Novel Aspects of Ciliary Beat Regulation in Human Bronchial Epithelial Cells

Ph.D. Theses

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1. INTRODUCTION

The epithelial surface of the respiratory tract is exposed to a large burden of microorganisms and other harmful material. A series of defense systems including mechanical, chemical and biological „barriers" protect the airways against these potentially injurious actions. Ciliary beat frequency (CBF) of ciliated epithelial cells is the primary determinant of mucus clearance, and thus the mechanical barrier function of the airways. Mucociliary dysfunction is a frequent consequence of chronic airway diseases; therefore the better understanding of the regulation of CBF could provide useful therapeutic tools in restoring normal clearance in such conditions. The present work focuses on some details of the regulation of CBF in human airway epithelial cells (HAEC).

External stimuli affecting ciliary beating in HAECs are transduced mainly by two intracellular second messengers, cAMP and intracellular calcium. Currently, transmembrane adenylyl cyclases (tmAC) are the only known sources of cAMP in HAECs. Another, distinct adenylyl cyclase activity was described in testis and called soluble adenylyl cyclase (sAC). Recent purification and cloning of sAC revealed that it is closely related to cyanobacterial adenylyl cyclases but not to tmAC. sAC differs from tmAC by its 10-fold lower affinity for substrate and its insensitivity to G-protein activation or forskolin. In contrast to tmACs, mammalian sAC is directly activated by HCO_3^- in a pH-independent manner and by Ca^{2+}. Sperm motility and hyperactivation has recently been shown to depend on sAC activity. Since airway cilia and sperm flagella share both structural and functional similarities, it is possible that sAC also plays a role in regulating airway ciliary beating. *One objective of these studies was to test the hypothesis that sAC is expressed and functioning in ciliated HAECs and co-localized to the cilia.*

Intracellular bicarbonate concentration ($[HCO_3^-]_i$), the regulator of sAC, is one of the most important regulators of pH_i as well. Therefore, when HCO_3^--related CBF changes are being investigated, it is necessary to identify whether CBF changes are caused by cAMP production (via sAC activation) or by pH_i-alterations (independently of sAC). *Considering that no publication has ever reported the relationship of pH_i to CBF, these studies also aimed to clarify this issue.*

Catecholamine derivatives, especially β2-adrenergic agonists are widely used bronchodilator drugs in obstructive airway diseases, but they effect is not restricted to airway smooth muscle cells. HAECs also express β2-adrenergic receptors and their stimulation by specific agonists enhances CBF through activation of tmAC, cAMP production, activation of cAMP-dependent protein kinase (PKA) and phosphorylation of an axonemal target protein. Actually, the group of β2-adrenergic agonists is one of the most effective ciliostimulant drugs. It has long been known that catecholamines can be metabolized only intracellularly (since the metabolizing enzymes reside in the cytoplasm). Furthermore, being organic cations, they cannot pass the plasmamembrane freely. Therefore, their inactivation is determined mainly by cellular uptake processes via catecholamine transporters, which is a critical determinant of the magnitude and duration of their effect on the target cell. For example, the inhibition of extraneuronal catecholamine uptake by glucocorticosteroids (GS) and consequent increase in extracellular catecholamine concentration has recently been shown to be responsible, at least in part, for GS-related vasoconstriction in bronchial arteries. A similar potentiating effect of GSs on β2-agonists-induced ciliostimulation also seems to be reasonable. However, the local cellular uptake and metabolism of these drugs is not known in airway epithelium. *Thus, in these studies we tested the catecholamine transporter expression profile of HAECs. Furthermore, pharmacological characteristics of NE transport as well as its inhibition by budesonide and methylprednisolone was also studied in a model of human bronchial arterial smooth muscle cells.*

2. AIMS

1. We aimed to clarify how isolated pH_i-changes influence CBF in HAECs, a key issue for further investigations of $[HCO_3^-]_i$ mediated CBF-effects. For this purpose, we performed simultaneous CBF and pH_i measurements on cultured HAECs.

