Interleukin 1-beta Up-regulates Na^+-K^+-Cl^- Cotransporter In Human Middle Ear Epithelial Cells.

Jung-Lim Kim

Department of Medical Science

The Graduate School, Yonsei University

Interleukin 1-beta up-regulates Na^+-K^+-Cl^- cotransporter in human middle ear epithelial cells.

Directed by Professor Joo-Heon Yoon

The Master's Thesis
submitted to the Department of Medical School,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Jung-Lim Kim

June 2006

This certifies that the Master's Thesis of Jung-Lim Kim is approved

---

Thesis Supervisor : Joo-Heon Yoon

Min Goo Lee : Thesis Committee Member #1

Myoung Hee Kim : Thesis Committee Member #2

The Graduate School

Yonsei University

June 2006

ACKNOWLEDGEMENTS

제 인생의 한 과정도 많은 분들의 도움으로 푸른 결실을 맺게 되었습니다. 대학 졸업 후, 좀 더 넓은 세상을 배워보고자 스스로 선택한 길이었던 석사 과정은 순간순간 선택의 기로에 서서 뜻하지 않은 결과와 경험으로 미래를 바라보기 위한, 단단한 초석을 만드는 시간이었습니다.

석사생활 동안에 저를 믿어주시고 이끌어주셨던 윤주헌 교수님께 감사 드립니다. 그리고 바쁘신 와중에도 무엇보다 많은 도움을 주신 이민구 선생님께 감사 드립니다. 또한 세심한 배려로 꼼꼼하게 지적해 주신 김명희 선생님께 감사를 드립니다. 제가 석사를 시작하는데 기초를 마련해 주시고 부족한 실험을 가르쳐 주시며 멀리서도 많은 관심과 배려를 보내주신 최재영 선생님과, 다른 실험실에서 와서 번거롭게 만들었을 텐데도 작은 도움까지 아낌이 없으셨던 남궁완 선생님과 다른 약리선생님들께 감사 드립니다. 일하고 공부하는 것에 지쳐있을 때 물심양면으로 신경 써 주신 김창훈 선생님, 김진국 선생님, 김현직 선생님, 조재훈 선생님, 이창훈 선생님께 감사 드립니다. 동고동락하며 제 석사 생활을 더 풍요롭게 해 준 실험실 식구들, 최혜정 선생님, 욱열오빠, 영덕오빠에게 감사 드립니다.

우연히 만난 인연이었으나 너무나도 특별했던 양띠들, 지현이와 영은이 둘 다 참 좋은 사람 만날 수 있길 바랍니다. 실험실 안에서나 밖에서나 같이 기뻐하고 슬퍼하며 친언니 이상으로 사랑하는 선영언니에게 감사 드립니다. 짧은 시간, 굵직한 인연으로 다가와 제게 힘이 되어준 지영언니, 보희, 선용, 요훈오빠 그리고 FEVER, DANZON 식구들 모두 감사 드립니다. 제 일에 치여 자주 신경 써 드리지 못했지만 자식을 위해 최선을 다 하시는 아버지와 동생 수현이에게 감사의 마음 전합니다.

무엇보다도 늘 애정 어린 눈빛으로 지친 제 마음에 맑은 샘물이 되어준 고양이, 바리와 고니 그리고 사랑과 헌신으로 자식이기 이전에 한 사람으로서 저의 평생을 함께 걸어주신 어머니에게 이 작은 결실을 바칩니다.

Table of Contents

List of Figures

List of Figures

Abstract

Interleukin 1-beta upregulates Na^+-K^+-Cl^- cotransporter in human middle ear epithelial cells.

Jung-Lim Kim

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Joo-Heon Yoon)

Disruption of periciliary fluid homeostasis is the main pathogenesis of otitis media with effusion (OME), which is one of the most common childhood diseases. Although the underlying molecular mechanisms are unclear, it has been suggested that the altered functions of ion channels and transporters are involved in the fluid collection of middle ear cavity of OME patients. In the present study, we analyzed effects of the inflammatory cytokine Interleukin-1ß(IL-1ß) on Na^+-K^+-$2Cl^-$- cotransporter 1 (NKCC1) in human middle ear cells. Intracellular pH (pH_i) were measured in primary

cultures of normal human middle ear epithelial (NHMEE) cells using a double perfusion chamber, which enabled us to analyze membrane-specific transporter activities. NKCC activities were estimated by the pH_i reduction due to bumetanide-sensitive intracellular uptake of NH_4^+. In addition, transepithelial fluid transport was measured with or without bumetanide-treatments. NKCC activities were observed in the basolateral membrane, but not in the luminal membrane of NHMEE cells. Correspondingly, immunoblotting of membrane-cultured NHMEE cells revealed the expression of NKCC1 on the basolateral membrane. Interestingly, IL-1ß treatments augmented the basolateral NKCC activities and increased NKCC1 expression. In addition, IL-1ß stimulated bumetanide-sensitive fluid transport in NHMEE cells. Furthermore, NKCC1 expression was increased in middle ear cells from the patients with OME when compared to samples from control individuals. The above results provide comprehensive evidence that the inflammatory cytokine IL-1β upregulates NKCC1 in the middle ear epithelial cells, which would be one of the important underlying mechanisms of fluid overcollection in patients with OME.

Key words : Na^+-K^+-Cl^- cotransporter, interleukin 1-beta, bumetanide, ouabain, $BaCl_2$, fluid transport

Interleukin 1-beta upregulates Na^+-K^+-Cl^- cotransporter in human middle ear epithelial cells.

Jung-Lim Kim

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Joo-Heon Yoon)

Ⅰ. INTRODUCTION

Maintaining a fluid-free middle ear cavity is a critical feature in the physiology of middle ear, which allows the transmission of sound vibrations from the eardrum to the inner ear. Epithelial cells lining middle ear cavity are a respiratory type epithelium and play an important role in maintaining fluid-free space by regulating periciliary fluid volume.[1] Disruption of periciliary fluid homeostasis has been thought to be one of main pathogenesis of otitis media with effusion (OME), which is one of the most common childhood diseases.[2] Although many evidences[3-5] indicated that ion channels and transporters are involved in the fluid transport and periciliary fluid volume

regulation, the exact underlying molecular mechanisms need to be determined.

Recent evidence suggests that the activities of basolateral Na^+-K^+-Cl^- cotransporter (NKCC) are the rate-limiting step of ion and fluid secretions in Cl^--secreting epithelia including airway epithelia.[13, 14] It is reported that middle ear epithelium possess characteristics of Cl^- secreting epithelia in a study using short-circuit current measurements.[3] NKCCs are a member of cation-chloride cotransporter family, which have 12 membrane-spanning domains [6], and now have been identified in many mammalian and non-mammalian tissues.[7, 8] They represent electroneutral transport systems with a presumed stoichiometry of 1 Na^+:1 K^+:2 Cl^- driven by the sum of the gradients of Na^+, K^+ and Cl^-. NKCCs serve a number of vital physiological functions including cell volume regulation[11] and cell proliferation.[12] Two isoforms of NKCC have been identified in human so far, NKCC1 and NKCC2. Among them, NKCC1 has been demonstrated to be expressed in Cl^- secreting epithelia such as salivary gland, intestine and human nasal epithelia where it is confined to the basolateral membrane, and is therefore considered to the secretory isoform of the transports.[9] The second isoform NKCC2 appears to be expressed only in the thick ascending limb of Henle's loop in the kidney, located in the apical membrane, and represents the absorptive isoform.[10]

Recently, accumulating evidences showed that cytokines can modulate

ion channels and transporters in various epithelia.[15-17] Inflammatory cytokines such as TNF-α and IL-1ß inhibit fluid absorption by suppression of Na^+ channel in airway epithelial cells.[15, 16] Cytokines also stimulate Cl^- secretion by activating Ca^{2+}-activated Cl^- channel in airway epithelia.[17] However, the effect of inflammatory cytokine on NKCC activity has not been studied. IL-1ß is one of the important cytokines in the inflammatory process [18], and increased levels of IL-1ß have been reported in the effusion of otitis.[19] Since the activity of NKCC is critical for Cl^--driven fluid secretions, we hypothesized that IL-1ß may modulate NKCC in middle ear epithelial cells.

