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

Nao Matsuyama1, Sumire Shibata1, Atsuko Matoba1, Tada-aki Kudo2, Jennifer Danielsson3, Atsushi Kohjitan4, Eiji Masaki1, Charles W. Emala3 and Kentaro Mizuta1,3*

Abstract

Background: Dopamine receptors comprise two subgroups, Gs protein-coupled “D1-like” receptors (D1, D5) and Gi-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 Gs-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, Gs-coupled receptor, cAMP, CREB, MUC5AC

* Correspondence: mizuta@m.tohoku.ac.jp
1Department of Dento-oral Anesthesiology, Tohoku University Graduate School of Dentistry, 4-1 Seiryo-machi, Aoba, Sendai, Miyagi 9808575, Japan
2Department of Anesthesiology, College of Physicians and Surgeons of Columbia University, New York, NY, USA
Full list of author information is available at the end of the article

© The Author(s). 2018 Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
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 Gs protein-coupled “D1-like” receptors (D1, D5 subtypes) and the Gi-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 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 Gs-coupled β2-adenoreceptor agonists, which are widely used as bronchodilators, but have been reported to increase mucin production via activation of β2-adenoreceptors 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-μm-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-μm 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 μg/ml leupeptin, 1 μM phenylmethylsulfonyl 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 μM) for indicated times (5–60 min). In separate experiments, NCI-H292 cells were initially pretreated with 10 μM H89 (PKA inhibitor; 30 min) or 5 μM U0126 (MEK inhibitor; 120 min) before treatment of the cells with A68930 (1 μM; 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 phenylmethylsulfonyl 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...
Biophosphorylation 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.

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 (Appli-ikan; 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 kit (Toyobo) 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 instructions. 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 (ΔΔCt) method.

Table 1 Primer sequences

| Target          | Sequence of Primer                  | Amplicon size (bp) |
|-----------------|------------------------------------|--------------------|
| Human MUCSAC    | 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 μM), dopamine (1 μM), 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 4°C. 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 ± 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 mucocutaneous carcinoma cells (NCI-H292 cells), human bronchial epithelial cells (16HBE14o- cells), primary cultured human bronchial epithelial cells, and primary

![Fig. 1](image-url) 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 μm. 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_4 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_ < 0.01, _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. 2** Representative immunoblot analyses using antibodies against the dopamine D₁ receptor using total protein prepared from freshly dissected native human tracheal epithelium (20 μg), primary cultured human airway epithelial cells (100 μg), the human bronchial epithelial cell line (16HBE14o-)(100 μg), the human pulmonary mucocoeidermoid carcinoma cell line (NCI-H292) (100 μg), and human airway smooth muscle cells (positive control) (100 μg). Reprobing of blots for GAPDH was performed to demonstrate relative lane loading. Each image is representative of at least 3 independent immunoblots.
cigarette smoke extract (CSE) (10%) significantly increased MUC5AC mRNA expression in NCI-H292 cells. The Gs protein-coupled β2 adrenergocceptor agonist isoproterenol (1 μM) 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 D1 receptor agonist-induced MUC5AC protein expression in NCI-H292 cells

We further investigated the effect of the dopamine D1 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 μM), A68930 (1 μM), and CSE (10%) (Fig. 6). These results suggest that activation of dopamine D1 receptor stimulates MUC5AC expression.

Discussion

The primary findings of the present study are that functional dopamine D1 receptors are expressed in human airway epithelium. Activation of the dopamine D1 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 D1 and D2 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 D1 receptor on human airway epithelium was identified by immunohistochemistry, while the dopamine D2 receptor protein was not detected. Immunoblot analyses further confirmed that the dopamine D1 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 D2 receptor was not expressed on airway epithelium [19], the dopamine D1 receptor would be the predominant dopamine receptor subtype expressed on human airway epithelium.

Thereafter, we further investigated whether functional dopamine D1 receptors could modulate airway epithelial function. The activation of Gs-coupled receptors including the dopamine D1 receptor and β2-adrenergocceptor stimulates cAMP accumulation via activation of adenylyl cyclase [3, 35, 36]. In accordance with these previous findings, we demonstrated that dopamine or the
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 of 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 Gs-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 Gs-coupled receptor (e.g.
β2-adrenoceptor) in airways. Although activation of β2-adrenoceptor on airway smooth muscle induces bronchodilation, β2-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 Gs-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 Gs-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

Acknowledgments
The authors thank Ayumi Goto and Fumioko Mizuta for technical assistance.

Funding
This work was supported by National Institutes of Health grants GM065281 (CWE) and HL122340 (CWE), a research grant from The Uehara Memorial Foundation (KM), and a research grant from Takeda Science Foundation (KM).

