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Physiomer® reduces the chemokine interleukin-;8 production by activated human respiratory epithelial cells

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Abstract

The authors have recently shown that the transcription factor nuclear factor-κB (NF-κB) is a central mediator in the NaCl-mediated interleukin (IL)-;8 production by human airway epithelial cells. In this study, it was investigated whether Physiomer®, an isotonic sea water-derived solution commercialized for cleaning the nasal mucosa, impaired the chemokine IL-;8 expression and secretion by human respiratory epithelial cells compared with that obtained with an isotonic 9% NaCl solution.

Primary human bronchial gland (HBG) epithelial cells were incubated either in Physiomer® or in a NaCl 9% solution and activated either with 20 ng·mL^{-1} tumour necrosis factor-;α, or IL-;1ß, respectively. Physiomer® significantly reduced the IL-;8 protein release in basal and activated HBG cells in comparison with that obtained with the 9% NaCl solution. In contrast to the effects of

Physiomer® observed on resting HBG cells, Physiomer® did not significantly reduce the level of phosphorylation of the NF-κB inhibitor protein IκBα or the steady-state IL-;8 messenger ribonucleic acid levels in activated HBG cells, suggesting that Physiomer® would have a post-transcriptional effect on IL-;8 expression in activated HBG cells.

The authors conclude that Physiomer® is potentially useful in the reduction of airway mucosal inflammation.

Airway epithelium actively participates in the airway homeostasis through a series of protective mechanisms including ciliary beating, secretion of mucus and release of inflammatory mediators in response to deleterious environmental stimuli 1. Epithelium lining the airways is bathed on its apical surface by a thin liquid layer containing macromolecules and ions, which is the first line of defence against inhaled allergens, bacteria and pollutants. Elevated Na^+ concentrations in airway fluids have been shown to significantly decrease the airway ciliary motility 2 and increase the glandular mucous exocytosis 3 in human airway epithelium. It has also been reported 4, 5 that airway epithelial cells are markedly implicated in the process of inflammatory cell recruitment since they contribute to the inflammatory cytokines network by producing the chemokines interleukin (IL)-;8, IL-;6, monocyte chemoattractant protein-;1 and the monocyte/macrophage/T-;cell regulated on activation, normal T-;cell expressed and secreted (RANTES), which directly or indirectly have paracrine and autocrine effects on the respiratory epithelium and its surrounding tissues. IL-;8 belongs to the C-;X-;C chemokine family, which plays a major role in the recruitment and activation of neutrophil degranulation to inflammatory sites in nasal and bronchial mucosa in patients suffering from upper respiratory viral infections 6, acute respiratory distress syndrome 7 and in airways of patients with cystic fibrosis 8. The authors and others have demonstrated that elevated extracellular salt content increased the production of cytokines, including IL-;8 9, 10 and IL-;18 11, by airway epithelial cells, inhibited the antibacterial peptides 12 and reduced neutrophil antimicrobial activity 13 in human airways. Based on these studies, the authors addressed the question of whether Physiomer®, an isotonic, sterile, undiluted sea water-derived solution containing a low final concentration of Na^+ (2,400 $mg \cdot L^{-1}$) commercialized for the cleaning of human nasal mucosa 14, 15, in comparison with an isotonic sterile, pyrogen-;free 9% NaCl solution (Na^+: 3,540 $mg \cdot L^{-1}$), would impair the levels of IL-;8 expression and secretion by unstimulated and tumour necrosis factor-;α (TNF-;α) or IL-;1ß-;stimulated human bronchial epithelial cells. The IL-;8 promoter regulation was also investigated, specifically evaluating the activation of the transcriptional nuclear factor-;κB (NF-;κB)/inhibitor protein IκBα system in human bronchial epithelial cells. Understanding such mechanisms is of great interest as it may lead to the development of novel therapeutic strategies in patients with inflamed airway tissues.