In the present study, we analyzed the effects of IL-1ß on NKCC in middle ear cells. NKCC activities were observed in the basolateral membrane of primarily cultured normal human middle ear epithelial (NHMEE) cells, and immunoblotting using specific antibodies revealed the expression of NKCC1 on the basolateral membrane of NHMEE cells. Interestingly, IL-1ß increased the basolateral NKCC activity and the expression of NKCC1. In addition, IL-1ß stimulated bumetanide-sensitive fluid transport in NHMEE cells. Furthermore, NKCC1 expression was increased in middle ear cells from the patients with OME when compared to samples from control individuals. These findings provide a new insight into understanding the pathogenesis of otitis media and offer new modalities in developing cytokine- or NKCC-based

treatments for diseases showing middle ear effusions.

Ⅱ. MATERIALS AND METHODS

1. Chemicals and Solutions

The pH-sensitive fluorescent probe, 2', 7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was purchased from Molecular Probes (Eugene, OR), and ammonium gluconate was from Pfaltz & Bauer (Waterbury, CT). All other chemicals including ouabain, bumetanide and $BaCl_2$ were purchased from Sigma. The standard HEPES-buffered perfusate was termed solution A and contained (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 D-glucose, and 10 HEPES (pH 7.4 with NaOH). The Na^+-free solution B was prepared by replacing Na^+ with N-methyl-D-glucamine$^+$ in solution A (pH 7.4 with Tris). The Cl^--free solution C contained (in mM) 140 Na^+-gluconate, 5 K^+-gluconate, 1 $MgSO_4$, 9.3 hemicalcium cyclamate, 10 D-glucose, and 10 HEPES (pH 7.4 with NaOH). In the measurements of NH_4^+ uptake, 20 mM Na^+ or N-methyl-D-glucamine$^+$ was replaced with equimolar NH_4^+ using NH_4Cl or NH_4^+-gluconate in each solution. The osmolarity of all solutions was adjusted to 310 mosM with the major salt prior to use.

2. Culture of NHMEE Cells

Primary cultures of NHMEE cells were prepared as described previously.[20] All procedures were approved by the Institutional Review Board of Yonsei Medical Center. Passage-2 NHMEE cells were plated on a collagen-coated semipermeable membrane with a 0.45-μm pore size (Transwell-clear; Costar Co., Cambridge, MA) at a density of 1.0 x 10^4 cells/cm^2 for intracellular pH (pH_i) measurement. The cells were maintained in a 1:1 mixture of bronchial epithelial growth medium and Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and all supplements.[21] Membranes bearing cultured cells (culture area, 0.3 cm^2) were floated on the culture medium in Petri dishes and incubated for 4–5 days until the cells formed a functionally polarized monolayer.

3. Measurements of Intracellular pH (pH_i) and NKCC Activity

pH_i was measured in the monolayers using the pH-sensitive fluorescent probe BCECF. Cells were loaded with BCECF for 10 min at room temperature in solution A containing 2.5 μM BCECF-AM and mounted in the miniature Ussing chamber. BCECF fluorescence was recorded and calibrated

using a previously described protocol.[22] Briefly, the fluorescence at excitation wave-lengths of 490 and 440 nm was recorded using a recording setup (Delta Ram; PTI Inc., Lawrenceville, NJ), and the 490/440 ratios were calibrated intracellularly by perfusing the cells with solutions containing 145 mM KCl, 10 mM HEPES, and 5 μM nigericin with the pH adjusted to 6.2–7.6.

NKCC activity was estimated from the pH_i decrease caused by the intracellular uptake of NH_4^+ using the methods of Evans and Turner [23] with a minor modification. As shown in Fig. 1A, when challenged with 20 mM NH_4Cl solutions, cells were rapidly alkalinized (0−2 s) due to the intracellular diffusion of NH_3. However, if the cells have NH_4^+-transporting machineries, the pH_i of the cells would be decreased due to the intracellular uptake of NH_4^+ (see Fig. 1B). As detailed in Results, most of the intracellular NH_4^+ uptake measured in $BaCl_2$ (5 mM)- and ouabain (100 μM)- containing perfusates was a function of NKCC in NHMEE cells. Typically, the first 10−40 s of the initial linear portion of the pH_i decreases due to $BaCl_2$- and ouabain-insensitive intracellular NH_4^+ uptake (shorter times were used for more rapid decreases) was fitted to a linear equation using the Felix software (version 1.4; PTI Inc.)

4. Fluorescent immunohistochemistry

Fully differentiated passage-2 NHMEE cells were grown on a transwell clear culture inserts (Costar, Cambridge, MA) on the air-liquid interface for 2 weeks after confluence. Fluorescent immunohistochemistry was done by using an anti-NKCC1 antibody. Briefly, cells were fixed with 4% paraformaldehyde for 24 hours, cryoprotected with sucrose, and stored in a deep freezer until required. Frozen samples were then sectioned at 10 μm and were exposed to NKCC11-P (rat NKCC1 control peptide) (Alpha Diagnostic International, San Antonio, TX, USA)at 1:500 dilutions. After an 1 h incubation in a humid chamber, NHMEE cells were washed 3 times for 10 minutes with PBS. The sections were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:200, Jackson Immunoresearch, PA) for 30 mim in a dark room, washed with PBS, and mounted with 10 μL glycerol. Images were obtained with a Zeiss LSM 510 confocal microscope. The specificity of immunostaining was verified using blocking peptide for anti-NKCC antibody.

5. Immunoblotting

NHMEE cells were grown to confluence in 6-well plates. The cells were lysed with 2x sample buffer (250 mM Tris-Cl, 2% SDS, 0.1 mol/liter

dithiothreitol, 0.02% bromphenol blue, 10% glycerol, pH 6.5). Equal amounts of whole cell lysates were resolved by 6% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were blocked with 5% skim milk in Tris-buffered saline (TTBS, 50 mM Tris-Cl, pH 7.5, 150 mM NaCl) for 2 h at room temperature. The membrane was then incubated overnight with T4 monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) in TTBS (0.5% Tween 20 in Tris-buffered saline). After washing with TTBS, the blot was further incubated for 45 min at room temperature with appropriate secondary antibody in TTBS and then visualized using the ECL system (Amersham Biosciences).

