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Interleukin-1β Upregulates Na^+-K^+-$2Cl^-$ Cotransporter in Human Middle Ear Epithelia

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Abstract Disruption of periciliary fluid homeostasis is the main pathogenesis of otitis media with effusion (OME), 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 the effects of a major cytokine interleukin (IL)-1β, which was known to be involved in the pathogenesis of OME, on Na^+-K^+-$2Cl^-$ cotransporter (NKCC) in human middle ear cells. Intracellular pH (pH_i) was measured in primary cultures of normal human middle ear epithelial (NHMEE) cells using a double perfusion chamber, which enabled us to analyze the membrane-specific transporter activities. NKCC activities were estimated by the pH_i reduction due to bumetanide-sensitive intracellular uptake of NH_4^+. In NHMEE cells, NKCC activities were observed only in the basolateral membrane, and immunoblotting using specific antibodies revealed the expression of NKCC1. Interestingly, IL-1β treatments augmented the basolateral NKCC activities and increased NKCC1 expression. In addition, IL-1β treatments stimulated bumetanide-sensitive fluid transport across the NHMEE cell monolayers. Furthermore, an elevated NKCC1 expression was observed in middle ear cells from OME patients when compared to those from control individuals. The above results provide in vitro and in vivo evidence that the inflammatory cytokine IL-1β upregulates NKCC1 in middle ear epithelial cells, which would be one of the important underlying mechanisms of excess fluid collection in OME patients. J. Cell. Biochem. 101: 576–586, 2007.

Key words: middle ear; human; Na^+-K^+-$2Cl^-$ cotransporter; interleukin-1β; otitis media with effusion

Maintaining a fluid-free middle ear cavity is a critical feature in the physiology of the middle ear, which allows the transmission of sound vibrations from the eardrum to the inner ear. Epithelial cells lining the middle ear cavity are a respiratory type epithelium and play an important role in maintaining a fluid-free space by regulating periciliary fluid volume [Portier et al., 2005]. Disruption of periciliary fluid homeostasis has been thought to be a major factor in the pathogenesis of otitis media with effusion (OME), which is one of the most common childhood diseases [Lim et al., 2000]. Although many studies indicate that ion channels and transporters are involved in fluid transport and periciliary fluid volume regulation [Herman et al., 1993; Furukawa et al., 1997], the exact underlying molecular mechanisms are still to be clarified.

Recent evidence suggests that activities of the basolateral Na^+-K^+-Cl^- cotransporter (NKCC) are the rate-limiting step for ion and fluid secretions in Cl^--secreting epithelia including airway epithelia [Gillie et al., 2001; Matthews, 2002]. It has been reported that middle ear epithelium possess characteristics of Cl^-

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secreting epithelia in a study using short-circuit current measurements [Furukawa et al., 1997]. NKCCs are members of a cation-chloride cotransporter family, which have 12 membrane-spanning domains, and have been identified in many mammalian and non-mammalian tissues [Haas and Forbush, 2000; Russell, 2000]. They represent electroneutral transport systems with a presumed stoichiometry of 1 Na^+:1 K^+:2 Cl^- driven by the sum of the Na^+, K^+, and Cl^- gradients. NKCCs serve a number of vital physiological functions including cell volume regulation and cell proliferation [Lytle and McManus, 2002; Panet et al., 2002]. Two NKCC isoforms have been identified to date, NKCC1 and NKCC2. 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 be the secretory isoform [Haas, 1994; Russell, 2000]. The second isoform, NKCC2, appears to be expressed only in the apical membrane of thick ascending limb of Henle's loop in the kidney, and represents the absorptive isoform [Boucher and Hviid, 1988].

Recently, accumulating evidences have shown that cytokines can modulate ion channels and transporters in various epithelia. Inflammatory cytokines such as TNFα and interleukin (IL)-1β inhibit fluid absorption by the suppression of epithelial Na^+ channel (ENaC) in airway epithelial cells [Barmeyer et al., 2004; Dagenais et al., 2004]. Cytokines also stimulate Cl^- secretion by activating Ca^{2+}-activated Cl^- channel in airway epithelia [Atherton et al., 2003; Naunyn, 2003]. However, the effects of inflammatory cytokines on NKCC remain controversial. IL-1β enhanced NKCC mRNA expression in vascular endothelial cell and lung tissue but not in heart tissue [Topper et al., 1997]. IL-1β is one of the important cytokines in the inflammatory process [Lin et al., 2005], and increased levels of IL-1β have been reported in the effusion of otitis [Yellon et al., 1991]. Since NKCC activity is critical for Cl^--driven fluid secretions, we hypothesized that IL-1β may modulate NKCC in middle ear epithelial cells.