Material and methods

Cell culture

Cell isolation and culture procedures of primary human bronchial gland (HBG) epithelial cells were performed on bronchial tissues collected from four patients (two males with primary pulmonary hypertension, aged 28 and 29 yrs, respectively, and two males with pulmonary idiopathic fibrosis, aged 40 and 61 yrs, respectively), as described previously 9. Briefly, HBG cells were isolated by enzymatic digestion from bronchial tissues and cultured in a Dulbecco's modified eagles medium (DMEM)/Ham's F12-mixture (50/50%, per cent volume in volume (v/v)) supplemented with 2% Ultroser G (a serum substitute from Sepracor, Villeneuve-;la-;Garenne, France) and antibiotics. After 4 weeks in primary culture, second- and third-passage HBG cells had proliferated and they exhibited characteristics of bronchial secretory gland epithelial cells, as previously described 16.

Enzyme-linked immunosorbent assay for interleukin-;8 determination

Second- and third-passage confluent HBG cells grown on type 1 collagen-coated coverslips were incubated for 16 h in a Ultroser G-;free control medium (DMEM/Ham's F12, alone) to ensure that cells were in a quiescent state. Following this incubation period, individual monolayers of HBG cells in six well-culture flasks were exposed for an additional 4-;h period to either Physiomer®, a sterile preparation of undiluted sea water brought to isotonicity by electrodialysis (also commercialized under the brand Rhinomert® and Hydrasense®, Goemar, Saint-Malo, France), or an isotonic sterile, pyrogen-free 9% NaCl solution (Delmas Perfusion, Chambrais-les-Tours, France), in the presence or absence of 20 ng·mL^{-1} of TNF-;α or 20 ng·mL^{-1} IL-;1ß (Calbiochem, Meudon, France), respectively. Immediately after the 4-;h period of cell exposure, supernatants and cell lysates were collected and stored at –80°C until tested for the presence of IL-;8, as described previously 9. The enzyme-linked immunosorbent assays (ELISAs) for IL-;8 detection, which were sensitive down to a level of 5 pg·mL^{-1}, were performed by following the manufacturer's instructions in commercially available ELISA kits (Biosource International, Camarillo, CA, USA). The uniformity of HBG cell monolayers was determined by quantifying the cell number per well. Cell viability of HBG cells was determined by trypan blue exclusion after all experimental interventions. All results were expressed as pg·mL^{-1} per viable 10^6 cells·h^{-1}.

Ribonucleic acid isolation

Nuclear extracts were prepared and analysed after HBG cells had been previously incubated either in Physiomer® or a 9% NaCl solution and stimulated either with 20 ng·mL^{-1} TNF-;α or 20 ng·mL^{-1} IL-;1ß, respectively for a period of 1 h. Total ribonucleic acid (RNA) was isolated from cells using RNeasy Mini kits (Quiagen, Courtaboeuf, France) according to the manufacturer's instruction. First-strand complementary deoxyribonucleic acid (cDNA) synthesis was performed in a 20 µL reaction mixture containing 1 µg RNA, 10 mM deoxynucleotide triphosphate (dNTP), 0.5 µg Oligo-deoxythymidine $(dT)_{12–18}$ primer, 10 mM ditheothreitol, 50 U ribonuclease (RNase) inhibitor, and 50 U avian myeloblastosis virus (AMV) reverse transcriptase (RT) (GIBCO-;BRL, Cergy-Pontoise, France), incubated at 65°C for 5 min, and then at 42°C for 50 min. The AMV RT was denatured at 70°C for 15 min. Reverse transcription reactions were amplified by polymerase chain reaction (PCR) in a 50 µL volume containing 2 µL of cDNA, 25 pmol of each primer, 1.5 mM $MgCl_2$, 2.5 U *Taq* polymerase (GIBCO-BRL), and 200 µM of dNTP mixture in Rnase-free distilled H_2O.

