Chapter 8

Airway mucosal mediation of chronic cough and cough hypersensitivity

Brian Button[1], Kian Fan Chung[2,3] and David A. Edwards[4,5]

[1]Marsico Lung Institute, University North Carolina at Chapel Hill, Chapel Hill, NC, USA. [2]National Heart & Lung Institute, Imperial College London, London, UK. [3]Royal Brompton and Harefield Hospital, Guy's and St Thomas' NHS Foundation Trust, London, UK. [4]Sensory Cloud Inc, Boston, MA, USA. [5]Center for Nanomedicine, Johns Hopkins University Medical School, Baltimore, MD, USA.

Corresponding author: David A. Edwards (dedwar81@jh.edu)

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Airway mucosal collapse during tidal breathing in extreme atmospheres is increasing as global warming promotes cough and cough hypersensitivity. It can be reversed by inhalation of alkaline aerosols of divalent salt ions endogenous to the airways. https://bit.ly/ERSM110

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Cough plays a critical role in the clearance of mucus in respiratory disease. Recent findings suggest that mucus may also play a key role in the origins of cough itself. Recent *in vitro* and *in vivo* studies indicate that protracted mucosal collapse or intermittent collapse over long periods of tidal breathing can initiate an inflammatory cascade that triggers cough by multiple pathways, upregulating the expression of genes associated with cough hypersensitivity. Recent clinical data in RCC patients, and experimental data collected in airway lining interface cultures of human bronchial epithelial cells, indicate that reversing mucosal collapse with salt ions and buffers endogenous to the airways, while mimicking airway surface liquid pH fluctuations that occur in healthy human airways, diminishes airway epithelial cell compression, reduces cough frequency in RCC, and potentially lessens cough hypersensitivity.

Introduction

Osmotic properties of mucin macromolecules play an exquisitely coordinated and dynamic role in maintaining airway hydration in the variable atmospheres common to the human breathing condition [1]. Overlapping and reversibly gelling near the air interface, mucin macromolecules form a stratified, reversible hydrogel that thins and thickens with fluctuations in air humidity [2]. This mucosal volume regulation modulates osmotic pressure near the apical epithelial membrane to regulate the osmotic flow of water into the airways and meet ever-changing while persistent evaporation demands (figure 1a) [3].

This coordination, which is vital to maintaining the mobility of mucus and supporting its role in the defence of the airways against inhaled particulate matter, can fail to assure water homeostasis in the relatively arid atmospheric conditions increasingly common to human

living. Mucosal collapse onto airway cilia, which restores water homeostasis in high evaporation conditions, results in loss of mucus mobility and compression of airway epithelia to promote cough and cough hypersensitivity (figure 1b) [2]. It also increases mucin sequestration of over 100 globular proteins in the mucin interactome [4] and thereby reduces the inherent osmotic pressure capacity of airway surface liquid (ASL) [2] and altering mucosal antimicrobial and antioxidant function.

