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Cigarette Smoke exposure Alters Phosphodiesterases in Human Structural Lung Cells

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Abstract

Cigarette smoke (CS), a highly complex mixture containing more than 4000 compounds, causes aberrant cell responses leading to tissue damage around the airways and alveoli which underlies various lung diseases. Phosphodiesterases (PDEs) are a family of enzymes that hydrolyze cyclic nucleotides. PDE inhibition induces bronchodilation, reduces the activation and recruitment of inflammatory cells, and the release of various cytokines. Currently, the selective PDE4 inhibitor roflumilast is an approved add-on treatment for patients with severe chronic obstructive pulmonary disease (COPD) with chronic bronchitis and a history of frequent exacerbations. Additional selective PDE inhibitors are being tested in pre-clinical and clinical studies. However, the effect of chronic CS exposure on the expression of PDEs is unknown.

Using mRNA isolated from nasal and bronchial brushes and lung tissues of never-smokers and current smokers, we compared the gene expression of 25 PDE coding genes. Additionally, the expression and distribution of PDE3A and PDE4D in human lung tissues was examined. This study reveals that chronic CS exposure modulates the expression of various PDE members. Thus, CS exposure may change the levels of intracellular cyclic nucleotides and thereby impact the efficiency of PDE-targeted therapies.

Abbreviations

CS, cigarette smoke; PDE, phosphodiesterase; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; COPD, chronic obstructive pulmonary disease.

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Introduction

Cigarette smoke (CS), which is a complex mixture of more than 4000 chemicals, is known to cause several respiratory ailments due to damage around the airways and alveoli (19). It has been demonstrated that CS exerts a variety of toxic effects on cellular functions in the lung, including but not limited to increased risk of protein and lipid oxidation, abnormal ceramide metabolism, endoplasmic reticulum stress, and cell death (4, 8, 26). Cyclic nucleotides are ubiquitous intracellular second messengers that, by acting in discrete subcellular microdomains, regulate a plethora of physiological and pathological processes in the lung including bronchodilation and cytokine release (1, 3, 7). Phosphodiesterases (PDEs), which are a family of enzymes that hydrolyze cyclic nucleotides, play important roles in inflammatory cell accumulation, cytokine and chemoattractant release, bronchoconstriction, vascular hypertrophy and remodeling (17, 27) These PDEs regulate their intracellular signals in a compartmentalized manner (17, 27). The superfamily of PDEs is composed of 11 families with distinct substrate specificities, molecular structures and subcellular localization. Depending on the substrate preference for either cyclic adenosine monophosphate (cAMP) and/or cyclic guanosine monophosphate (cGMP), PDEs are divided into 3 groups: cAMP-specific PDEs (PDE4, PDE7, and PDE8), cGMP-specific PDEs (PDE5, PDE6, and PDE9) and dual-specific PDEs (PDE1, PDE2, PDE3, PDE10 and PDE11) (17, 27). Each PDE family has at least one, often multiple coding genes, resulting in more than 21 genes (18).

Earlier studies indicated that altered gene/ protein PDE isoform levels were correlated with respiratory disease pathophysiology (ie. PDE3 and PDE4) (11, 24, 27). PDE inhibition has benefits in structural lung cells, including preventing CS-induced epithelial dysfunction (14, 15, 22), inducing airway smooth muscle relaxation (5, 28), and preventing emphysema (12, 16). Current therapies focus primarily on PDE3 and PDE4 inhibitors (27). For example, the selective PDE4 inhibitor roflumilast is approved as add-on treatment for severe chronic obstructive pulmonary disease (COPD) patients with chronic bronchitis and a history of frequent exacerbations. Additional selective PDE inhibitors are being tested in pre-clinical and clinical studies (18, 27). However, the impact of chronic CS exposure on the expression of PDEs is ill defined. The aim of our study was to investigate the effect of chronic CS exposure

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on PDE gene expression and protein distribution in nasal epithelium, bronchial epithelium and lung tissue in current and never smokers.