Primers for the amplification of IL-;8 were (sense, 5'-;ATGACTTCCAAGCTGGCCGTG-;3'; and antisense, 5'-;TTATGAATTCTCAGCCCTCTTCAAAAACTTCTC-;3'; GenBank Y000787 17), and for 28 S were (sense, 5'-;GTTCACCCACTAATAGGGAACGTGA-;3'; and antisense 5'-;GGATTCTGACTTAGAGGCCTTCAGT-;3'). The amplification profile was 25 cycles for IL-;8 and 19 cycles for 28 S (used as control for loading differences) of 15-;s denaturation at 94°C, 20-;s annealing at 66°C, and 10-;s extension at 72°C. After amplification, PCR products were separated by size on a 10% acrylamide gel, labelled by SYBR® gold probe (Molecular Probes, Eugene, OR, USA), and analysed by a fluorescent image analyser (Fujifilm Co., Courbevoie, France). As a negative control, RNA was omitted from the reverse transcription and PCR amplification (data not shown).

Preparation of nuclear extracts and electrophoretic mobility shift assay

Nuclear extracts were prepared and analysed after HBG cells had been incubated in either Physiomer® or a 9% NaCl solution for a period of 1 h, as previously described 9. The consensus κB deoxyribonucleic acid (DNA) sequence was used for the electrophoretic mobility shift assay (5'AGTTGAGGGGACTTTCCCAGGC3', Promega Corp., Madison, WI, USA). The oligonucleotides were radiolabelled by the T4 polynucleotide kinase enzyme (Pharmacia Biotech, Paris, France) with α^{32}P-;adenosine triphosphate (ATP). Nuclear extracts (4 µg) were incubated with 50 kcpm of ^{32}P-;labelled NF-κB oligonucleotide in binding reaction mixture. The protein-DNA complexes were electrophoresed on a nondenaturing 5% polyacrylamide gel. In competition studies and supershift assays, a 100-fold molar excess of unlabelled oligonucleotides or 1 µg of antibodies was added to the binding reaction mixture as indicated, prior to addition of the labelled 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-κBI (p50) and the Rel (p65) RelA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), prior to the addition of the labelled κB probe, as previously described 9.

Cell extracts and Western blot analysis

HBG cells were exposed to either Physiomer® or the 9% NaCl solution and stimulated with either 20 ng·mL^{-1} TNF-;α or 20 ng·mL IL-;1ß for a period of 1 h, respectively; harvested by scraping; centrifuged (300×*g*, 5 min, 4°C) and total protein was extracted (30 min, 4°C) in radio immunoprecipitation assay buffer. Equal amounts of protein were electrophoresed under denaturing conditions using 4–15% polyacrylamide gels (Pharmacia Biotech, Orsay, France) and blotted onto a nitrocellulose membrane (Millipore, Bedford, MA, USA). The level of phosphorylated IκBα was analysed by Western blot using a polyclonal phosphospecific anti-IκBα (New England Biolabs, Beverly, MA, USA) antibody, which detects IκBα only when activated by phosphorylation at the amino acid Ser-32. Proteins were visualized using horseradish peroxidase-conjugated donkey antirabbit immunoglobulin-;G (IgG; Boehringer Mannheim, Mannheim, Germany) and the enhanced chemiluminescence detection kit (Amersham Life Science, Arlington Heights, IL, USA). Densitometric analyses of Western blots were performed on a Fuji imaging densitometer (Fujifilm Co.) and the intensities of bands were compared on the basis of adjusted volume (mean optical density×area in mm^2).

Statistical analysis
Results were expressed as mean±sd. Each data point was performed at least in triplicate, and each cell culture experiment performed at least three times. Data were subjected to analysis of variance (ANOVA) and unpaired t-;test for between-group comparison. p-;Values of <0.05 were considered significant.

Results

Chemokine interleukin-;8 secretion
After a 4-;h incubation period in either Physiomer® or a 9% NaCl solution in the presence or absence of TNF-;α or IL-;1ß, observations of cell cultures by light microscopy demonstrated that these agents did not lead to any obviously noticeable damage in the cultured HBG cells. Viability of HBG cells exceeded 97%, as determined by trypan blue exclusion after all experimental interventions (data not shown). Figure 1 shows that exposure of HBG cells to Physiomer® for a 4-;h period resulted in a statistically significant ($p<0.001$) 2.1-fold reduction, compared with the 9% NaCl solution, of the amount of immunoreactive IL-;8 released. After the 4-;h incubation period, the intracellular IL-;8 content was 20 pg·mL^{-1} and undetectable (<5 pg·mL^{-1}) in Physiomer® and the 9% NaCl solution, respectively.

