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Reduction of chemokine IL-8 and RANTES expression in human bronchial epithelial cells by a sea-water derived saline through inhibited nuclear factor-κB activation

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Abstract

The NaCl content of airway surface fluid is believed to be of central importance in lung pathology. To test whether the Na^+ concentration could influence the inflammatory response in human bronchial epithelial cells (BECs), we investigated the interleukin (IL)-8 and RANTES expression in BECs exposed to an isotonic sea-water derived low Na^+ (ISW) saline compared to isotonic 0.9% NaCl saline. Exposure of BECs to ISW saline caused a significant decrease in IL-8 and RANTES gene expression and protein production as compared to that observed with 0.9% NaCl saline. Furthermore, we observed a concomitant reduction of phosphorylated IκBα associated with a marked inhibition of NF-κB–DNA binding activity in BECs exposed to ISW saline as compared to 0.9% NaCl saline. These findings support a new role for Na^+ in the pathogenesis of airway inflammatory disorders. Therapies targeted at lowering Na^+ level in airway epithelium may be beneficial in treating inflammatory lung diseases.

Keywords: Airways; Epithelial cells; Inflammation; Chemokines; NF-κB; Seawater; NaCl

Osmotic stress, caused by altered extracellular NaCl level, is a highly relevant challenge to normal cell function in mammalian tissues such as kidney [1], liver [2], and airways [3]. Osmotic regulation is a result of an activation of osmosensitive NF-κB and MAP kinases signaling pathways [2,4,5]. Activation of MAP kinase pathways by hypertonicity has been demonstrated in different epithelial cell lines [6,7]. A hyperosmotic stress increases the expression and production of interleukin (IL)-8, IL-18 and regulated on activation normal T cell expressed and secreted (RANTES) of cultured human respiratory [8,9] and intestinal epithelial cells [10].

The epithelial cells that form a barrier lining the airways are key regulators of neutrophil trafficking into the airway lumen. High levels of IL-8 and RANTES in nasal and bronchial mucosa contribute to the massive recruitment of neutrophils and eosinophils to inflammatory sites [11–13]. In airway epithelium, activation of NF-κB causes an enhanced expression of most of genes encoding inflammatory chemokines and generated neutrophilic lung inflammation [14,15]. NF-κB dimers exist as inactive complexes in the cytoplasm of unstimulated cells due to their interaction with a family of inhibitory proteins collectively designated IκBs. These IκBs are phosphorylated by cellular kinase complexes known as IκB kinases α/β [16,17]. The best-studied member of the IκB family, IκBα, binds to the nuclear translocation sequence of p65 and sequesters NF-κB in the cytoplasm. The key regulatory steps in the signal-induced control of NF-κB include the phosphorylation and degradation rates of IκBα [18,19]. Cytosolic inhibitor factor IκBγ appears to be a major regulator of NF-κB, as indicated by the degradation of IκBα matching the translocation of NF-κB to the nucleus [19], and binds to DNA

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regulatory elements of target genes such as those for cytokines [20].

Changes in the level of NaCl of the airway surface fluid have been implicated in the pathogenesis of various forms of inflammatory lung diseases included cystic fibrosis [21] and allergic inflammation in bronchial asthma [22]. We and others have shown that in cystic fibrosis, high IL-8 production of airway epithelial cells was associated with high NF-κB activity [23–25]. Several authors have proposed that nebulized hypertonic saline may have a beneficial effect on bronchial mucociliary clearance and lung function in cystic fibrosis patients [26–28]. However, other studies have shown that hypertonic saline in airways can be deleterious by inhibiting the activity of endogenous antibacterials at the airway surface [21,29], reducing neutrophil antimicrobial activity [30] and increasing secretory and exudative responsiveness of human nasal airway in vivo [31]. We recently reported that elevated IL-8 and RANTES production by human primary bronchial epithelial cells (BECs) in culture could be rapidly reduced when cells initially placed in a hypertonic medium were shifted to an isotonic medium [32]. We therefore hypothesized that lowering Na^+ level in isotonic saline could reduce the chemokine expression in airway epithelial cells. Undiluted seawater brought to low Na^+ content and isotonicity by electrodialysis has been shown to favor in vitro respiratory epithelium regeneration [33] and to be safe for the cleaning of nasal cavities following endonasal surgery [34].