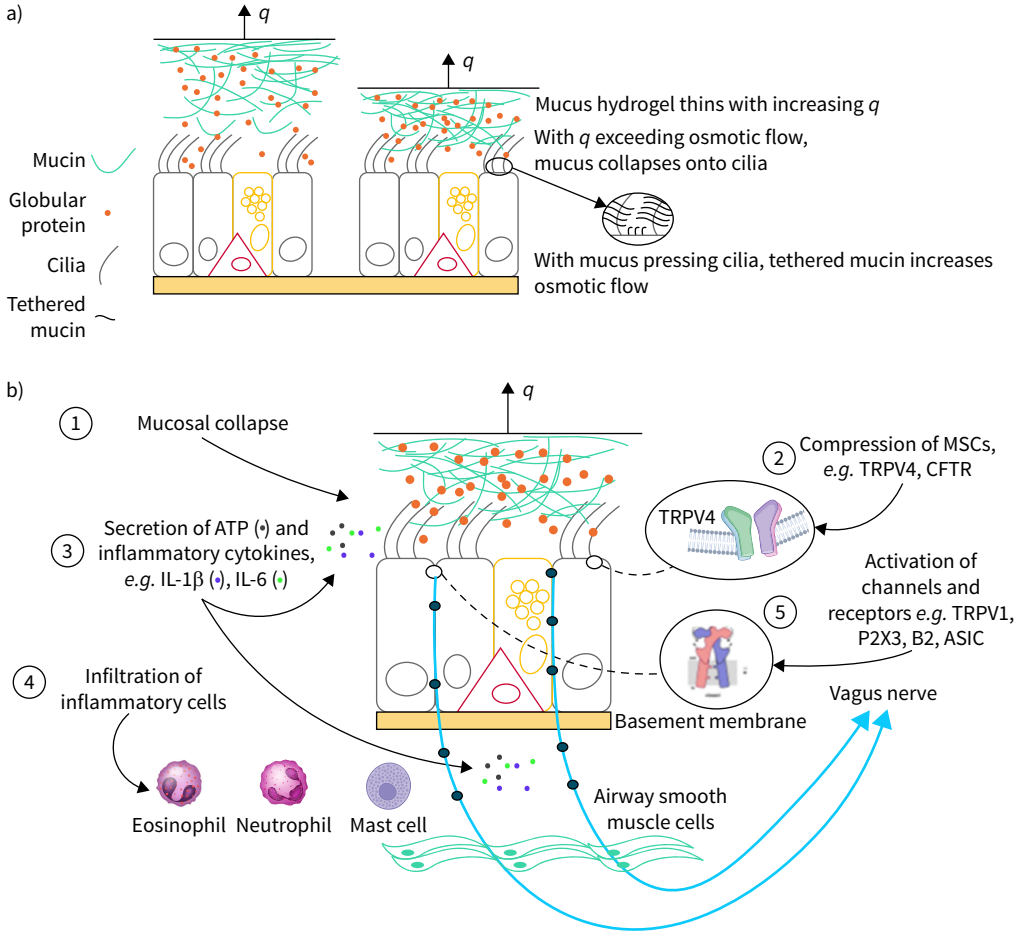

FIGURE 1 The airway hydration function and dysfunction of mucosal collapse. a) During slow nasal breathing, and in most tidal breathing scenarios in mild atmospheres, globular protein and tethered mucin provide sufficient time-averaged osmotic pressure to pull water into the airway surface liquid at or near a rate equal to evaporation rate. However, on tidal mouth breathing of the relatively arid atmospheres common to indoor living, and increasingly likely with global warming and heat waves, an inability to draw water into the airways with sufficient speed leads to mucus collapse onto cilia, and reduction of the mesh size between tethered mucin to a dimension where elevated osmotic pressure re-establishes water homeostasis. b) Mucosal collapse (1) compresses mechano-sensitive ion channels (MSCs) (2) to trigger secretion of ATP, inflammatory cytokines and chemokines, sodium/chloride imbalances and acidification (3), and, with prolongation, the infiltration of inflammatory cells (4), thereby establishing an airway milieu of chemical triggers of afferent sensory neurons (5) characteristic of cough hypersensitivity. *q*: evaporation rate; CFTR: cystic fibrosis transmembrane conductance regulator; TRPV4: transient receptor potential cation channel, subfamily V, member 4; TRPV1: transient receptor potential cation channel, subfamily V, member 1 ; ASICs: acid-sensing ion channels.

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Mucosal collapse is a risk inherent to many conditions of chronic respiratory disease as well as to all human airways on the tidal mouth breathing of hot (>35°C), cold (<0°C) and dry (<40% relative humidity) air of any temperature. Exercise [5], exposure to high altitude [6] and high levels of particulate matter with aerodynamic diameter <2.5 μm ($PM_{2.5}$) [7] are among additional physiological and atmospheric conditions that amplify the threat of mucosal collapse and cough provocation.