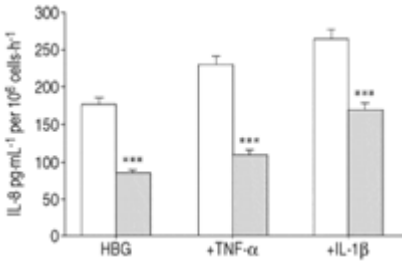

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Fig. 1.— Levels of interleukin (IL)-;8 production in cultured human bronchial gland (HBG) cells after exposure to either Physiomer® (◥) or the 9% NaCl solution (□). Basal production of HBG cells (unstimulated state) and cells stimulated with 20 ng·mL^{-1} tumour necrosis factor-;α (TNF-;α) and IL-;1ß are shown. Values in enzyme-linked immunosorbent assays (ELISAs) of IL-;8 levels in 4-;h supernatants represent mean±sd of HBG cell cultures obtained from four different patients, each assayed in triplicate. ***: $p<0.001$, compared with the 9% NaCl solution.

Effects of tumour necrosis factor-;α or interleukin-;1ß stimulation on the interleukin-;8 expression and release by human bronchial gland cells

The IL-;8 release was analysed after stimulation with either 20 ng·mL^{-1} TNF-;α or IL-;1ß solubilized in either Physiomer® or the 9% NaCl solution for a 4-;h incubation period, respectively. Interestingly, the induction of IL-;8 secretion by HBG cells in response to cytokine stimulation was significantly ($p<0.001$) reduced in Physiomer® compared with the 9% NaCl solution. As shown in figure 1✦, exposure of HBG cells to TNF-;α induced a significant increase in IL-;8 release ($p<0.05$) in the presence of 9% NaCl solution (177–230 pg·mL^{-1} per 10^6 cells·h^{-1}) and in the presence of Physiomer® (85–110 pg·mL^{-1} per 10^6 cells·h^{-1}). Similarly, exposure of HBG cells to IL-;1ß induced a significant increase ($p<0.05$) in IL-;8 release in the presence of 9% NaCl solution (177–270 pg·mL^{-1} per 10^6 cells·h^{-1}) and in the presence of Physiomer® (85–170 pg·mL^{-1} per 10^6 cells·h^{-1}). After the 4-;h incubation period, similar and undetectable intracellular IL-;8 levels (<5 pg·mL^{-1}) were observed when TNF-;α- or IL-;1ß-;activated HBG cells were exposed to Physiomer® and to the 9% NaCl solution. These findings clearly demonstrate that Physiomer® significantly ($p<0.001$) attenuated the TNF-;α and IL-;Iß-;induced release of IL-;8 by HBG cells (by a factor of 2.0 and 1.5, respectively) when compared with that obtained in the 9% NaCl solution.

The lower basal IL-;8 release observed with Physiomer® was associated with a significant ($p<0.05$) decrease in steady-state IL-;8 messenger ribonucleic acid (mRNA) level of unstimulated HBG cells in comparison with the 9% NaCl solution, after a 1-;h incubation period (fig. 2✦, lane 2 compared to lane 1). With IL-;1ß-;stimulated HBG cells, similar and significantly ($p<0.001$) increased IL-;8 mRNA levels were observed when cells were exposed to Physiomer® and the 9% NaCl solution for a period of 1 h (fig. 2✦, lanes 3 and 4).

