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The dopamine D₁ receptor is expressed and induces CREB phosphorylation and MUC5AC expression in human airway epithelium

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Abstract

Background: Dopamine receptors comprise two subgroups, G_s protein-coupled “D₁-like” receptors (D₁, D₅) and G_i-coupled “D₂-like” receptors (D₂, D₃, D₄). In airways, both dopamine D₁ and D₂ receptors are expressed on airway smooth muscle and regulate airway smooth muscle force. However, functional expression of the dopamine D₁ receptor has never been identified on airway epithelium. Activation of G_s-coupled receptors stimulate adenylyl cyclase leading to cyclic AMP (cAMP) production, which is known to induce mucus overproduction through the cAMP response element binding protein (CREB) in airway epithelial cells. We questioned whether the dopamine D₁ receptor is expressed on airway epithelium, and whether it promotes CREB phosphorylation and MUC5AC expression.

Methods: We evaluated the protein expression of the dopamine D₁ receptor on native human airway epithelium and three sources of cultured human airway epithelial cells including primary cultured airway epithelial cells, the bronchial epithelial cell line (16HBE14o-), and the pulmonary mucoepidermoid carcinoma cell line (NCI-H292) using immunohistochemistry and immunoblotting. To characterize the stimulation of cAMP through the dopamine D₁ receptor, 16HBE14o- cells and NCI-H292 cells were treated with dopamine or the dopamine D₁ receptor agonists (SKF38393 or A68930) before cAMP measurements. The phosphorylation of CREB by A68930 in both 16HBE14o- and NCI-H292 cells was measured by immunoblot. The effect of dopamine or A68930 on the expression of MUC5AC mRNA and protein in NCI-H292 cells was evaluated by real-time PCR and immunofluorescence staining, respectively.

Results: The dopamine D₁ receptor protein was detected in native human airway epithelium and three sources of cultured human airway epithelial cells. Dopamine or the dopamine D₁-like receptor agonists stimulated cAMP production in 16HBE14o- cells and NCI-H292 cells, which was reversed by the selective dopamine D₁-like receptor antagonists (SCH23390 or SCH39166). A68930 significantly increased phosphorylation of CREB in both 16HBE14o- and NCI-H292 cells, which was attenuated by the inhibitors of PKA (H89) and MEK (U0126). Expression of MUC5AC mRNA and protein were also increased by either dopamine or A68930 in NCI-H292 cells.

Conclusions: These results suggest that the activation of the dopamine D₁ receptor on human airway epithelium could induce mucus overproduction, which could worsen airway obstructive symptoms.

Keywords: Dopamine D₁ receptor, G_s-coupled receptor, cAMP, CREB, MUC5AC

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Background

Dopamine is a predominant catecholamine neurotransmitter in the mammalian central nervous system [1–4] but it also plays a role in modulating peripheral physiologic actions such as renal and cardiovascular functions through specific dopamine receptor subtypes expressed in peripheral organs and tissues [3, 5–8]. The dopamine receptors belong to the superfamily of G protein-coupled receptors (GPCR), and five different receptor subtypes (D₁-D₅) have been divided into two subgroups, the G_s protein-coupled “D₁-like” receptors (D₁, D₅ subtypes) and the G_i-coupled “D₂-like” receptors (D₂, D₃, D₄ subtypes) [3, 9]. Dopamine, by acting on the dopamine D₁-like receptor, stimulates adenylyl cyclase activity to increase intracellular cyclic AMP (cAMP) levels [10], which stimulate cAMP-dependent protein kinase (PKA) [11]. PKA phosphorylates a range of target proteins including the cAMP response element binding protein (CREB) [12–14].

In airways, dopamine is localized in the lung [15], and acts as a neurotransmitter in addition to its role as a noradrenaline precursor [16]. Dopamine D₁ and D₂ receptors are expressed on lung alveolar type I cells, which line most of the alveolar surface, and contribute to lung fluid homeostasis [17]. In addition, either inhaled or intravenously administered dopamine has bronchodilatory effects in human healthy and asthmatic subjects [18]. We have previously shown that dopamine D₁ and D₂ receptors are expressed on airway smooth muscle itself, and that the dopamine D₁ receptor modulates airway smooth muscle tone through adenylyl cyclase/cAMP production [19, 20], which would favor airway relaxation in asthmatics. Although, the dopamine D₂ receptor was not detected on airway epithelial tissue [19], the functional expression of the dopamine D₁-like receptor on airway epithelium remains poorly characterized.

In respiratory diseases including asthma, COPD, and cystic fibrosis, mucus hypersecretion is a recognized component of the pathophysiology. Airway epithelium is the predominant source of mucus, which contributes to airway narrowing and obstruction. MUC5AC, which is induced by phosphorylation of CREB [21, 22], is predominantly expressed in respiratory epithelium and constitutes 95–98% of the mucin secreted in the human airway [23]. Interestingly, the dopamine D₁-like receptor agonist SKF83959 significantly exacerbated bronchial mucus production in ovalbumin-sensitized mice [24], which would in theory, therapeutically contrast with its direct relaxation of airway smooth muscle [20]. Similar contrasting findings have been reported with G_s-coupled β₂-adrenoceptor agonists, which are widely used as bronchodilators, but have been reported to increase mucin production via activation of β₂-adrenoceptors on airway epithelial cells [25]. These findings led us to

hypothesize that functional dopamine D₁-like receptors are expressed on airway epithelium and promote mucus production through cellular cAMP's activation of the PKA-CREB-MUC5AC axis.

In the present study, protein expression of the dopamine D₁-like receptor was examined in native human airway epithelial tissue and cultured human airway epithelial cells. In addition, effects of the dopamine D₁ receptor on cAMP production, CREB phosphorylation, and MUC5AC expression were assessed to confirm their physiological role in airway epithelium.

Methods

Materials

Protease inhibitor cocktail III was purchased from EMD Millipore (Billerica, MA). Antibiotic-antimycotic mix, DMEM/F-12 medium, fetal bovine serum (FBS), and RPMI-1640 medium were purchased from Thermo Fisher Scientific (Waltham, MA). A68930 and SCH39166 were purchased from Tocris Bioscience (Bristol, UK). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Cell culture

Primary cultured normal human bronchial epithelial cells (CC-2541; Lonza, Walkersville, MD) were grown in Clonetics™ BEGM BulletKit (CC-3170, Lonza) supplemented with the following growth supplements: bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, transferrin, insulin, retinoic acid, triiodothyronine, and gentamicin/amphotericin-B at the concentrations recommended by the manufacturer. 16HBE14o- cells, a human bronchial epithelial cell line which was kindly gifted from Dr. Tilla S. Worgall (Columbia University, New York NY), were grown in minimal essential medium supplemented with 10% FBS and 200 μg/ml geneticin (G418). NCI-H292 cells (CRL-1848; American Type Culture Collection, Manassas, VA), a human pulmonary muco-epidermoid carcinoma cell line, were cultured in RPMI-1640 medium containing 5% FBS. Primary cultured human airway smooth muscle cells (HASM; cc-2576, Lonza) were grown in DMEM/F12 culture medium, supplemented with 10% FBS and an antibiotic-antimycotic mix (100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B). All the cells were incubated at 37°C in humidified 95% air/5% CO₂.

Preparation of human trachea

Studies were approved by Columbia University's Institutional Review Board (IRB) and deemed not human subjects research under 45 CFR 46. Human trachea was obtained from discarded regions of healthy donor lungs harvested for lung transplantation at Columbia University.