2. To analyze the signaling mechanism by which pH_i regulates CBF, we used specific inhibitors of cAMP- and Ca^{2+}-dependent signaling pathways and also measured intracellular Ca^{2+}-concentration ($[Ca^{2+}]_i$). Furthermore, we measured CBF on basolaterally permeabilized HAECs under the direct control of pH_i.

3. We also tested whether sAC mRNA and protein is expressed in HAECs. In order to determine the subcellular localization of sAC, we performed immunofluorescence studies on human tissue sections and cultured HAECs. Our preliminary physiological experiment aimed to investigate sAC's effect on CBF.

4. The expression profile of HAECs for catecholamine transporter mRNAs was also investigated.

5. Finally we examined the pharmacologic characteristics of cellular catecholamine uptake, including the effect of GSs on it as well as the mechanism by which GSs inhibit cellular catecholamine uptake.

3. METHODS

Cell cultures. Human tissue was obtained from organ donors whose lungs were rejected for transplant through the Life Alliance Organ Recovery Agency of the University of Miami. Primary submerged and air liquid interface (ALI) cultures of bronchial epithelial cells and primary submerged cultures of bronchial arterial smooth muscle cells (SMC) were prepared using standard procedures.

Simultaneous measurement of CBF and pH_i or $[Ca^{2+}]_i$. Cells were incubated either with the pH-sensitive dye BCECF/AM or with the Ca^{2+}-sensitive dye fura-2/AM. They were visualized with a 60x water immersion objective (Nikon) on an upright Nikon E600FN microscope. CBF from a single cell was measured with infrared DIC imaging. pH_i or $[Ca^{2+}]_i$ from the same single cell was estimated using ratiometric epifluorescence imaging. pH_i measurements were calibrated by the high K^+ / nigericin method. For simultaneous measurements of epifluorescence and CBF from the same single cell, the microscope was equipped with a Nikon multi-image module. CBF was measured every ~3 seconds, whereas $[Ca^{2+}]_i$ and pH_i were estimated every 10 – 20 seconds.

Selective permeabilization of the basolateral membrane of cells grown at the ALI. The basolateral surface of the ALI culture was exposed to 10,000 U/ml *Staphylococcus aureus* alpha-toxin for 30 min at room temperature. (For selected experiments, 0,05% saponin was used instead of alpha-toxin.) The

composition of the basolateral solution mimicked cytoplasmic environment containing ATP and high concentration of potassium.

RT-PCR. Total RNA was extracted from HAECs cells grown and redifferentiated at the ALI and freshly isolated bronchial arterial smooth muscle cells. Specific primers for sAC and four different catecholamine transporters were designed using the GeneBank database. PCR products were gel purified and sequenced. For cloning the cDNA-fragments of sAC-variants, they were inserted into pGEM-T Easy vector, gel electrophoresed and sequenced.

Immunohisto- and immunocytochemistry. Human tracheal rings and HAEC ALI cultures were incubated with sAC-specific monoclonal antibodies. For visualizing the axonemes and nuclei, acetylated anti-tubulin antibodies and DAPI were used, respectively. A nonspecific monoclonal antibody was used as a control. Confocal microscopy was used for immunolocalization of sAC in ALI cultures.

Western blotting for sAC. Protein extracts from ALI cultures of HAECs were obtained by standard methods. Immunoblotting using sAC-specific monoclonal antibodies was kindly performed by L. R. Levin and coworkers (Cornell University, New York, NY, USA).

Single-cell, fluorometric NE uptake measurements. To measure NE uptake, intracellular NE was visualized using a sucrose-potassium phosphate-glyoxylic acid (SPG) method described for tissue slices and adapted by us for use in isolated vascular SMCs. Fluorescence of individual SMCs exposed to the SPG solution was measured with the same imaging system and software used for fluorometric pH_i and $[Ca^{2+}]_i$ measurements. Since we have shown that fluorescence is nearly linear to the intracellular NE concentration over a wide concentration range, we report here the fluorescence only without converting it to NE concentration.