To evaluate in vivo expression of NKCC1, five pooled samples of middle ear epithelial cells, each from 4-5 patients, were obtained by harvesting from 21 OME patients during Type II ventilation tube insertion. Two pools of normal middle ear mucosa are also harvested from 9 patients during cochlear implantation who have sensory neural hearing loss. Epithelial cells from the middle ear mucosa were isolated by treatment with 1% Pronase (type XIV protease; Sigma Chemical Co., St.Louis, MO) for 18–20 h at 4°C. In order to remove fibroblasts, endothelial cells, and muscle cells, isolated cells were placed in a plastic dish and incubated for 1 h at 37°C. Isolated epithelial cell clusters were digested into single cells by incubating them with

0.25% trypsin:EDTA, and then used for immunoblotting.

6. Measurement of fluid absorption capacity

To evaluate the fluid transport activity across the NHMEE cells, we measured remained volume after application of 100μl of fluid to the luminal side.[25] Briefly, NHMEE cells were cultured in a 12 well-sized Costar Transwell insert for at least 35 days as previously described (37 °C, 5% CO_2 in a humidified atmosphere). The luminal surface at air-liquid interface of fully differentiated NHMEE cells was washed with PBS three times and the surface liquid was completely aspirated. Then, 100 μl of Krebs bicarbonate Ringer (KBR) solution containing 2% blue dextran (BD), a cell-impermeant fluid volume marker dye, was added. After 1, 4, 8, 12, 24 and 48 h incubations in the humidified chamber, microaliquots (2–5μl) of luminal liquid were sampled. BD concentration was measured optically and remaining fluid volume was calculated as previously described.[25]

7. Statistical Analysis

The results of multiple experiments are presented as means ± S.E. Statistical analysis was carried out by analysis of variance or Student's t test as appropriate. $p < 0.05$ was considered statistically significant.

Ⅲ. RESULTS

1. NH_4^+ transport in NHMEE cells

NKCC activities were measured using NH_4^+ as a K^+ surrogate. As an initial step, NH_4^+ transport in the luminal and the basolateral membranes of polarized NHMEE cells was measured. The basal pH_i of NHMEE cells was 7.12 ± 0.06 in Hepes-buffered solution A, and the application of 20 mM NH_4Cl to the luminal side evoked an increase in pH_i followed by a sustained pH_i increase (Fig. 1A). These results indicate that only NH_3 can pass through the luminal membrane of NHMEE cells. At pH 7.4 and 37 °C, 20 mM NH_4Cl solution contains about 0.6 mM NH_3 (pKa ; 8.9). The charged NH_4^+ ion cannot pass through the lipid bilayer of plasma membrane, whereas nonpolar NH_3 can freely diffuse into the cells. When it enters the cells, NH_3 rapidly converts into NH_4^+ by picking up H^+ until equilibrium is reached. Therefore, pH_i increases due to a reduced H^+ concentration. However, if the cell membrane expresses an NH_4^+-transporting process, NH_4^+ can directly enter the cells and decrease pH_i since NH_4^+ donates H^+ to achieve equilibrium with NH_3. Interestingly, applications of 20 mM NH_4Cl to the basolateral side induced a rapid pH_i decrease, which suggests that the basolateral membranes of NHMEE cells have strong NH_4^+-transporting activities (Fig. 1B). The

average value of the initial pH_i reduction caused by basolateral challenge with 20 mM NH_4Cl was 0.281± 0.012 ΔpH unit/min.

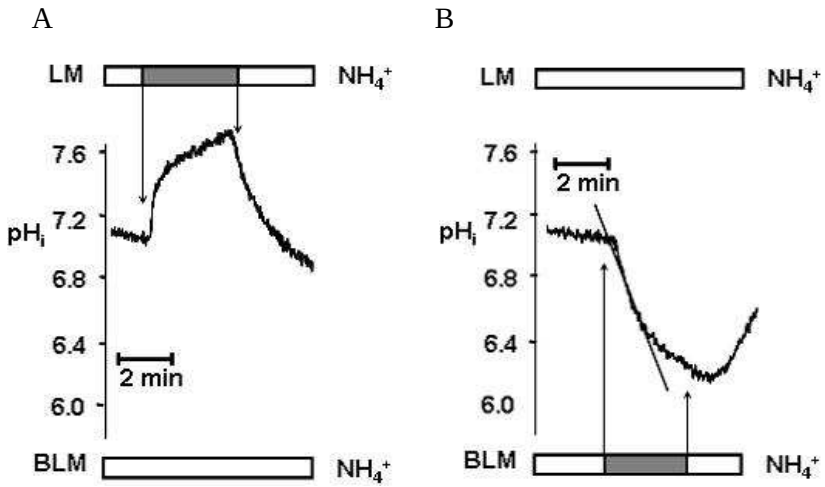


Fig. 1. NH_4^+ transport in the luminal and the basolateral membranes of NHMEE cells. The pHi of NHMEE cells was measured using BCECF as described in Experimental Procedures. The membrane-specific NH_4^+-transporting activities of polarized NHMEE cells were analyzed using a double perfusion chamber with separate applications of NH_4Cl (20 mM) to the luminal and basolateral sides. A, Application of NH_4Cl to the luminal side evoked an abrupt increase in pH*i* followed by a sustained pH*i* increase. B, Application of NH_4Cl to the basolateral side induced a rapid decrease in pH*i* indicating the presence of strong NH_4^+-transporting activities in the BLM. LM, luminal membrane; BLM, basolateral membrane.

2. Characterization of the basolateral NH_4^+-transporting mechanisms

To date, several transporters are known to transport NH_4^+, particularly via K^+ binding sites.[26] Therefore, we characterized the basolateral NH_4^+-transporting activities of NHMEE cells. As shown in Fig. 2, we first analyzed the Na^+ and Cl^- dependence of the NH_4^+-transporting mechanisms. The basolateral application of NH_4Cl in Na^+-free solutions significantly reduced NH_4^+-transporting activities by 54% compared to results in Na^+-containing solutions. Interestingly, basolateral NH_4^+-transporting activities were completely disappeared in Cl^- free solution (Fig. 2).

We further evaluated the characteristics of basolateral NH_4^+-transporting activities using blockers of K^+ channels or Na^+-, K^+-coupled transporters (Fig. 3) Inhibition of K^+ channels with the non-specific K^+ channel blocker Ba^{2+} (5mM) did not affect NH_4^+-transporting activities. On the other hand, the Na^+, K^+ ATPase inhibitor ouabain (100 μM) partially inhibited the basolateral NH_4^+-transport by 26%. Importantly, the addition of the NKCC inhibitor bumetanide (100 μM) inhibited the basolateral NH_4^+-transport by 71% (Fig. 3). Therefore, results of inhibitor profiles together with the results of ion dependency suggest that bulk of the basolateral NH_4^+-transport is mediated by NKCC in NHMEE cells. Subsequently, the expression of NKCC1 in NHMEE cells was verified by

immunocytochemistry. Confocal microscopic images using NKCC11-P (rat NKCC1 control peptide) antibody revealed that NKCC1 was predominantly expressed in the basolateral surface of cultured NHMEE cells.