Methods

Bronchial and nasal brushings collection, RNA extraction and microarray processing

The Study to Obtain Normal Values of Inflammatory Variables From Healthy Subjects (NORM; NCT00848406) included healthy smokers and never smokers as previously described (10). The study was approved by the University Medical Center Groningen ethics committee and all subjects provided their written informed consent. The characteristics of healthy smokers and never smokers are summarized in **Table 1A**. Nasal and bronchial epithelium was collected at the same time, using a Cyto-Pak CytoSoft nasal brush (Medical Packaging Corporation, Camarillo, Calif) or a Cellebrity bronchial brush (Boston Scientific, Marlborough, Mass). Microarrays were used for genome wide gene expression profiling. Methods for RNA extraction, labeling and microarray processing have been described previously (10). PDEs were also measured in lung tissue samples by microarray, which has been previously described (2). In the current study we focused on a subset of samples collected as part of the Groningen cohort. All the microarray data was analyzed using the Bioconductor-limma package in R software version 3.5.1.

To identify PDEs differentially expressed in matched nasal and bronchial brushes between current (n=41) and never smokers (n=36), we ran a linear model using limma (R statistical software) correcting for age and gender; while for the lung tissue samples we compared current smokers (n=165) and never smokers (n=39), using a linear model correcting for age and gender. Clinical characteristics of subject groups are tabulated in **Table 1B**.

Immunoblotting

Human lung tissue was obtained from eleven non-COPD control individuals without airway obstruction with different smoking statuses (5 never smokers and 6 current smokers) (**Table 1C**) according to the Research Code of the University Medical Center Groningen (http://www.rug.nl/umcg/onderzoek/researchcode/index) and national ethical and professional guidelines ("Code of conduct; Dutch federation of

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biomedical scientific societies"; http://www.federa.org). RIPA buffer (65 mM Tris, 155 mM NaCl, 1% Igepal CA-630, 0.25% sodium deoxycholate, 1 mM EDTA, pH 7.4 and a mixture of protease inhibitors: 1 mM Na_3VO_4, 1 mM NaF, 10 µg/mL leupetin, 10 µg/mL pepstatin A, 10 µg/mL aprotinin) was used to lyse tissue. Equal amounts of total protein were loaded for 10% SDS–polyacrylamide gel electrophoresis. After transferring to a nitrocellulose membrane, primary antibodies anti-PDE3A (kindly provided by Chen Yan, rabbit polyclonal antibody, 1:1000) (23), anti-PDE4D (kindly provided by Prof. Marco Conti, ICOS 4D, rabbit monoclonal antibody, 1:2000) (20, 21) and anti-GAPDH (HyTest, 1:10,000) were incubated at 4°C overnight, followed by secondary antibody (anti-mouse, IgG, 1:5,000 or anti-rabbit, IgG, 1:5,000, Sigma) incubation at room temperature for one hour. The antibodies specificity was indicated previously (28). Protein bands were developed on film using Western detection ECL-plus kit (PerkinElmer, Waltman, MA). ImageJ software was used for densitometric analyses (28).

Immunohistochemistry

Human lung tissue (**Table 1C**) sections were stained with primary antibodies anti-PDE3A (Santa Cruz, goat polyclonal antibody, 1:100), anti-PDE4D (kindly provided by Prof. George Baillie, sheep polyclonal antibody, 1:4500) (13) overnight at 4°C. The following day, tissue sections were incubated with HRP-conjugated anti-sheep and anti-goat antibodies for 2 hours (1:100, DAKO).

For color development, NovaRed (Vector Laboratories) was applied on slides and hematoxylin was used as a counterstain. Images were captured using a slide scanner (Nanozoomer 2.0 HT, Hamamatsu Photonics) with 20× magnification. Semi-quantification of the staining intensity in the epithelium and smooth muscle around airways from never smokers (n=87 airways from 12 donors) and current smokers (n=24 airways from 8 donors) was performed by 4 blinded observers on a scale from [0] to [3].