Chronic compression of airway epithelial cells promotes the secretion of mucin from airway goblet cells [7], elevates the MUC5AC:MUC5B ratio [8] and activates mechano-sensitive ion channels (MSCs) [3]. The MSCs activated include transient receptor potential cation channel, subfamily V, member 4 (TRPV4) (promoting ATP secretion) [9], cystic fibrosis transmembrane conductance regulator (CFTR) (promoting acidification) [10, 11] and Piezo-type mechanosensitive ion channel component 1 (promoting IL-6 secretion) [12]. This initiates a cascade of inflammatory consequences that include the infiltration of eosinophils, lymphocytes and neutrophils [2]. With the associated elevation of inflammatory-cell secreted cough triggers [13–16], this mucosal-mediated inflammatory cascade appears to promote cough by most recognised cough pathways (figure 2). While avoidance or reversal of acute mucosal collapse can be achieved by behavioural changes or re-engineered indoor atmospheres (*e.g.* by indoor humidification) [17], reversal of chronic mucosal collapse requires therapeutic intervention to relieve epithelial cell compression and de-activate MSCs [3].

We review current understanding of the mucosal origins of CC, beginning with an overview of mucus composition, structure and function. We review recent findings of mucus collapse and transpiration using *in vitro* and *in vivo* models of human airways, mucosal factors in productive and unproductive cough, environmental and behavioural factors in mucosal-mediated CC, and strategies for treatment that are advancing in human clinical trials.

Mucus composition, structure and function

Airway mucus is a critical component of the lung's mechanical host defence system, forming a protective hydrogel that facilitates the clearance of inhaled particles and pathogens *via* mucociliary clearance (MCC) and cough clearance [18]. Normal human airway mucus is composed of approximately 97.5% water, 0.9% salts, 1.1% globular proteins and 0.5% high molecular weight mucin polymers, primarily MUC5B and MUC5AC [19]. These components collectively determine mucus biophysical properties, such as viscosity and elasticity, which are essential for efficient clearance.

MUC5B and MUC5AC are the dominant secreted mucins in human airways [19, 20]. MUC5B, primarily secreted by Club cells in the superficial epithelium and submucosal glands, is a linear multimer of macromonomers (up to 250 MDa) and is critical for basal MCC in healthy lungs [21]. Its constitutive secretion *via* small granules (∼100 nm) ensures a continuous mucus layer. MUC5AC, in contrast, is minimally expressed in healthy lungs while it is upregulated in response to stressors (*e.g.*, dry air, allergens and infections), leading to goblet cell metaplasia and enhanced secretion from the large (∼1 μm) mucin-containing granules [22]. MUC5AC may form linear or trimeric structures, contributing to increased mucus viscosity under pathological conditions [23]. Gene-targeted mouse studies demonstrate that MUC5B knockout results in absent MCC and airway infections, while MUC5AC knockout shows no basal defects but impairs stress responses [24, 25].

While mucins are the major structural proteins in mucus, globular proteins (∼1.1% w/w) are the main contributor to osmotic or water-drawing capacity of mucus [26]. These proteins