Immunochemical detection of a plasma membrane binding site for corticosterone in bronchial arterial SMC. Cultured human bronchial arterial SMCs were incubated with 1 μM BSA, 1 μM corticosterone-21-hemisuccinate-BSA, or 1 μM corticosterone-21-hemisuccinate-BSA plus 100 μM corticosterone, followed by incubation with rabbit anti-BSA IgG (primary

antibody) and TRITC-conjugated goat anti-rabbit IgG (secondary antibody). TRITC fluorescence was imaged using the imaging system described above and an appropriate excitation/emission filter set.

4. RESULTS

4.1 Regulation of human airway ciliary beat frequency by pH_i

For the first set of experiments, the ammonium prepulse technique was used to achieve a change in pH_i of intact cells without changing the pH of the bathing solution. Baseline CBF and pH were 7.2 ± 0.2 Hz and 7.49 ± 0.02, respectively (n = 63 cells from 7 different organ donors). Transient alkalization and acidification of the cytoplasm achieved by two minutes of ammonium exposure resulted in a parallel change in CBF (**Figure 1**): pH_i reached a maximum of 7.78 ± 0.02 ($p < 0.0001$ compared to baseline) whereas CBF peaked at 9.4 ± 0.2 Hz ($p < 0.001$ compared to baseline). Similarly, intracellular acidification of 7.24 ± 0.02 pH units resulted in a fall in CBF to 5.8 ± 0.2 Hz (19 ± 2% below original baseline).

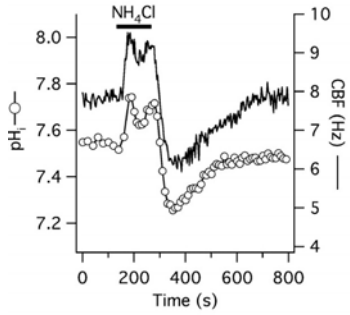

Figure 1: CBF responses to changing pH_i during ammonium prepulse. Simultaneous CBF and pH_i recording from a single, submerged, BCECF-loaded human tracheobronchial epithelial cell. Intracellular alkalization and acidification by transient exposure to 10 mM NH_4Cl result in pH_i-coupled changes in CBF.

Similar results were detected when pH_i was changed by removing of CO_2/HCO_3^- from the extracellular solution. As a consequence, cytoplasmic CO_2 left the cell by crossing the plasmamembrane freely, making the cytoplasm more alkaline (**Figure 2**).

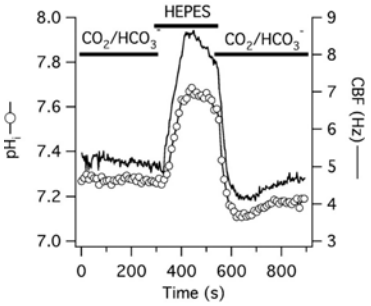

Figure 2: CBF responses to changing pH_i during removal of external CO_2. Simultaneous CBF and pH_i recording from a single, submerged, BCECF-loaded human tracheobronchial epithelial cell, mounted into a closed chamber and perfused with CO_2/HCO_3^--buffered solution in exchange with CO_2/HCO_3^--free, Hepes-buffered solution (pH = 7.4 for both solutions).

Next, we aimed to clarify the mechanism by which pH_i influences CBF. Potential targets of pH_i-changes could be both the kinase/phosphatase system and $[Ca^{2+}]_i$. Alternatively, pH_i-changes could directly act on outer dynein arm, the ciliary frequency-sensitive location of the ciliary axoneme. The kinase system was tested in two different ways: by inhibiting PKA either with H-7, a broad based serine-threonine kinase inhibitor or H-89, a specific PKA-inhibitor and by pre-stimulation of PKA using forskolin (**Figure 3**). Neither inhibition nor stimulation of PKA prevented pH_i-induced CBF changes, suggesting that PKA is not involved in the pH-dependent CBF regulation. Similarly to PKA, phosphatase inhibitors (cyclosporine + okadaic acid) also failed to alter the CBF-response to ammonium prepulse.

