Differentiation of human bronchial epithelial cells: Role of hydrocortisone in development of ion transport pathways involved in mucociliary clearance

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Abstract

Glucocorticoids strongly influence the mucosal-defense functions performed by the bronchial epithelium and inhaled corticosteroids (ICS) are critical in the treatment of patients with inflammatory airway diseases such as asthma, COPD and cystic fibrosis. A common pathology associated with these diseases is reduced mucociliary clearance, a defense mechanism involving the coordinated transport of salt, water and mucus by the bronchial epithelium, ultimately leading to the retention of pathogens and particles in the airways and to further disease progression. In the present study, we investigated the role of hydrocortisone (HC) in differentiation and development of the ion transport phenotype of normal human bronchial epithelial (NHBE) cells under air-liquid interface (ALI) conditions. NHBE cells differentiated in the absence of HC (HC0) showed significantly less benzamil-sensitive short-circuit current compared to controls as well as a reduced response after stimulation with the selective β2-adrenergic receptor (AR) agonist salbutamol. Apical membrane localization of ENaCα subunits were similarly reduced in HC0 cells compared to controls, supporting a role of HC in the trafficking and density of sodium channels in the plasma membrane. Additionally, glucocorticoid exposure during differentiation regulated the transcription of CFTR and β2-AR mRNAs and appeared to be necessary for the expression of CFTR-dependent anion secretion in response to β2-agonists. HC had no significant effect on surface cell differentiation but did modulate the expression of mucin mRNAs. These findings indicated that glucocorticoids support mucosal defense by regulating critical transport pathways essential for effective mucociliary clearance.
Introduction

The airway epithelium establishes a boundary between the internal and the external environment that protects against potential injury and infection caused by inhaled particles, debris and microbial pathogens (17, 53, 61). In order to maintain the health of the lungs and surrounding tissues, the epithelium utilizes numerous defense mechanisms that complement the barrier function of the epithelium. These include the expression of a broad array of innate immune receptors including Toll-like, NOD-like and RIG-I receptors which facilitate the expression and secretion of multiple defense molecules, cytokines and chemokines essential for recruitment and activation of immune cells (38, 53). Furthermore, the airway epithelium produces antimicrobial agents such as reactive oxygen species, defensins, iron chelating proteins, and interferons that effectively prevent infection by inhaled pathogens without activation of an adaptive immune response (38, 53). Chronic inflammation of the airways due to prolonged or repeated exposure to noxious agents can result in epithelial remodeling and fibrosis that leaves the airways more susceptible to infection due to disruption of innate immune processes (54, 66, 70). Loss of epithelial integrity compromises normal mucosal defense, underscoring the important relationship between structure and function of the airway epithelium (30, 55).

The pseudostratified airway epithelium is composed of multiple cell types (57). Multipotent, progenitor basal cells are transit-amplifying cells that reside along the basal lamina of the airway which differentiate into surface cells (29). Basal cells are instrumental in the maintenance of normal epithelial structure and function as well as driving orderly regeneration after injury (59, 60). Differentiated surface cells can be divided into two distinct lineages: secretory and ciliated. Secretory cells synthesize and secrete gel-forming polymeric mucins that absorb water to form mucus (1, 41). Ciliated cells propel the mucus gel towards the pharynx and
out of the lungs by the directional, synchronized beating of cilia on the cell surface, effectively removing entrapped particles and pathogens from the lungs by a process known as mucociliary clearance (46, 67, 68).

The mucus gel rests atop a thin fluid layer called the airway-surface liquid (ASL). The depth of the ASL is maintained through coordinated regulation of transcellular sodium absorption and anion secretion pathways within the epithelium, as well as paracellular ion and water fluxes (9, 32). The efficacy of mucociliary clearance depends on the integrity of these and other transport pathways to preserve the height of the ASL, thus facilitating effective ciliary beating (4, 71). Respiratory disorders such as cystic fibrosis (CF), asthma and chronic obstructive pulmonary disease (COPD) are all associated with defective or ineffective mucociliary clearance that ultimately leads to chronic inflammation, airway remodeling and permanent loss of lung function (10, 13, 21, 23, 35, 49, 73).

Current treatment strategies for patients with asthma, COPD and other inflammatory airway disorders emphasize a combined therapy involving inhaled corticosteroid (ICS) and long-acting β2-agonists (LABA) aimed at reducing inflammation and increasing airway caliber (6). LABAs, such as Salmeterol and Formoterol, are effective long-acting inhaled bronchodilators (7, 62, 72). These drugs stimulate increases in cAMP which activates protein kinase A (PKA) dependent signaling pathways in airway smooth muscle, leading to relaxation and a decrease in airway resistance (8). The therapeutic benefit of ICS treatment is attributed to transrepression of specific genes induced by proinflammatory transcription factors such as NF-κB and AP-1, limiting airway hyperresponsiveness (26, 36, 66, 75). Other effects of corticosteroids on airway epithelial cells suggest a role in surface cell diversity by increasing the number of ciliated
epithelial cells and decreasing mucus hypersecretion, therefore promoting mucociliary clearance (19, 42).

