydroceramide species (right vertical axis; each species is defined by sphingosine saturation and by the length and saturation of the fatty acid chain) grouped by COPD GOLD classification; red denoting high relative levels and green low levels; each column represents a sample from an individual subject. (D) Representative images of ceramide abundance (brown) in the lung parenchyma determined by IHC with anticeramide antibody of lung sections from the indicated study groups. The interstitial lung disease (ILD) group that included respiratory bronchiolitis and idiopathic pulmonary fibrosis (IPF) with usual interstitial pneumonitis (UIP). Size bar=50 µm. (E,F) Linear correlations between distal lung ceramide detected by immunostaining in COPD samples and active caspase-3 immunostaining in adjacent lung tissues (E) and alpha-1 antitrypsin (AAT) levels in plasma (F). CerK, ceramide kinase; CoA, coenzyme A; COPD, chronic obstructive pulmonary disease; GCS, glucosylceramide synthase; GOLD, Global Initiative for Chronic Lung Disease; S1P, sphingosine-1 phosphate; Smk, smoker; SphK, sphingosine kinase; SPP, S1P phosphatase.

Lung S1P levels also differed by COPD stage, being increased in more severe COPD (figure 2A). Interestingly, S1P correlated, as expected, with ceramide levels in the smokers at risk, but not in COPD lungs (figure 2B), suggesting a disruption of ceramide-to-S1P metabolism controlled by sphingosine kinase-1 (SphK1) in the latter group. Indeed, COPD, but not ILD lungs, had significantly decreased SphK1 activity (figure 2C). A multiple regression

Table 1 Demographic and clinical data

	Smokers at risk	COPD (all)	ILD	P value	Statistical test
Patients (n)	16	69	10		
Age (years)	66±13.5	62.8±8.5	60.5±10.3	0.6	Kruskal-Wallis
Gender (males %)	7 (46.6)	34 (49.2)	5 (50)	0.9	χ^2
Race (Caucasian %)	15 (93.7)	66 (95.6)	8 (80)	0.2	χ^2
Race (AA %)	0	1 (1.45)	1 (10)		
Race (Asian %)	0	1 (1.45)	1 (10)		
BMI (kg/cm^2)	29.4±4	25.8±4.2	28.2±6.7	**0.01**	Kruskal-Wallis
Smoking status: current/ex-smokers	0/16	3/66	0	0.6	χ^2
Smoking history (pack-year)	36±26.6	54±27.7	0	0.1	Kruskal-Wallis
SpO_2 at rest	96	90.7±3.5	91.3±5		Kruskal-Wallis
FVC pre (% predicted)	96.2±13.3	63.5±19.6	54.6±21.81	**<0.0001**	Kruskal-Wallis
FEV_1 pre (% predicted)	95.3±15.5	35.8±21.5	61.1±23.1	**<0.0001**	Kruskal-Wallis
FVC post (% predicted)	95.9±9.9	72.5±10.1	68.7±27.7	**0.0007**	Kruskal-Wallis
FEV_1 post (% predicted)	97±11.7	42.2±22.9	77±29.4	**<0.0001**	Kruskal-Wallis
FEV_1/FVC pre (%)	99.2±9.2	54.1±19.5	112.8±7.4	**<0.0001**	Kruskal-Wallis
FEV_1/FVC post (%)	101.7±6.9	56.3±20.3	112.7±6.16	**<0.0001**	Kruskal-Wallis
Carbon monoxide transfer factor (% predicted)	84.3±13.2	49.4±20.7	46.5±22.8	**<0.0001**	Kruskal-Wallis
6MWT (feet)	317±170.3	302.9±98.8	240.6±189.3	0.5	Kruskal-Wallis
BODE	2.0±1.3	5.3±2.3	3.7±2.6	**<0.0001**	Kruskal-Wallis
SGRQ	16.2±16.7	47±20.7	57±23.8	**0.0014**	Kruskal-Wallis
O_2 therapy (%)	0	34 (49.2)	4 (40)	0.1	χ^2
Asthma (%)	0	16 (23.1)	1 (10)	0.4	χ^2
Emphysema (%)	0	48 (69.5)	0	**<0.0001**	χ^2
Lung fibrosis (%)	0	0	5 (50)	**<0.0001**	χ^2