Human tissue was transported to the laboratory in cold (4 °C) M199 cell culture media. The exterior of human trachea was carefully dissected free of adherent connective tissue under a microscope. The tissue sample was used for immunohistochemistry and immunoblot.

Immunohistochemistry

Human tracheal rings were fixed with 4% paraformaldehyde/1% glutaraldehyde in 0.1 M phosphate buffer for 4 h at 4 °C and were then dehydrated through a graded ethanol series. The tracheal rings were embedded in paraffin and cut into 10- μ m-thick sections. The tracheal ring sections were deparaffinized in xylene and rehydrated in descending grades of alcohol. Heat-mediated antigen retrieval was performed in Tris-EDTA buffer (10 mM Tris-base and 1 mM EDTA, pH 9.0) for 2 min using a pressure cooker. Endogenous peroxidase activity was blocked with 3% H₂O₂ for 15 min. Sections were blocked with 10% normal goat serum in phosphate-buffered saline with 0.1% Triton X-100 (PBST) for 30 min, then avidin-biotin blocking was performed as previously described [26]. The slides were kept in humidified chamber with primary antibody against the dopamine D₁ receptor protein (rabbit monoclonal 1:2000; 2192-1, Epitomics, Burlingame, CA) or dopamine D₅ receptor protein (rabbit polyclonal 1:2000; sc-25,650, Santa Cruz Biotechnology, Santa Cruz, CA) in 2% normal goat serum in PBST for overnight at 4 °C. The immunoreactivity of these antibodies directed against the dopamine D₁ or D₅ proteins were previously confirmed in control tissues during immunohistochemistry [20]. A parallel tracheal ring section was incubated with an isotype-specific rabbit IgG antibody (Thermo Fisher Scientific) as a negative control. The slides were then washed three times with PBST and primary antibodies were detected using biotinylated anti-rabbit antibodies (Vector Laboratories, Burlingame, CA) at a concentration of 1:200. After incubation with ABC-HRP complex (Vector Laboratories) for 30 min, the antigen-antibody complex was visualized with the peroxidase substrate kit (DAB) (SK-4100, Vector Laboratories). The sections were counterstained with hematoxylin (Vector Laboratories), dried, dehydrated in ascending grades of alcohol, and cover slipped using Poly-mount mounting medium (Polysciences, Warrington, PA).

Immunoblot analysis

Freshly dissected native human airway epithelium was homogenized (Tekmar Ultra Turrax T25 high-speed homogenizer set at top speed for 30 s) in cold (4 °C) buffer (50 mM Tris, 10 mM HEPES, pH 7.4, 1 mM EDTA with a 1:200 dilution of protease inhibitor cocktail III). The homogenate was filtered through 125- μ m Nitex mesh and centrifuged twice at 500 g for 15 min. The

supernatant was transferred into new tubes and centrifuged at 50,000 g for 30 min at 4 °C. The final membrane pellet was resuspended in the same buffer for protein concentration determinations and stored at -80 °C.

For analysis of dopamine D₁ receptor expression, confluent cultures of either primary cultured normal human bronchial epithelial cells, 16HBE14o- cells, NCI-H292 cells, or primary cultured human airway smooth muscle cells were rinsed with ice-cold phosphate-buffered saline (PBS), and mechanically scraped from the surface of the T75 culture flask in the presence of protease inhibitor cocktail III. Cells were pelleted (500 g, 10 min, 4 °C) and lysed in ice-cold lysis buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Nonidet P-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 μ g/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride, 1:200 dilution of protease inhibitor cocktail III]. Lysed cells were centrifuged (15,000 g, 15 min, 4 °C) and an aliquot of the supernatant was subjected to protein analysis and storing at -80 °C. For analysis of CREB phosphorylation, 16HBE14o- cells or NCI-H292 cells were serum-starved for 24 h, and then treated with dopamine D₁ receptor agonist A68930 (1 μ M) for indicated times (5–60 min). In separate experiments, NCI-H292 cells were initially pretreated with 10 μ M H89 (PKA inhibitor; 30 min) or 5 μ M U0126 (MEK inhibitor; 120 min) before treatment of the cells with A68930 (1 μ M; 20 min). After treatment, the cells were washed twice with ice-cold PBS, and lysed in ice-cold RIPA buffer (Cell signaling Technology (CST), Danvers, MA) supplemented with 1 mM phenylmethanesulfonyl fluoride and a 1:200 dilution of protease inhibitor cocktail III. Each lysed cell sample was harvested and centrifuged at 15000 g for 15 min at 4 °C, and an aliquot of the supernatant was subjected to protein analysis. The protein concentration of each sample was determined using Pierce BCA reagents (Thermo Fisher Scientific), using BSA as a control, and samples were stored at -80 °C. Each sample was solubilized by heating at 95 °C for 10 min in sample buffer (final concentrations: 50 mM Tris HCl pH 6.8, 2.5% SDS, 6% glycerol, 2.5% 2-mercaptoethanol, and bromophenol blue) before use. Lysates were electrophoresed (10% Mini-Protean TGX™ precast gel; Bio-Rad, Hercules, CA) and transferred to PVDF membranes using a Trans-Blot Turbo™ transfer system (Bio-Rad). The PVDF membrane was blocked for 1 h at room temperature with 5% membrane blocking agent (RPN418; GE Healthcare, Waukesha, WI) in Tris-buffered saline with 0.1% Tween 20 (TBST). Membranes were then probed with antibodies directed against the dopamine D₁ receptor protein (rabbit monoclonal 1:1000; 2192-1, Epitomics) or the dopamine D₅ receptor protein (rabbit polyclonal 1:500; sc-25,650, Santa Cruz

Biotechnology) overnight at 4 °C. For the CREB phosphorylation study, the membranes were probed with antibodies directed against the anti-phospho CREB (rabbit monoclonal 1:1000; CST #9198), or anti-CREB (rabbit monoclonal 1:1000; CST #9197) overnight at 4 °C. After washing three times with TBST, membranes were incubated for 1 h at room temperature with HRP-labeled secondary anti-rabbit antibodies (1:5000; GE Healthcare, NA934V). The signals from the immunoreactive bands were detected by ECL Prime (GE Healthcare) and the signal was captured using a chemiluminescent image analyzer (LAS 4000 Mini; GE Healthcare). The same PVDF membranes were stripped and reprobed with the antibody against the GAPDH protein (rabbit monoclonal 1:2000, CST #5174) to demonstrate the variation in protein loading on the gels. For analysis of CREB phosphorylation, the band intensities were measured using Image J software (NIH) and were expressed as a ratio of the phosphorylated/total CREB protein.

cAMP assays

Cyclic AMP (cAMP) production in 16HBE14o- and NCI-H292 cell lines was measured using a HitHunter™ cAMP Assay for Small Molecules kit (DiscoverX, Fremont, CA) according to the manufacturer's instructions. Briefly, the cells grown in white-walled 96-well plates and were washed twice with warm PBS (37 °C). The cells were incubated with dopamine (1 μM), or the dopamine D₁-like receptor agonists (A68930 or SKF38393) (1 μM) for 20 min at 37 °C. The concentration and duration of the dopamine D₁-like receptor agonists used in this study was determined based on previous studies [20, 27]. In separate experiments, the cells were pretreated with the dopamine D₁-like receptor antagonists [SCH23390 (1 μM) or SCH39166 (1 μM)] or vehicle (PBS) for 30 min followed by incubation with A68930 (1 μM) for 20 min at 37 °C. Then the cAMP antibody reagent followed by the cAMP working solution (mixture of enzyme donor/lysis buffer/Emerald II/Galacton) was added to each well and incubated for 60 min at room temperature. Cells were further incubated with the enzyme acceptor reagent for 3 h at room temperature, and luminescence signals were detected using a multimode microplate reader (Appliskan, Thermo Fisher Scientific). The data from the triplicate wells were averaged for each sample.