In the present study, we addressed the question whether an isotonic sea-water derived low Na^+ (ISW) saline could reduce the regulation of IL-8 and RANTES expression of BECs, compared to isotonic 0.9% NaCl saline. Molecular mechanisms through NF-κB signaling were also investigated in BECs after their exposure to both isotonic saline solution.

Materials and methods

Cell culture. Cell isolation and culture procedures of BECs were performed on bronchial tissues collected from adult patients undergoing lung transplantation, as previously described [23]. Tissues were obtained from two patients with pulmonary hypertension and two patients with pulmonary idiopathic fibrosis. BECs were isolated by enzymatic digestion from bronchial submucosa and grown on type 25 cm² culture flasks in DMEM/Ham's F-12 mixture (50/50%, v/v) supplemented with 2% Ultroser G (a serum substitute from Sepracor, Villeneuve-la-Garenne, France), 50 U/ml penicillin G, and 100 μg/ml streptomycin. Confluent BECs exhibited characteristics of bronchial secretory gland epithelial cells, as previously described [23].

Cell viability and cytotoxicity. Confluent BECs cultured in six-well [illegible] [redacted] [illegible] respectively). [redacted]

(major ions are: Na^+, 1575 mg/L; Cl^-, 5480 mg/L; Mg^{2+}, 1135 mg/L; Ca^{2+}, 300 mg/L; and K^+ 37 mg/L) by electrodialysis. The ISW saline was obtained from Goemar Laboratories (St. Malo, France).

BEC viability was evaluated by trypan blue exclusion method after exposure to either 0.9% NaCl or ISW saline for 4 h incubation. The cytotoxic activity of 0.9% NaCl and ISW saline on BECs cells was measured using a commercial colorimetric assay, based on the cleavage of the tetrazolium salt WST-1 (Roche Diagnostics, France) to formazan by mitochondrial dehydrogenases. The mitochondrial enzyme activity leads to an increase in the amount of formazan formed, which is directly correlated to the metabolic active cells [35]. Briefly, 96-well plate cultured confluent BECs were preincubated in Ultroser G-free medium containing actinomycin D (1 μg/ml) for 3 h. Culture medium was then removed and replaced by either ISW or 0.9% NaCl saline for the desired incubation times. The plate was shaken thoroughly for 1 min and the absorbance of the samples was measured at 450 nm with a reference wavelength at 620 nm using a microplate reader.

Assay of IL-8 and RANTES cytokines. Confluent BECs cultured in six well-culture plates were incubated in Ultroser G free medium (DMEM/Ham's F-12) for 16 h. Following this period, BECs were exposed to either the ISW or 0.9% NaCl saline for 2 and 4 h incubations. In some experiments, BECs were stimulated with 20 ng/ml IL-1β which was solubilized in either ISW or 0.9% NaCl saline for 2 and 4 h incubations. Levels of IL-8 and RANTES in cell supernatants were determined using ELISA kits (R&D Systems, Abington, United Kingdom), following the manufacturer's instructions.

RNA extraction and reverse transcriptase-PCR. Total cellular RNA from BECs exposed to either ISW or 0.9% NaCl saline for 1 h incubation was extracted using RNeasy (Quiagen, France) following the manufacturer's instructions. The precipitated RNA was resuspended in sterile water and quantified by absorbance at 260 nm. The expression level of RANTES, IL-8, and β-actin was determined by reverse transcriptase-PCR. Single-strand cDNAs were synthesized from 1 μg of total RNA using oligo(dT) priming and Moloney murine leukemia virus reverse transcriptase (Gibco, Cergy Pontoise, France) according to the manufacturer's instructions. The resulting cDNA samples were amplified by PCR. The PCRs were carried out at 94 °C initial denaturing temperature for 5 min, followed by 30 cycles and 35 cycles for β-actin and IL-8 denaturing temperature for 30 s, 60 °C for annealing temperature for 50 s, and 72 °C extending temperature for 45 s with a final extension for 7 min. For RANTES, the PCRs were carried out at 94 °C initial denaturing for 3 min, followed by 40 cycles denaturing temperature for 40 s, 62 °C for annealing temperature for 30 s, and 72 °C extending temperature for 30 s with a final extension for 7 min.