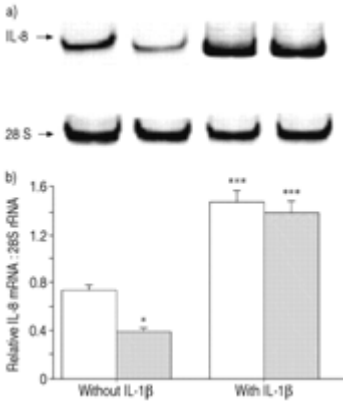

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Fig. 2.— Representative reverse transcriptase polymerase chain reaction (RT-PCR) analysis demonstrating that Physiomer® decreases interleukin (IL)-;8 messenger ribonucleic acid (mRNA) expression only in unstimulated human bronchial gland cells. Cells were incubated for a period of 1 h with or without 20 ng·mL^{-1} IL-1ß solubilized in Physiomer® (◥) or the 9% NaCl solution (□). a) Total RNA was extracted and amplified by RT-PCR for IL-;8 mRNA transcripts. The authors used 28 S ribosomal ribonucleic acid (rRNA) to control for loading differences. b) Graph representing the average IL-;8 mRNA densitometry values (mean±sem), corrected for respective 28 S rRNA densitometry values, of three separate experiments. *: $p<0.05$; ***: $p<0.001$.

Nuclear factor-κB activation in response to Physiomer® and 9% NaCl solution
It was of interest to determine whether or not Physiomer® and the 9% NaCl solution differently affected constitutive NF-κB activation in HBG cells. Nuclear extracts obtained from HBG cells incubated in each saline condition were prepared and incubated with an end ^{32}P-labelled DNA oligonucleotide containing the recognition site

for NF-κB. Compared with HBG cells maintained in Physiomer® (fig. 3, lane 3), a higher NF-κB-DNA binding activity was demonstrated in the nuclear protein extracts from HBG cells maintained in the 9% NaCl solution (fig. 3, lane 4), with a mean increase of 2.5-fold, as evaluated by densitometric analyses (data not shown). The specificity of NF-κB-DNA binding was confirmed in competition experiments with a 100-fold excess of unlabelled cold κB NF-κB oligonucleotide, which led to a complete inhibition of binding activity (fig. 3, lane 1). Moreover, supershift assays confirmed the presence of p65 subunits of NF-κB (fig. 3, lane 2).

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Fig. 3.— Nuclear factor-κB (NF-κB) deoxyribonucleic acid (DNA) binding activity in unstimulated human bronchial gland (HBG) cells after their exposure to either Physiomer® or the 9% NaCl solution. Electrophoretic mobility shift analysis (EMSA) binding activity in nuclear protein extracts from HBG cells after exposure to either Physiomer® (lane 3) or to the NaCl 9% solution (lane 4), respectively. To demonstrate the specificity of binding of the NF-κB oligonucleotide, a 100-fold M excess of unlabelled NF-κB (lane 1, cold κB) was used to compete with the labelled NF-κB probe. The addition of antibody to RelA (p65 subunit) component (lane 2, p65) caused a supershift, as indicated. The results are representative of HBG cell cultures obtained from four different patients.

IκBα phosphorylation in response to Physiomer® and the 9% NaCl solution

To evaluate the levels of IκBα phosphorylation in HBG cells when cells were previously exposed to either Physiomer® or the 9% NaCl solution for a 1-;h period, a phospho-specific anti-IκBα-antibody that detects IκBα protein was used, only when activated by phosphorylation at the Ser-32 residue (fig. 4). Image analysis of digitized Western blots of phosphorylated IκBα (IκBα-;P) showed a significant 30% decrease ($p<0.01$) in HBG cells maintained in Physiomer®, compared with the value obtained in the 9% NaCl solution (fig. 4, lane 2 compared to lane 1). With IL-1ß-stimulated HBG cells, similar and significantly increased IκBα-;P levels were observed when cells were exposed to Physiomer® and the 9% NaCl solution, for a 1-;h period (fig. 4, lanes 3 and 4).

