Effect of luminal osmolarity on ion content of connective tissue in rat trachea after epithelial damage

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ABSTRACT: The authors investigated the physical role of the airway epithelium in response to changes in the airway surface fluid's composition by superperfusing the lumen with nonisotonic solutions. Morphological studies and measurements of changes in ion content in the underlying connective tissue (CT) were carried out.

The study used an experimental model of isolated rat trachea. The trachea was mounted in an extraluminal organ bath with Ringer's solution, whereas the lumen was perfused with a fluid in which the NaCl concentration was varied. The tissue was fixed for electron microscopy or frozen for X-ray microanalysis.

X-ray microanalysis showed that the Na and Cl content of the CT increased with increasing luminal NaCl concentrations. This increase was significantly larger when the tight junctions had been damaged by exposure to ethylene glycol tetra-acetic acid. At high luminal NaCl concentrations, electron microscopy showed that a significant influx of fluid into the CT had occurred in tracheae with damaged epithelia. Damage to the epithelium also resulted in ultrastructural changes in myofibroblasts, increased diameter of capillaries, and thickening of the basement membrane.

The epithelium evidently plays a crucial role in the regulation of the ion content of the connective tissue in the airway wall, and epithelial damage may explain the greater sensitivity to provocation with hypersomolar sodium chloride solutions or airway dehydration observed in patients with asthma or cystic fibrosis.


Homeostasis of the epithelial cell layer plays a key role in maintaining the integrity and physiological processes of the airways. To function as a barrier, the cells of the epithelium must be capable of maintaining its structural and functional integrity. This task is performed by cell contacts including tight junctions (TJ), intermediate junctions, desmosomes, and gap junctions. The primary role of the TJ is to form a selective permeability barrier between apical and basolateral compartments of the extracellular space. Maintaining equilibrium between these two compartments may be important in preserving the regular function of the airway wall. The epithelial-mesenchymal interactions are significant in the pathogenesis of asthma [1].

The viscosity and ion content of the airway surface fluid (ASF), which lines the airway of the respiratory tract, is fundamental to the mechanics of the cilia and mucus and may play a role in bacterial defence [2]. However, the composition of the ASF is still uncertain. It has been claimed that the composition of the ASF is altered in cystic fibrosis (CF) patients who lack a functional Cl⁻ channel, the CF transmembrane conductance regulator (CFTR). Evaporative water loss from the airway mucosa, possibly due to exercise or cold air, induces a transient change in osmolarity in the epithelial fluid and this change is a potent stimulus to bronchoconstriction [3]. Changes in osmolarity in the airways can lead to a release of inflammatory mediators and dry air hyperventilation that can result in airway narrowing. This airway resistance is proposed to be a basic defence mechanism that limits peripheral airway exposure [4], seen in CF patients and exaggerated in asthmatics. In response to an increase in osmolarity, basophils and mast cells are activated and secrete histamine [5].

Healthy subjects do not show any bronchoconstrictive responses during inhalation of hypertonic saline, whereas subjects with symptoms of asthma do. Biopsies from asthma patients show evidence of destruction of the epithelium at all levels of the airways, with ciliated cells being the most destroyed cell type [6]. Therefore, the authors suggest that the respiratory epithelium may be involved in the protective responses of the airways to nonisotonic solution. While much is known about transepithelial ion and water transport in intact airway epithelium, this is not the case for airways with damaged epithelium. The aim of this study was therefore to investigate the effect of changes in the ionic composition of the ASF on airways with a damaged (rather than intact) epithelium, since this is more relevant with regard to
asthma and other airway diseases. Rat trachea with either undamaged or damaged epithelium was subjected to hypo-osmolar and different degrees of hyperosmolar solutions (produced by altering the NaCl concentration) and changes in ion content in the connective tissue (CT) were measured. Several studies have found the ASF normally to be isotonic [7] or slightly hypertonic [8], but other studies have shown that ASF in healthy subjects is hypotonic [9]. The authors, therefore, varied the NaCl concentration in the experimental fluid in the lumen 0.4–1.4%. The physical role of the airway epithelium in response to nonisotonic solutions was investigated by morphological studies and by measuring the changes in ion content in the underlying CT.