Primers used for human IL-8 were: 5′-CTCTTGGCAGCCTT CCTGAT-3′; 5′-ACTTCTCCACAACCCTCTGC-3′ and the expected size of PCR product was 248 pb. Primers for RANTES were: 5′-GCTGTCATCCTCATTGCTAC-3′; 5′-TCCATCCTAGCTCATCTC CA-3′ and the expected size of PCR product was 288 pb. As a control for cDNA synthesis, β-actin-specific primers: 5′-CATGCCATCCT GCGTCTGGA-3′; 5′-CCACATCTGCTGGAAGGTGG-3′ were used. The PCR products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. The intensity of each band was compared with the adjusted volume (mean optical density × area on mm² by densitometric analysis.

Preparation of nuclear extracts and electrophoretic mobility shift assay. Nuclear extracts were prepared and analyzed from BECs exposed to either the ISW or 0.9% NaCl saline for 1 h incubation. Nuclear extracts were incubated with ³²phosphate (p)-labeled NF-κB oligonucleotides. The consensus κB DNA sequence used for the electrophoretic mobility shift assay (EMSA) was 5′-AGTTGAGGGGA [illegible] [redacted] [illegible] [redacted]

unlabeled oligonucleotides or 1 μg Ab was added to the binding reaction mixture, prior to the addition of the labeled κB probe. Identification of the different NF-κB heterodimeric proteins was carried out by incubating the nuclear extracts with polyclonal antibodies against the NF-κB proteins NF-κB1 (p50) and Rel A (p65) (Santa Cruz Biotechnology, France), prior to the addition of the labeled κB probe.

Cell extracts and Western blotting. BECS cells were exposed to either ISW or 0.9% NaCl saline for 4 h incubation. Following this incubation period, cells were washed once with ice-cold PBS and 500 μl of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP40, 1% Triton X-100, Na^+ deoxycholate, 0.1% SDS, 5 mM iodoacetamide, and 0.1 M PMSF) was added for 15 min at 4 °C. Cellular extracts were harvested and centrifuged for 5 min at 12,500*g* at 4 °C. The supernatant was removed and an aliquot was used for protein measurement using the Bio-Rad protein assay kit. Equal amounts (15 μg) of protein were loaded and separated by SDS–PAGE (10% acrylamide). Western blots were prepared by transferring the protein onto nitrocellulose membranes for 1 h at 120 V. Immunoblotting was performed by first saturating the nitrocellulose membrane for 1 h in PBS containing 0.1% Tween in PBS and 5% powdered milk. The level of phosphorylated IκBα was analyzed using a polyclonal phospho-specific anti-IκBα (New England Biolabs, Beverly, USA) antibody that detects IκBα only when activated by phosphorylation at Ser-32. Proteins were visualized using horseradish peroxidase-conjugated donkey anti-rabbit IgG (Boehringer Mannheim, Mannheim, Germany) and the enhanced chemiluminescence detection kit (Amersham Life Science, Arlinghton Heights, IL, USA).