In the present study, we analyzed the effects of IL-1β on NKCC in middle ear cells. NKCC activities were observed in the basolateral membrane of primary cultured normal human middle ear epithelial (NHMEE) cells, and immunoblotting using specific antibodies revealed the expression of NKCC1 in the cells. Interestingly, IL-1β increased the basolateral NKCC activity and the expression of NKCC1. Furthermore, IL-1β stimulated bumetanide-sensitive fluid transport in NHMEE cells, and NKCC1 expression was increased in middle ear cells of OME patients. 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 associated with middle ear effusions.

MATERIALS AND METHODS

Chemicals and Solutions

The pH-sensitive fluorescent probe, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was purchased from Molecular Probes (Eugene, OR), and ammonium gluconate from Pfaltz & Bauer (Waterbury, CT). All other chemicals including ouabain, bumetanide, and $BaCl_2$ were purchased from Sigma (St. Louis, MO). 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). For 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.

Culture of NHMEE Cells

Primary cultures of NHMEE cells were prepared as described previously [Yoon et al., 2000; Choi et al., 2002]. All procedures were approved by the Institutional Review Board of Yonsei Medical Center. Passage-2 NHMEE cells were plated on a collagen-coated semi-permeable membrane with a 0.45-μm pore size (Transwell-clear; Costar Co., Cambridge, MA) at a density of 1.0×10^4 cells/cm^2 for intracellular pH (pH_i) measurement. The cells were maintained in a 1:1 mixture of Dulbecco's

modified Eagle's medium and bronchial epithelial growth medium (BEGM; Clonetics Corp., San Diego, CA) and further supplemented with epidermal growth factor (EGF; 25 ng/mL; Collaborative Research, Bedford, MA), all-*trans* retinoic acid (5×10^{-8} mol/L; Sigma), bovine serum albumin (1.5 mg/mL; Sigma), and bovine pituitary extract (1% vol/vol; Pel Freez, Rogers, AR) [Yoon et al., 2000]. 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.

Measurements of Intracellular pH (pH_i) and NKCC Activity

pH_i was measured in the cell monolayers using the pH-sensitive fluorescent probe BCECF using a previously described protocol [Namkung et al., 2003]. Briefly, cells were loaded with BCECF for 10 min at room temperature in solution A containing 2.5 μM BCECF-AM and mounted in a miniature Ussing chamber (August Krogh Institute, Copenhagen, Denmark). The fluorescence at excitation wavelengths 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 [1997] with a minor modification. As shown in Figure 1A, when challenged with 20 mM NH_4Cl-containing solutions, cells were rapidly alkalinized (0–2 s) due to the intracellular diffusion of NH_3. However, if the cells have NH_4^+-transporting activities, the pH_i of the cells would decrease due to intracellular uptake of NH_4^+ (see Fig. 1B). As detailed in Results section, 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 pH_i decrease 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.).

Fluorescent Immunohistochemistry

Passage-2 NHMEE cells were grown on transwell clear culture inserts (Costar) on an air–liquid interface for 2 weeks after confluence. Fluorescent immunohistochemistry was performed using an anti-NKCC1 antibody (NKCC11-A; Alpha Diagnostic International, San Antonio, TX). Briefly, cells were fixed with 4% paraformaldehyde for 24 h, cryoprotected with sucrose, and stored in a deep freezer until required. Frozen samples were then exposed to

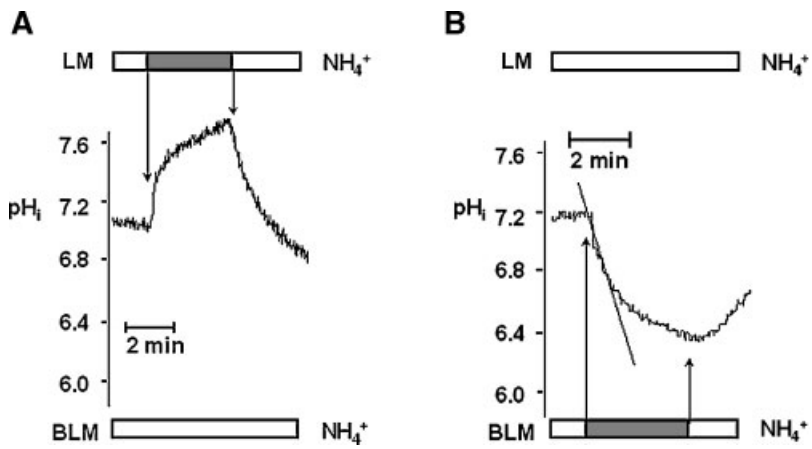

Fig. 1. NH_4^+ transport in the luminal and basolateral membranes of NHMEE cells. The pH_i of NHMEE cells was measured using BCECF as described in Materials and Methods section. The membrane-specific NH_4^+-transporting activities of polarized NHMEE cells were analyzed using a double perfusion chamber 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.