Statistical analyses

Lung homogenate data were analyzed using GraphPad Prism 6 (GraphPad, La Jolla, USA) and presented as mean ± SEM. The statistical significance of the data was examined using two-tailed unpaired Students t test for normally distributed data or by

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by either Mann-Whitney comparison or Kolmogorov-Smirnov comparison. For all data a $p < 0.05$ was considered statistically significant.

Results

In the nasal epithelium of current smokers, PDE4A, PDE7A, and PDE8A were significantly decreased compared to never smokers ($p<0.05$), whereas PDE10A were significantly increased ($p<0.05$) **(Fig. 1, Table 2)**. In bronchial epithelium from current smokers PDE1A, PDE3A, PDE4D, PDE5A, PDE7A, PDE7B, PDE8A, PDE8B, and PDE11A were significantly downregulated ($p<0.05$) (**Fig. 1**), while PDE4C, PDE6A, PDE6B, and PDE9A were upregulated ($p<0.05$) in the current smokers compared to never smokers **(Fig. 1)**. In total lung tissue, only 4 PDE genes were changed, with a decrease ($p<0.05$) of PDE1A and PDE11A and an increase ($p<0.05$) of PDE4D and PDE6A in current smokers versus never smokers **(Fig. 1)**.

Since PDE3 and PDE4 are pharmaco-therapeutic targets for obstructive lung disease (6), we further studied these PDEs at the protein level. To investigate the influence of CS on the protein expression, we used total lung homogenates of never and current smokers. Protein expression of PDE3A and PDE4D did not differ across the groups in total lung homogenates **(Fig. 2A)**.

To dissect the cell type distribution of PDE3A and PDE4D, immunostainings for these PDE isoforms were performed. As shown in **Fig. 2B**, PDE3A and PDE4D were expressed in airway epithelium and airway smooth muscle in both never smokers and current smokers. PDE3A was also strongly expressed in vascular smooth muscle. In current smokers, PDE3A and PDE4D increased in airway epithelium compared to never smokers (**Fig. 2B**), but no difference was observed in airway smooth muscle.

Discussion

This study is the first to report differences of PDE family member mRNA levels in response to CS exposure in patients. Using nasal and bronchial epithelium as well as total lung tissue, our study shows that the gene expression of multiple PDEs in current smokers is changed compared to that of never smokers. Importantly, the

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gene expression changes of a number of PDE members was reflected in two study groups, including PDE1A (decreased in bronchial epithelium and lung tissue), PDE6A (increased in bronchial epithelium and lung tissue), PDE7A (decreased in nasal epithelium and bronchial epithelium) and PDE11A (decreased in bronchial epithelium and lung tissue). Studies in the lung with focus on PDE1A, PDE6A, PDE7A and PDE11A are largely lacking. Our data suggest that these PDE isoforms are of central importance in the changes induced by CS exposure. Strikingly, PDE4D had a contrasting pattern of change (decreased in bronchial epithelium and increased in lung tissue), possibly pointing to an alternative regulatory role for this PDE in the different compartments of the respiratory tract or alternatively cell type specific expression and the shift in these cell types may cause the shift in expression levels during smoke exposure.

Alterations in expression of PDEs are linked to pulmonary disorders. Acute CS extract exposure increased the gene expression of PDE3B and PDE4D and the protein expression of PDE3A and PDE4D in human airway smooth muscle cells (28). In whole lung tissue of mice, acute CS exposure induced a higher PDE4 activity, accompanied by an increase in both gene and protein expression of PDE4B and PDE4D (28). These studies reflect the increase we saw in PDE4D in lung tissue but not the nasal or bronchial epithelium, possibly suggesting the PDE4 lung tissue signal is driven by mesenchymal cells rather than the epithelial cells. In concert, in asthmatic airway smooth muscle cells, isoproterenol-induced cAMP production was decreased due to enhanced PDE4D protein expression, in comparison to non-asthmatic airway smooth muscle cells (24). Acute CS exposure did not alter the gene and protein expression of PDE3A in human bronchial epithelial cells (28). In our study, a decrease of PDE3A mRNA was observed in the bronchial epithelium of current smokers compared to never smokers, which highlights the chronic influence of CS exposure on the gene expression of PDE3A. In contrast, an increased PDE3A protein expression was found in airway epithelium in current smokers, pointing to a possible differential effect of CS on gene and protein regulation of PDE3A. Gene expression of PDE4D was decreased in the bronchial epithelium, but was increased in lung tissue. In agreement, protein expression of PDE4D was increased in airway epithelium in current smokers. As protein expression of PDE3A and PDE4D were not different in total lung homogenates, changes in CS-induced PDE expression are