Statistically significant values are marked in bold.
AA, African American; BMI, body mass index; BODE, BMI, Obstruction (FEV_1), Dyspnea (modified Medical Research Council Dyspnea Scale) and Exercise capacity (6MWD); COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; ILD, interstitial lung disease; 6MWT, 6-Minute Walk Test; SGRQ, St. George's Respiratory Questionnaire; SpO_2, oxygen saturation in the peripheral blood.

model identified that the best fit that included SphK1 activity were emphysema (volume by CT scan), age, smoking history (pack-years) and carbon monoxide transfer factor (TLco) (% predicted) ($R^2=0.925$, $F(4, 4)=25.68$, $p<0.0041$). This model suggests that for each 1% decrease in predicted TLco, there was ~5% of decrease in SphK activity. In contrast, the activity of S1P lyase was significantly decreased in COPD lungs (figure 2D).

DISCUSSION

We identified an imbalanced ceramide and S1P metabolism in COPD lungs, with reduction in the metabolic flux at the level of SphK1 that was linked to emphysema phenotype. This agrees with integrated metabolomic and genomic analysis of plasma of a distinct COPD cohort[4] and together with preclinical data strongly implicate the sphingolipid metabolism in emphysema pathogenesis.

We report the novel finding of decreased SphK activity in COPD lungs. This contrasts with highly variable but similar *SPHK* 1–2 expression and SphK activity reported among 25 COPD (many actively smoking) and 24 non-smoker controls,[7] measured using D-erythro-sphingosine and P^{32}-adenosine triphosphate. This may be due to a distinct methodology, a larger number of subjects including mostly ex-smokers and lack of never smoker controls used in our study.

Surprisingly, we measured increased lung ceramide in mild and moderate COPD and increased lung S1P mostly in severe COPD. This may indicate that proapoptotic ceramide accumulation (associated with decreased SphK1 activity) precedes alveolar tissue destruction and that severely emphysematous lungs may be enriched with cells capable of prosurvival adaptive responses that limit excessive ceramide while preserving S1P levels (associated with decreased S1P lyase activity). Indeed, ceramide in the distal lung correlated with active capase-3, another apoptosis marker, and inversely correlated with alpha-1 antitrypsin a lung-protective serpin with antiapoptotic activity.[8] Notably, ceramide augmentation in mice is sufficient to increase alveolar cell apoptosis and airspace enlargement.[1]

Limitations of our study include a relatively small sample size for such a heterogeneous disease, which may explain the relatively weak correlation coefficients noted. Further, a lung cancer field effect may have confounded results in several subjects. We included ILD/IPF as a diseased comparison group because it shares with COPD smoking as a major risk factor and because