Materials and methods

Animals

Sprague Dawley rats (female, 3–4 weeks old, 200–300 g) were purchased from B&K Universal (Sollentuna, Sweden) and housed in a conventional animal care facility at the Biomedical Center, Uppsala University, Uppsala, Sweden, 1 week before experiment. The protocol was approved by the Regional Committee on Animal Experiments, Uppsala.

Study design and preparation

Rats were anaesthetized intraperitoneally by sodium pentobarbital (0.15 mL-100 g−1), and their tracheae were immediately excised and incubated in standard Ringer’s solution (containing (in mM) 140 NaCl, 5 KCl, 5 hydroxyethyl pipеразин-етиленсульфоная кислота (HEPES), 1 MgCl2, 1.5 CaCl2, and 5 D-glucose) aerated with 95% oxygen (O2) and 5% carbon dioxide (CO2) at 37°C. The tracheae were dissected free from surrounding tissues and transversely cut. The distal/proximal half would arbitrarily serve as a control (standard Ringer’s solution) and the other half would be the experiment (nonisotonic solution). An equal number of distal and proximal halves were used as controls.

The trachea halves were mounted horizontally on stainless steel catheters (1 mm diameter) at each extreme, extended to their approximate in situ length, and sutured at each end with surgical string (based on [10, 11]). The intraluminal side was perfused (2 mL·min−1) (VS-2×2 10 R and phase-shifted by 70% pump, Altea AB, Stockholm, Sweden), whereas the extraluminal side was incubated in standard Ringer’s solution as described above. The lumen was exposed to various solutions and the tissue was equilibrated for 10 min for each solution.

The lumen was exposed to either the standard Ringer’s solution having 0.9% NaCl (290 mOsm) or the Ringer’s solution with a different salt concentration: 0.4% NaCl (130 mOsm), 1.0% NaCl (320 mOsm), 1.2% NaCl (380 mOsm), 1.4% NaCl (450 mOsm) for 10 min aerated with 95% O2/5% CO2. To investigate the importance of the epithelium as a physical barrier, the TJ’s were disrupted and the lumen exposed to different osmotic solutions. For the damaged trachea, the TJ on the epithelial cells in the lumen were disrupted with 10 mM ethylene glycol tetra-acetic acid (EGTA) (Sigma, St Louis, MO, USA) before perfusion with nonisotonic and isotonic solutions. Small tissue pieces were cut lengthwise after the trachea was dismounted from the apparatus, and: 1) rapidly frozen in liquid propane cooled with liquid nitrogen and thereafter stored in liquid nitrogen pending preparation for X-ray microanalysis; or 2) fixed in 2.5% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer (Agar Scientific, Stansted, UK) for 1 day (morphological studies). The undamaged epithelium perfused with isotonic solution served as a control.

Determination of elemental content in the subepithelial connective tissue

X-ray microanalysis was used to quantify the ion content in the subepithelial CT. Tissue pieces were cut into semithin (3–4 μm) cryosections in a conventional cryostat at −30°C [12], mounted on a carbon specimen holder over a layer of thin Formvar film (Merck, Darmstadt, Germany) and freeze-dried for 24 h. To avoid charging in the electron microscope, the sections were coated with a conductive carbon layer after the specimen was successively warmed to room temperature. The sections were analysed in the scanning-transmission electron microscope mode at 100 kV (Hitachi 7100, Hitachi, Tokyo, Japan) with an Oxford Instruments ISIS energy-dispersive spectrometer system (Oxford Instruments, Oxford, UK). Quantitative analysis was carried out based on the peak-to-continuum ratio after correction for extraneous background [13] and comparison to a standard with known elemental content. Spectra were acquired for 50 s. Analysis was performed on the subepithelial CT and the electron beam was focused on the extracellular matrix. Forty spectra were collected from each specimen and averaged.