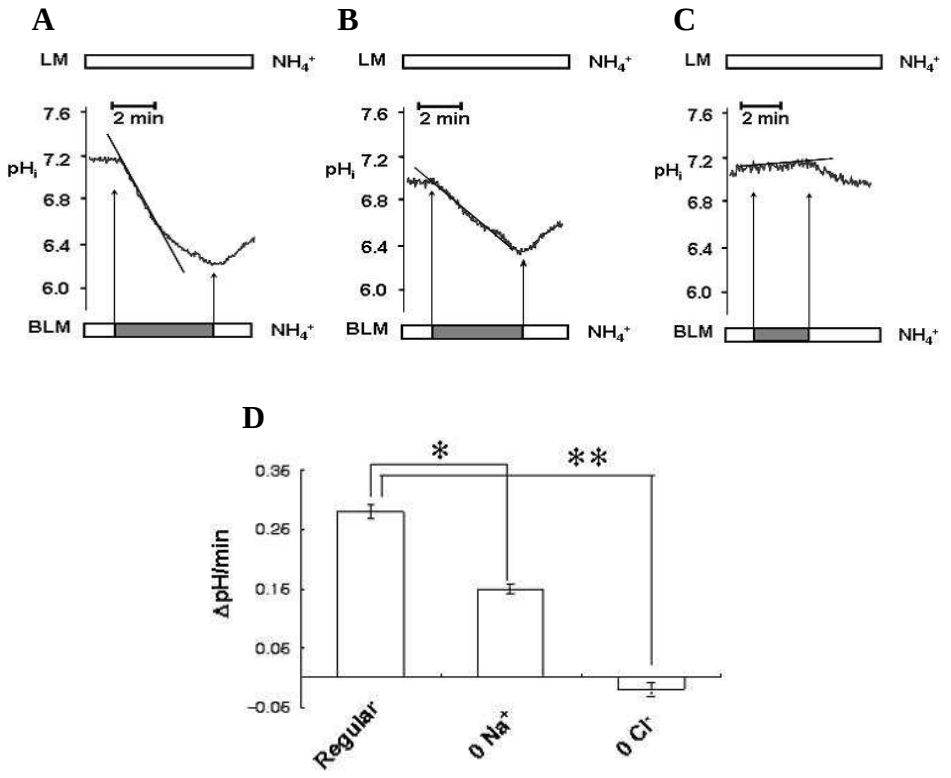


Fig. 2. Na^+ and Cl^- dependence of the basolateral NH_4^+-transporting system. The effects of Na^+ or Cl^- removal on basolateral NH_4^+-transport were analyzed in NHMEE cells. Representative traces of control, Na^+, or Cl^- removal are presented in A, B and C, respectively, and a summary (each n=6) is shown in D. Incubations in Na^+-free solutions reduced NH_4^+-transport by

54% and removal of Cl^- abolished the basolateral NH_4^+-transporting activity. Compositions of solution A, B, and C are detailed in Experimental Procedures. *P< 0.05, **P< 0.01, difference from control.

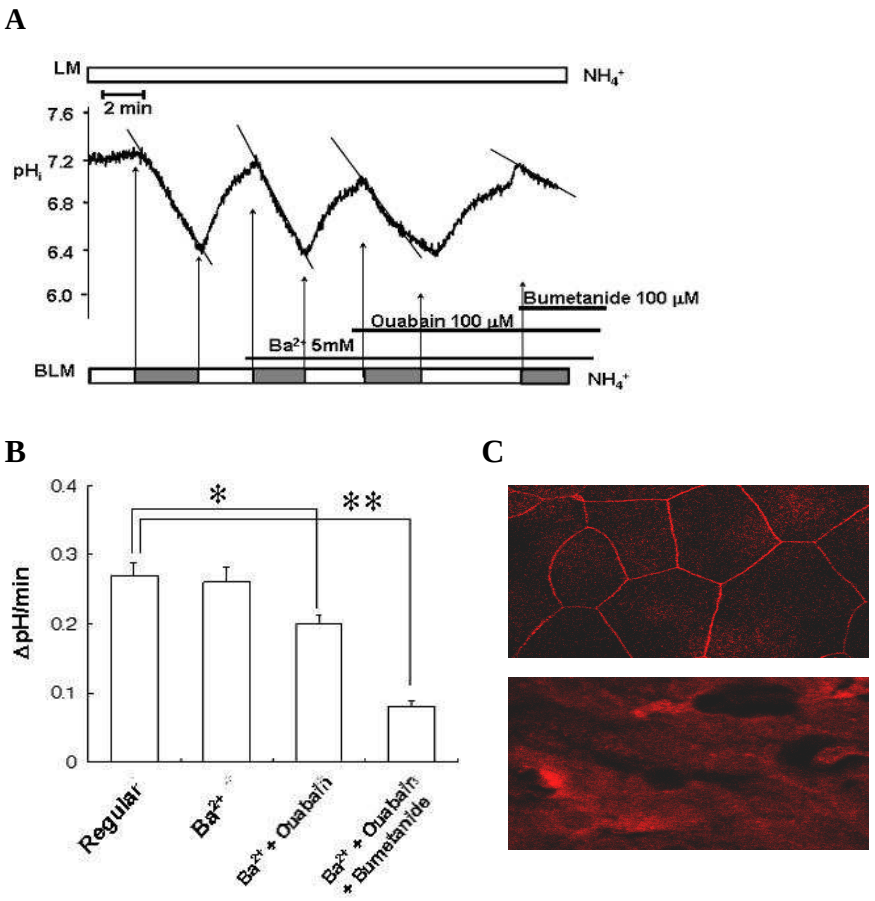


Fig. 3. Functional and molecular expression of NKCC1 in NHMEE cells.

Basolateral NH_4^+-transport was measured with solutions containing non-specific K^+ channel inhibitor (BaCl2), Na^+/K^+ ATPase inhibitor (ouabain), and NKCC inhibitor (bumetanide). A, A representative trace showing the

effects of $BaCl_2$ (5mM), ouabain (100 M) and bumetanide (100 M). B, Summarized results of six experiments. C, Immunolocalization of NKCC1 in NHMEE cells using NKCC11-P (rat NKCC1 control peptide) antibody. *P< 0.05, **P< 0.01, difference from control.

3. Upregulation of NKCC1 by IL-1β treatment

Accumulating evidence suggests that cytokines are involved in the modulation of transepithelial ion transport in various epithelia.[15-17] To investigate the effects of IL-1β on NKCC, we treated NHMEE cells basolaterally with 10 ng of IL-1β for 12 h and measured pH_i in response to basolateral application of NH_4^+ (Fig. 4). Bumetanide-sensitive NH_4^+-uptake under $BaCl_2$- and ouabain-pretreated conditions was considered as NKCC activity. Interestingly, treatment of IL1-β increased the basolateral NKCC activity by 112% (Control, 0.170± 0.022 ΔpH unit/min; IL-1β treated, 0.361± 0.032 ΔpH unit/min).

Next, protein expression of NKCC1 was investigated in order to identify the underlying molecular mechanisms of the IL-1β-induced increase of NKCC activity in NHMEE cells (Fig. 5). Protein samples were collected after 4, 12, and 24 h-treatment with IL-1β and the expression of NKCC1 was compared with samples from non-treated cells. Of interest, IL-1β increased

the expression of NKCC1 in a time dependent manner which reached a plateau at 12 h after the treatment.