Preparation of cigarette smoke extract

Cigarette smoke extract (CSE) was prepared by a modification of a previously published method [28]. Smoke of four commercial filtered cigarettes (Marbolo Red, Philip Morris, Richmond, VA; 1.0 mg nicotine; 12 mg Tar) was bubbled through 50 mL of PBS using a vacuum pump. This 100% CSE solution was adjusted to pH 7.4 and

filtered through a 0.2 μm-pore filter to remove bacteria and large particles.

Cell viability assay

NCI-H292 cell viability after 48 h treatment with CSE (10% or 20%) was measured using an MTT assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Briefly, NCI-H292 cells were seeded in a 96-well plate (5000 cells/well) in RPMI-1640 medium containing 0.5% FBS for 24 h. Then, the cells were incubated with or without CSE (10% or 20%) for 48 h in a final volume of 100 μl/well. Ten microliters of MTT reagent were added to each well and the cells were re-incubated for 4 h at 37 °C in a CO₂ incubator. The medium was aspirated, and 100 μl of the crystal dissolving solution were added to each well. The absorbance of each sample was measured at 570 nm using a multimode microplate reader (Appliskan; Thermo Fisher Scientific). The results were expressed as a percentage of surviving cells over control (no treatment) cells.

Quantitative RT-PCR

Total RNA was extracted from cultured NCI-H292 cells after 48 h treatment with dopamine (1 μM), A68930 (1 μM), isoproterenol (1 μM), or CSE (10%) using the RNeasy Mini Kit (QIAGEN, Valencia, CA). Total RNA was transcribed into cDNA using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) in accordance with the manufacturer's instructions. Quantitative real-time PCR on the CFX96 Real-Time PCR Detection System (Bio-Rad) was performed using Thunderbird SYBR qPCR kit (Toyobo) according to manufacturer's instruction. Primer sequences for *MUC5AC* and *GAPDH* were shown in Table 1. The specificity of amplification was confirmed by melting curve analysis. The Ct value determined by the CFX manager Software (Bio-Rad) for all samples was normalized to the housekeeping gene *GAPDH*, and the relative fold induction against untreated controls was computed by the comparative Ct ($\Delta\Delta Ct$) method.

Table 1 Primer sequences

Target	Sequence of Primer	Amplicon size (bp)
Human MUC5AC	FP: 5'- GGA GGA AGC TGG CCC TGC TCT GG-3'	116
	RP: 5'- AGA GAG GGC AGG GTG GTG CTT GT-3'	
Human GAPDH	FP: 5'- CCA GGG CTG CTT TTA ACT CTG GTA AAG TGG ATA-3'	173
	RP: 5'- CAT CGC CCC ACT TGA TTT TGG AGG GA -3'	

FP forward primer, RP reverse primer

Immunofluorescence staining

Immunofluorescence staining of MUC5AC protein in NCI-H292 cells was carried out according to the previously described method [29] with some modifications. Briefly, NCI-H292 cells were seeded on an 8-chamber microscope slide and serum-starved for 24 h. After starvation, cells were exposed to A68930 (1 μ M), dopamine (1 μ M), or CSE (10%) for 48 h. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and washed 3 times with PBS. After permeabilization (0.2% Triton X-100 in PBS for 5 min) and blocking (1% bovine serum albumin in 0.1% Triton X-100 in PBS for 15 min), cells were incubated with Alexa Fluor 488-conjugated MUC5AC antibody (sc-21,701 AF488, Santa Cruz Biotechnology) overnight at 4C. After cells were washed twice with PBS, the slide was coverslipped with ProLong gold antifade-reagent with DAPI (Thermo Fisher Scientific), and visualized with an inverted fluorescent microscope (DMI-4000; Leica Microsystems, Wetzlar, Germany). Digitized images were captured with MetaMorph software (Molecular Devices, Sunnyvale, CA). When capturing the images, we kept constant the duration of image capture (300 ms), the image intensity gain, the image enhancement, and the image black level among the samples.

Statistical analysis

The data were analyzed with two-tailed paired Student's t-test when comparing means between two groups or repeated measures of ANOVA followed by Bonferroni post

test when comparing multiple groups using GraphPad Prism 6 for Mac OS X software (GraphPad Software, La Jolla, CA). Data are presented as mean \pm SEM; $P < 0.05$ was considered significant.

Results

Immunohistochemical detection of dopamine D₁-like receptor expression in human tracheal epithelium

Initially, we examined the protein expression of the dopamine D₁ and D₅ receptor in human tracheal epithelium by immunohistochemistry. Light microscopic immunohistochemical staining for the dopamine D₁ receptor was observed throughout the epithelial layer of human trachea (indicated by brown color) (Fig. 1a). In contrast, no staining of the dopamine D₅ receptor was detected in the airway epithelial layer (Fig. 1c). Consecutive sections exposed to rabbit IgG isotype-specific control antibodies yielded no staining (Fig. 1b and d).

Immunoblot analysis of the dopamine D₁ receptor in human airway epithelium

We further examined whether the dopamine D₁ receptor protein was expressed in human airway epithelial tissue and cells by immunoblot. A single immunoreactive band of the appropriate molecular mass for the dopamine D₁ receptor (75 kDa) was identified in freshly dissected human tracheal epithelium, human pulmonary mucocarcinoma cells (NCI-H292 cells), human bronchial epithelial cells (16HBE14o- cells), primary cultured human bronchial epithelial cells, and primary

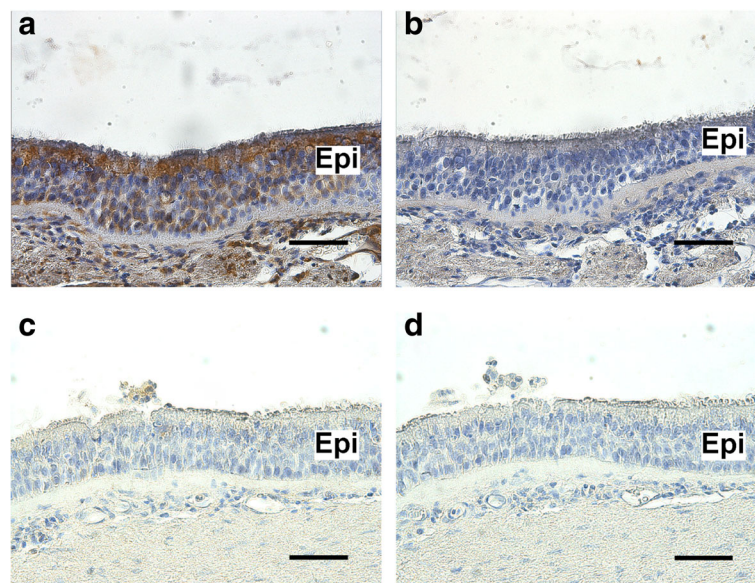


Fig. 1 Immunohistochemical detection of dopamine D₁-like receptor expression in human tracheal epithelium. **a** and **c** Representative immunohistochemical staining of dopamine D₁ receptor (**a**) and dopamine D₅ receptor (**c**) in paraformaldehyde/glutaraldehyde-fixed human tracheal epithelium. **b** and **d** anti-rabbit IgG isotype negative control in serial section of human tracheal epithelium. All sections were counterstained with hematoxylin. Calibration bars: 50 μ m. Epi, airway epithelium. Images are representative of at least 3 independent immunohistochemical analyses from human trachea

cultured human airway smooth muscle cells (positive control) (Fig. 2). These results suggest that the dopamine D₁ receptor is expressed on human airway epithelium of both trachea and bronchi as well as human airway smooth muscle.