Morphological studies

Trachea pieces were fixed as described above. After being washed in 0.1 M cacodylate buffer, the pieces were postfixed in 1% OsO4 (Agar Scientific) in cacodylate buffer for 20 min. A second washing in buffer was followed by dehydration in a graded series of ethanol, and the pieces were finally embedded in Agar 100 Resin (Agar Scientific). Sections were cut (50 nm), contrasted with uranyl acetate/lead citrate and examined in a Hitachi H7100 transmission electron microscope at 75 kV.

Analysis

Elemental ion concentrations were expressed as mmol·kg−1 of dry weight. One-way analysis of variance was performed. Bonferroni’s Multiple Comparison Test was used to determine the significance of
different concentrations of NaCl in the luminal fluid and a paired t-test was used to determine the significance between damaged and undamaged samples. A p-value of <0.05 was considered significant. A non-linear regression model for a best-fit line was performed to analyse the ion ratio data.

The thickness of the basement membrane and the cross-sectioned area of the blood vessel were expressed in μm after being measured at ×150,000 and ×7,500 magnification, respectively. The mean was calculated and a paired t-test was used to determine the significance between samples. A p-value of <0.05 was considered significant.

**Results**

**Morphology**

The trachea perfused with standard Ringer’s solution showed an intact epithelial layer (fig. 1a) with intact TJ and a tight, intact CT. The EGTA-perfused trachea showed disrupted TJ and widened intercellular spaces between the epithelial cells (fig. 1b). The epithelial layer was sometimes detached from the CT.

Varying the osmolarity of the luminal fluid within the range of 0.4–1.4% NaCl did not appear to cause damage to the connections between the epithelial cells in undamaged epithelia. Perfusion of the damaged epithelium with hyperosmolar solution resulted in major structural changes in the CT (fig. 1c), which had a much more irregular structure than the undamaged trachea with epithelia exposed to the same luminal NaCl concentration (fig. 1d). With a damaged epithelial layer, the damaged CT extended deeply into the airway wall, eventually up to the cartilage rings. A gradient of damage in the CT was noted, with more extensive damage just below the epithelium and much less damage close to the cartilage.

In the undamaged trachea, mesenchymal cells (myofibroblasts) close to the cartilage were flattened,

Fig. 1. – Transmission electron micrographs of rat trachea. a) Undamaged epithelium, 0.9% NaCl; note the intact tight junctions and the very narrow intercellular spaces (arrows). b) Damaged epithelium, 0.9% NaCl; note the widened intercellular spaces, some epithelial cells are detached. c) Connective tissue in trachea with damaged epithelium, 1.4% NaCl; note swelling of the connective tissue (CT) compartment. d) CT in trachea with undamaged epithelium, 1.4% NaCl, intact CT with tight structure. Internal scale bars=2.5 μm.
while they had a more round shape closer to the epithelium. Damaging the TJ with EGTA did not affect the morphology of the myofibroblasts (fig. 2a). However, the myofibroblasts close to the epithelium in the connective tissue of damaged trachea subjected to hypertonic solutions were shrunken and misshaped (fig. 2b). The ultrastructure of the cells close to the cartilage was not noticeably affected by the hypertonic conditions.

The capillaries in the trachea with undamaged and damaged epithelium under isotonic conditions did not differ in size, whereas the capillaries in the trachea with damaged epithelium exposed to hypertonic solution had a significantly larger diameter (fig. 3). The basement membrane of the trachea with damaged epithelium was significantly thicker than the basement membrane of the trachea with undamaged epithelium (fig. 4). The damaged trachea perfused with hypertonic solution showed epithelium denudation and the thickness of the basement membrane could not be determined.

**X-ray microanalysis**

When the luminal fluid was isotonic, damage to the epithelium resulted in insignificant changes in the Na and Cl concentration in the CT compartment (fig. 5). The changes in the concentrations of Na and Cl in the CT with varying concentrations of NaCl in the luminal fluid are shown in figure 6. When the luminal fluid was clearly hypertonic (>1.0%), damage to the epithelium resulted in a marked increase of the Na and Cl concentration in the CT. Increasing the

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**Fig. 2.** Transmission electron micrographs of rat trachea. Myofibroblast in a) undamaged trachea (note the round shape) and b) in damaged trachea exposed to 1.4% NaCl (note the irregular shape). Internal scale bars=1 µm.