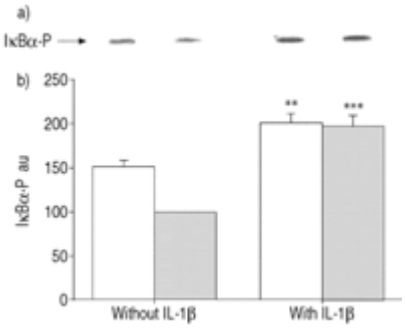

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Fig. 4.— Expression of phosphorylated IκBα (IκBα-;P) protein levels in unstimulated and interleukin (IL)-1ß stimulated human bronchial gland (HBG) cells when exposed to either Physiomer® or the 9% NaCl solution. a) Equal amounts of cytoplasmic protein from HBG cells in each condition were analysed for IκBα-;P levels by Western blotting. Data of densitometric analyses, expressed in arbitrary units (au) from data obtained with Physiomer® (value 100, lane 2), were combined with three studies obtained from four different patients. b) IκBα-;P levels obtained from IL-;1ß stimulated HBG cells were compared with those obtained from unstimulated HBG cells incubated in either Physiomer® (◥) or 9% NaCl solution (❑), respectively. **: $p<0.01$; ***: $p<0.001$.

Discussion

Previous reports have clearly shown that elevation of the extracellular salt content in human airway fluids can lead to significantly decreased antibacterial defences and increased inflammatory responses in human respiratory tissues [9–13]. The present study was designed to analyse whether the IL-;8 expression and secretion by activated respiratory epithelial cells could be attenuated by decreasing extracellular Na^+ concentration. The presented findings clearly show that Physiomer® (containing a low concentration of Na^+ reduced to 2,400 mg·L^{-1}), in comparison with the 9% NaCl solution, significantly reduced the IL-;8 protein release in both unstimulated and TNF-;α or IL-;1ß-;stimulated HBG cells. In unstimulated HBG cells, intracellular IL-;8 content was higher in Physiomer® than with 9% NaCl solution. However, the total IL-;8 content (*i.e.* intracellular IL-;8 plus released IL-;8 level) expressed by Physiomer®-treated HBG cells was lower than that in HBG cells exposed to 9% NaCl solution, suggesting that Physiomer® had a post-transcriptional effect on IL-;8 expression in activated HBG cells. In contrast to the effects of Physiomer® observed on unstimulated HBG cells, compared to the 9% NaCl solution, Physiomer® does not significantly reduce the level of IκBα-;P, nor the steady-state IL-;8 mRNA levels in stimulated HBG cells. Compared to the 9% NaCl solution (Na^+: 3,540 mg·L^{-1}; $C1^-$: 5,460 mg·L^{-1}), Physiomer® contains a final concentration of Na^+: 2,400 mg·L^{-1}; $C1^-$: 6,100 mg·L^{-1}; other ions such as $SO4^{2-}$: 2,900 mg·L^{-1}; Mg^{2+}: 1,200 mg·L^{-1}; Ca^{2+}: 350 mg·L^{-1}; K^+: 90 mg·L^{-1}; and a totally

preserved concentration of mineral salts and trace elements 18. Regarding the transcriptional mechanism, the authors have shown that the reduction of basal IL-;8 release by bronchial epithelial cells exposed to Physiomer® is associated with a significant decrease in steady-state IL-;8 mRNA level and with a concomittant reduced IκBα-;P level, causing a significant decrease of NF-κB DNA binding activity, which was less marked in the 9% NaCl solution. The mechanisms by which elevated Na^+ concentration (*i.e.* in the 9% NaCl solution) increases the IL-;8 expression appear to involve the regulation of NF-κB/Iκ-Bα complex. This assertion is well supported by recent studies that demonstrate that p38 mitogen-activated protein kinase and IκB kinase α/ß kinases activation play an all important role in the control of IL-;8 expression and secretion mediated by high extracellular NaCl concentrations in peripheral blood mononuclear cells, THP-;1 monocyte-like cells 19 and human respiratory epithelial cells 9, 10, 20. Further investigations are now required to define more precisely whether either the low Na^+ concentration *per se* or other ions present in Physiomer® affect the IL-;8 production in human bronchial epithelial cells differently through differential activation of NF-κB/IκBα pathway.

In conclusion, Physiomer® is potentially useful in the reduction of human respiratory mucosal inflammation. Although the present findings remain to be investigated in *in vivo* situations, for example, on the human nasal mucosa, the presented data may be informative with respect to inflammatory processes, in which excessive sodium chloride-induced interleukin-;8 secretion by respiratory epithelial cells plays a determinant role in the regulation of human nasal mucosal inflammation.

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