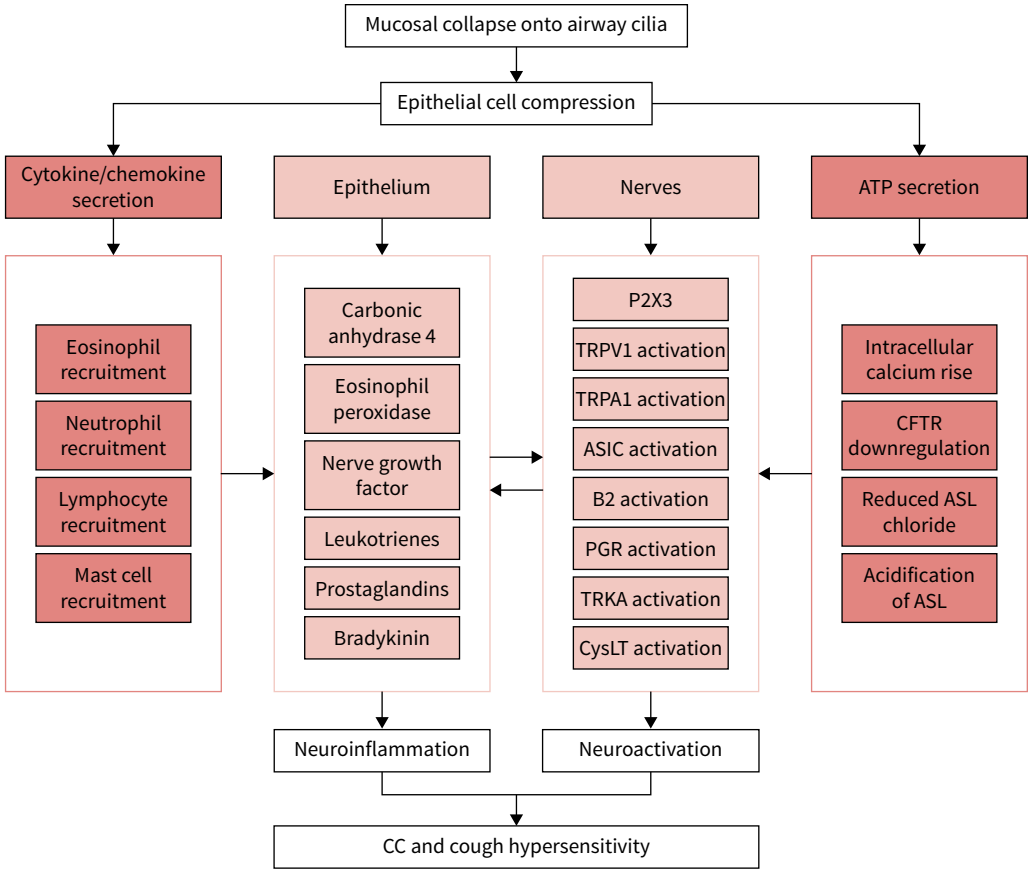

FIGURE 2 The mucus-transpiration inflammatory cascade and epithelium-nerve interactions. In the larynx and trachea, mucosal collapse promotes epithelial cell compression, activation of various channels and receptors, release of inflammatory mediators and cytokines that interact with the peripheral sensory nerves in the airway mucosa to induce cough hypersensitivity and cough. Mucin hypersecretion may be an additional source of hypersensitivity in most CC aetiologies. TRPV1: transient receptor potential cation channel, subfamily V, member 1; TRPA1: transient receptor potential cation channel, subfamily A, member 1; ASIC: acid-sensing ion channel; PGR: prostaglandin receptor; TRKA: tropomyosin receptor kinase A; CysLT: cysteinyl leukotriene; CFTR: cystic fibrosis transmembrane conductance regulator; ASL: airway surface liquid.

include enzymes, antimicrobial peptides and signalling molecules, and have a wide array of functions [27]. Salts (0.9% w/w, primarily NaCl) maintain isotonicity with plasma, facilitated by high airway epithelial water permeability mediated by aquaporins (AQP3, AQP4, AQP5) [28]. This isotonic environment ensures proper hydration of the ASL, which comprises the mucus layer and the periciliary layer and glycocalyx (PCL-G) [29].

PCL-G contains a space-filling, brush-like layer that is dominated by membrane-bound (tethered) mucins (including MUC1, MUC4, MUC16 and MUC20) anchored to epithelial cell surfaces, microvilli and cilia [21]. These mucins, along with other glycopolymers, form a dense network that prevents secreted mucins from penetrating interciliary spaces, maintaining a distinct mucus layer [17]. The osmotic properties of the PCL, driven by tethered mucins and globular proteins, interact with the mucus layer to regulate ASL height and clearance efficiency. The “gel-on-brush” model describes this interaction, where osmotic moduli of the mucus and PCL layers correlate with MCC rates [18].