anti-NKCC1 antibody at a 1:100 dilution after a three-time wash with PBS. After 1 h incubation in a humid chamber, NHMEE cells were washed three times for 10 min with PBS. The sections were then incubated with rhodamine-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:200, Jackson Immunoresearch, PA) for 30 min 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 a blocking peptide for the anti-NKCC1 antibody (NKCC11-P; Alpha Diagnostic International).

Immunoblotting

NHMEE cells were grown to confluence in 6-well plates. The cells were lysed with 2× sample buffer (250 mM Tris-Cl, 2% SDS, 0.1 mol/L 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 Anti-NKCC1 monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) in TTBS. After washing with TTBS containing 0.5% Tween 20, the blot was further incubated for 45 min at room temperature with the appropriate secondary antibody in TTBS, and then visualized using ECL reagents (Amersham Biosciences).

To evaluate in vivo expression of NKCC1, five pooled samples of middle ear epithelial cells, each from 4 to 5 patients, were obtained by harvesting from 21 OME patients during a Type II ventilation tube insertion. Two pools of normal middle ear mucosa were also harvested during cochlear implantation from nine patients who have sensory neural hearing loss. Epithelial cells from the middle ear mucosa were isolated by treatment with 1% Pronase (type XIV protease; Sigma) for 18–20 h at 4°C. In order to remove fibroblasts and endothelial cells, isolated cells were placed in a plastic dish and incubated for 1 h at 37°C. The unattached epithelial cells were collected and cell clusters were digested into single cells by incubating them with 0.25% trypsin/EDTA, and then used for immunoblotting. The staining intensities of immunoblots were analyzed using imaging software (MCID version 3.0; Brock University, St. Catharine's, Ontario, Canada).

Measurement of Fluid Absorption Capacity

To evaluate the fluid transport activity across the NHMEE cells, the remaining fluid volume was measured after application of 100 μL of fluid to the luminal side. Briefly, NHMEE cells were cultured in a 12-well Costar Transwell insert for about 2 weeks until monolayer was formed as previously described (37°C, 5% CO_2 in a humidified atmosphere) [Matsui et al., 1998]. The luminal surface of differentiated NHMEE cells cultured in air–liquid interface conditions 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, was added. After 1, 4, 8, 12, 24, and 48 h incubations in the humidified chamber, microaliquots (2–5 μL) of luminal liquid were sampled. BD concentration was measured optically and the remaining fluid volume was calculated as previously described (Matsui et al., 1998).

Statistical Analysis

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

RESULTS

NH_4^+ Transport in *NHMEE* Cells

NKCC activities were measured using NH_4^+ as a K^+ surrogate. NH_4^+ transport across the luminal (apical) and basolateral membrane of polarized NHMEE cells was analyzed by measuring pH_i changes. 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 instant 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, 20 mM NH_4Cl solution contains about 0.6 mM NH_3 (pK_a: 8.9, 37°C). The charged NH_4^+ ion cannot pass through the lipid bilayer of the plasma membrane, whereas nonpolar NH_3 can freely diffuse into the cells. When it enters the cells, NH_3 rapidly converts to

NH_4^+ by acquiring H^+ until equilibrium is reached. Therefore, pH_i increases due to a reduced H^+ concentration. However, if the cell membrane has 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 membrane of NHMEE cells has 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.

Characterization of the Basolateral NH_4^+-Transporting Mechanisms

To date, several transporters are known to transport NH_4^+, particularly via K^+ binding sites [Bergeron et al., 2003]. Therefore, we set out to characterize the basolateral NH_4^+-transporting activities of NHMEE cells. As shown in Figure 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 reduced NH_4^+-transporting activities by 54% as compared to those in Na^+-containing solutions (Fig. 2B). Interestingly, basolateral NH_4^+-transporting activities completely disappeared upon the application of Cl^--free solution (Fig. 2C).