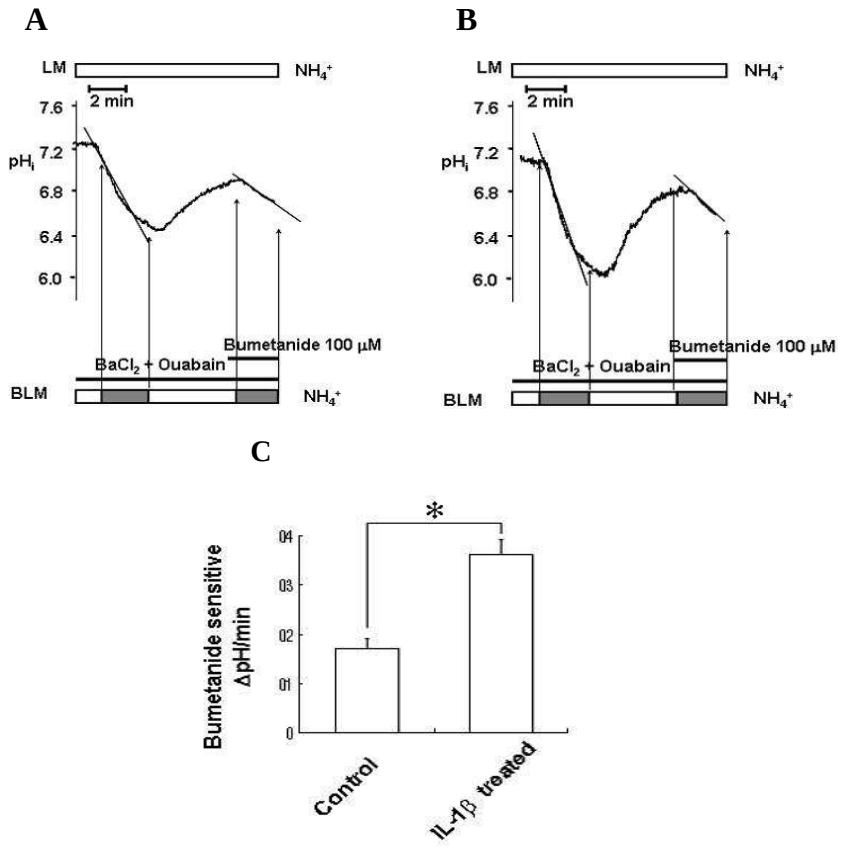


Fig. 4. Augmentation of NKCC1 activity by IL-β treatment. Basolateral NH_4^+-uptake was measured after IL-1β treatment (10 ng/ml for 12 h). Representative traces of control and IL-1β-treated cells are presented in panels A and B, respectively. To inhibit non-specific K^+ channel- and, Na^+/K^+ ATPase-mediated NH_4^+ uptake, $BaCl_2$ (5 mM) and ouabain (100 μM) was

pre-added to the basolateral solutions. C, Effects of basolateral IL-1β stimulation on NKCC1 was determined by comparing the bumetanide-sensive portion of the basolateral NH_4^+ uptake (ΔpH unit/min).

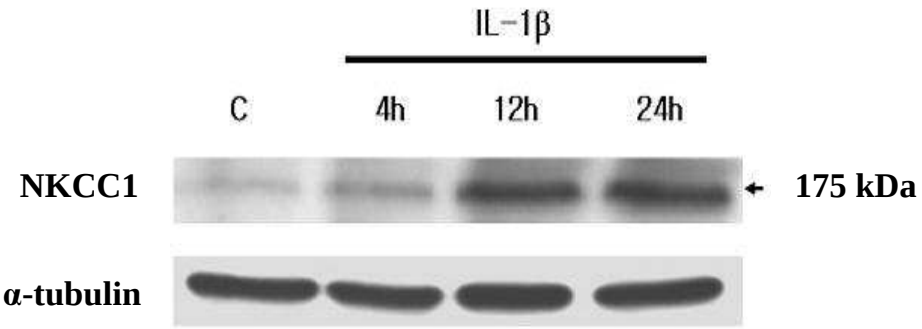


Fig. 5. Upregulation of NKCC1 expression by IL-1β treatment. Immunoblotting of protein extracts of NHMEE cells after treatment of IL-1β for designated times was performed using T4 anti-NKCC1 monoclonal antibody. Equal amounts of protein (50 μg) were loaded on to each lane. Immunoblotting of α-tubulin was used for the determination of total protein loading.

4. Augmentation of NKCC-mediated fluid transport by IL-1β treatment

In order to identify the physiologic impact of IL-1β-induced NKCC1 upregulation, we next measured fluid transport across the NHMEE cell. In general, cultured airway epithelia possess both absorptive and secretory functions. In resting states, middle ear epithelia absorb fluids by a Na^+-dependent mechanism. When 100 μl of KBR solution (0.1% DMSO) was applied on the luminal surface, cells absorbed the fluids with time and 65.8 ±1.9 μl of fluids are remained at 24 h after the application. Interestingly, addition of bumetanide (100 μM) in basolateral membrane of NHMEE cells increased the fluid absorption in a dose-dependent manner (Fig. 6). The observed transepithelial fluid absorption/secretion is a net result of absorptive and secretory functions in epithelial cells. In most epithelia, basolateral NKCC1 plays an important role in Cl^--driven fluid secretion by maintaining $[Cl]_i$. Therefore, we interpreted the above data as a result of decreased NKCC-mediated fluid secretion, which in turn increased the observed fluid absorption in NHMEE cells.

Subsequently, we evaluated the effect of IL-1β on NKCC-dependant fluid transport across NHMEE cell. When the cells were pretreated with IL-1β basolaterally for 12 h (10 ng/ml), the transepithelial fluid absorption was significantly reduced (Fig. 7A). Treatment of bumetanide (100μM) increased

fluid absorption in both control and IL-1β treated sample. Interestingly, the decreased fluid absorptions by IL-1β treatments were completely reversed by bumetanide. Thus, bumetanide-sensitive fluid transport at 24 h after the fluid application was increased from 14.1 ± 1.8 μl to 28.3 ± 2.1 μl by IL-1β treatment (Fig. 7B). These results indicate that IL-1β augments NKCC1-dependent fluid transport in NHMEE cells.