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restricted to distinct lung compartments. In addition to altered regulation of PDE3A and PDE4D, we now show that chronic CS exposure could also modulate the gene expression of other PDE members, for which the functions are largely unknown and urgently require more investigations.

The PDE4 inhibitor roflumilast is approved for the treatment of patients with severe COPD (25), however unwanted side effects including nausea and vomiting still limit its oral administration (9). Dual inhibition of PDE3 and PDE4 acted as an add-on tool further enhancing their therapeutic benefits (9, 27). Here we show that PDE4 was the only PDE subfamily for which gene expression changes were observed in all 3 groups (nasal epithelium, bronchial epithelium and lung tissue), the gene expression of PDE4A, PDE4C and PDE4D (not PDE4B) were significantly changed. In contrast, only the gene expression of PDE3A was significantly decreased in the bronchial epithelium. We report here on a change in protein expression of both PDE3A and PDE4D in the airway epithelium of current smokers. Therefore, targeting PDE3A and PDE4D specifically might potentially increase the therapeutic benefit for patients with fewer side effects, however, clearly more preclinical experiments are needed.

This is the first study to show that chronic CS exposure leads to alterations in PDE expression in different cell types in the lung. Further investigation will expand our understanding of the contribution of a defined subset of PDEs to mechanisms driving lung diseases and elucidate the possibility of using PDEs subfamilies as potential pharmaceutical targets for treating COPD depending on patients' smoking status.

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Figure legend

Figure 1. Comparison of gene expression of PDE isoforms in current smokers versus never smokers. (A) The difference of PDE isoforms was compared in nasal epithelium (red points), bronchial epithelium (blue points) and lung tissues (black points). All the points above black solid line are considered as significant change. The left side of the black dotted line indicate genes decreased in current smokers compared to never-smokers, whereas the right side indicate genes increased in current smokers compared to never-smokers.

Figure 2. (A) Protein expression of PDE3A and PDE4D in lung homogenates of never smokers (n=5) and current smokers (n=6). (B) Representative images of PDE3A and PDE4D staining. Arrows indicate airway epithelium and smooth muscle. Semi-quantitative staining intensity around airways of never smokers (n=87, airways from 12 donors) and current smokers (n=24, airways of 8 donors). PDE, brown staining (NovaRed); hematoxylin counterstain.

Table 1A. **Clinical characteristics NORM study**

	All (N=77)	Never-smokers (N=36)	Current smokers (N=41)
Age, yr	36.06 (16.23)	34.89 (17.10)	37.10 (15.55)
BMI kg/m^2	23.73 (3.50)	23.52 (3.76)	23.91 (3.29)
Gender, Male/Female	41/36	16/20	25/16
Pack years****	8.68 (13.4)	0	16.30 (14.63)
FEV_1% predicted	108.14 (10.49)	109.75 (10.24)	106.73 (10.62)
Reversibility % from baseline	3.82 (3.05)	3.82 (3.50)	3.82 (2.64)
FEV_1/FVC	83.06 (6.37)	84.54 (6.57)	81.75 (5.97)
RV % predicted	93.74 (17.46)	94.78 (21.62)	92.83 (12.99)
TLC % predicted	104.04 (9.42)	105.44 (9.19)	102.80 (9.56)
RV/TLC % predicted	85.62 (12.38)	85.25 (15.59)	85.95 (8.84)

BMI, body mass index; FEV_1, forced expiratory volume in one second; FEV_1/FVC, forced expiratory volume in one second/ forced vital capacity; RV, residual volume; TLC, total lung capacity; RV/TLC, residual volume/ total lung capacity.
*The mean and standard deviation are shown for continuous variables. Unpaired T-test showed no significant difference between the two groups except **** Significant at p < 0.0001.*