Although effective at reducing inflammatory immune reactions and airway hyperresponsiveness, the effects of combined therapy on the airway epithelium are not as well characterized. Furthermore, hydrocortisone (HC) is one of the constituents of cell culture media that is commonly used for maintaining NHBE cells in culture. Therefore, the overall goal of the present study was to investigate the role of glucocorticoids in the development of the ion transport phenotype of bronchial epithelial cells during differentiation. We hypothesized that hydrocortisone (HC) is necessary for in vitro differentiation of airway basal cells into a pseudostratified epithelium with ciliated and secretory surface cells. Additionally, we hypothesized a role for HC in the development of normal transepithelial ion-transport pathways essential for mucociliary clearance. The results of the present study demonstrate that early glucocorticoid exposure affects the development and maintenance of specific ion-transport pathways required for mucociliary clearance. These findings indicate that ICS treatment supports mucosal defense through direct interactions with the airway epithelium.

Materials and Methods

Materials

Retinoic acid, Benzamil hydrochloride, CFTRinh-172, Salbutamol hemisulfate salt, 8-(4-Chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt (8-CPT-cAMP), 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate (DIDS), and Uridine triphosphate (UTP) were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Paraformaldehyde, 16% solution was purchased from VWR (Radnor, PA, USA).
Cell Culture

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza (Basel, CHE) and expanded in bronchial epithelial cell growth medium (BEBM + SingleQuots containing 1.4 μM hydrocortisone), also from Lonza. Cells were plated at low density on 0.4 μm pore-size Snapwell polyester membranes and maintained under liquid-liquid interface growth conditions with complete BEGM until cells reached confluency (day 0). Hydrocortisone (HC; 1.4μM) was withdrawn from HC0 and HC8 cells at day 0 and 8, respectively. Retinoic acid (RA; 500nM) was added to BEGM for 48hrs to promote differentiation (1). Air-liquid interface (ALI) culture conditions were initiated at day 2 by removing apical media and basolateral growth media was replaced with differentiation media (DMEM/F12 + SingleQuots) containing 100 nM RA. Cells were harvested at four different time points: days 0, 4, 8 and 24. Transepithelial resistance (TER) measurements were made every-other day for 24 days before media change using “chopstick” electrodes and a voltohmmeter (EVOM: World Precision Instruments (WPI), New Haven, CT, USA). All cells were grown at 37°C in a humidified CO₂ atmosphere.

Quantitative RT-PCR

RNA was isolated using the Rneasy Mini Kit from Qiagen (Hilden, DEU). cDNA was produced with the QuantiTect Reverse Transcription Kit with gDNA Wipeout also from Qiagen. Taqman PCR probes were purchased through Life Technologies (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative PCR amplification of 5ng cDNA was performed on an Applied Biosystems 7300 Real-Time PCR system. Baseline and threshold values were set according to manufacturer’s instructions. Relative expression was quantified using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the reference gene (65).
Immunofluorescence and Western blot analysis

Cells were grown on Snapwell polyester membranes as described and fixed in 4% PFA for 20 min. Cell membranes were permeated using 0.3% Triton-X and blocked with a PBS + 3% BSA solution for at least 1 hr. Cells were incubated with the primary antibody overnight at 4°C diluted in 3% BSA solution. Following incubation, cells were rinsed three times in PBS and incubated with the secondary antibody for 1 hr at room temperature. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Monolayers were excised and mounted on microscope slides using VECATSHIELD HardSet mounting media (Vector Laboratories, Burlingame, CA, USA). Images were captured using an Olympus FV1000 Confocal Microscope. Primary antibodies were purchased from Abcam (ENaC α, α1-NaKATPase, CFTR, β2-AR, ZO1; Cambridge, GBR) or R&D Systems (Prss8/CAP1; Minneapolis, MN, USA) and secondary antibodies were purchased from Invitrogen (Alexa Fluor 488, 568, 647; Carlsbad, CA, USA).

Total protein was collected using Pierce IP Lysis Buffer and was quantified with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). 10 or 25ng total protein was loaded on NuPAGE 4-12% Bis-Tris Gels with Chameleon Duo Pre-stained protein ladder (Li-Cor Biosciences, Lincoln, NE, USA) and separated using electrophoresis in MOPS SDS Running Buffer (200V, 50 min). Proteins were transferred onto activated Immobilon-FL PVDF membranes (Millipore Corporation, Billerica, MA, USA) and blocked in Odyssey Blocking Buffer overnight at 4°C (Li-Cor Biosciences). Primary antibodies were diluted in Odyssey Blocking Buffer containing 0.2% Tween-20 and incubated overnight, washed five times in PBS+0.1% Tween-20, and then incubated with IRDye secondary antibodies diluted in Odyssey
Blocking Buffer for 40 mins (Li-Cor Biosciences). Blots were visualized with an Odyssey CLx Imager and analyzed using Image Studio Lite (Li-Cor Biosciences). β-actin primary antibodies were purchased from Santa Cruz Biotechnology (sc-69879/sc-130656, Dallas, TX, USA).

Electrophysiology

Short-circuit current (I\text{sc}) measurements were performed on high-resistance monolayers (>700 $\Omega \cdot \text{cm}^2$) mounted in Ussing chambers bathed on both sides with standard saline solution containing (in mM) 130 NaCl, 6 KCl, 1.5 CaCl\text{2}, 1 MgCl\text{2}, 20 NaHCO\text{3}, 0.3 Na H\text{2}PO\text{4}, and 1.3 Na\text{2}HPO\text{4}, pH 7.4, and maintained at 37°C with 95% O\text{2}-5% CO\text{2} gassing.