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Efficient mucus clearance by cilia and coughing requires precise regulation of mucin secretion and ASL hydration. Club cells constitutively secrete MUC5B, supplemented by submucosal gland MUC5B during cough reflexes [21]. Purinergic signalling (*via* P2Y2R and A2b receptors) coordinates ion transport, balancing Na^+ absorption (*via* the epithelial sodium channel (ENaC)) and Cl^- secretion (*via* CFTR and calcium-activated chloride channels) to maintain optimal mucus hydration (~2% organic solids) in the transient conditions of cyclical breathing [30]. On time average over many breaths, ASL, however, retains ionic isotonicity, such that it is the persistent osmotic pressure of globular protein (~350 Pa) and tethered mucin (~180 Pa) that ensures the time-averaged water flux needed to counter net, time-averaged evaporative losses [3]. This natural osmotic water draw capacity of hydrated ASL can be overwhelmed in conditions of high evaporation rates or excessive sequestration of globular protein within a highly concentrated mucin interactome. The dehydrated mucus layer consequently collapses onto cilia, reducing the correlation length or mesh size between tethered mucin, and increasing the tethered mucin osmotic pressure (>180 Pa) until water homeostasis is re-established (figure 1b) [3].

The mucus-mediated inflammatory cascade action on mucosal airway sensory nerves

Depending on the "thirstiness" of air, quantified by vapour pressure deficit (VPD) [31], a measure of atmospheric aridity equal to the difference between water saturation pressure and vapour pressure, human airways exhale between approximately 100 mL and 1 L of water per day [32, 33]. Nasal humidification of inhaled air assures that, in this process and in nonextreme atmospheres, the evaporation rate from human airways does not exceed the natural osmotic water draw of fully hydrated ASL [3]. On human mouth breathing, by which the larynx is exposed to a moisture content close to atmospheric [34], high evaporation rates can outstrip the osmotic water draw capacity of healthy ASL, resulting in mucosal collapse. Particularly on exposure to laryngeal VPD ~1.5 kPa, as occurs on the tidal mouth breathing of dry air (relative humidity <40%), cold air (<0°C) and hot air (>35°C), compressions of epithelial cells in the first 6 generations [34] of the human airways can exceed the kPa threshold that has long been observed to promote inflammation [2, 3].

Airway epithelial cell compression by mucosal collapse triggers MSCs in airway epithelia to stimulate the acute secretion of ATP (in the case of TRPV4 [9]), chemokines such as C-C motif chemokine ligand 4 (in the case of Piezo-type mechanosensitive ion channel component 1) [13] and a range of inflammatory cytokines (including IL-1β, IL-4, IL-6, IL-8, IL-33, TNF-α and IFN-γ) [35]. Sustained or intermittently elevated cytokine and chemokine concentrations result in the infiltration of eosinophils, neutrophils, lymphocytes and mast cells [3]. ATP elevation in the ASL directly provokes cough by activation of P2X3 receptors on afferent sensory neurons (figure 2). In addition, certain inflammatory cytokines such as IFN-γ can directly induce cough hypersensitivity [36]. Acidification of the ASL – promoted by ENaC sodium-permeation enhancement in response to compression (as has been observed in cardiac cells) [37], combined with downregulation of CFTR in response to ATP elevation [38] – further promotes cough by activation of acid-sensitive ion channels and TRPV1 receptors [39]. Secretion by eosinophils and neutrophils of nerve growth factor [40] can trigger cough by activation of TRPV1 and tropomyosin receptor kinase A (TrkA) receptors [41]. Eosinophils, neutrophils and mast cells also secrete cysteinyl leukotrienes, which appear to provoke cough by a cysteinyl leukotriene receptor pathway [42] and neuropeptides [43] in the tachykinin family, namely neurokinin-A, neurokinin-B and substance P.

Infiltration of inflammatory cells and chronic elevation of chemokines, cytokines and neuropeptides promote neuroinflammation [43] by multiple pathways [44], including increased

sensitisation of airway nerves [45], increase in airway nerve density, [45, 46] glial cell activation [47], and the upregulation of nociceptor channels [48] and MSCs [3] implicated in cough hypersensitivity. Increased airway sensitisation, and associated loss of neural inhibitory control [43] are signature features of cough hypersensitivity and CC [48].