Table 1B: **Characteristics of subject groups**

	Never smokers	Current smokers

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Male subjects, no. (%)	20 (51)	86 (52)
Age, yr	47.5 (12.82)	57.8 (10.53)
Pack years	0	30 (17.44)
FEV_1% predicted	60.91 (31.74)	66.96 (31.62)

FEV_1= Forced Expiratory Volume in one second, FEV_1 % predicted = FEV_1 percentage predicted

Table 1C. **Patients characteristics: immunoblotting**

	Never smokers	Current smokers
Number of subjects	5	6
Age, yr	54.6 (45.0-69.0)	56.2 (47.0-65.0)
Male/Female	4/1	2/4
Pack years	0	44.4 (14.0-75.0)
FEV_1% Predicted	95.2 (70.0-130.0)	95.0 (74.0-111.0)
FEV_1/FVC%	79,1 (73.0-86.0)	78.3 (62.4-92.0)

FEV_1, forced expiratory volume in one second; FEV_1/FVC, forced expiratory volume in one second/ forced vital capacity; FEV_1% predicted and FEV_1/FVC% were measured post bronchodilators.

Table 1D. **Patients characteristics: immunohistochemistry**

	Never smokers	Current smokers
Number of subjects	12	8
Age, yr	62.2 (40.0-81.0)	56.5 (45.0-63.0)
Male/Female	3/9	2/6
Pack years	0	37.1 (15.0-81.0)
FEV_1% Predicted	99.9 (80.0-116.0)	92.6 (67.9-105.9)
FEV_1/FVC%	77.8 (71.8-84.0)	77.9 (71.5-90.1)

FEV_1, forced expiratory volume in one second; FEV_1/FVC, forced expiratory volume in one second/ forced vital capacity; FEV_1% predicted and FEV_1/FVC% were measured pre bronchodilators.

Table 2.**Transcriptional differences of PDE in comparison**

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	Nasal Brush			Bronchial Brush			Lung Tissue		
	logFC	P.Value	adj.P.Val	logFC	P.Value	adj.P.Val	logFC	P.Value	adj.P.Val
PDE1A	0.07	5.09E-01	0.782716	-0.40	5.94E-06	1.05E-04	-0.46	3.40E-04	6.36E-02
PDE1B	-0.10	7.13E-02	0.309923	-0.21	6.39E-02	1.32E-01	-0.02	7.99E-01	9.53E-01
PDE1C	-0.01	8.38E-01	0.941114	0.07	2.48E-01	3.46E-01	0.13	1.83E-01	6.62E-01
PDE2A	-0.01	8.56E-01	0.94893	0.10	2.74E-01	3.72E-01	-0.17	1.13E-01	5.86E-01
PDE3A	0.01	8.89E-01	0.961216	-0.26	5.39E-04	3.62E-03	-0.07	1.90E-01	6.70E-01
PDE3B	-0.02	8.34E-01	0.940098	0.08	3.29E-01	4.28E-01	0.12	2.35E-01	7.09E-01
PDE4A	-0.25	2.48E-06	0.001174	0.06	2.02E-01	2.98E-01	0.06	2.29E-01	7.04E-01
PDE4B	-0.06	7.05E-01	0.885564	-0.08	3.16E-01	4.14E-01	0.13	3.05E-01	7.55E-01
PDE4C	0.06	2.26E-01	0.553695	0.19	1.90E-02	5.44E-02	0.18	1.79E-01	6.58E-01
PDE4D	0.08	9.38E-02	0.357909	-0.15	6.57E-03	2.48E-02	0.27	1.90E-02	3.47E-01
PDE5A	0.15	1.99E-01	0.520577	-0.17	1.01E-02	3.43E-02	-0.08	3.16E-01	7.61E-01
PDE6A	0.05	1.81E-01	0.498468	0.18	6.51E-04	4.16E-03	0.19	1.18E-02	2.89E-01
PDE6B	0.01	9.02E-01	0.96435	0.24	1.92E-08	9.65E-07	0.16	6.82E-02	5.14E-01
PDE6C	0.05	3.07E-01	0.633937	0.00	9.84E-01	9.89E-01	-0.18	6.59E-02	5.08E-01
PDE6D	-0.05	5.19E-01	0.789229	-0.13	3.17E-02	7.94E-02	-0.01	8.23E-01	9.59E-01
PDE6G	-0.02	6.66E-01	0.866589	0.11	2.98E-01	3.96E-01	NA	NA	NA
PDE6H	0.05	2.89E-01	0.618107	0.04	3.40E-01	4.39E-01	-0.02	7.14E-01	9.32E-01
PDE7A	-0.13	1.14E-02	0.127596	-0.57	1.54E-10	1.66E-08	-0.17	1.98E-01	6.75E-01
PDE7B	-0.25	7.58E-02	0.319725	-0.57	2.30E-08	1.12E-06	-0.08	5.00E-01	8.57E-01
PDE8A	-0.14	2.77E-02	0.1939	-0.27	5.81E-05	6.28E-04	0.04	6.51E-01	9.14E-01
PDE8B	-0.18	4.07E-01	0.715006	-0.24	2.25E-02	6.18E-02	0.13	1.34E-01	6.10E-01
PDE9A	0.09	1.10E-01	0.388535	0.15	2.38E-03	1.14E-02	0.06	4.58E-01	8.40E-01
PDE10A	0.28	6.19E-03	0.092195	0.15	8.34E-02	1.58E-01	-0.19	7.41E-02	5.25E-01
PDE11A	-0.01	7.51E-01	0.907926	-0.21	5.57E-05	6.09E-04	-0.12	8.00E-04	9.34E-02
PDE12	0.01	8.87E-01	0.960349	-0.16	4.45E-02	1.02E-01	0.06	3.17E-01	7.62E-01