Statistics

All values reported in the Results are expressed as the mean ± SEM. For the qRT-PCR experiments reported in figures 2, 6, 7, 8 and 9, an asterisk (*) represents significant differences between day zero and each subsequent day of differentiation as determined by an ANOVA followed by Dunnett’s test for comparisons with a common control within each treatment condition. The open circles (o) represent significant differences between either the HC0 condition or the HC8 condition compared to the corresponding day of differentiation in the control group as determined by ANOVA followed by a Tukey-Kramer multiple comparisons test. In figures 4, 5 and 9 differences in blocker-sensitive or agonist-sensitive I\text{sc} are shown with either an asterisk (*) representing significant differences between HC0 and control conditions or a circle (o) which represents a significant differences between HC0 and HC8 conditions as determined by ANOVA followed by a Tukey-Kramer multiple comparisons test. A value of $p<0.05$ was considered significant.
Results

NHBE cells differentiate into a ciliated-pseudostratified epithelium

NHBE cells were expanded then differentiated on polyester Snapwell membranes according to the protocol described in the Methods. The cells were maintained under liquid-liquid conditions in complete BEGM growth media until they reached confluence (day 0). 1.4μM hydrocortisone (HC), supplied in the BEGM SingleQuots, was withdrawn from HC0 and HC8 cells at day 0 and 8, respectively. Apical media was withdrawn from control, HC0 and HC8 cells at day 2 and cells were maintained under ALI conditions for the duration of the experiment. Transepithelial resistance (TER) was measured every-other day as shown in Figure 1A. HC0 cells exhibited greater increases in TER at all days between 2 and 10 compared to control and HC8 cells. Under all three conditions, the mean TER value calculated using measurements from all of the days after day 8 exceeded 1000 Ω·cm² (mean TER ± SEM d8-d24 (Ω·cm²): control = 1055 ± 85.9, HC0 = 1161 ± 79.8, HC8 = 1149 ± 93.5). Tight junction (TJ) formation was visualized by immunocytochemistry targeting the cytoskeletal scaffolding protein ZO1 (74). As shown in Figure 1B, ZO1 was localized at the lateral borders of control and HC0 cells at day 24, indicating mature TJ structures.

We examined changes in expression of specific mRNAs associated with differentiation of bronchial basal cells by qRT-PCR. The basal cell transcription factor p63 (Figure 2A) was expressed at the highest level in control cells at day 0, but exhibited a significant reduction in expression at days 4, 8 and 24. More p63 was detected in HC0 and HC8 cells at days 8 and 24 than the corresponding days in control monolayers indicating a role for HC in the expression of p63. Transcription of cytokeratin 6a (KRT6a: Figure 2B), a type II intermediate filament protein highly expressed in the airway basal cells (27), also showed HC dependent down-regulation in
differentiated control cells. However, HC0 and HC8 cells expressed a greater amount of KRT6a mRNA at day 24 than control (CT = 25.4 ± 0.8, 24.3 ± 0.5, 28.7 ± 0.3, respectively). These results suggest that differentiation in the presence of HC reduced the abundance of basal cell markers within the epithelium but that basal cells are still present within differentiated, pseudostratified monolayers by day 24.

Next, the transcription of genes associated with airway surface cells was investigated. FoxJ1 (Figure 2C), a master transcription factor of ciliogenesis (76), as well as mucins Muc5ac (Figure 2D) and Muc5b (Figure 2E), were not detected at day 0 but were observed at days 4, 8 and 24 in control cells, indicating the presence of ciliated and secretory surface cells in differentiated monolayers. This was confirmed by immunocytochemistry (Figure 3A and B). HC0 and HC8 cells expressed higher levels of FoxJ1 mRNA at day 24 compared to control monolayers (CT = 28.8 ± 0.5, 28.3 ± 0.5, 31.5 ± 0.3, respectively). Muc5ac was not detected in HC0 cells at day 24. Interestingly, transcription of Muc5b showed HC-dependent up-regulation in control cells, however it is worth noting that this does not necessarily indicate increased protein expression or mucus secretion in HC treated monolayers. HC withdrawal (HC8) cells exhibited reduced expression of Muc5ac and Muc5b at days 8 and 24 compared to control, similar to HC0 cells (Figure 2E). Moreover, detection of Muc5b by immunocytochemistry at the apical surface of day 24 differentiated HC0 monolayers was reduced compared to control and HC8 cells (Figure 3C and D). The secretoglobin protein Uteroglobin or Clara Cell 10 protein (SCGB1A1, Figure 2F) also showed robust HC dependent expression during differentiation in the presence of HC, but significantly reduced expression at days 8 and 24 in HC0 cells and at day 24 in HC8 monolayers. This result suggested an increase in Clara cell abundance within control monolayers during differentiation that required HC for sustained expression.
Differentiated NHBE monolayers were mounted in Ussing chambers to investigate ion transport function by characterizing ENaC and CFTR dependent transport pathways. Day 8 control and HC8 cells had statistically greater total and benzamid-sensitive short circuit current (Isc) compared to HC0 monolayers, reflecting a greater level of basal Na⁺ absorption (Figure 4). HC had no effect on the magnitude of the CFTR_{inh-172}-sensitive Isc, indicating that the level of basal anion secretion was unaffected by the absence of hydrocortisone. Day 24 HC8 monolayers displayed a similar phenotype as HC0 cells (Figure 5). Total and benzamid-sensitive currents were significantly reduced in HC0 and HC8 cells compared to control.