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. NW, 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.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
1Department of Dento-oral Anesthesiology, Tohoku University Graduate School of Dentistry, 4-1 Seiryo-machi, Aoba, Sendai, Miyagi 9808575, Japan.
2Department of Oral Physiology, Tohoku University Graduate School of Dentistry, Sendai, Japan.
3Department of Anesthesiology, College of Physicians and Surgeons of Columbia University, New York, NY, USA.
4Department of Dental Anesthesiology, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan.

Received: 4 January 2018 Accepted: 20 March 2018
Published online: 02 April 2018

References
1. Goto Y, Grace AA. The dopamine system and the pathophysiology of schizophrenia: a basic science perspective. Int Rev Neurobiol. 2007;87:41–68.
2. 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.
3. Missale C, Nash SR, Robinson SW, Jaber M, Caron MG. Dopamine receptors: from structure to function. Physiol Rev. 1998;78:189–225.
4. 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.
5. 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.
6. Hussain T, Lokhandwala MF. Renal dopamine receptors and hypertension. Exp Biol Med (Maywood). 2003;228:134–42.
7. Ricci A, Mignini F, Tomassoni D, Amenta F. Dopamine receptor subtypes in the human pulmonary arterial tree. Auton Autacoid Pharmacol. 2006;26:361–9.
8. Zizzo MG, Mule F, Mastropaolo M, Serio R. D1 receptors play a major role in the dopamine modulation of mouse ileum contractility. Pharmaco Res. 2010;61:371–8.
9. Neve KA, Seemans JK, Trantham-Davidson H. Dopamine receptor signalling. J Recept Signal Transduct Res. 2004;24:165–205.
10. Billington CK, Hall IP. Novel cAMP signalling paradigms: therapeutic implications for airway disease. Br J Pharmacol. 2012;166:401–10.
11. Delghandi MP, Johannessen M, Moens U. The cAMP signalling pathway activates CREB through PKA, p38 and MSK1 in NH 3T3 cells. Cell Signal. 2005;17:1343–51.
12. Naqi 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. Calabrese P, Piccioni 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. Michoud MC, Amyot R, Jeanneret-Grosjean A. Dopamine effect on extracellular signal-regulated kinases of the basal ganglia. Br J Pharmacol. 1997;130:374–85.

16. Peiser C, Trevisani M, Groneberg DA, Dinh QT, Lencer D, Amadesi S, Aviado DM, Sadavongvivad C. Pharmacological significance of biogenic amines in the lungs: noradrenaline and dopamine. Br J Pharmacol. 1997;130:374–85.

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 D1 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-4 induction of MUC5AC gene expression is mediated by CREB and NF-kB 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 OC, Lee MY, Kim HS, Kim JC, Oh SR, Ahn KS. Melatonin inhibits MUC5AC production via suppression of MAPK signalling in human airway epithelial cells. J Pineal Res. 2014;56:390–8.

24. Gong S, Li J, Ma L, Li K, Wang G, Liu Y, Ji X, Liu X, Chen P, Ouyang G. Cigarette smoke extract modulates respiratory defence mechanisms through effects on T-cells and airway epithelial cells. Respir Med. 2006;100:819–27.

25. Barnes PJ. Beta-adrenergic receptors and their regulation. Am J Respir Crit Care Med. 1995;152:838–60.

26. Billington CK, Ojo OO, Penn RB, Ito S. cAMP regulation of airway smooth muscle function. Pulm Pharmacol Ther. 2013;26:112–20.

27. Nguyen LP, Lui R, Parra S, Omoluabi O, Hanania NA, Tsurim MJ, Knoll BJ, Dickey BF, Bond RA. β2-adrenoceptor signalling is required for the development of an asthma phenotype in a murine model. Proc Natl Acad Sci U S A. 2009;106:2435–2440.

28. Di Benedetto G, Manara-Shediac FS, Mehta A. Effect of cyclic AMP on ciliary activity of human respiratory epithelium. Eur Respir J. 1991;4:789–95.

29. 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.

30. Schmid A, Bai G, Schmid N, Zaccole M, Ostromski LE, Conner GE, Fregnin 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.

31. Salathe M. Effects of beta-agonists on airway epithelial cells. J Allergy Clin Immunol. 2002;110:S275–81.

32. Spurzem JR, Gupta J, Veys T, Kneifl KR, Rennard SI, Wyatt TA. Activation of protein kinase a accelerates bovine bronchial epithelial cell migration. Am J Physiol Lung Cell Mol Physiol. 2002;282:L1108–16.

33. 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;125:106–17.