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Figure 1

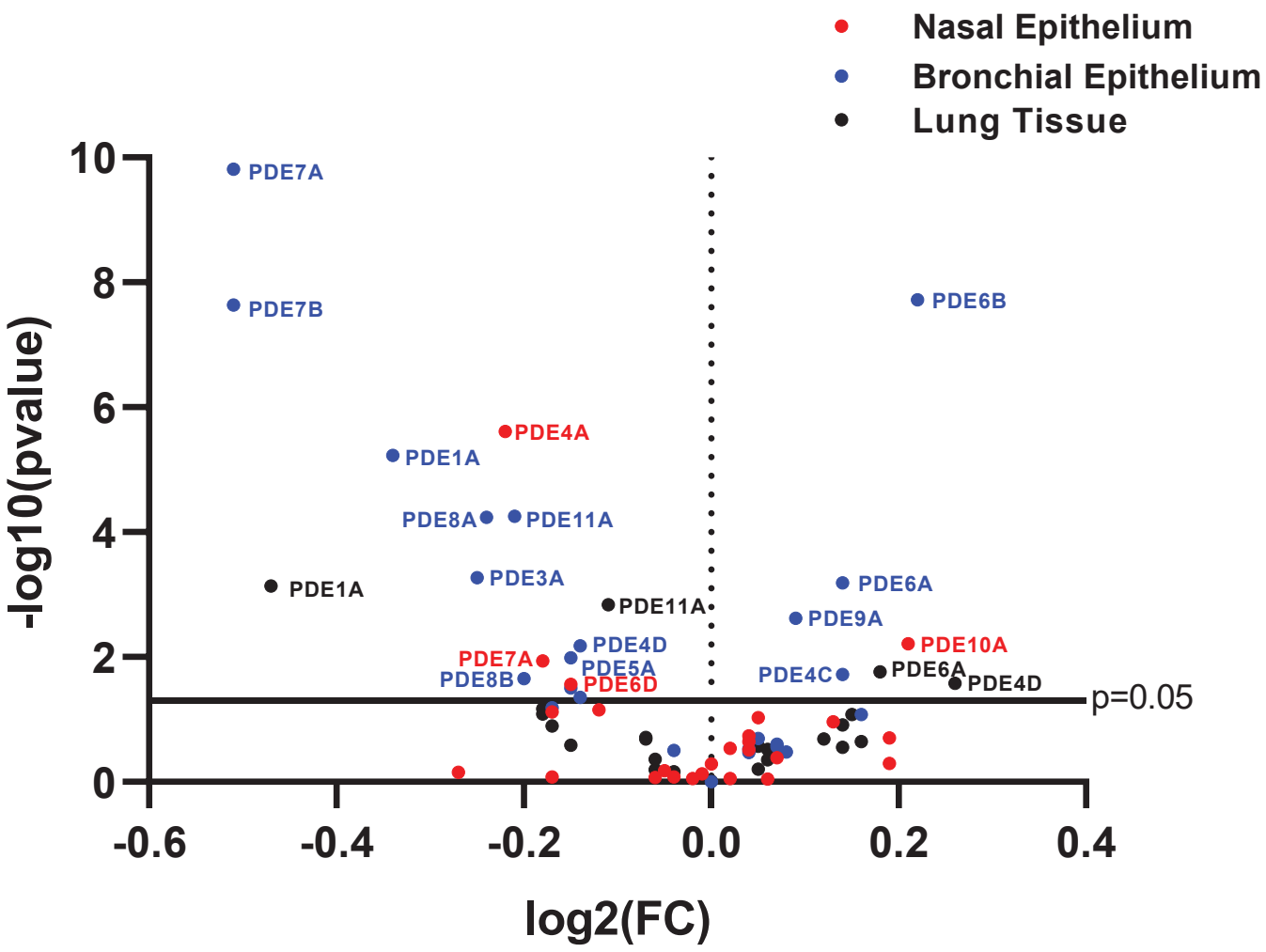