Immunocytochemistry revealed the localization of ENaCα and the α₁-subunit of Na⁺-K⁺ ATPase at the apical and basolateral membranes, respectively, of differentiated NHBE monolayers (Figure 6A). ENaCα localized at cilia-like structures on surface cells of control monolayers. Surface ENaCα was reduced in HC0 cells. ENaCα was detected just below the apical membrane in the same focal plane as the Na-K ATPase α₁ subunit in HC0 and HC8 cells, implicating a role for HC in trafficking of ENaC to the apical surface and into the cilia through insertion of sub-apical membrane vesicles. Furthermore, qRT-PCR analysis of ENaCα and γ mRNAs displayed HC dependent increases at day 8 (Figure 6B and D). HC did not alter the localization of the Na⁺-K⁺ ATPase at the basolateral membrane as shown in Figure 6A. However, ATP1A1 mRNA was reduced in the absence of HC but showed no change in HC8 cells compared to control (Figure 6E), indicating that the initial 8 days of exposure to HC was sufficient to augment expression of the α₁ subunit.

Earlier studies have demonstrated that ENaCα and γ subunits are subject to cleavage by
proteases that enable complete activation of the channel (33). Therefore, we examined whether glucocorticoid exposure was involved in regulating the expression of known proteases involved in ENaC processing. Our results showed an increase in mRNA expression of furin and channel-activating protease 1 (CAP1) during differentiation in control versus HC0 monolayers at days 8 and 24, although mRNA for both enzymes was still expressed at high levels in HC0 cells (Figure 6F and G). However, similar levels of the 37 kDa N-terminal cleavage product of ENaCα was observed in control and HC0 monolayers at days 8 and 24 (Figure 6H) (43). Serum and glucocorticoid-regulated kinase 1 (SGK1) mRNA, a known modulator of surface ENaC expression, was significantly reduced in HC0 monolayers at days 4 and 8 compared to control cells (Figure 6H). ATP1α1 protein was observed at days 8 and 24 in HC0 and control cells (Figure 6H).

Anion secretion increases following β2-AR stimulation

Signaling pathways coupled to ion transport in airway surface epithelial cells are integral to mucociliary clearance. We investigated the role of HC in development of normal β2-adrenergic receptor regulation of CFTR-dependent anion secretion using the short-acting selective β2 agonist salbutamol. Previous experiments (Figures 4 & 5) demonstrated that the transport phenotype of differentiated NHBE monolayers treated with HC was similar at day 8 and 24. Therefore, day 16 monolayers were pre-treated with benzamil before being stimulated with 10µM salbutamol. Treatment with CFTRinh-172 after salbutamol stimulation inhibited the Isc in control, HC0 and HC8 monolayers (Figure 7A and B). HC0 and HC8 monolayers displayed less of an increase in Isc after addition of salbutamol compared to control monolayers (Figure 7B). qRT-PCR analysis of β2-AR transcription (ADRB2) revealed HC dependent
expression at days 8 and 24 (Figure 7C). CFTR mRNA expression showed a time-dependent
decrease in HC0 cells while HC8 expression remained similar to control at day 24 (Figure 7D).
Western blot analysis also revealed a time-dependent decrease in total CFTR abundance in HC0
cells. However, ADBR2 protein abundance did not appear to be reduced in the absence of HC.
Immunocytochemistry results presented in Figure 8A and B showed co-localization of the β2-AR
and CFTR in cilia-like structures of control monolayers, but less distinct cilia localization in
HC0 and HC8 monolayers.

To confirm that the salbutamol-activated Isc was dependent on functional CFTR
expressed in the apical membrane, human Δ508 cystic fibrosis bronchial epithelial cells (CFBE)
were differentiated according to the protocol used for control NHBE cells. CFBE monolayers
displayed a reduced benzamil sensitive current compared to control (1.04±0.16 µA) and were
unresponsive to salbutamol as well as 8-(4-Chlorophenylthio)-adenosine-3',5'-cyclic
monophosphate (8-CPT-cAMP), a membrane permeable analog of cAMP known to activate
CFTR-dependent anion secretion (Figure 8C). As a positive control, CFBE cells were
subsequently stimulated with a P2Y2 receptor agonist (UTP) known to activate Ca2+-dependent
Cl secretion in airway epithelial cells (45). Although CFTR-dependent anion secretion could not
be activated by agents that increase cAMP, CFBE monolayers did exhibit UTP-dependent
increases in Isc that could be blocked by DIDS, a disulfonic stilbene compound that inhibits
Ca2+-dependent Cl channels (51). CFBE monolayers also appeared to exhibit colocalization of
the β2-AR with CFTR (Figure 8D) although more extensive co-immunoprecipitation experiments
would be needed to confirm this interpretation. However, CFTR expression was not localized in
cilia-like structures of ΔF508 monolayers, appearing instead within the perinuclear region below
the apical membrane.
In vitro differentiation of NHBE cells under ALI conditions provides a unique opportunity to study signaling molecules that regulate the development of a physiologically responsive, pseudostratified epithelium. The technique has been employed by many groups to study the effects of cigarette smoke, pathogenesis of influenza infection, bronchial wound healing, and as a model for airway drug transport (18, 25, 44, 48, 69). The precise culture conditions used to differentiate bronchial basal cells into a monolayer containing secretory and ciliated cells are varied. Addition of retinoic acid to culture medium is a common practice which promotes pseudostratification and suppresses keratinizing squamous differentiation (15, 24, 50). Moreover, nearly all NHBE cell culture media contain glucocorticoids but the rationale for supplementation is poorly understood. Corticosteroids are well-studied modulators of multiple processes across a variety of tissues but their role in the differentiation of airway basal cells into a tissue capable of transepithelial salt and water transport has not been systematically investigated.