Dopamine D₁ receptor agonist-induced cAMP activity in 16HBE14o- cells and NCI-H292 cells

The dopamine D₁ receptor induces production of cAMP through the stimulation of adenylyl cyclase, which is activated by the G_s protein [30]. Therefore, we examined whether activation of the dopamine D₁ receptor increases intracellular cAMP levels in human airway epithelial cells (16HBE14o- cells and NCI-H292 cells). Dopamine (1 μM) as well as dopamine D₁-like receptor agonists (SKF38393 or A68930; 1 μM) significantly increased intracellular cAMP levels in 16HBE14o- cells (dopamine; $P < 0.001$, SKF38393; $P < 0.001$, A68930; $P < 0.01$, $n = 6$) and NCI-H292 cells (dopamine; $P < 0.01$, SKF38393; $P < 0.01$, A68930; $P < 0.01$, $n = 6$) (Fig. 3a). Cyclic AMP production induced by A68930 (1 μM) was significantly reversed by pretreatment with the dopamine D₁ receptor antagonists SCH23390 (1 μM) (16HBE14o- cells: $P < 0.001$, $n = 6$; NCI-H292 cells: $P < 0.05$, $n = 6$) or SCH39166 (1 μM) (16HBE14o- cells: $P < 0.001$, $n = 6$; NCI-H292 cells: $P < 0.05$, $n = 6$) (Fig. 3b).

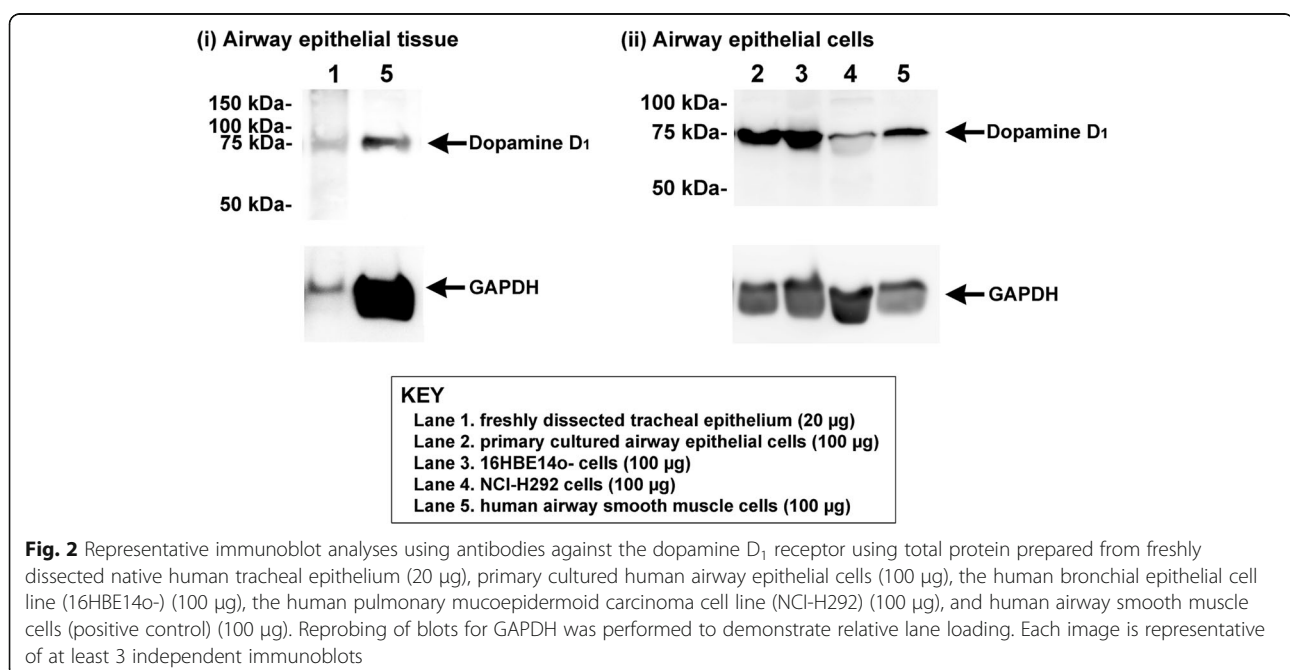
Dopamine D₁ receptor agonist-induced CREB phosphorylation in 16HBE14o- cells and NCI-H292 cells

The increase of cAMP after dopamine D₁ receptor activation induces the activation of protein kinase A (PKA), which induces phosphorylation of CREB [14]. MEK-ERK

signaling also contributes to CREB phosphorylation [13]. In addition, the dopamine D₁ receptor activates MEK-ERK signaling through β-arrestin [31]. Therefore, we examined whether the dopamine D₁ receptor agonist A68930 phosphorylates CREB through PKA and/or MEK in 16HBE14o- cells and NCI-H292 cells. A68930 (1 μM, 20 min) significantly increased phosphorylation of CREB in 16HBE14o- cells ($P < 0.05$, $n = 3$) and NCI-H292 cells ($P < 0.05$, $n = 7$) (Fig. 4a and b). The phosphorylation reached maximal levels at 20–30 min and then slowly declined to basal levels within 60 min in 16HBE14o- cells, while the increased phosphorylation was maintained at 60 min in NCI-H292 cells. To confirm that A68930 phosphorylates CREB through PKA or MEK, NCI-H292 cells were pretreated with the PKA inhibitor H89 (10 μM; 30 min) or the MEK inhibitor U0126 (5 μM; 120 min). A68930 (1 μM; 20 min)-stimulated CREB phosphorylation in NCI-H292 cells was significantly inhibited by H89 ($P < 0.001$, $n = 4$) or U0126 ($P < 0.05$, $n = 4$) (Fig. 4c). These results confirm that the dopamine D₁ receptor agonist-induced phosphorylation of CREB proceeds through both PKA and MEK/ERK signaling.