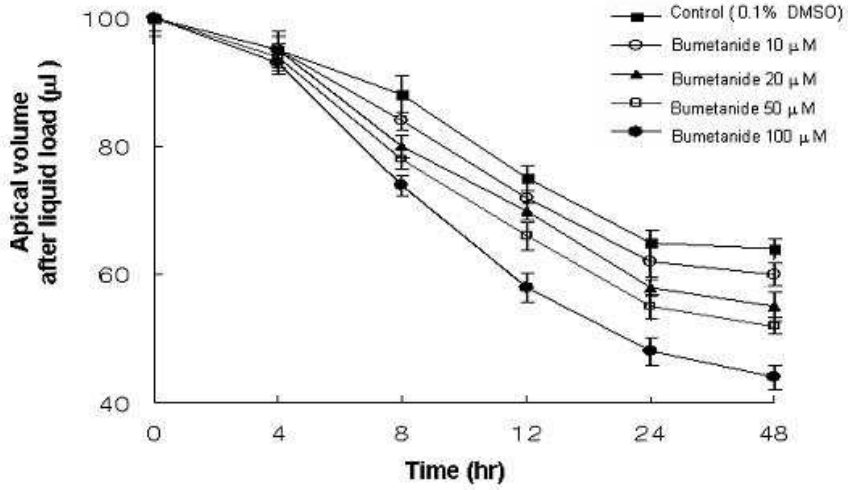
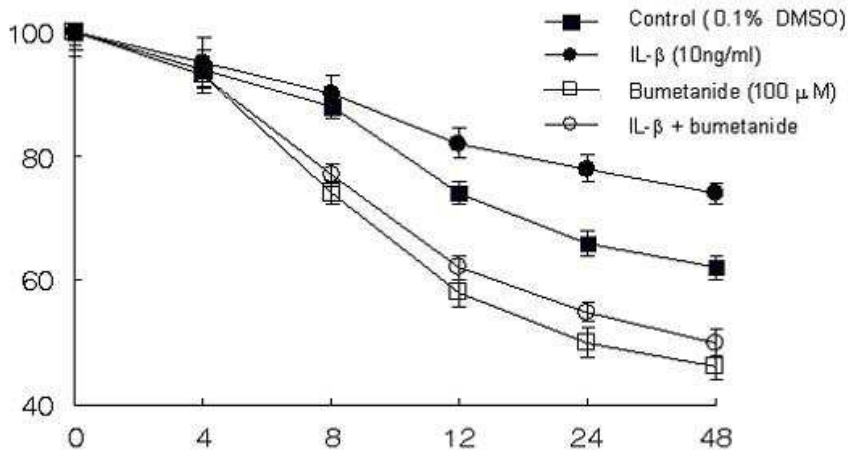


Fig. 6. Role of NKCC in the fluid transport of NHMEE cells. One hundred microliter of KRB solution (0.1% DMSO) with or without various concentrations of bumetanide was applied on the luminal surface and the remained fluid volume was measured at indicated time. Bumetanide treatments inhibited fluid absorption in a dose dependant manner.

A



B

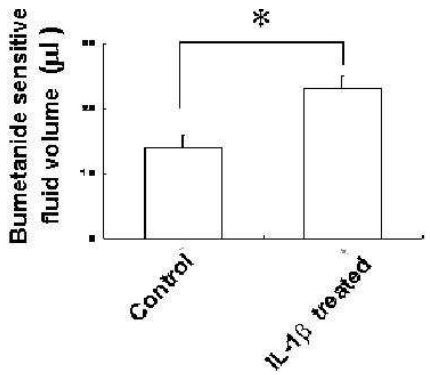


Fig. 7. Augmentation of bumetanide–sensitive fluid transport by IL-1β. A, After application of 100 μl of KRB solution (0.1% DMSO) on control and IL-1β-treated cells with or without bumetanide, remained fluid volume were measured at indicated time. B. Bumetanide-sensitive fluid volume 24 h after the luminal fluid application was calculated (n=5).

5. Upregulation of NKCC1 in the middle ear epithelial cells from otitis media patients

Lastly, we tried to investigate whether the levels of NKCC1 expression in the middle ear epithelial cells are altered in patients with OME. Middle ear epithelial cells from one patient are too small to perform immunoblotting. Therefore, we collected middle ear epithelial cells from 4-5 patients and mixed as one pool. As showed in Fig. 8, NKCC1 expressions were highly increased in the epithelial cell pools from OME patients compared with those from control subjects (Fig. 8). These results suggest that middle ear inflammation indeed increases the expression of NKCC1 in epithelial cells.

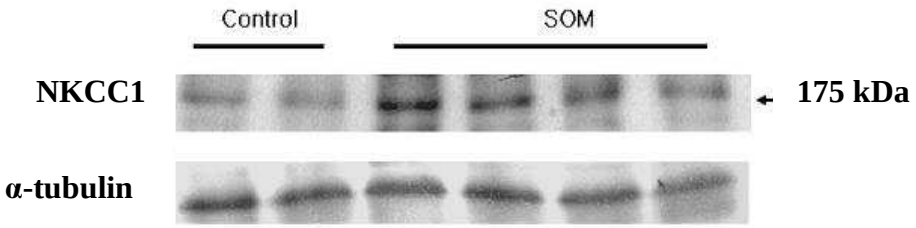


Fig. 8. Upregulation of NKCC1 in the middle ear epithelial cells from otitis media patients. Immunoblotting of NKCC1 was performed with the protein extracts of middle ear epithelial cells obtained from OME patients (4 pools from 21 patients) and was compared with samples from control subjects (2 pools form 9 patients).

Ⅳ. DISCUSSION

Otitis media with effusion, defined as the accumulation of middle-ear effusion behind an intact tympanic membrane, is one of the most common causes of childhood hearing loss in developed countries, potentially leading to language deficits. Although multifactoiral pathogenic mechanisms such as negative middle ear pressures and bacterial infections are suggested, the exact molecular mechanisms of fluid collection are not well understood. Hypersecretion of the periciliary fluid would be one of the important mechanisms of fluid collection in the middle ear cavity.[2] It is generally assumed that the fluid secretion in respiratory-type epithelium is driven by the Cl^- transport from basolateral to apical sides.[3] However, the specific electrolyte transport mechanisms, which generate the driving force for the fluid transport, and the location of the associated transporters in the polarized middle ear epithelia remained to be elucidated.

Our investigation addressed the questions whether NKCCs are expressed in middle ear epithelial cells. By measuring pH_i changes due to NH_4^+ uptake, we demonstrated the presence of a bumetanide-sensitive K^+ uptake in the basolateral membrane of cultured NHMEE cells. Bulk of the K^+ uptake depends on the presence of extracellular Na^+ and Cl^- and is also

sensitive to bumetanide. All these findings correspond to the characteristics of NKCC. We also obtained evidence for the expression of NKCC1 protein in the basolateral surface of NHMEE cells by immunohistochemistry. These data are similar to the results from nasal [27] and tracheobronchial [28] epithelia where NKCC1 plays one of the major driving forces of $[Cl]_i$ uptake in the basolateral membrane. However, the characteristics of overall K^+ transport system in NHMEE cells are somewhat different from other airway epithelia. The basolateral NH_4^+ uptake in NHMEE cells was not completely inhibited by the bumetanide in the presence of $BaCl_2$ and oubain, and was more dependent to Cl^- than Na^+ (Fig. 2 and 3). These data indicated that there are other K^+ transporting systems, such as K^+-Cl^- cotransporters (KCCs) may exist in the basolateral membrane of NHMEE cells. Indeed, we detected mRNAs of KCC1 and KCC4 in NHMEE cells using RT-PCR (data not shown). However, since electrochemical gradients of KCCs are set to exit of K^+ and Cl^- ions, they would not contribute to the transepithelial Cl^- and fluid secretion toward luminal side.