Mucosal factors in productive and unproductive cough

Productive cough relies on coordinated mucin secretion and ASL water flux to maintain mucus transportability [49]. MUC5B secretion from submucosal glands and superficial epithelia, coupled with fluid secretion, ensures a hydrated mucus layer (∼1–2% organic solids) with low viscosity and elasticity [50]. Water flux, driven by the combination of (breath-to-breath) CFTR-mediated Cl^- secretion and ENaC-regulated Na^+ absorption [51] and (over many breaths) globular protein/tethered mucin osmotic pressure [3], maintains ASL volume temporally and in the steady state. During cough, vagal stimulation enhances submucosal gland secretion, reducing mucus concentration and facilitating airflow-mediated clearance [52]. Studies in porcine trachea demonstrate that inhibiting Cl^- or HCO_3^- secretion impairs mucus transport [53], by disallowing the temporal ASL hydration that prevents mucosal collapse and potentially retards ciliary beating by other mechanisms [54]. Similarly, studies in humans reveal that mucus transport is impaired by exposure to cold, dry, and hot air, by limiting the ASL hydration that prevents mucosal collapse in the steady state [2, 4].

Mucosal collapse and cilia dysfunction are persistent features of CC aetiologies associated with muco-obstructive lung diseases, such as cystic fibrosis (CF) [54, 55], COPD [56], non-CF bronchiectasis [57] and primary ciliary dyskinesia. [58] Increased mucin secretion (particularly MUC5AC) and defective ion transport (*e.g.*, CFTR dysfunction in CF) can raise mucus organic solids content in these conditions up to 8–10% [59], increasing mucus viscosity and elasticity. Hyper-concentrated mucus forms adherent plaques, increasing the adhesive energy required to detach mucus from epithelial surfaces during productive cough [60], while chronic activation of the mucosal-mediated inflammatory cascade eventually promotes cough hypersensitivity and CC [7].

Vulnerability to bacterial colonisation is a hallmark feature of hyper-concentrated mucus, as in CF and protracted bacterial bronchitis [61, 62]. A natural defence against bacterial infection in healthy airways involves the fluctuation of ASL pH that occurs in the natural breathing cycle (figure 3a) [63]. Carbon dioxide concentrations in the ASL rise to ∼5.6% on exhalation. Catalysed by carbonic anhydrase 12 tethered to the apical epithelial membrane, carbon dioxide reacts with water to produce bicarbonate, thereby elevating healthy ASL pH to ∼9 on inhalation. Carbon dioxide levels fall over 140-fold on inhalation, such that ASL pH falls back to ∼7 during the exhalation cycle [63]. Alkalinity near pH 9 (above the pKa of the primary cysteine crosslinks of MUC5B and MUC5AC [64]) disrupts mucin–mucin and mucin–globular protein electrostatic interactions, liberating antimicrobial globular proteins sequestered in dehydrated mucus, and providing a surge of water flux as globular protein accumulates in the PCL-G [4]. This temporary osmotic water flux occurs precisely when the mucus layer thins owing to the elevated water evaporation burden of inhalation (figure 3a).

Reversing mucosal collapse and disrupting the mucus interactome to treat CC

Efforts to reverse the state of mucosal collapse and recover mucus clearance by the inhalation of hypertonic aerosols have frequently provoked – rather than relieved – cough in human subjects [65]. Hypertonic saline (HS) and mannitol, the most common rehydrating aerosol compositions [66, 67], suffer from a drawback inherent to hypertonic aerosols that include a