We further evaluated the characteristics of the basolateral NH_4^+-transporting activity 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+} (5 mM) did not affect NH_4^+-transporting activities. On the other hand, the Na^+/K^+ ATPase inhibitor ouabain (100 μM) partially inhibited basolateral NH_4^+-transport by 26%. Importantly, addition of the NKCC inhibitor bumetanide (100 μM) inhibited basolateral NH_4^+ transport by 71% (Fig. 3). Therefore, the inhibitor profile results together with the results of ion dependency suggest that the bulk of basolateral NH_4^+ transport is mediated by NKCC in NHMEE cells.

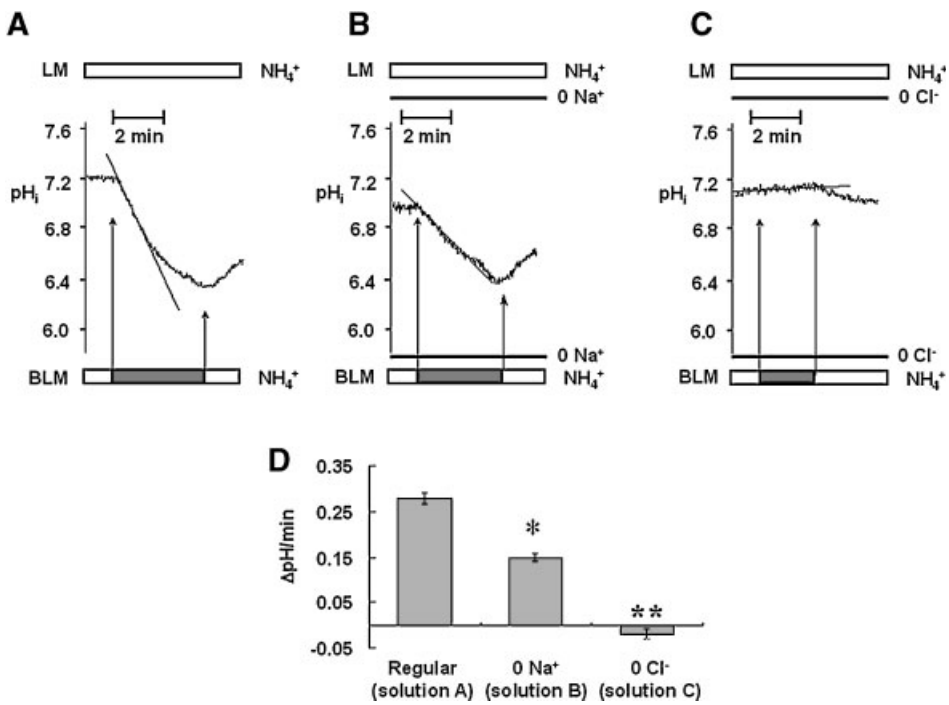

Fig. 2. Na^+ and Cl^- dependence of the basolateral NH_4^+-transporting system. The effects of Na^+ or Cl^- removal on basolateral NH_4^+-transport were analyzed in NHMEE cells. Representative traces of control, Na^+-, or Cl^--removal are presented in **panels A**, **B**, and **C**, respectively, and a summary (each n = 6) is shown in **panel 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 solutions used in panels A, B, and C are detailed in Materials and Methods section. $^*P < 0.05$, $^{**}P < 0.01$, difference from control.