In a wide variety of secretory epithelia, including those of the intestines [29] and salivary glands [30], the activity of basolateral NKCC1 is the rate-limiting step of transepithelial Cl^- fluxes. Previous animal studies have also shown the

importance of NKCC1 in the fluid transport of airway epithelia.[31, 32] In fetal sheep and guinea-pig lung, the addition of the loop diuretics bumetanide or furosemide to fetal lung liquid caused slowing of lung-liquid secretion or increasing in liquid absorption. We evaluated the role of NKCC in the fluid transport of middle ear cells by measuring the remaining fluid volume 24 h after the luminal application of 100 μM of fluid. Airway epithelia possess both absorptive and secretory functions. For example, surface epithelial cells predominantly absorb fluids by a Na^+-dependent mechanism and cells in submucosal glands secrete bulk of fluids by a Cl^--driven mechanism. In general, cultured airway epithelia show both absorptive and secretory functions depending on the situations. Several factors are involved in the net direction of transepithelial fluid transport. In addition to the relative strengths of secretory and absorptive mechanisms, hydrostatic pressure and osmotic activity also affect the driving force of fluid transport. In our experimental conditions, the resting state NHMEE cells dominantly absorb luminal fluid with time-dependent manner up to 24 h. Importantly, fluid absorption was substantially increased by basolateral NKCC1 inhibition (Fig. 6). These data strongly suggest that NKCC1 significantly contributes to electrolyte and fluid transport across middle ear epithelia even in the resting state.

NKCCs are known to be regulated by many factors including

osmolarity changes of the cell, and several extracellular and intracellular signal molecules such as vasoactive intestinal polypeptide, ATP and cAMP.[34-36] Recently, there has been an increasing body of evidence supporting the important role of cytokines in the modulation of ion channels and transporters.[15-17] IL-1β is one of the major proinflammatory cytokines produced by macrophages. It has been shown that IL-1β altered Na^+ absorption and fluid transport in the airway epithelia[16] IL-1β also increased bumetanide-sensitive short circuit current in human tracheal epithelial cells.[37] Moreover, previous reports demonstrated that the effusion of otitis media contains high levels of IL-1ß.[18] These findings prompted us to investigate the effects of IL-1β on NKCC activity in middle ear cells. As shown in Fig. 4 and 5, IL-1β increased the bumetanide-sensitive K^+ transport and the expression of NKCC1 in the basolateral membrane of NHMEE cells. The activation of NKCC1 by IL-1β would lead to a increased $[Cl]_i$, which would increase the driving force of fluid secretion in epithelial cells. Within our knowledge, these results provide for the first time conclusive evidence for the activation of NKCC1 by IL-1β and its possible involvement in the middle ear effusions associated with otitis media. Consequently, we confirmed that NKCC1 is upregulated in the middle ear epithelial cells from OME patients. The above results imply that cytokines can upregulate NKCC1 in the middle ear epithelia,

which may in turn induce the fluid hypersecretion in OME patients. Such an up-regulation of NKCC1 by proinflammatory cytokines might be involved in hypersecretions observed in other inflammatory airway disease such as sinusitis and lower airway tract diseases. The exact molecular mechanisms involved in the IL-1β-induced upregulation of NKCC1 and the signaling pathways that activates NKCC1 in middle ear inflammation need to be determined in future studies.

Ⅴ. Conclusion

We found that NKCC1 is present in the basolateral membrane of and contribute to fluid transport across middle ear epithelial cells. More importantly, IL-1β increased NKCC1 expression and augmented NKCC-mediated fluid transport in middle ear epithelia. In addition, NKCC1 expression was increased in middle ear cells from the patients with OME. The above results suggest that upregulation of NKCC1 by inflammatory cytokines would be an important underlying mechanism of fluid overcollection in patients with OME.

Ⅵ. References

1. Portier, F, Kania, ., Planès, ., Hsu, WC, Couette, ., Huy, P et al. Enhanced sodium absorption in middle ear epithelial cells cultured at air-liquid interface. Acta. Otolaryngol 2005; 125: 16-22.

2. Lim, DJ, Chun YM, Lee HY, Moon SK, Chang KH, Li JD et al. Cell biology of tubotylpanum in relation to pathogenesis of otitis media. Vaccine 2000; 19: 17-25.

3. Furukawa M, Ikeda K, Yamaya M, Oshima T, Sasaki H, Takasaka T. Effects of extracellular ATP on ion transport and [Ca^{2+}]i in Mongolian gerbil aiddle ear epithelium. Am J Physiol 1997; 272**:** 827-836.

4. Herman P, Tan CT, van den Abbeele T, Escoubet B, Friedlander G, Huy PT. Glucocorticosteroids increase sodium transport in middle ear epithelium. Am J Physiol. 1997; 272: 184-190.

5. Herman P, Cassingena R, Friedlander G, Soler P, Grodet A, Huy PT et al. Middle ear cell line that maintainsvectorial electrolyte transport. J Cell Physiol 1993; 154: 615-622.

6. Haas M, Forbush B 3^{rd}. The Na-K-Cl cotransporter of secretory epithelia. Annu Rev Physiol 2000; 62: 515-534.

7. Russell JM. Sodium-Potassium-Chloride Cotransporter. Physiol Rev 2000; 80: 211–276.

8. Haas M. The Na-K-Cl cotransporters. Am J Physiol 1994; 267: 869–885.

9. Boucher RC, Larsen EH. Comparison of ion transport by cultured secretory and absorptive canine airway epithelia. Am J Physiol 1988; 254: 535–547.

10. Ginns SM, Knepper MA, Ecelbarger CA, Terris J, He X, Coleman RA et al. Immunolocalization of the secretory isoform of Na-K-Cl cotransporter in rat renal intercalated cells. J Am Soc Nephrol 1996; 7: 2533-2542.

11. Lytle C, McManus T. Coordinate modulation of Na-K-2Cl cotransport and K-Cl cotransport by cell volume and chloride. Am J Physiol Cell Physiol 2002; 283: 1422-1431.

12. Yang H, Wang Z, Miyamoto Y, Reinach PS. Cell signaling pathways mediating epidermal growth factor stimulation of Na:K:2Cl cotransport activity in rabbit corneal epithelial cells. J Membr Biol 2001; 183: 93-101.

13. Gillie DJ, Pace AJ, Coakley RJ, Koller BH, Barker PM. Liquid and ion transport by fetal airway and lung epithelia of mice deficient in sodium-potassium-2-chloride transporter. Am J Respir Cell Mol Biol 2001; 25: 14-20.

14. Matthews JB. Molecular regulation of Na^+-K^+-$2Cl^-$ cotransporter (NKCC1) and epithelial chloride secretion. World J Surg 2002; 26: 826-830.

15. Barmeyer C, Amasheh S, Tavalali S, Mankertz J, Zeitz M, Fromm M et al. IL-1beta and TNFalpha regulate sodium absorption in rat distal colon.

Biochem Biophys Res Commun 2004; 317: 500-507.

16. Dagenais A, Frechette R, Yamagata Y, Yamagata T, Carmel JF, Clermont ME et al. Downregulation of ENaC activity and expression by TNF-alpha in alveolar epithelial cells. Am J Physiol Lung Cell Mol Physiol 2004; 286: 301-311.

17. Atherton H, Mesher J, Poll CT, Danahay H. Preliminary pharmacological characterisation of an interleukin-13-enhanced calcium-activated chloride conductance in the human airway epithelium. Naunyn Schmiedebergs Arch Pharmacol 2003; 367: 214-217

18. Monton A, Torres C, El-Ebiary M, Filella X, Xaubet A, de la Bellacasa J. Cytokine expression in severe pneumonia: a bronchoalveolar lavage study. Crit Care Med 1999; 27: 1745-1753.