**Fig. 3.** Morphological analysis of capillary in undamaged and damaged rat trachea after exposure to isotonic or hypertonic luminal fluid. Data are expressed in µm and mean diameter of capillary±SEM. ***: p<0.001.

**Fig. 4.** Morphological analysis of basement membrane in undamaged and damaged rat trachea after exposure to isotonic luminal fluid. Data are expressed in nm and mean thickness of basement membrane±SEM. **: p<0.01.
concentration of NaCl in the luminal fluid from 0.9 to 1.4% produced only minor effects on the Na and Cl concentrations in the CT when the epithelium was undamaged, but caused a dramatic increase when the epithelium was damaged. Increasing the NaCl concentration in the CT with a damaged epithelium showed that the ion content of Na and Cl increased significantly at 1.2% NaCl (p<0.05 and p<0.001, respectively), and the Cl concentration increased significantly at 1.4% (p<0.001). When the luminal fluid was very hypo-osmolar, damage to the epithelium had little effect on the concentrations of Na and Cl in the CT. Whether or not the epithelium was damaged had no significant influence on the potassium (K) concentration in the CT.

**Discussion**

By superperfusing the lumen with nonisotonic solutions, it was possible to mimic the effect of changes in the ion composition of the ASF. By disrupting the TJ in the epithelium, the authors were able to model the damaged epithelium seen in asthma and other respiratory diseases. The damaged epithelium was obtained by breaking intercellular TJ with EGTA, since extracellular Ca$^{2+}$ is required for the formation and stabilization of intercellular junctions [14] and the addition of EGTA led to a depletion of Ca$^{2+}$.

Morphological evidence indicates that the epithelial barrier is severely damaged in asthmatics [15] and thus, must have lost part of its protective function. The mechanism by which epithelial damage occurs has not yet been fully explained. It is possible that damage to TJ [16] and influx of ASF through widened intercellular spaces [17] cause further disintegration of the epithelium. Disrupting TJ by perfusion of the trachea by EGTA may thus be a relevant model of the epithelial damage seen in asthma. Morphological studies were performed to investigate the effects of nonisotonic fluids on the rat trachea epithelium and underlying CT with and without intact TJ. The rat trachea was hardly altered upon nonisotonic stress when the epithelium was undamaged. However, after disrupting the TJ, the epithelial cells were separated by widened intercellular spaces and the basement membrane was significantly thickened. The matrix and mesenchymal cells of the CT were structurally irregular when the epithelium was damaged and nonisotonic solution was perfused through the lumen. This damage to the CT was increased with increasing hyperosmolarity of the luminal solution and
extended deep into the airway wall up to the cartilage, showing a gradient with more extensive damage at the epithelial side.

Major alterations found in the airways of asthmatics are thickened basement membrane [18, 19] and bronchial vessel dilation (reviewed by [20]). The authors found that there was a significant change in the thickness of the basement membrane after epithelial damage to the rat trachea that was due to the disruption of the TJ. Measurement of the thickness of the basement membrane after the disruption of the epithelium and exposure to hypertonic solutions was not possible because of the denudation of the epithelium. The myofibroblasts were modified in the damaged trachea that was exposed to hypertonic solution. It is known that airway epithelial shedding affects myofibroblasts [21].

To investigate the changes in the physiological environment in the CT, X-ray microanalysis was used. Exposure of undamaged rat trachea to hyperosmolar solution caused a slight increase in the concentrations of Na and Cl in the CT compartment. This is in agreement with earlier studies on rabbit airways [22, 23]. When the intact epithelium is exposed to hypertonic solutions, the most extensive ionic changes take place in the epithelium [22] and there are minimal changes in ion content in the CT upon nonisotonic stress. When the epithelium is damaged, however, a hyperosmolar NaCl solution gives rise to a significantly more pronounced increase in the NaCl concentration in the CT compartment. It can be concluded that the intact epithelium is instrumental in protecting the CT layer from the effect of changes in osmolarity in the ASF. In asthmatics, where the airway epithelium is damaged, exposure to nebulized high concentrations of NaCl may be expected to have a greater impact on the NaCl concentrations in the CT compartment in the airway wall. This may, in part, explain the greater sensitivity of asthmatics to stimulation with hyperosmolar NaCl.