Detection of both ciliated and secretory cells at day 24 occurred in both HC0 and HC8 cells and did not appear to be different from control. However, HC did reduce transcription of mRNAs associated with secretory cells. Relative expression of Muc5ac, Muc5b and SCGB1A1 were all reduced in the absence of HC. Previous studies have reported suppression of goblet cell abundance in the presence of glucocorticoids (40, 58). Although not necessarily indicative of surface cell-type abundance, our data suggests increased transcription of mucin proteins in the presence of HC. One possible explanation may involve HC dependent down-regulation of basal-cells occurring in parallel with an increase in surface cell expression as indicated by reductions in the levels of p63 and KRT6a and increased expression of surface cell markers. HC0
monolayers expressed greater relative amounts of p63 and KRT6a at later time-points than control cells which may be indicative of basal cell proliferation, potentially leading towards squamous metaplasia (3).

We also identified a role for HC in determining the magnitude of basal benzamil-sensitive Na\(^+\) absorption in well-differentiated NHBE cells. Reduced sodium absorption was observed at day 8 and 24 in NHBE cells differentiated in the absence of HC (HC0). Furthermore, surface expression of ENaC\(\alpha\) was reduced at day 24, although the \(\alpha\) subunit was detected by immunocytochemistry just below the apical membrane, suggesting localization within sub-apical membrane vesicles. In contrast, cells differentiated in the presence of HC exhibited ENaC\(\alpha\) localization within cilia-like structures, consistent with previous results showing localization of ENaC within motile cilia of native airways as well as K2P channels in cilia of differentiated HBE cells (22, 77). Additionally, withdrawal of HC after eight days of differentiation (HC8) resulted in a similarly reduced benzamil-sensitive Isc at day 24, although unlike HC0 monolayers, ENaC\(\alpha\) expression within the apical membrane was comparable to monolayers that were differentiated in the continuous presence of HC. One potential explanation for the reduced levels of Na\(^+\) transport associated with HC0 monolayers is an effect of HC on mRNA expression of ENaC subunits. Glucocorticoids have been previously shown to directly regulate ENaC transcription and in this study, HC-dependent regulation of \(\alpha\)ENaC and \(\gamma\)ENaC, but not \(\beta\)ENaC mRNA was observed when HC was removed at the start of the differentiation protocol. Earlier studies demonstrated dexamethasone/hydrocortisone-dependent up-regulation of all three ENaC subunits in human middle ear (39) and human mammary epithelial cells (11) respectively as well as dexamethasone but not aldosterone regulation of \(\beta\)ENaC and \(\gamma\)ENaC mRNA expression in bovine mammary epithelial cells (56). Interestingly, dexamethasone did not stimulate an increase
in ENaCα or γ protein expression in CFBE41o- cells, an immortalized human bronchial epithelial cell line stably expressing wt CFTR or the ΔF508 CFTR mutation (63).

Furthermore, HC could also regulate ENaC function by altering the expression of enzymes involved in proteolytic processing of the channel. Previous investigations have shown that ENaCα and γ are subject to proteolytic cleavage, resulting in functionally mature sodium channels (33). Multiple proteases have been reported to mediate ENaC processing and we hypothesized a role of HC in regulating the expression of these enzymes based on previous reports showing that aldosterone directly controls the expression of prostasin, also known as channel-activating protease 1 (CAP1). Our results demonstrated that removal of HC prior to the start of differentiation reduced mRNA expression of two proteases known to regulate ENaC processing, including furin, which cleaves the extracellular loop of ENaCα and γ, and CAP1, which cleaves the extracellular loop of the γ subunit (12). Although we observed a reduction in ENaCα mRNA in the absence of HC, we detected similar total abundance of cleaved ENaCα by western blot, indicating a mechanism other than transcription is responsible for the reduced benzamil-sensitive current observed in HC0 and HC8 monolayers.

The observation that HC0 monolayers exhibited reduced ENaC protein expression within the apical membrane supports a role for HC in the trafficking of channel subunits to the apical surface. ENaC retrieval from the plasma membrane is known to be regulated by the ubiquitin-protein ligase, Nedd4-2. Nedd4-2 activity is itself controlled by serum/glucocorticoid-regulated kinase 1 (SGK1). SGK1-mediated phosphorylation of Nedd4-2 reduces its interaction with ENaC leading to an increase in channel density in the apical membrane (20, 37). Therefore, in this study we tested the hypothesis that removal of HC from the media at the start of differentiation reduces SGK1 mRNA expression, which in turn reduces ENaC surface density.
SGK1 mRNA expression was reduced in the absence of HC compared to control and HC8 monolayers, consistent with previous studies on HC stimulation of ENaC surface expression through regulation of the SGK1-Nedd4-2-ENaC axis in middle ear epithelial cells (39).