19. Yellon RF, Leonard G, Marucha PT, Craven R, Carpenter RJ, Lehmann WB et al. Characterization of cytokines present in middle ear effusions. Laryngoscope 1991; 101: 165-169.

20. Choi JY, Kim CH, Lee WS, Kim HN, Song KS, Yoon JH. Ciliary and secretory differentiation of normal human middle ear epithelial cells. Acta Otolaryngol 2002; 122: 270-275.

21. Yoon JH, Kim KS, Kim SS, Lee JG, Park IY. Secretory differentiation of serially passaged normal human nasal epithelial cells by retinoic acid:

expression of mucin and lysozyme. Ann Otol Rhinol Laryngol 2000; 109: 594-601.

22. Namkung W, Lee JA, Ahn W, Han W, Kwon SW, Ahn DS et al. Ca^{2+} activates cystic fibrosis transmembrane conductance regulator-and Cl^--dependent HCO_3 transport in pancreatic duct cells. J Biol Chem 2003; 278: 200–207.

23. Evans RL, Turner RJ. Upregulation of Na(+)-K(+)-2Cl- cotransporter activity in rat parotid acinar cells by muscarinic stimulation. J Physiol 1997; 499: 351-359.

24. Lytle C, Xu JC, Biemesderfer D, Forbush B 3rd. Distribution and diversity of Na-K-Cl cotransport proteins: a study with monoclonal antibodies. Am J Physiol Cell Physiol 1995; 269: 1496–1505.

25. Matsui H, Grubb B, Tarran R, Randell S, Gatzy J, Davis C et al. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airway disease. Cell 1998; 95: 1005-1015.

26. Bergeron MJ, Gagnon E, Wallendorff B, Lapointe JY, Isenring P. Ammonium transport and pH regulation by K^+-Cl^- cotransporters. Am J Physiol Renal Physiol 2003; 285: 68-78.

27. Shin JH, Namkung W, Choi JY, Yoon JH, Lee MG. Purinergic stimulation induces Ca^{2+}-dependent activation of Na^+-K^+-$2Cl^-$ cotransporter in human

nasal epithelia. J Biol Chem 2004; 279: 18567-74.

28. Grubb BR, Lee E, Pace AJ, Koller BH, Boucher RC. Intestinal ion transport in NKCC1-deficient mice. Am J Physiol 2000; 19: 707-718.

29. Evans RL, Park K, Turner RJ, Watson GE, Nguyen HV, Dennett MR et al. Severe impairment of salivation in $Na^+/K^+/2Cl^-$ cotransporter (NKCC1)-deficient mice. J Biol Chem 2000; 275: 26720-26726.

30. Cassin S, Gause G, Perks AM. The effects of bumetanide and furosemide on lung liquid secretion in fetal sheep. Proc Soc Exp Biol Med 1986; 181: 427-431.

31. Carlton DP, Cummings JJ, Chapman DL, Poulain FR, Bland RD. Ion transport regulation of lung liquid secretion in fetal lambs. J Dev Physiol 1992; 17: 99-107.

32. Xu JC, Lytle C, Zhu TT, Payne JA, Benz E Jr, Forbush B 3rd. Molecular cloning and functional expression of the bumetanide-sensitive Na-K-Cl cotransporter. Proc Natl Acad Sci U S A 1994; 91: 2201-2205.

33. Haas M. 1995. Activation of Na-K-Cl cotransport in airway epithelial cells by apical UTP, cell shrinkage, and reduced $[Cl]_i$: role of protein phosphorylation. FASEB J 1995; 9: 599.

34. Lytle C, Forbush B 3rd. Regulatory phosphorylation of the secretory Na-KCl cotransporter: modulation by cytoplasmicCl. Am J Physiol 1996;

270: 437–448.

35. Dharmsathaphorn K, McRoberts JA, Masui H, Mandel KG. Vasoactive intestinal peptide-induced Cl secretionby a colonic epithelial cell line. Direct participation of a basolaterally localized Na,K,Cl cotransport system. J Clin Invest 1985; 75: 462–471.

36. Gray T, Coakley R, Hirsh A, Thornton D, Kirkham S, Koo JS et al. Nettesheim PRegulation of MUC5AC mucin secretion and airway surface liquid metabolism by IL-1beta in human bronchial epithelia. Am J Physiol Lung Cell Mol Physiol 2004; 286: 320-330.

국문요약

사람 중이점막세포 내에서 Interleukin-1 beta에 의한 Na^+-K^+-$2Cl^-$ cotransporter의 증가 연구

(지도교수 윤 주 헌)

연세대학교 대학원 의과학과

김 정 림

삼출성중이염은 감기나 비염, 급성중이염 등이 생긴 후에 적절한 치료가 되지 않아 만성발열이나 통증 등 염증의 증상이 없이 고막 안에 삼출액이 차 있는 경우로 소아의 가장 흔한 청력장애의 원인이 되고 있다. 삼출액의 발생을 그 성분 중에 가장 많은 염증성 cytokine 으로 알려져 interleukin-1ß(IL-1ß)가 삼출성 중이염 환자의 중이강 내에서 주변 세포에 체적과 분비, 물 수송에 관여하는 기능성 막 단백질에 미치는 어떤 영향으로 예상했다.

종래의 연구에서 IL-1ß 의 세포생리학적인 기능은 아직 연구가 미비한 실정이다. 그래서 기도점액에서 세포 체적과 분비기능이

있는 Na^+-K^+-Cl^- cotransporter 1(NKCC1)이 조직 특이성이 비슷한 중이점막상피세포에서 발현하는지의 여부와 IL-1ß 에게서 받는 영향을 분석하였다.

정상인의 중이점막상피세포를 채취하여 P2 단계까지 배양한 후 H^+를 세포막의 안팎으로 강제 이동시키며 pH 변화를 통해 세포의 기저외측막에서 NKCC1 의 실직적인 기능을 확인하면서 동시에 IL-1ß 를 처리하였을 때 그 기능이 유의성 있게 증가하는 것을 확인하였다.

또한 단백질 수준에서 IL-1ß 를 시간 별로 처리한 시료와 삼출성 중이염 환자군에서 채취한 시료가 동일한 결과를 보였으며 NKCC1 을 특이적으로 저해하는, bumetanide 를 처리하여 실질적으로 물 수송 양이 줄어드는 결과로 보아 NKCC1 이 기도상피세포와 마찬가지로 정상사람 중이 점막상피세포에서도 그 기존의 역할을 하는 것으로 보여진다. 세포 첨부측에 일정량의 Kreb's solution 을 넣어주고 IL-1ß 를 처리했을 때 물의 분비량이 대조군에 비해 유의성 있게 증가하였으며 이를 중이 내 삼출액의 발생원인으로 추정한다.

이와 같은 연구들이 염증성 cytokine 에 의한 기능성 막단백질의 이상으로 야기되는 삼출성 중이염의 병리 생리를 연구하는데 기초가 될 것이다.

핵심 되는 말 : Interleukin-1 beta, 정상인 중이점막세포(NHMEE), NKCC, 삼출성 중이염, pH*i* 측정, BCECF-AM