Dopamine D₁ receptor agonist-induced MUC5AC mRNA expression in NCI-H292 cells

CREB was previously shown to mediate the transcriptional regulation of MUC5AC in airway epithelial cells including NCI-H292 cells [21, 22, 32]. We examined whether dopamine or the dopamine D₁ receptor agonist A68930 induces MUC5AC mRNA expression in NCI-H292 cells. Dopamine (1 μM), A68930 (1 μM), and



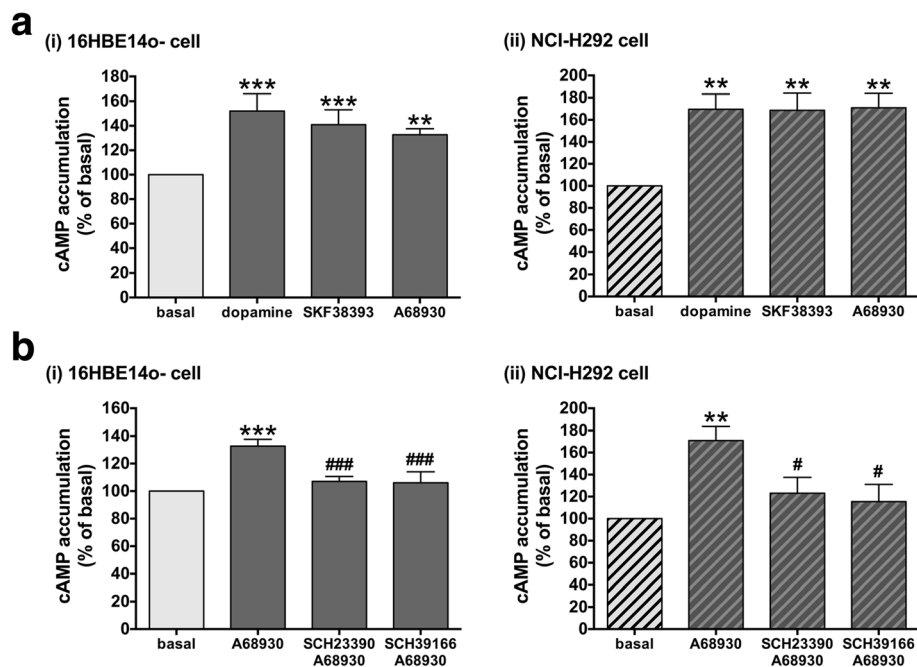


Fig. 3 a The effects of dopamine (1 μ M) or the dopamine D_1 receptor agonists (A68930 or SKF38393; 1 μ M respectively) on cAMP production in cultured (i) 16HBE14o- cells and (ii) NCI-H292 cells. $n = 6$. **b** The effect of dopamine D_1 -like receptor selective antagonists SCH23390 or SCH39166 on A68930-stimulated cAMP production in cultured (i) 16HBE14o- cells and (ii) NCI-H292 cells. Cells were pretreated with SCH23390 (1 μ M) or SCH39166 (1 μ M) for 30 min prior to A68930 (1 μ M) treatment for 20 min. $n = 6$. Data represent means \pm SEM. ** $P < 0.01$ and *** $P < 0.001$ compared with basal. # $P < 0.05$ and ### $P < 0.001$ compared with A68930 alone

cigarette smoke extract (CSE) (10%) significantly increased *MUC5AC* mRNA expression in NCI-H292 cells. The G_s protein-coupled β_2 adrenoceptor agonist isoproterenol (1 μ M) also significantly induced *MUC5AC* mRNA expression (Fig. 5a). Previous studies in airway epithelial cells have employed final CSE concentrations ranging from 1 to 30% [33, 34]. MTT cell viability analyses confirmed that 48 h treatment of NCI-H292 cells with 10% CSE or even higher concentrations (20%) of CSE did not reduce NCI-H292 cell viability, suggesting that treatment with CSE at 10% or 20% has no cytotoxicity (Fig. 5b).

Dopamine D_1 receptor agonist-induced *MUC5AC* protein expression in NCI-H292 cells

We further investigated the effect of the dopamine D_1 receptor agonist A68930 or dopamine on *MUC5AC* protein expression using an immunofluorescent assay. Consistent with the mRNA data of *MUC5AC*, *MUC5AC* protein expression in NCI-H292 cells were increased by dopamine (1 μ M), A68930 (1 μ M), and CSE (10%) (Fig. 6). These results suggest that activation of dopamine D_1 receptor stimulates *MUC5AC* expression.

Discussion

The primary findings of the present study are that functional dopamine D_1 receptors are expressed in human

airway epithelium. Activation of the dopamine D_1 receptor stimulated cAMP production, CREB phosphorylation, and mRNA and protein expression of *MUC5AC* in human airway epithelial cells.

In airways, we have previously reported that both dopamine D_1 and D_2 receptors are expressed on airway smooth muscle itself, and regulate airway smooth muscle force [19, 20]. In the present study, protein expression of the dopamine D_1 receptor on human airway epithelium was identified by immunohistochemistry, while the dopamine D_5 receptor protein was not detected. Immunoblot analyses further confirmed that the dopamine D_1 receptor is expressed in freshly dissected native human tracheal epithelium, primary cultured human bronchial epithelial cells and cell lines derived from airway epithelium (16HBE14o- cells and NCI-H292 cells). Since the dopamine D_2 receptor was not expressed on airway epithelium [19], the dopamine D_1 receptor would be the predominant dopamine receptor subtype expressed on human airway epithelium.

Thereafter, we further investigated whether functional dopamine D_1 receptors could modulate airway epithelial function. The activation of G_s -coupled receptors including the dopamine D_1 receptor and β_2 -adrenoceptor stimulates cAMP accumulation via activation of adenylyl cyclase [3, 35, 36]. In accordance with these previous findings, we demonstrated that dopamine or the

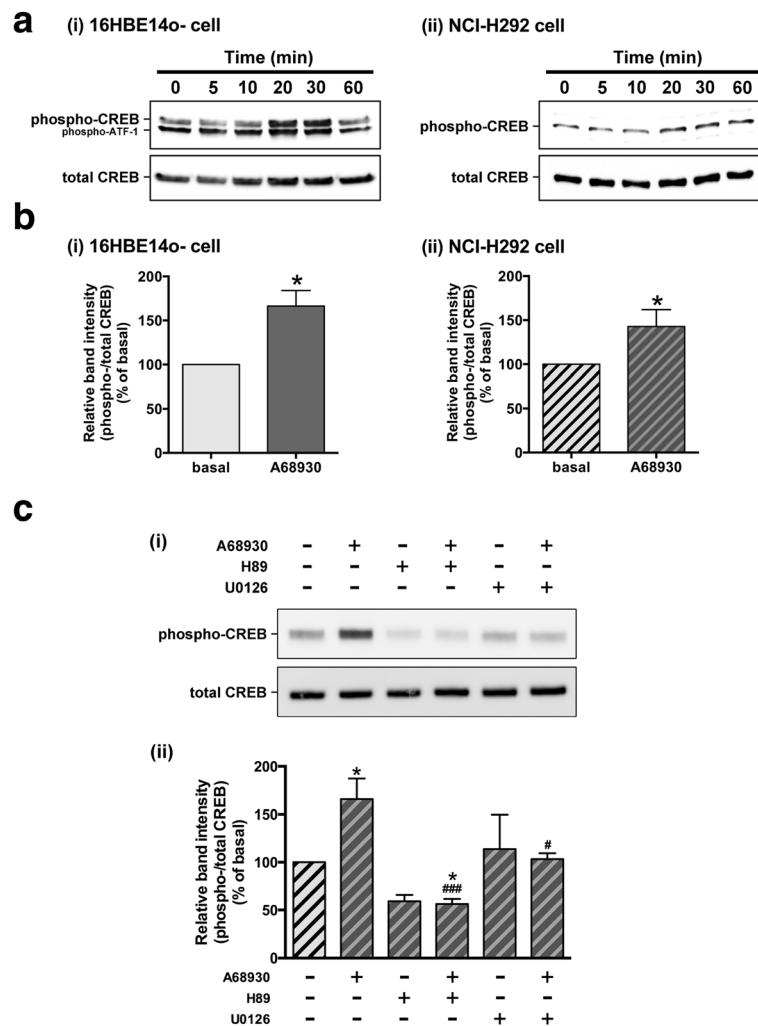
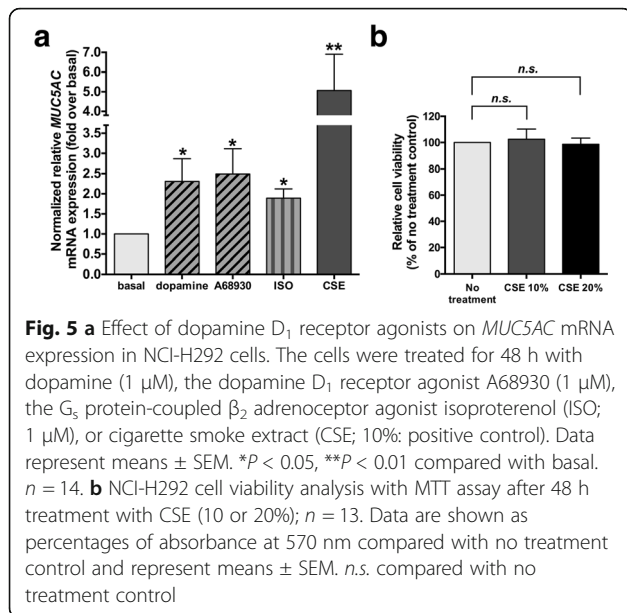