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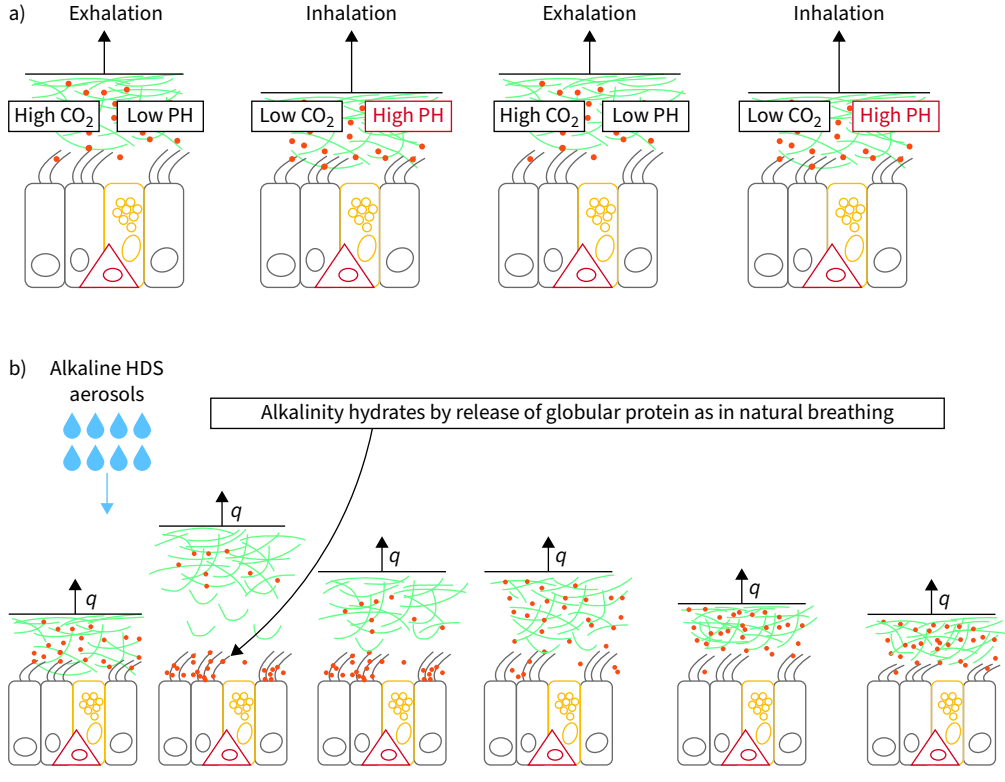

FIGURE 3 Alkalinity modulation of airway hydration and stress relief. a) Alkalinity on inhalation coincides with mucosal thinning. Healthy hydrated human airways naturally modulate airway surface liquid pH by carbonic anhydrase 12 catalysis of carbon dioxide (CO_2) during exhalation to achieve pH ~9 on inhalation owing to a transient sharp rise in bicarbonate concentrations. This pH elevation liberates globular proteins otherwise sequestered in the mucin interactome to act as osmotic pumps precisely during the most dehydrating phase of the breathing process. b) Alkaline hypertonic divalent salts (HDS) aerosols aim to achieve therapeutic relief from chronic mucosal collapse by mimicry of the natural pH swings of healthy human airways. The combination of globular protein release from the mucin interactome and slow clearance of the divalent cation (Mg^{2+}) results in a multi-hour rehydration and stress relief that is further prolonged in airways with hyper-secreted mucin.

cell-permeating cation (*e.g.*, sodium) or lack a cell-permeating anion (*e.g.*, chloride) [68]. Such aerosols, by elevating intracellular sodium and diminishing extracellular chloride, promote rapid and transient acidification of the ASL, inducing the cough reflex [69]. Hypertonic divalent salts (HDS) of magnesium and calcium chloride avoid this drawback [69]. Given that Mg^{2+} and Ca^{2+} exit the ASL by a paracellular pathway, rather than translocating across the apical epithelial membrane, as does Na, these HDS avoid the cough reflex that can occur on inhalation of HS (or mannitol). As the paracellular transport pathway is also significantly slower than the trans-membrane pathway, HDS aerosols also hydrate the ASL longer than HS [69].