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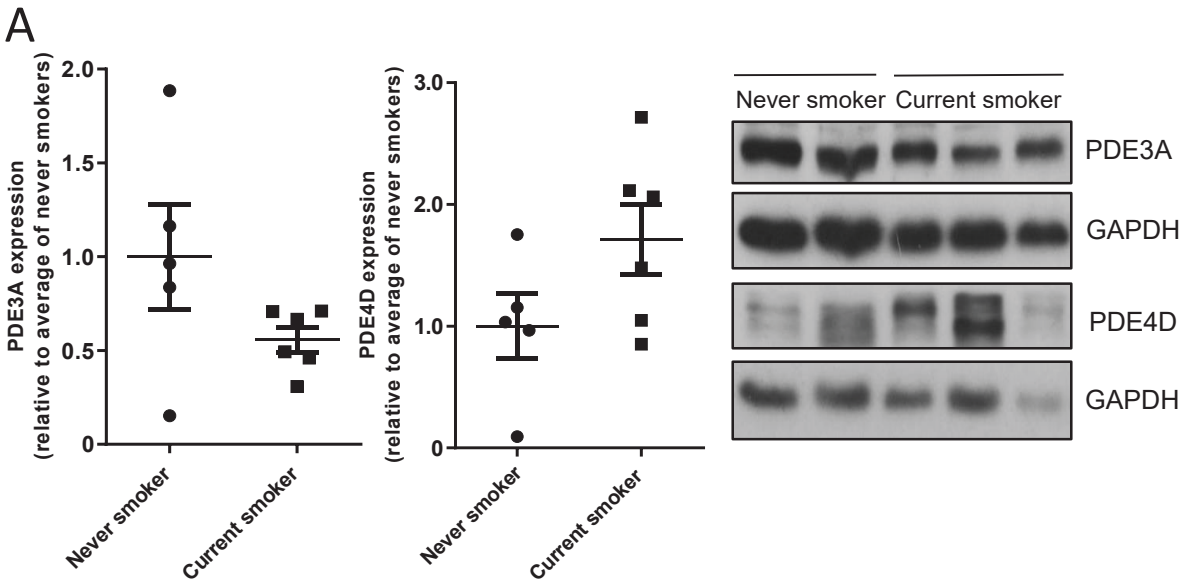