Fig. 4 Effects of the dopamine D₁ receptor agonist A68930 on the phosphorylation of CREB in cultured airway epithelial cells. Cells were stimulated with A68930 (1 μ M), and subsequently cell lysates were processed to detect phosphorylated (upper panels) and total (lower panels) levels of CREB by immunoblot. **a** Representative immunoblot analyses of the time-course effect of the dopamine D₁ receptor agonist A68930 (1 μ M) on CREB phosphorylation in cultured (i) 16HBE14o- cells and (ii) NCI-H292 cells. Phosphorylated activating transcription factor 1 (ATF-1) is a CREB family member that is also recognized by the phospho-CREB antibody and closely correlates with CREB [13]. **b** Effect of A68930 (1 μ M, 20 min) on CREB phosphorylation in (i) 16HBE14o- cells ($n = 3$) and (ii) NCI-H292 cells ($n = 7$). **c** Effects of inhibitors of PKA or MEK on A68930-stimulated CREB phosphorylation in NCI-H292 cells ($n = 4$). Cells were pretreated with the PKA inhibitor H89 (10 μ M, 30 min), or the MEK inhibitor U0126 (5 μ M, 120 min) before treatment with A68930 (1 μ M, 20 min). Data represent means \pm SEM. * $P < 0.05$ compared with basal. # $P < 0.05$ and ### $P < 0.001$ compared with A68930 alone

dopamine D₁ receptor agonists (SKF38393 or A68930) stimulated cAMP production in 2 different cell lines of human airway epithelial cells (16HBE14o- cells and NCI-H292 cells), which was significantly reversed by pretreatment the cells with the dopamine D₁-like receptor antagonists (SCH23390 or SCH39166). These findings suggest that stimulation of the dopamine D₁ receptor on human airway epithelial cells induces intracellular cAMP accumulation.

A major signaling target of cAMP is PKA, which can translocate to the nucleus and subsequently phosphorylate CREB. The present study demonstrated that the

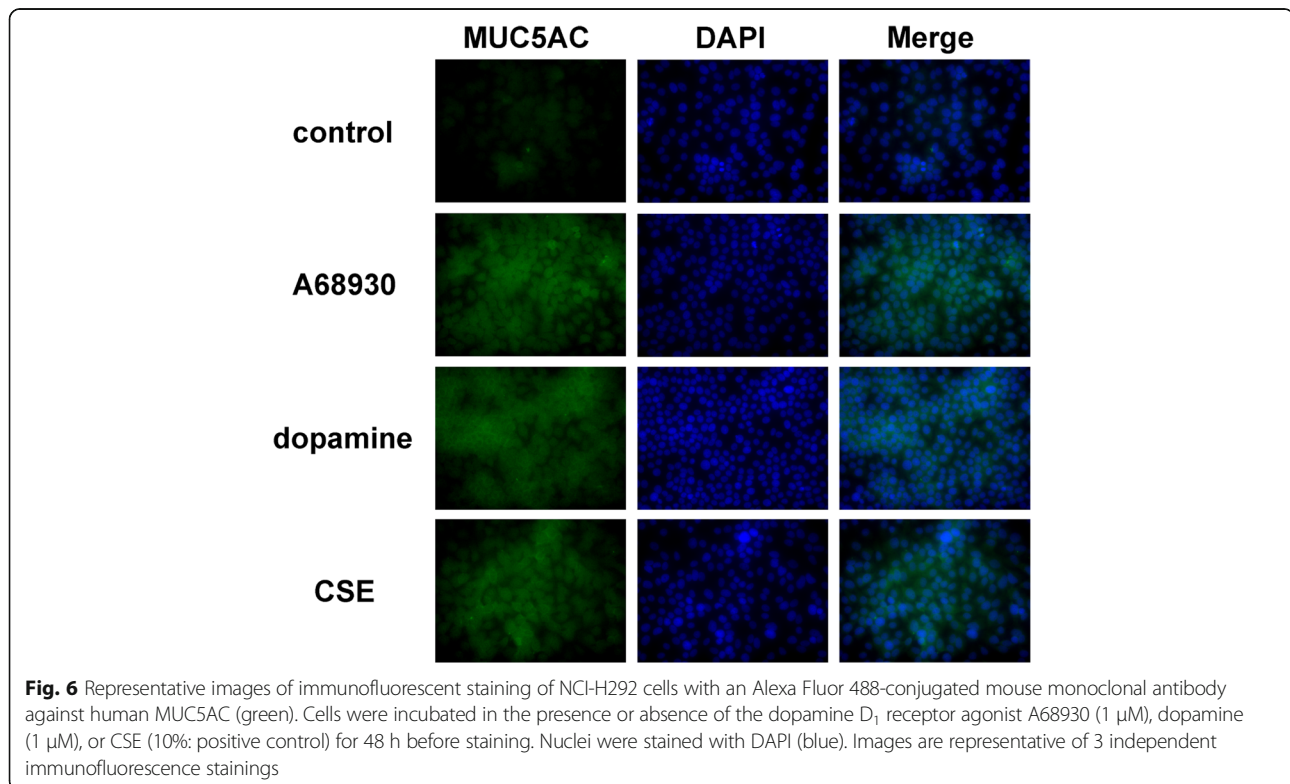
dopamine D₁ receptor agonist A68930 induced phosphorylation of CREB in 16HBE14o- cells and NCI-H292 cells. This phosphorylation reached maximum levels at 20–30 min which is consistent with our findings that A68930 significantly induced cAMP accumulation at 20 min in 16HBE14o- cells and NCI-H292 cells. Furthermore, A68930-induced CREB phosphorylation was blocked by the PKA inhibitor H89, suggesting that activation of the dopamine D₁ receptor induces CREB phosphorylation through classical cAMP-PKA signaling. Other principal signaling cascades responsible for CREB phosphorylation include the MEK-ERK pathway [13].