Results

Cell viability of BECs in ISW and 0.9% NaCl saline

Confluent cultures of BECs were placed in Ultroser G-free DMEM/F12 medium for 16 h and then exposed to either ISW saline or 0.9% NaCl saline for a 4 h-incubation period. Viability and cytotoxicity were assayed by two approaches: one evaluated viable cells by the trypan blue exclusion test (Fig. 1A) and the other assayed the formazan dye produced by metabolically active cells using the WST-1 proliferation reagent assay (Fig. 1B). Viability of BECs did not significantly change and exceeded 75% in ISW saline, up to the 4 h period. In contrast, only 40% and 20% of viable BECs were observed in 0.9% NaCl saline after 2 h and 4 h incubation, respectively (Fig. 1A). As shown in Fig. 1B, significantly lower cytotoxicity of ISW saline for BECs was measured when compared to that observed with 0.9% NaCl saline ($p < 0.05$ for 2 h incubation and $p < 0.001$ for 4 h incubation). By optical phase contrast microscopy, higher cell death induced by 0.9% NaCl saline was documented with a reduced number of adhered BECs and intercellular junctions which was not observed with ISW saline, after a 4 h incubation (Fig. 1C).

IL-8 and RANTES protein and mRNA expression of BECs in ISW and 0.9% NaCl saline

Next, we examined the protein and mRNA expression of IL-8 and RANTES of BECs maintained in both

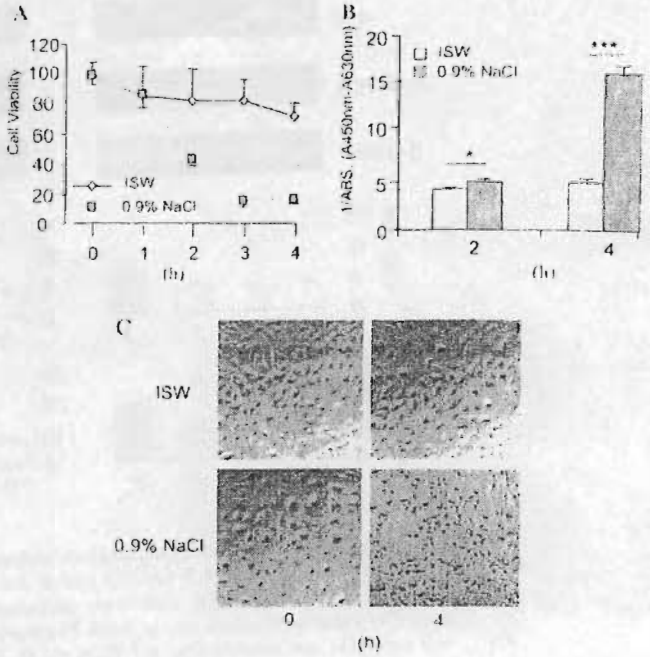

Fig. 1. Viability of BECs (A) and cytotoxicity (B) of ISW saline and 0.9% NaCl saline for 1–4 h incubation periods. Viability in (A) was determined by trypan blue exclusion method. Cytotoxicity in (B) was determined by the cell proliferation reagent WST-1 method after incubation of BECs in either ISW saline or 0.9% NaCl saline. Values represent means ± SD of BEC cultures obtained from at least 4 patients, each assayed in triplicate. Representative microscopic images (C) of BECs are shown before (0 h) and after 4 h incubation in either the ISW saline or 0.9% NaCl saline at 37 °C (original magnification 200×). $^*p < 0.05$, $^{***}p < 0.001$, compared to ISW saline.

ISW and 0.9% NaCl saline. The reduced number of viable BECs observed in 0.9% NaCl saline after a 4 h incubation (Fig. 1A) led us to report data on protein production after a 2 h-incubation period. In these conditions, Fig. 2A shows that exposure of BECs to ISW saline resulted in a statistically significant ($p < 0.001$) 2.5-fold and 3.5-fold reduction of the spontaneous