In addition to regulation of ENaC function, the mineralocorticoid hormone aldosterone has been shown to directly stimulate Na\(^+\)-K\(^+\) ATPase activity and transcription in distal nephron and collecting duct epithelial cells resulting in increased sodium absorption and water retention (64). Similarly, dexamethasone has been shown to regulate Na\(^+\)-K\(^+\) ATPase activity in corneal endothelial cells and to increase transcription of the β\(_1\) subunit of the enzyme in renal carcinoma cells (31, 34). Therefore we investigated whether HC was involved in regulating ATP1A1 localization and expression during differentiation of NHBE cells. Our results demonstrated reduced transcription of ATP1A1 mRNA in the absence of HC but no effect on membrane localization of the protein. The reduced expression and protein abundance of the α\(_1\) subunit of the pump was consistent with the decrease in ENaC mRNA and surface expression of ENaCα subunits in HC0 monolayers and may have contributed to the lower rate of basal Na\(^+\) transport. Interestingly, once differentiation was initiated and HC exposure sustained for 8 days (HC8 monolayers), withdrawal of HC for the next 16 days did not reduce ATP1A1 mRNA expression. A similar effect was observed for ENaCα, β and γ mRNA expression in HC8 monolayers, supporting continuous expression of ENaC subunits within the apical membrane at day 24. Transcriptional regulation by HC involves histone modifications which may have effects lasting longer than the time period of HC exposure and may explain sustained gene transcription of ENaC and ATP1A1 after HC withdrawal (5). Curiously, despite the sustained expression of ENaC and ATP1A1 mRNA subunits and their appropriate membrane localization, basal Na\(^+\) transport in HC8 monolayers at day 24 was reduced to a similar level as observed in HC0
monolayers. At this time the mechanistic basis for the reduction in basal ENaC-dependent Isc is unknown, but one possible explanation could be a decrease in basolateral K$^+$ conductance, which would reduce the driving force for transepithelial Na$^+$ absorption.

Previous studies have suggested that ICS potentiates LABA efficacy by enhancing the functional response to β$_2$-AR agonists (2, 52). In the present study, qRT-PCR results indicated that HC plays a role in regulating the transcription of CFTR mRNA. Although the basal CFTR$_\text{inh-172}$-sensitive Isc was not affected by HC, salbutamol stimulated currents did show differences between control and HC-deficient conditions measured at day 16. The increase in Isc following addition of salbutamol was blocked by the addition of CFTR$_\text{inh-172}$ and was not observed in ΔF508 CFTR expressing monolayers which lacked functional CFTR. Earlier studies have shown that CFTR forms a signaling complex with β$_2$-ARs and here we show co-localization of CFTR and β$_2$-AR in NHBE cells and in ΔF508 CFTR expressing monolayers. Although this protein-protein interaction between CFTR and the β$_2$-AR appeared to show no dependence on HC, the relative abundance of β$_2$-AR mRNA was HC-dependent such that in the absence of HC, mRNA transcription was significantly reduced, although β$_2$-AR protein expression was sustained at levels comparable to control conditions. Glucocorticoid effects on β$_2$-receptors in airway epithelial cells and other tissues have been previously reported (2, 14, 16). These studies suggested a role for glucocorticoids as a permissive hormone in regulating the expression and density of β$_2$-ARs in plasma membranes, including airway smooth muscle cells (28, 47). Furthermore, glucocorticoids have been reported to protect airway smooth muscle from pro-asthmatic effects of LABAs through indirect suppression of phosphodiesterase E4 (52). Although basal CFTR currents were not different between control and HC0 conditions, salbutamol-induced CFTR currents in HC0 and HC8 cells were significantly lower than control
monolayers. The reduced Isc response in HC0 cells may have been due to the decrease in β2-AR expression, however mRNA levels in HC8 cells were comparable to control monolayers, so other mechanisms are likely to be involved in these cells.

Summary and Conclusions

The key findings of this study that relate to the effects of glucocorticoids during differentiation include i) enhanced mRNA expression of ENaCα, β and γ subunits, ii) coordinated up-regulation of ENaC processing (Furin/CAP1) enzymes as well as SGK1, iii) increased expression of CFTR and β2 adrenergic receptors, iv) colocalization of ENaC, CFTR and β2 adrenergic receptors within the cilia of surface cells, v) increased Na-K ATPase α1 subunit expression and vi) increased CFTR-dependent anion secretion evoked by stimulation with β2 adrenergic receptor agonists. These results support the conclusion that glucocorticoid treatment facilitates normal differentiation and development of ion transport protein expression and function critical for mucociliary clearance. Furthermore, glucocorticoid exposure during differentiation appears to be necessary for the expression of apical β2-ARs that regulate CFTR-dependent anion secretion in response to inhaled β2-agonists. Thus the combined clinical use of glucocorticoids and LABAs for the treatment of asthma and other inflammatory airway diseases would be expected to promote epithelial restitution and mucociliary clearance necessary for maintaining mucosal barrier function and innate defense against airway pathogens. Inclusion of glucocorticoids in media used for differentiating airway progenitor cells may also be necessary for producing a fully functional pseudostratified airway epithelium for use in the bioengineering of human airways and perhaps ultimately, intact human lungs.
Figure Legends

Figure 1. NHBE cells develop tight junctions and form electrically tight monolayers during differentiation.