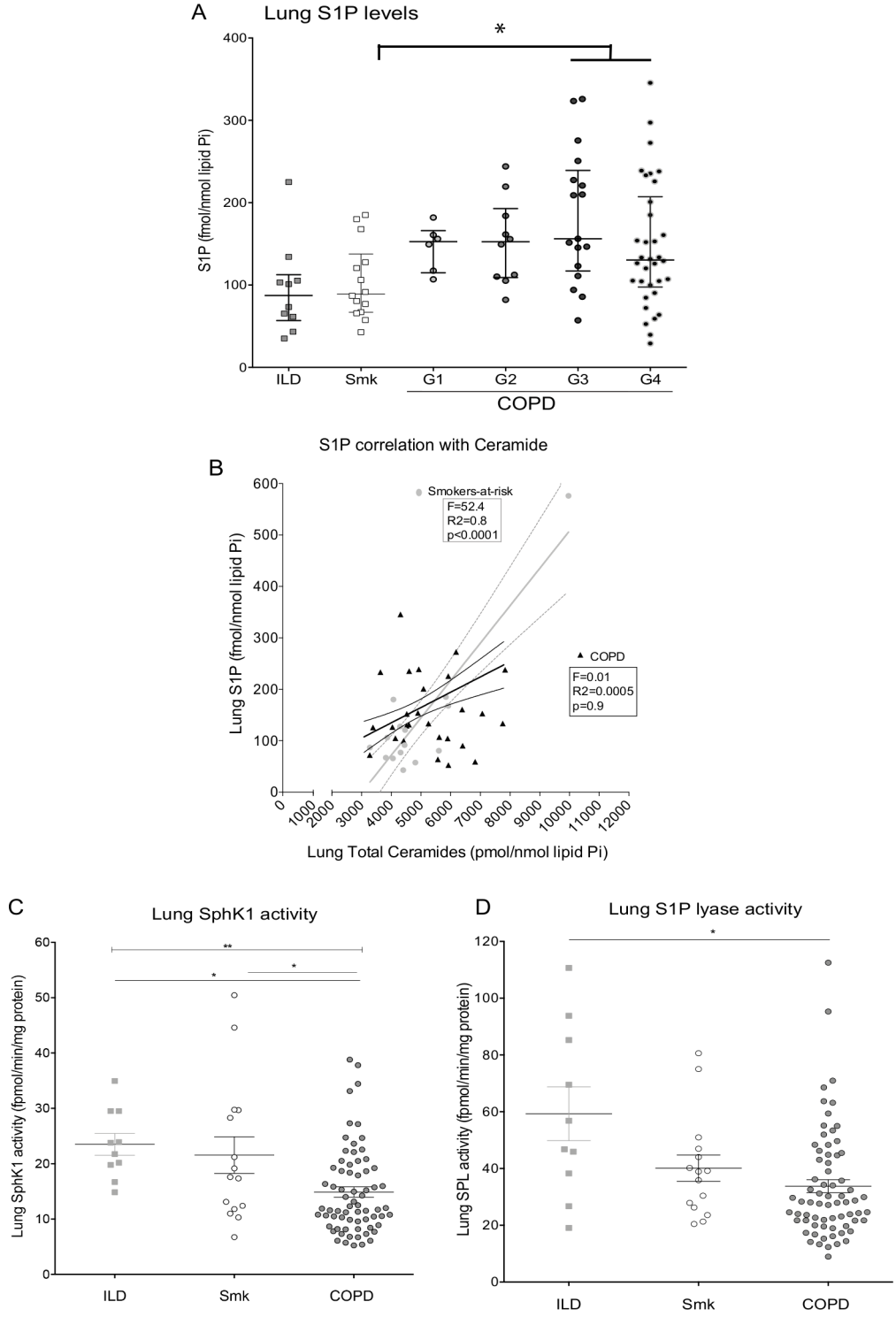

Figure 2 Lung S1P and metabolic enzymes. (A) S1P levels in lung homogenates (median and IQR, each data point representing an individual subject; ANOVA, Kruskal-Wallis; overall p=0.008; *p<0.05 Smk versus GOLD 3 (G3) and G4). (B) Linear regression (Pearson) analyses between lung ceramide and S1P levels in the indicated groups. (C) Lung sphingosine kinase-1 (Sphk1) activity measured by mass spectrometry in the indicated groups. ANOVA: **p<0.01, intergroup comparisons: *p<0.05. (C) Lung S1P lyase activity measured by mass spectrometry in the indicated groups. ANOVA: **p=0.01, intergroup comparisons: *p<0.05. ANOVA, analysis of variance; COPD, chronic obstructive pulmonary disease; ILD, interstitial lung disease; S1P, sphingosine-1 phosphate; Smk, smokers.

COPD lungs can also exhibit fibrosis. We identified increased ceramide associated with increased SphK1 activity in IPF lungs similar to previous reports,[9 10] distinctly different from the increased SphK1 activity in COPD lungs.

We conclude that decreased metabolism of ceramide to S1P is linked to the pathology of COPD and emphysema phenotype.

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Contributors EVB designed and performed experiments and analysed and interpreted data. KAS analysed data and participated in manuscript writing. KSS coordinated experiments and participated in manuscript writing. IB performed data analyses. AM performed statistical analyses. IP designed and coordinated experiments, performed data analysis and interpretation and wrote the manuscript.

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