Deposition of nonalkaline HDS aerosols promotes longer hydration than HS aerosols on dehydrated airway lining interface cultures of human bronchial epithelial cells while not exceeding the 3 h period beyond which downregulation of MSC channels associated with cough hypersensitivity has been observed. Prolonged therapeutic hydration of 4–6 h can, however, be achieved by combing hypertonic divalent magnesium salts with alkalinity above the pKa of cysteine–in a mimicry of the high pH fluctuation that occurs in healthy airways to liberate sequestered globular protein from the dehydrated mucin interactome (figure 3b).

As a therapeutic modality for the treatment of CC, alkaline HDS aerosols are currently in Phase 2 clinical development. In an exploratory clinical trial that aimed to clarify the safety and efficacy of HDS aerosols for treatment as a function of their alkaline pH [70], 12 RCC patients wore continuous cough monitors for 1 week of baseline, 1 week of nasal saline control and 1 week of active HDS aerosol nasal inhalation. Significant control-adjusted cough rate suppression treatment efficacy was observed from Day 1 for the participants (n=5) who self-administered an HDS aerosol (SC001) of pH 9 while not for those (n=7) who received an HDS aerosol of pH 8. Those receiving the HDS pH 9 experienced placebo-adjusted efficacy of cough rate (35%) (p=0.01) and cough bout (34%) (p=0.001) suppression from Day 3. No drug-related treatment emergent adverse events were observed [70]. A randomised, double-blinded, placebo-controlled crossover study (REACH) was recently completed on the alkaline HDS therapeutic aerosol SC0023 (4.3% $MgCl_2$ buffered to pH 9). The trial involved continual cough monitoring to explore placebo-adjusted cough rate suppression efficacy during and post-treatment for up to 4 weeks (ClinicalTrials.gov NCT07003347).

Discussion

Airway mucus function and dysfunction is fundamentally associated with its ability to facilitate the humidification of inhaled air while assuring airway water homeostasis [1–3]. The process by which airway mucus manages this water balance in the variable environmental and physiological conditions of human breathing is essentially identical to the process by which the surface of a leaf remains moist on a dry, hot and windy day [2]. Macromolecular building blocks of hydrogel barriers accumulate near the evaporating surface. This accumulation sets up osmotic pressure gradients that pull water across the hydrogel at the rate of evaporation while compressing cellular matter in proportion to the evaporation rate. When the rate becomes too high, at a threshold VPD of ∼1.5 kPa for leaves as for human airways, cellular compression becomes inflammatory [2, 3]. Leaves wilt [71], while human upper airways inflame, eventually developing conditions of cough hypersensitivity [1]. Restoring and maintaining topical hydration (droughts kill plants while so do floods [72]) may be as essential to healthy airways as it is to healthy plants.

Human breathing atmospheres have dramatically changed over the last century, with the human population moving to indoor atmospheres that are heated in the winter and increasingly cooled in the summer [73]. Global warming and the rising incidence and duration of heat wave events is now confronting mammalian airways with an existential evaporative or “VPD” threat of larger magnitude [74]. Crop failure rates are predicted to increase threefold to fourfold by the end of the 21st century from their current rate of 5–10% without dramatic curbs on carbon emissions [75]. Given the commonality of the inflammatory threat to plants and to airways, similar worsening of human respiratory diseases such as CC and asthma may be anticipated.

Human airways have evolved a remarkable capability to regulate water into and out of the airways for the purpose of humidifying inhaled air in variable environmental conditions. Mimicking this water management process in a therapeutic aerosol, notably fortifying the natural role of mucosal water mediation in atmospheres that are considerably more extreme than the pre-industrial condition, may lead to solutions for prophylaxis and treatment that, by their safety and simplicity, will be potentially accessible to the human population as the century unfolds [3].

Mucosal transpiration phenomena, and their roles in airway hydration and the development of the inflammatory state, are a recent discovery. Among the many questions that remain to be answered are those pertaining to the impact of acute and chronic mucosal collapse on innervation of the airway mucosa, signal transduction to the CNS, and CNS plasticity.

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