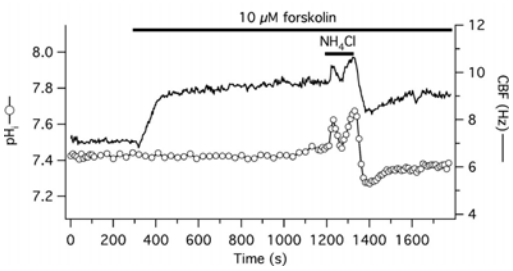

Figure 3: Pre-stimulation of PKA with forskolin does not prevent pH_i-dependent CBF changes. cAMP produced by tmAC upon forskolin exposure results in an acceleration in ciliary beating. Alkalization by ammonium prepulse has an additional stimulatory effect on CBF.

Then, the potential role of changing $[Ca^{2+}]_i$. upon pH_i-transients was studied. First, simultaneous measurement of $[Ca^{2+}]_i$ and CBF from the same single cells revealed no significant change in $[Ca^{2+}]_i$ during ammonium prepulse. Furthermore, emptying internal Ca^{2+}-stores with thapsigargin and inhibiting the influx of external Ca^{2+} using nominally Ca^{2+}-free bathing solutions did not modify CBF-response on pH_i-alterations (**Figure 4**).

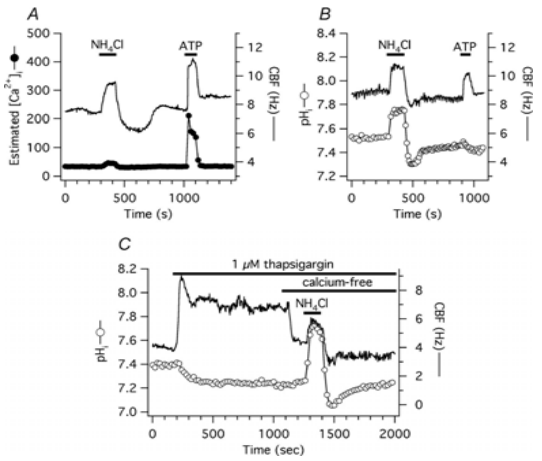

Figure 4: pH_i-mediated changes in CBF are not due to $[Ca^{2+}]_i$ variations. (A) Simultaneous CBF and $[Ca^{2+}]_i$ recording from a fura-2-loaded cell presented a negligible effect on estimated $[Ca^{2+}]_i$, upon NH_4Cl exposure ($[Ca^{2+}]_i$ is given in nM). Purinergic stimulation with 10 μM ATP as a control, however, resulted in a transient increase of both estimated $[Ca^{2+}]_i$ and CBF. (B) Experiment shown in panel A was repeated with BCECF instead of fura-2. NH_4Cl caused a significant change in pH_i, while ATP did not. (C) Emptying internal Ca^{2+}-stores with thapsigargin and removal of Ca^{2+} from bathing solution did not prevent pH_i-related CBF changes.

Finally, we measured CBF under direct control of cytoplasmic pH. After selective permeabilization of the basolateral membrane using *Staphylococcus aureus* alpha-toxin, the basal compartment of the ALI chamber was perfused with solutions titrated to pH 6.8, 7.2, 7.6 and 8.0. CBF was 3.9 ± 0.3 Hz, 5.7 ± 0.4 Hz, 7.0 ± 0.3 Hz, and 7.3 ± 0.3 Hz at pH 6.8, 7.2, 7.6, and 8.0, respectively (n = 18).