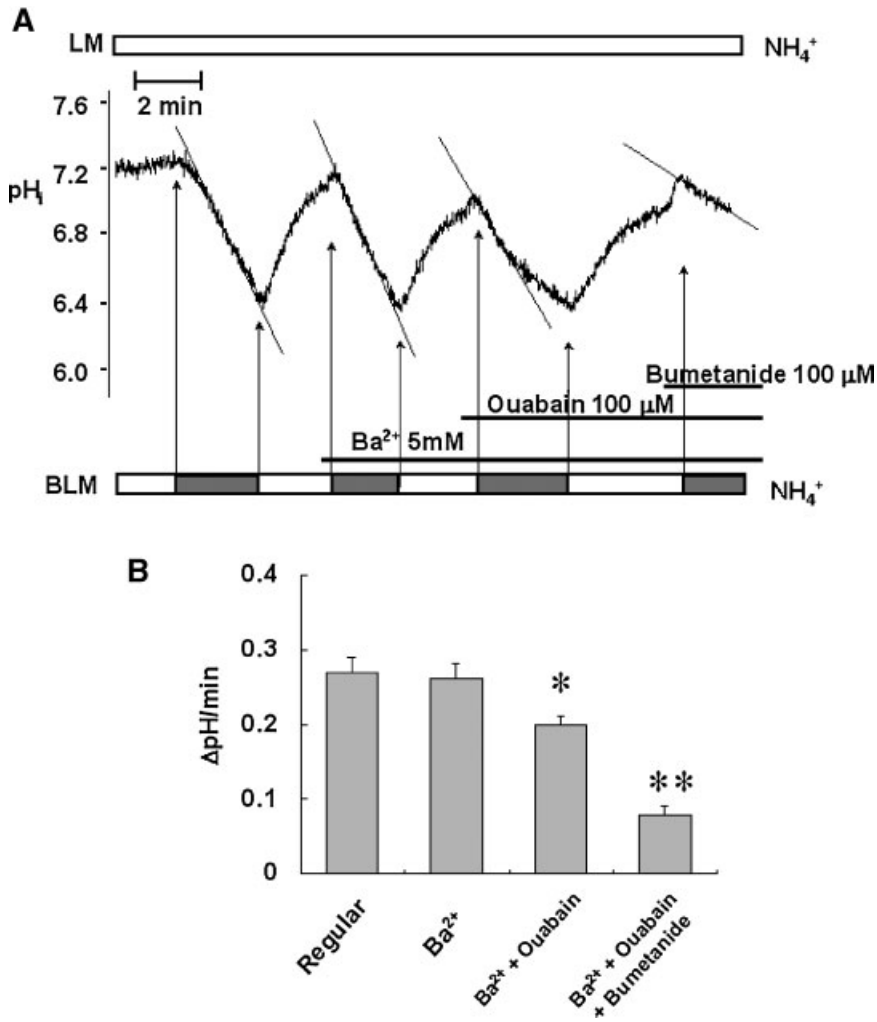

Fig. 3. Functional expression of NKCC in NHNE cells. Basolateral NH_4^+-transport was measured with solutions containing a non-specific K^+ channel inhibitor ($BaCl_2$), a Na^+/K^+ATPase inhibitor (ouabain), and a NKCC inhibitor (bumetanide). **A**: A representative trace showing the effects of $BaCl_2$ (5 mM), ouabain (100 μM), and bumetanide (100 μM). **B**: Summarized results of six experiments. $^*P < 0.05$, $^{**}P < 0.01$, difference from control.

Upregulation of NKCC by IL-1β Treatment

Accumulating evidence suggests that cytokines are involved in the modulation of transepithelial ion transport in various epithelia [Barmeyer et al., 2004; Dagenais et al., 2004]. To investigate the effects of IL-1β on NKCC, we treated NHMEE cells basolaterally with 10 ng of IL-1β for 12 h and then measured the pH_i in response to basolateral application of NH_4^+ (Fig. 4A–C). Bumetanide-sensitive NH_4^+ uptake under $BaCl_2^-$ and ouabain-pretreated conditions was considered as an indicative of NKCC activity. Interestingly, treatment of IL-1β increased the basolateral NKCC activity by 112% (Control, 0.170 ± 0.022 ΔpH unit/min; IL-1β treated, 0.361 ± 0.032).

A reverse-transcription PCR using RNAs from NHMEE cells revealed that NHMEE cells express mRNAs of NKCC1, but not NKCC2 (data not shown). Therefore, 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. 4D). Protein samples were collected after 4, 12, and 24 h treatments with IL-1β, and the expression of NKCC1 was compared with samples from non-treated cells. Importantly, IL-1β upregulated the expression of NKCC1 in a time-dependent