A. NHBE cells grown under standard differentiation conditions develop and maintain transepithelial resistances (TERs) over a 24 day period. HC0 cell TERs exhibited greater increases at earlier time points compared to control and HC8 cells. The asterisk (*) represents significant differences between the HC0 condition and Control/HC8 conditions, whereas open circles indicate a significant difference between Control and HC8 conditions as determined by ANOVA followed by a Tukey-Kramer multiple comparisons test (n = at least 6 for each time point). Differentiation conditions are graphically represented below the graph. B. Localization of ZO1 immunofluorescence along the basolateral membrane in control, HC0 and HC8 cells. Inset panels are 30μm x 30μm images of day 24 monolayers.

Figure 2. Differentiated NHBE cells express mRNAs associated with bronchial basal cells and surface cells.

A-C. qRT-PCR analysis showing mRNA expression of consensus bronchial basal cell markers p63, cytokeratin 6a (KRT6a) and FoxJ1 in undifferentiated NHBE cells (day 0) and differentiated NHBE monolayers (n = 6 for each condition). Data was normalized to GapDH and C_T values were 23.1±0.19, 23.1±0.56, 23.7±0.28 for control, HC0, and HC8 d24 respectively. Blue and white striped bars represent undifferentiated control data at day 0 (n = 6 for each bar). D-F. mRNA expression of bronchial surface cell markers were not detected or expressed at low levels in undifferentiated NHBE cells. However, by day 4, Muc5ac, Muc5b and Scgb1a1 were detected (n = 6 for each bar).
Figure 3. Immunocytochemistry of differentiated NHBE cells reveals a pseudostratified epithelium with ciliated and mucin-containing cells.

A. NHBE control monolayers contained surface cells expressing Muc5b (green) and acetylated α-tubulin (red), characteristic of cilia during differentiation. Nuclei (blue) were labeled with DAPI. B. Enlarged image identified in the white box of day 24 acetylated α-tubulin labeled cilia (in part A) on the apical surface of differentiated monolayers. C. HC0 and HC8 monolayers contained both ciliated and Muc5b-expressing cells. D. Orthogonal views of differentiated cells showed pseudostratification and apical localization of acetylated α-tubulin.

Figure 4. Day 8 HC0 cells have reduced benzamil-sensitive current but normal CFTR inh-172-sensitive current compared to hydrocortisone treated controls.

A-C. Representative I_{sc} traces of day 8 differentiated monolayers treated with 5 μM benzamil, a selective blocker of epithelial sodium channels (ENaC), and 20 μM CFTR inh-172, a selective CFTR blocker. D. Histogram summarizing I_{sc} results reported in A-C (n = 6 for each condition).

Figure 5. Day 24 HC0 and HC8 cells have reduced total and benzamil-sensitive currents compared to control.

A-C. Representative I_{sc} traces of day 24 differentiated monolayers treated with 5 μM benzamil and 20 μM CFTR inh-172. D. Histogram summarizing the I_{sc} results reported in A-C (n = 6 for each condition).
Figure 6. HC0 cells express lower ENaC mRNA levels and have less apically localized ENaCα subunits compared to control and HC8 cells.

A. Immunocytochemistry comparing control, HC0 and HC8 monolayers at day 24 showing localization of ENaCα (green) and Na⁺-K⁺ ATPase α1 subunit (red) at the apical and basolateral membranes, respectively. Left panels show detection of ENaCα at the apical membrane. Right panels show detection of Na⁺-K⁺ ATPase α1 along the lateral boarder just beneath the apical membrane of the same monolayer. Nuclei (blue) were labeled with DAPI. B-E. qRT-PCR analysis showing the relative expression of ENaCα, β, and γ subunits as well as ATP1A1mRNAs (n = 6 for each bar). F-H. qRT-PCR analysis of mRNAs associated with ENaC proteolytic processing (FURIN, CAP1) and trafficking (SGK1) (n = 6 for each bar). I. Western blot analysis of cleaved ENaCα and ATP1α1 in control and HC0 cells (10 ng total protein). The ENaCα antibody recognizes a transmembrane region downstream of the amino-terminus of full length ENaCα.

Figure 7. Differentiated NHBE monolayers display increased I_sc responses after apical treatment with the selective β₂-AR agonist salbutamol.

A. Representative I_sc trace of day 16 monolayers treated apically with benzamil (5 μM) then the selective β₂-AR agonist salbutamol (10 μM), then CFTR_inh-172 (20μM). Apical addition of salbutamol increased the I_sc in both control HC0 and HC8 monolayers, consistent with stimulated anion secretion. B. Histogram summarizing benzamil sensitive, salbutamol activated and CFTR-dependent I_sc results reported in A. An asterisk (*) represents significant differences between treatment and control conditions (n = 4). C-D. qRT-PCR analysis of β₂-AR (ADRB2) and CFTR mRNAs (n = 6 for each bar). HC0 monolayers showed reduced ADRB2 and CFTR expression at
days 8 and 24. **E.** Western blot analysis of CFTR and ADBR2 in control and HC0 cells (25 ng total protein). CFTR protein abundance appeared to be reduced in HC0 cells at days 8 and 24.