4.2 sAC expression and cellular distribution in human airway epithelial cells

Gel-electrophoresis of sAC-specific RT-PCR products using RNA extracted from HAECs suggested three bands in the expected size (243 bps) range. Subsequent cloning, purification and sequencing of the RT-PCR products revealed a 243 bp fragment of human testicular sAC cDNA and two variants.

Western blot analysis of whole cell extract of HAECs grown at the ALI using sAC-specific monoclonal antibodies revealed a band close to the 50 kDa marker, matching the band of sAC standard protein, which is supposed to be the product of posttranslational cleavage of the full length (187 kDa) protein.

Immunofluorescence studies on human tracheal sections using sAC specific monoclonal mouse antibody and, for visualizing the ciliary axonemes, acetylated anti-tubulin antibody demonstrated that sAC is expressed in the ciliary cells along with the cilia. Immunofluorescence study of HAECs cultured at the ALI was also performed in order to construct confocal images for better intracellular localization of sAC. Using the same sAC specific monoclonal antibody applied for the tracheal tissue, confocal microscopy confirmed that sAC is expressed in cultured ciliated cells as well The merged image clearly proves that sAC is located on the ciliary shaft co-localizing with the axonemal tubulin and in the apical membrane.

To demonstrate that sAC expressed in HAECs is functioning and involved in CBF-regulation, we performed preliminary experiments to test the effect of cytosolic HCO_3^- on CBF. In order to overcome the difficulties of controlling $[HCO_3^-]_i$ by externally applied bicarbonate without changing pH_i, the basolateral surface of a polarized HAEC culture was partially permeabilized by the detergent saponin. The basolateral membrane was perfused with an ATP containing buffer followed by an ATP-free solution, containing 0.05% saponin and CBF of twelve ciliated cells was recorded simultaneously. Those cells, which remained intact (i.e., no change in CBF upon ATP-removal) responded on CO_2/HCO_3^--containing buffers with slowing ciliary beating because of intracellular acidification (as expected based on the experiment shown in **Figure 2**). On the contrary, CO_2/HCO_3^- augmented CBF of permeabilized cell in the same culture, possibly via sAC-activation.

4.3 Catecholamine transporter mRNA expression profile in human airway epithelial cells. Pharmacological characterization of norepinephrine uptake

mRNA was extracted from cultured HAECs and human bronchial arterial smooth muscle cells (SMC). Because non-neuronal cells may express various and/or multiple transport systems for NE, RT-PCR reactions were initiated to examine the presence of norepinephrine transporter (NET), organic cation transporter-1 and -2 (OCT-1, OCT-2), and extraneuronal monoamine transporter (EMT) mRNAs. Gel-purification and DNA-sequencing of RT-PCR products revealed that both HAECs and human bronchial SMCs expressed OCT-1 and EMT. Interestingly, HAECs also expressed NET mRNA.

To study the pharmacologic characteristics of OCT-1 and/or EMT mediated NE transport and to determine whether it is blockable by GSs, we used human bronchial arterial smooth muscle cells expressing both transporters. NE uptake was measured in these cells in the absence or presence of 10 μM corticosterone. This concentration was chosen to inhibit EMT but not OCT-1 (and OCT-2) in a significant amount. The amount of uptake blockable by 10 μM corticosterone was defined as EMT-mediated. The experiments proved the time- and concentration-dependence of NE uptake (K_m approx. 240 μM). The pharmacological inhibitor profile of NE uptake was further investigated. Desipramine (inhibitor of NET) did not decrease NE uptake significantly, whereas 1 μM corticosterone and 1 μM O-methyl-isoprenaline (both are inhibitors of EMT) inhibited NE uptake by 63.2 ± 6.9% and 57.8 ± 2.9%, respectively ($p < 0.05$ versus control for both). Together with NE transport kinetics data, these inhibitor characteristics suggest that a significant amount of NE uptake is mediated by EMT in human bronchial arterial SMCs.