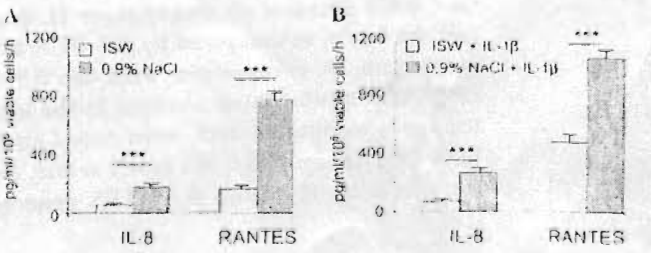

Fig. 2. Levels of IL-8 and RANTES protein production were evaluated in BECs exposed to either ISW saline or 0.9% NaCl solution for 2 h incubation. Spontaneous production of BECs (A) and stimulated BECs (B) with 20 ng/ml IL-1β is shown. Values in enzyme-linked immunosorbent assays (ELISA) of IL-8 and RANTES levels (expressed as pg/ml/viable 10^6 cells/h) represent means ± SD of BEC cultures obtained from four patients, each assayed in triplicate. $^{***}p < 0.001$, compared to ISW saline.

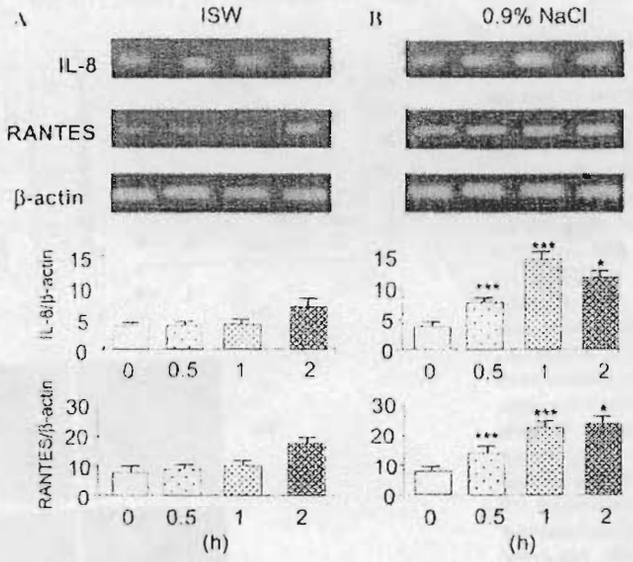

Fig. 3. Representative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of IL-8 RANTES and β-actin messenger ribonucleic acid (mRNA) in BECs. Cells were incubated for 0.5, 1, and 2 h period in either ISW saline (A) or 0.9% NaCl saline (B). Total RNA was extracted and amplified by RT-PCR for IL-8, RANTES, and β-actin mRNA transcripts. We used β-actin mRNA to control for loading difference. Graph represents the average IL-8 and RANTES mRNA densitometry values, of four separate experiments. $^{*}p < 0.05$, $^{***}p < 0.001$, compared to ISW saline.

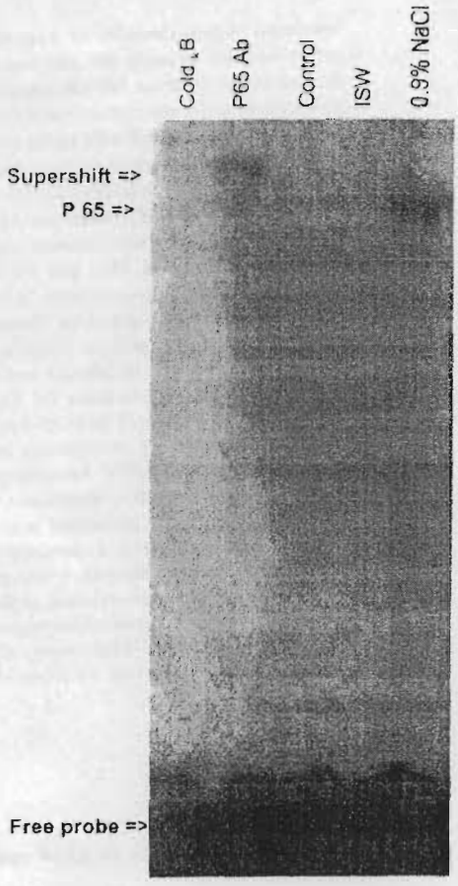

Fig. 4. Nuclear factor-κB (NF-κB) deoxyribonucleic acid (DNA) binding activity of BECs incubated in either DMEM/F12 medium (lane 3, control) or ISW saline (lane 4) or 0.9% NaCl (lane 5) saline for 1 h incubation. To demonstrate the specificity of NF-κB oligonucleotides, a 100-fold excess of unlabeled NF-κB (lane 1, cold κB) was used to compete with the labeled NF-κB probe. The addition of antibody to RelA (p65 subunit) component (lane 2, p65 Ab) caused a supershift, as indicated. The results are representative of BEC cultures obtained from four patients.