**Figure 8.** CFTR and $\beta_2$-AR co-localize at the apical membrane of differentiated NHBE monolayers.

**A.** Immunocytochemistry shows co-localization of CFTR (red) and $\beta_2$-AR (green) at the apical membrane in day 24 differentiated control, HC0 and HC8 monolayers. Co-localization of CFTR and $\beta_2$-AR was detected in cilia-like structures. Nuclei (blue) were labeled with DAPI. **B.** Orthogonal views of day 24 differentiated cells showing apical localization of CFTR and $\beta_2$-ARs within cilia. **C.** Representative $I_{sc}$ trace of day 16 ΔF508 CFBE monolayers treated apically with benzamil (5 μM) then the selective $\beta_2$-AR agonist salbutamol (10 μM). 8-CPT-cAMP (10 μM) was then added to the apical and basolateral compartments but no change in $I_{sc}$ was detected. Stimulation with UTP (20 μM) produced an increase in $I_{sc}$ that was blocked by the disulfonic stilbene derivative DIDS (100 μM), a Ca$^{2+}$-activated Cl channel blocker. **D.** Immunocytochemistry shows localization of CFTR (red) and $\beta_2$-AR (green) in the perinuclear region below the apical membrane in differentiated ΔF508 CFBE monolayers. Localization of CFTR and $\beta_2$-AR was not detected in cilia-like structures. Nuclei (blue) were labeled with DAPI.

**References**


64. **Salyer SA, Parks J, Barati MT, Lederer ED, Clark BJ, Klein JD, and Khundmiri SJ.** Aldosterone regulates Na(+), K(+) ATPase activity in human renal proximal tubule cells through mineralocorticoid receptor. *Biochim Biophys Acta* 1833: 2143-2152, 2013.


Figure 1

A

![Graph showing TER (Ω·cm²) over days for different conditions.](Image)

- Control
- -HC0
- -HC8

**Legend:**
- Sub Conf
- Confluent
- Growth
- Differentiation Media
- Liq/Liq
- ALI
- 500
- 100nM RA

**Control**:
- 1.4 μM HC
- d0
- HC0

**HC8**:
- 1.4 μM HC
- d8
- 0 μM HC

B

**Imaging Data**

- Cont
- HC0
- HC8

For each condition, images are shown at d8 and d24.
Figure 2

(A) Relative expression of p63 over time for Cont, HC0, and HC8.

(B) Relative expression of KRT6a over time for Cont, HC0, and HC8.

(C) Relative expression of FOXJ1 over time for Cont, HC0, and HC8.

(D) Relative expression of MUC5AC over time for Cont, HC0, and HC8.

(E) Relative expression of MUC5B over time for Cont, HC0, and HC8.

(F) Relative expression of SCGB1A1 over time for Cont, HC0, and HC8.
Figure 4

A) Control

B) HC8

C) HC0

D) Bar graph showing ΔIsc (μA/cm²) with error bars: Control, Benzamil, CFTRinh-172.
Figure 5

A control

B Benzamil (5 μM)
Benzamil (5 μM)

CFTRinh-172 (20 μM)

HC8

μA \text{ min}

μA \text{ min}

C HC0

D

Δ \text{Isc} (\mu A/cm^2)

Benzamil

CFTRinh-172

Control

HC0

HC8

*
Figure 6

A

B

C

D

E

F

G

H

I

ENaCα

ATP1α1

FURIN

CAP1

SGK1

Cont

HC0

HC8

Control

HC0

HC8

ENaCα

ENaCβ

ENaCl

0 4 8 24

0 4 8 24

0 4 8 24

0 4 8 24

0.01

0.1

1

0.01

0.1

1

0.0001

0.01

0.1

1

37 kDa

100 kDa

43 kDa

β-actin

40 μm

15 μm

37 kDa

100 kDa

43 kDa

β-actin
Figure 7

A

Benzamil (5μM) → CFTR_{inh-172} (20μM) → Salbutamol (10μM)

μA
min

B

ΔIsc (μA/cm²)

Control
HC0
HC8

Benzamil
Salbutamol
CFTR_{inh-172}

C

ADRB2

Relative Expression

Cont
HC0
HC8

0 4 8 24 0 4 8 24 0 4 8 24

0.0001
0.001
0.01
0.1
1

D

CFTR

Relative Expression

Cont
HC0
HC8

0 4 8 24 0 4 8 24 0 4 8 24

0.0001
0.001
0.01
0.1
1

E

CFTR
ADBR2
β-actin

Cont 0 8 24 HC0 0 8 24

168 kDa
47 kDa
43 kDa
Figure 8

A) CFTR merge

B) Control, HC0, HC8

C) Benzamil (5 μM), Salbutamol (10 μM), 8-CPT-cAMP (10 μM), DIDS (100 μM)

D) CFTR merge

μA/min

UTP (20 μM)