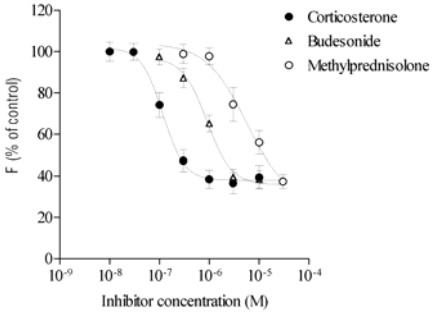

Figure 5: Inhibition of NE uptake by GSs in human bronchial arterial SMCs. Cells were exposed to 50 μM NE for 5 min in the presence of corticosterone, budesonide, or methylprednisolone. Data are shown as a percentage of NE uptake measured in the absence of inhibitors. Shown are means ± SEM for triplicate experiments with n of cells = 25-50 for each.

To test whether GSs used in the clinical practice are also able to inhibit cellular NE uptake, we investigated the effects of budesonide and methylprednisolone. Similarly to corticosterone, budesonide and methylprednisolone inhibited NE uptake after 5 min of incubation (**Figure 5**). The calculated apparent IC_{50} values for these compounds were 0.12 μM, 0.89 μM, and 5.56 μM, respectively.

The rapid inhibition of NE uptake by corticosterone suggests a non-genomic GS effect. To prove this hypothesis, i.e. that classical genomic pathway (binding to cytoplasmic receptors thereby initiating changes in transcription and protein

synthesis of target genes) was not involved in corticosterone's rapid action, either 10 μM RU486 (a cytoplasmic GS receptor antagonist), 100 μM actinomycin D (transcription inhibitor), or 10 μM cycloheximide (protein synthesis inhibitor) was added to the incubation medium 30 min prior to the addition of 50 μM NE and 1 μM corticosterone. None of the three drugs changed significantly the corticosterone-dependent NE uptake inhibition. To further confirm that corticosterone acts at the plasmamembrane, corticosterone was prevented from entering the cell by conjugation to the membrane-impermeant carrier protein BSA. Conjugation of corticosterone did not change its inhibitory potency on NE uptake. Finally, a specific plasmamembrane binding site for corticosterone was demonstrated in human bronchial arterial SMCs. For this purpose, plasmamembrane-bound corticosterone-BSA conjugate was visualized using rabbit IgG antibodies against BSA and TRITC-labeled goat anti-rabbit IgG secondary antibodies. Fluorescent labeling was absent in control cells that were incubated in 1 μM BSA-containing medium for 5 min whereas cells incubated in 1 μM corticosterone-BSA conjugate for 5 min were specifically labeled. Competition with 100 μM corticosterone completely inhibited binding of corticosterone-BSA conjugate to the cells.

5. CONCLUSIONS

Our experiments presented here provide novel information on the regulation of ciliary beating in HAECs.

1. Here we have shown the first time that intracellular acidification attenuates, while intracellular alkalization augments ciliary beating in HAECs. Relatively small changes in pH_i result in significant changes in CBF.

2. Phosphorylation/dephosphorylation events and $[Ca^{2+}]_i$ transients do not mediate pH_i-induced CBF changes. pH_i may act directly on the ciliary motile machinery.

3. RT-PCR, Western blot, immunohisto- and immunocytochemical studies revealed the first time that ciliated HAECs express sAC, a recently cloned unique adenylyl cyclase, which is regulated by certain cytosolic ions like HCO_3^- and Ca^{2+}, but independent of pH_i-changes and G-protein regulation.

4. Immunofluorescence studies in human tracheal tissue and cultured HAECs have shown that sAC is localized to the cilia and in the apical membrane of ciliated HAECs. Our preliminary functional studies suggest that sAC may be involved in the regulation of CBF.

5. HAECs express mRNA for extraneuronal (OCT1, EMT) and neuronal (NET) catecholamine transporters.

6. Using a model of airway vascular smooth muscle cell expressing OCT1 and EMT, we have shown that NE uptake is acutely and reversibly blockable by a non-genomic action of various GSs including budesonide and methylprednisolone.

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