release of IL-8 and RANTES protein, respectively, as compared to the 0.9% NaCl saline. To mimic the in vivo situation, in which the pro-inflammatory cytokine IL-1β is considered to be a pivotal cytokine in the initiation of early inflammatory process in response to infection, it was important to analyze the effect of this cytokine on IL-8 and RANTES production by BECs exposed to either ISW saline or 0.9% NaCl saline. Interestingly, the induction of both IL-8 and RANTES production by BECs in response to IL-1β stimulation was also significantly ($p < 0.001$) reduced in ISW saline compared with that obtained in 0.9% NaCl saline (Fig. 2B).

The lower spontaneous IL-8 and RANTES release by BECs exposed to ISW was associated with a significant ($p < 0.05$) decrease in steady-state IL-8 and RANTES mRNA levels, as measured by RT-PCR after 0.5 and 1 h incubation, in comparison with the 0.9% NaCl saline (Fig. 3). No substantial changes in the levels of β-actin, used as a loading control, were noted upon exposure to both ISW saline and 0.9% NaCl saline, suggesting that the effects on IL-8 and RANTES gene expression are specific.

NF-κB activity of BECs in ISW and 0.9% NaCl saline

Because NF-κB is a central regulator of many inflammatory gene expressions including IL-8 in airway epithelial cells, we measured the NF-κB activity by EMSA using nuclear extracts from BECs exposed to either ISW saline or 0.9% NaCl saline (Fig. 4). Strikingly, exposure of BECs to 0.9% NaCl saline induced a NF-κB–DNA binding complex (Fig. 4, lane 5) that was not observed in ISW saline-treated BECs and control (Fig. 4, lanes 4 and 3, respectively). Supershift assays confirmed the presence of p65 subunits of NF-κB (Fig. 4, lane 2), and the specificity of NF-κB–DNA binding was confirmed in competition experiments with a 100-fold molar excess of unlabeled (cold κB) NF-κB oligonucleotide, which led to a complete inhibition of binding activity (Fig. 4, lane 1).

IκBα phosphorylation level of BECs in ISW and 0.9% NaCl saline

NF-κB exists as an inactive form bound to the inhibitor protein IκBα in the cytoplasm, and the

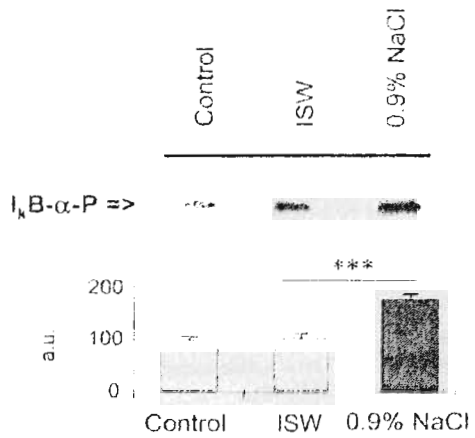

Fig. 5. Expression of phosphorylated IκBα (IκB-P) protein levels after 1 h of exposure to either DMEM/F12 (lane 1, control) or ISW saline (lane 2) or 0.9% NaCl (lane 3) saline. Equal amount of cytoplasmic protein from BECs obtained in each condition was analyzed for IκB-P level by Western blotting. Graph was obtained after densitometric analysis, expressed in arbitrary units (a.u.) of three separate experiments. $^{***}p < 0.001$, compared to ISW saline.

degradation of IκBα by its phosphorylation must occur in order to translocate to the nucleus. Therefore, to investigate the mechanism involved in the activation of NF-κB, we examined the level of phosphorylated IκBα in BECs after their exposure to either ISW saline or 0.9% NaCl saline for 1 h incubation. By Western blot analysis using an antibody specific to phosphorylated IκBα on Ser^{32} (Fig. 5), we showed a significant ($p < 0.001$) 60% decrease of the phosphorylated IκBα in BECs exposed to ISW saline compared to that obtained with the 0.9% NaCl saline.