Previous studies suggested that the dopamine D₁ receptor activates MEK-ERK signaling through β-arrestin [31]. In the present study, the MEK inhibitor U0126 significantly inhibited dopamine D₁ receptor-mediated CREB phosphorylation. Collectively, activation of dopamine D₁ receptor induces CREB phosphorylation through both cAMP/PKA and MEK/ERK signaling.

CREB increases the transcription of *MUC5AC* in NCI-H292 cells [21]. Our findings demonstrated that dopamine or the dopamine D₁ receptor agonist A68930 induced expression of *MUC5AC* mRNA and *MUC5AC* protein, the most prominent mucin in airways. These results were consistent with the previous findings from Gong et al. [24] that the dopamine D₁-like receptor agonist SKF83959 significantly exacerbated bronchial mucus production. They also showed that the dopamine D₁-like receptor antagonist SCH23390 attenuated mucus production in the ovalbumin-sensitized mice, although they speculated that attenuated mucus production after blockade of dopamine D₁-like receptor signaling by SCH23390 was predominantly mediated by decreased IL-17 secretion. In contrast, our results suggest that G_s-coupled dopamine D₁ receptor signaling could contribute to *MUC5AC* expression through cAMP/CREB pathways without involving inflammatory mediators.

Although activation of the dopamine D₁ receptor expressed on airway smooth muscle induced airway relaxation [20], the present study suggests that activation of the dopamine D₁ receptor on airway epithelium could worsen asthma symptoms by mucus overproduction. These paradoxical effects of dopamine D₁ receptor activation on airways would hamper the clinical use of a dopamine D₁ receptor agonist as a novel treatment option of asthma and COPD. Similar paradoxical findings have been reported in other G_s-coupled receptor (e.g.



β_2 -adrenoceptor) in airways. Although activation of β_2 -adrenoceptor on airway smooth muscle induces bronchodilation, β_2 -adrenoceptor on airway epithelial cells increase mucus production, which exacerbates the symptoms of asthma and COPD [25, 37]. In contrast, it is well established that cAMP stimulates ciliary motility of airway epithelium [38–40]. These findings point out the possibility that activation of the dopamine D₁ receptor on airway epithelium might enhance mucociliary clearance, which is beneficial for asthma and COPD patients with impaired airway mucociliary clearance. Moreover, elevation of cAMP levels and activation of PKA through G_s-coupled receptor also contributes to bronchial epithelial wound repair and regulation of cystic fibrosis transmembrane regulator (CFTR) activity in airway epithelium [41–43]. Thus, activation of the dopamine D₁ receptor on multiple cell types in the airway could affect multiple beneficial and potentially detrimental airway effects and it is unclear what the net effect would be in the setting of allergic lung inflammation and bronchoconstriction. A limitation of the present study is that we have used submerged NCI-H292 cells to examine dopamine D₁ receptor-mediated MUC5AC expression. Submerged cells in culture may not recapitulate the phenotype of in vivo airway epithelium as closely as primary cultured bronchial epithelial cells grown at an air-liquid interface. Further studies are required to identify the possible diverse roles of dopamine D₁ receptor in the normal physiology and pathophysiology of the airway.

Conclusions

In summary, our major findings in this study are that G_s-coupled dopamine D₁ receptors are expressed in human airway epithelium, and stimulate cAMP production, CREB phosphorylation, and MUC5AC expression. These results combined with our previous findings [20], suggest that activation of the dopamine D₁ receptor in airways can have complex net effects from the activation of the dopamine D₁ receptor on multiple cell types. While activation of the dopamine D₁ receptor on airway smooth muscle could have a bronchodilatory therapeutic benefit, the activation of the dopamine D₁ receptor on airway epithelium could induce mucus overproduction which may be counteracted by a beneficial effect on ciliary activity.

Abbreviations

cAMP: cyclic AMP; CFTR: Cystic fibrosis transmembrane regulator; CREB: cAMP response element binding protein; CSE: Cigarette smoke extract; HASM: Human airway smooth muscle; PBS: Phosphate-buffered saline; PKA: Protein kinase A

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Availability of data and materials

Please contact author for data requests.

Authors' contributions

CWE and KM conceived and designed the study. NM, SS, AM, TK, JD, AK, and KM performed the experiments. NM, SS, AM, TK, EM, CWE, and KM analyzed the data. CWE and KM wrote the paper. All authors reviewed, revised and approved final version of the manuscript.

Ethics approval and consent to participate

Human trachea was obtained from discarded regions of healthy donor lungs harvested for lung transplantation at Columbia University (deemed not human subjects research by Columbia University's Institutional Review Board).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

- Goto Y, Grace AA. The dopamine system and the pathophysiology of schizophrenia: a basic science perspective. *Int Rev Neurobiol.* 2007;78:41–68.
- Pivonello R, Ferone D, Lombardi G, Colao A, Lamberts SW, Hofland LJ. Novel insights in dopamine receptor physiology. *Eur J Endocrinol.* 2007;156(Suppl 1):S13–21.
- Missale C, Nash SR, Robinson SW, Jaber M, Caron MG. Dopamine receptors: from structure to function. *Physiol Rev.* 1998;78:189–225.
- Hasbi A, O'Dowd BF, George SR. Heteromerization of dopamine D2 receptors with dopamine D1 or D5 receptors generates intracellular calcium signaling by different mechanisms. *Curr Opin Pharmacol.* 2010;10:93–9.
- Ozono R, O'Connell DP, Wang ZQ, Moore AF, Sanada H, Felder RA, Carey RM. Localization of the dopamine D₁ receptor protein in the human heart and kidney. *Hypertension.* 1997;30:725–9.
- Hussain T, Lokhandwala MF. Renal dopamine receptors and hypertension. *Exp Biol Med (Maywood).* 2003;228:134–42.
- Ricci A, Mignini F, Tomassoni D, Amenta F. Dopamine receptor subtypes in the human pulmonary arterial tree. *Auton Autacoid Pharmacol.* 2006;26:361–9.
- Zizzo MG, Mule F, Mastropaolo M, Serio R. D1 receptors play a major role in the dopamine modulation of mouse ileum contractility. *Pharmacol Res.* 2010;61:371–8.
- Neve KA, Seamans JK, Trantham-Davidson H. Dopamine receptor signaling. *J Recept Signal Transduct Res.* 2004;24:165–205.
- Billington CK, Hall IP. Novel cAMP signalling paradigms: therapeutic implications for airway disease. *Br J Pharmacol.* 2012;166:401–10.
- Delghandi MP, Johannessen M, Moens U. The cAMP signalling pathway activates CREB through PKA, p38 and MSK1 in NIH 3T3 cells. *Cell Signal.* 2005;17:1343–51.