Hypertonicity of the ASF may not only occur after experimental provocation, but it has also been suggested to be an important mechanism in exercise-induced asthma [24]. Similarly, patients with allergic rhinitis, another disease in which extensive epithelial damage is found, react more strongly to provocation with hypertonic saline than healthy controls [25]. Furthermore, CF patients, epithelial damage is common in their respiratory tract [27], even if this is not a primary feature of the disease.

It is generally accepted that the ion content of the CT of the airway walls is determined by interaction between the CT compartment and the circulation, and that the ASF does not play a role in this. This may be true for airways with intact epithelia. The present data suggest, however, that in damaged epithelia, the situation is different from normal and that the role of the ASF cannot be neglected. The morphological changes in the CT show a clear gradient from the epithelial side to the cartilage side. This indicates that the main factor responsible for the changes in the CT is passive influx of fluid from the lumen. Since there was no circulation in this in vitro system, the changes in capillary diameter were likely to be caused by the ionic changes in the CT.

The present results show that a hyperosmolar ASF in combination with a damaged epithelium leads to increased Na and Cl concentrations in the CT compartment, which may have adverse effects. Polymorphonuclear neutrophil (PMN) apoptosis and lysis are accelerated by a high Cl concentration [28], which can promote inflammation since a high Cl concentration can also alter PMN recruitment and clearance. Furthermore, accelerated PMN lysis would increase the amount of toxic PMN cytoplasm released into the airways and promote lung injury. Mast cells are known to be activated by hyperosmolar solutions [29] and it has been suggested that the beneficial effects of nedocromil sodium on allergic airways are mediated by inhibition of chloride fluxes [30]. Airway sensory nerves are stimulated by hyperosmolar solutions and this may lead to vasodilatation [31]. Hyperosmolar solutions also stimulate nociceptive nerves and promote substance P release in airways [32]. Increasing the osmolarity of the ASF can block the relaxant effect of nitric oxide inhalation on airway smooth muscle [22].

The K content of the CT is, in part, due to the K present in the CT matrix, but also to K present in the cells of the CT. Even though the authors specifically attempted to analyse the CT matrix, the cell processes often were so small that they could not be seen in the image, and therefore could not be avoided during analysis. However, although there is a tendency for K to increase with increasing luminal osmolarity, changes in K are not statistically significant.

The experimental approach of superfusion of nonisotonic solution intraluminally through the rat trachea introduces a relatively large volume of solution into the tracheal lumen. It can be argued that this does not correspond to the total volume of a nonisotonic fluid that is added to airway surfaces in vivo by an inhaled aerosol route. However, since in vivo conditions would replenish an ASF that is dehydrated, the authors’ ex vivo model mimicked this, replenishing by an excess volume of nonisotonic solution.

NaCl was used to vary the osmolarity, because a modification in NaCl is what would naturally occur in the respiratory tract during dehydration upon respiration of dry air (seen in exercise-induced asthma) or defective ion transport (as a result of a faulty CFTR seen in CF). Mannitol, however, would be a practical solute for looking at the sole effects of changes in osmolarity alone; indeed, it has been used in clinical tests of airway hyperresponsiveness [33].

The authors’ data provide a better understanding of the role of the epithelium and how a change in the airway surface fluid composition can affect the connective tissue component of the airway wall, especially when the epithelium is damaged, such as is the case in cystic fibrosis and asthma. Through the use of an experimental model of isolated rat trachea to study the physical role of the airway epithelium in the regulation of ion content in the connective tissue, the authors have concluded that: 1) airway epithelial cells
play a major role in protecting the connective tissue via tight junctions; and 2) luminal hyperosmolality increases the sodium and chloride content in the connective tissue, which may lead to the activation of inflammatory cells by transforming the ion environment in the connective tissue.

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References