Discussion

Airway epithelium plays a pivotal role in the regulation of ionic content of the connective tissue in the airway wall, and modulation of the composition of the airway surface liquid is likely to provide promising target for new therapies of airway inflammatory diseases. In this report, our findings clearly show that lowering Na^+ concentration in an isotonic sea-water derived (ISW) saline leads to a better viability of BECs compared to that observed with the 0.9% NaCl saline. We demonstrate that resting and IL-1β-stimulated BECs exposed to ISW saline markedly reduce IL-8 and RANTES protein secretion as compared to 0.9% NaCl saline. We also show that the reduction of constitutive IL-8 and RANTES secretion is paralleled with a significant decrease in both steady-state IL-8 and RANTES mRNA levels. This decrease of IL-8 and RANTES expression in BECs exposed to ISW saline is associated with a reduced cytoplasmic expression of phosphorylated IκBα, resulting in inhibited NF-κB–DNA binding activation, which was less marked in the 0.9% NaCl saline.

NF-κB is a key determinant of the respiratory epithelial inflammatory cascade and occupies a central role in the transcriptional activation of IL-8 and RANTES in BECs [36,37]. Recent evidence has implicated NF-κB as an important osmosignaling molecule that is activated in response to hyperosmolarity in nonrespiratory cell types, i.e., renal epithelial cells and endothelial cells [38]. A recent report by Nemeth et al. [39] showed that hyperosmotic stress induces NF-κB activation and IL-8 production in human intestinal epithelial cells. Interestingly, these authors demonstrate that the hyperosmolarity-induced increase in IL-8 production was, in a large part, mediated by activation of Na^+/H^+ exchangers and not by an osmotic-dependent mechanism. In our study, similar mechanisms may operate in BECs, as we demonstrated that decreased Na^+ level in isotonic saline markedly reduced both NF-κB–DNA binding activation and IL-8 and RANTES expression and production. Our results provide evidence that NF-κB is one of the central osmosignaling factors that mediate the production of IL-8 and RANTES by human airway epithelium. In addition to NF-κB, a variety of other transcription factors (AP-1, NF-AT, and CREB) have been shown to convey the effects of salt-induced stress on gene expression [40,41]. Further studies are currently being performed to examine whether these factors might also contribute to the enhanced inflammatory response of BECs caused by extracellular Na^+ level.

Human airway mucosa is a semipermeable membrane across which osmotic equilibration occurs [42]. In proximal airways, the Na^+ content in airway surface liquid has been reported to be either unchanged [42] or elevated [43] in cystic fibrosis disease. Reports that elevated Na^+ level decreases airway mucociliary motility by inhibiting P2X channels [44], inhibits the activity of β-defensin antibacterial peptide [29], and now that Na^+ regulates NF-κB activity and inflammatory gene expression in BECs indicate that Na^+ level in the airway liquid may be a fundamental physiological component of respiratory function. Hypertonic and 0.9% NaCl saline aerosols are currently used to increase the volume of airway surface liquid and mucociliary clearance in airways of patients with chronic obstructive pulmonary disease and cystic fibrosis [27,28]. However, from the data reported in the present study, it is likely that therapeutic strategies using inhalation of saline solution with a reduced Na^+ content such as ISW saline might be more appropriate to reduce inflammation in the respiratory tract.

In summary, the present study provides evidence that decreasing the extracellular Na^+ content of airway epithelium can reduce the inflammatory cascade, a process that involves inhibition of NF-κB activation.

Acknowledgments

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