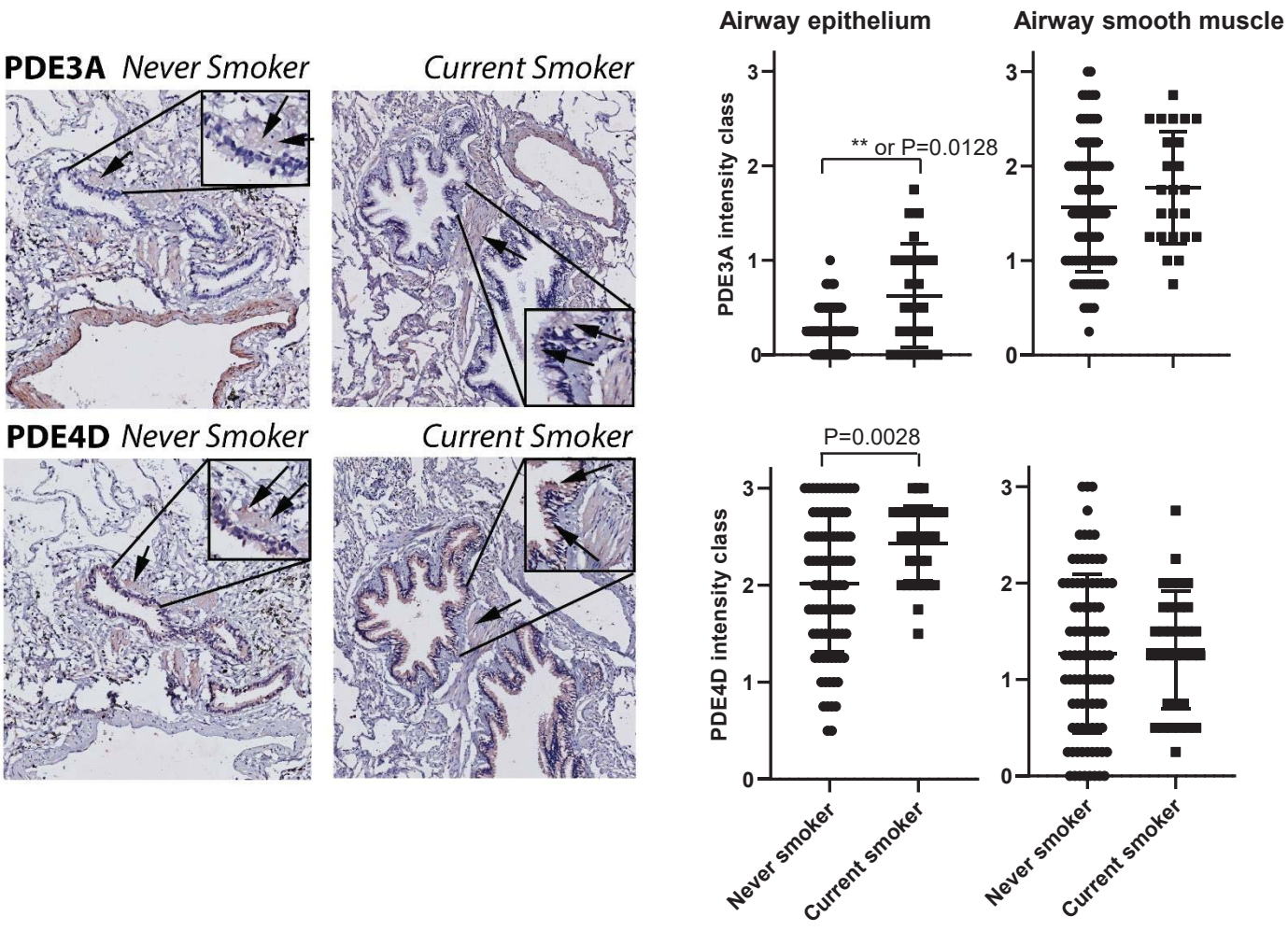
Figure 2





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