12. Naqvi S, Martin KJ, Arthur JS. CREB phosphorylation at Ser133 regulates transcription via distinct mechanisms downstream of cAMP and MAPK signalling. *Biochem J*. 2014;458:469–79.
13. Barlow CA, Barrett TF, Shukla A, Mossman BT, Lounsbury KM. Asbestos-mediated CREB phosphorylation is regulated by protein kinase a and extracellular signal-regulated kinases 1/2. *Am J Physiol Lung Cell Mol Physiol*. 2007;292:L1361–9.
14. Calabresi P, Picconi B, Tozzi A, Ghiglieri V, Di Filippo M. Direct and indirect pathways of basal ganglia: a critical reappraisal. *Nat Neurosci*. 2014;17:1022–30.
15. Aviado DM, Sadavongvivad C. Pharmacological significance of biogenic amines in the lungs: noradrenaline and dopamine. *Br J Pharmacol*. 1970;38:374–85.
16. Peiser C, Trevisani M, Groneberg DA, Dinh QT, Lencer D, Amadesi S, Maggiore B, Harrison S, Geppetti P, Fischer A. Dopamine type 2 receptor expression and function in rodent sensory neurons projecting to the airways. *Am J Physiol Lung Cell Mol Physiol*. 2005;289:L153–8.
17. Helms MN, Self J, Bao HF, Job LC, Jain L, Eaton DC. Dopamine activates amiloride-sensitive sodium channels in alveolar type I cells in lung slice preparations. *Am J Physiol Lung Cell Mol Physiol*. 2006;291:L610–8.
18. Michoud MC, Amyot R, Jeanneret-Grosjean A. Dopamine effect on bronchomotor tone in vivo. *Am Rev Respir Dis*. 1984;130:755–8.
19. Mizuta K, Zhang Y, Xu D, Masaki E, Panettieri RA, Jr., Emala CW. The dopamine D₂ receptor is expressed and sensitizes adenylyl cyclase activity in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol*. 2012;302:L316–L324.
20. Mizuta K, Zhang Y, Xu D, Mizuta F, D'Ovidio F, Masaki E, Emala CW. The dopamine D₁ receptor is expressed and facilitates relaxation in airway smooth muscle. *Respir Res*. 2013;14:89.
21. Kim CH, Kim KE, Yoon JH, Song KS. Upregulation of *MUC5AC* gene expression by IL-4 through CREB in human airway epithelial cells. *J Cell Biochem*. 2009;108:974–81.
22. Chen Y, Garvin LM, Nickola TJ, Watson AM, Colberg-Poley AM, Rose MC. IL-1 β induction of *MUC5AC* gene expression is mediated by CREB and NF- κ B and repressed by dexamethasone. *Am J Physiol Lung Cell Mol Physiol*. 2014;306:L797–807.
23. Shin IS, Park JW, Shin NR, Jeon CM, Kwon OK, Lee MY, Kim HS, Kim JC, Oh SR, Ahn KS. Melatonin inhibits *MUC5AC* production via suppression of MAPK signaling in human airway epithelial cells. *J Pineal Res*. 2014;56:398–407.
24. Gong S, Li J, Ma L, Li K, Zhang L, Wang G, Liu Y, Ji X, Liu X, Chen P, Ouyang R, Zhang S, Zhou Z, Wang CY, Xiang X, Yang Y. Blockade of dopamine D₁-like receptor signalling protects mice against OVA-induced acute asthma by inhibiting B-cell activating transcription factor signalling and Th17 function. *FEBS J*. 2013;280:6262–73.
25. Al-Sawalha N, Pokkunuri I, Omoluabi O, Kim H, Thanawala VJ, Hernandez A, Bond RA, Knoll BJ. Epinephrine activation of the β_2 -adrenoceptor is required for IL-13-induced mucin production in human bronchial epithelial cells. *PLoS One*. 2015;10:e0132559.
26. Mizuta K, Zhang Y, Mizuta F, Hoshijima H, Shiga T, Masaki E, Emala CW Sr. Novel identification of the free fatty acid receptor FFAR1 that promotes contraction in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol*. 2015;309:L970–82.
27. Grenader A, Healy DP. A68930 is a potent, full agonist at dopamine₁ (D₁) receptors in renal epithelial LLC-PK₁ cells. *Br J Pharmacol*. 1992;106:229–30.
28. Yang M, Chen P, Peng H, Zhang H, Chen Y, Cai S, Lu Q, Guan C. Cigarette smoke extract induces aberrant cytochrome-c oxidase subunit II methylation and apoptosis in human umbilical vascular endothelial cells. *Am J Physiol Cell Physiol*. 2015;308:C378–84.
29. Kurakula K, Hamers AA, van Loenen P, de Vries CJ. 6-Mercaptopurine reduces cytokine and *Muc5ac* expression involving inhibition of NF κ B activation in airway epithelial cells. *Respir Res*. 2015;16:73.
30. Bibb JA. Decoding dopamine signaling. *Cell*. 2005;122:153–5.
31. Chen J, Sidhu A. The role of D₁ dopamine receptors and phospho-ERK in mediating cytotoxicity. *Commentary. Neurotox Res*. 2005;7:179–81.
32. Song KS, Lee WJ, Chung KC, Koo JS, Yang EJ, Choi JY, Yoon JH. Interleukin-1 β and tumor necrosis factor- α induce *MUC5AC* overexpression through a mechanism involving ERK/p38 mitogen-activated protein kinases-MSK1-CREB activation in human airway epithelial cells. *J Biol Chem*. 2003;278:23243–50.
33. Shao MX, Nakanaga T, Nadel JA. Cigarette smoke induces *MUC5AC* mucin overproduction via tumor necrosis factor- α -converting enzyme in human airway epithelial (NCI-H292) cells. *Am J Physiol Lung Cell Mol Physiol*. 2004;287:L420–7.
34. Glader P, Moller S, Lijja J, Wieslander E, Lofdahl CG, von Wachenfeldt K. Cigarette smoke extract modulates respiratory defence mechanisms through effects on T-cells and airway epithelial cells. *Respir Med*. 2006;100:818–27.
35. Barnes PJ. Beta-adrenergic receptors and their regulation. *Am J Respir Crit Care Med*. 1995;152:838–60.
36. Billington CK, Ojo OO, Penn RB, Ito S. cAMP regulation of airway smooth muscle function. *Pulm Pharmacol Ther*. 2013;26:112–20.
37. Nguyen LP, Lin R, Parra S, Omoluabi O, Hanania NA, Tuvim MJ, Knoll BJ, Dickey BF, Bond RA. β_2 -adrenoceptor signaling is required for the development of an asthma phenotype in a murine model. *Proc Natl Acad Sci U S A* 2009;106:2435–2440.
38. Di Benedetto G, Manara-Shediak FS, Mehta A. Effect of cyclic AMP on ciliary activity of human respiratory epithelium. *Eur Respir J*. 1991;4:789–95.
39. Wyatt TA, Forget MA, Adams JM, Sisson JH. Both cAMP and cGMP are required for maximal ciliary beat stimulation in a cell-free model of bovine ciliary axonemes. *Am J Physiol Lung Cell Mol Physiol*. 2005;288:L546–51.
40. Schmid A, Bai G, Schmid N, Zaccolo M, Ostrowski LE, Conner GE, Fregien N, Salathe M. Real-time analysis of cAMP-mediated regulation of ciliary motility in single primary human airway epithelial cells. *J Cell Sci*. 2006;119:4176–86.
41. Salathe M. Effects of beta-agonists on airway epithelial cells. *J Allergy Clin Immunol*. 2002;110:S275–81.
42. Spurzem JR, Gupta J, Veys T, Kneiff KR, Rennard SJ, Wyatt TA. Activation of protein kinase a accelerates bovine bronchial epithelial cell migration. *Am J Physiol Lung Cell Mol Physiol*. 2002;282:L1108–16.
43. Monterisi S, Favia M, Guerra L, Cardone RA, Marzulli D, Reshkin SJ, Casavola V, Zaccolo M. CFTR regulation in human airway epithelial cells requires integrity of the actin cytoskeleton and compartmentalized cAMP and PKA activity. *J Cell Sci*. 2012;125:1106–17.

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