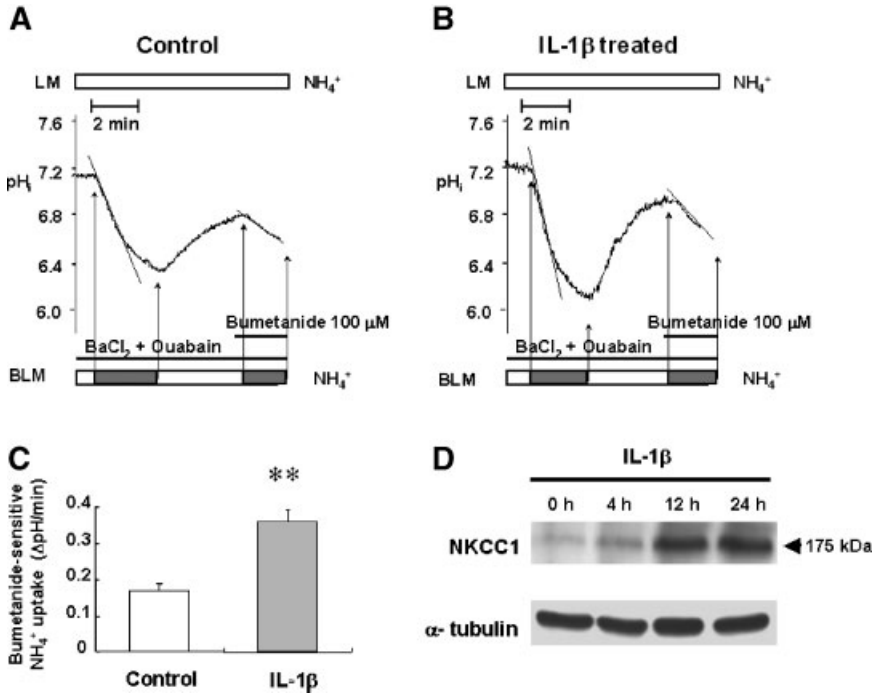

Fig. 4. Upregulation of NKCC1 activity by IL-1β treatment. Basolateral NH_4^+-uptake was measured after IL-1β treatment (10 ng/mL for 12 h). Representative traces of control and IL-1β treated cells are presented in **panels A** and **B**, respectively. To inhibit non-specific K^+ channel- and Na^+/K^+ ATPase-mediated NH_4^+ uptake, $BaCl_2$ (5 mM) and ouabain (100 μM) were pre-added to the basolateral solutions. **C**: Effects of basolateral IL-1β treatment on NKCC1 was determined by comparing the bumetanide-sensitive portion of the basolateral NH_4^+ uptake (ΔpH unit/min). **D**: Immunoblotting of protein extracts from NHMEE cells after treatment with IL-1β for designated times was performed using T4 anti-NKCC1 monoclonal antibody. Equal amounts of protein (50 μg) were loaded in each lane. Immunoblotting of α-tubulin was used for the determination of total protein loading.

manner, which reached a plateau 12 h after the treatment. NKCC1 expression was increased to 4.7 ± 1.2 fold ($n = 3$) by 12 h treatments of IL-1β in NHMEE cells. Subsequently, the expression of NKCC1 in NHMEE cells was verified by immunocytochemistry. Confocal microscopic images using anti-NKCC1 polyclonal antibody revealed that NKCC1 was predominantly expressed on the lateral surfaces of cultured NHMEE cells and its expression was increased after 12-h treatment of IL-1β (Fig. 5).

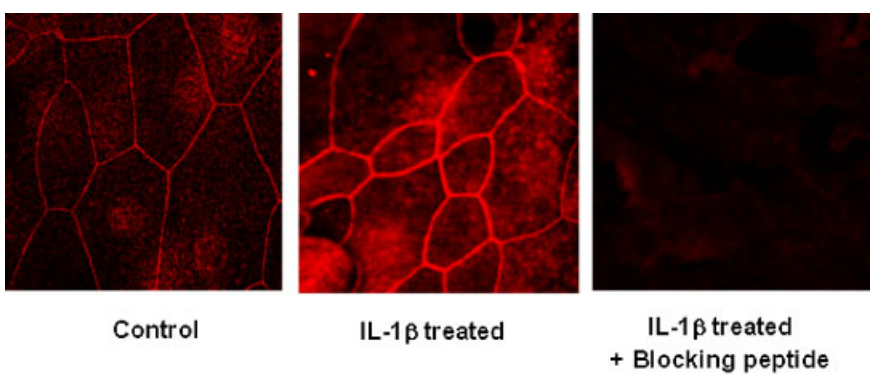

Fig. 5. Upregulation of NKCC1 expression by IL-1β treatment. Immunolocalizations of NKCC1 in NHMEE cells using a NKCC1 antibody. Note that NKCC1 shown as lateral staining in NHMEE cells was increased after 12 h IL-1β treatment (middle) compared with control (left), which was absent in preparations incubated with blocking peptide (right). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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 the fluid transport across NHMEE cells. In general, cultured airway epithelia possess both absorptive and secretory functions. In the resting state, middle ear epithelia absorb fluids by a Na^+-dependent mechanism. When 100 μL of KBR solution was applied on the luminal surface, cells absorbed the fluid in a time-dependent manner and 65.8 ± 1.9 μL of fluid remained 24 h after application. Interestingly, the addition of bumetanide (100 μM) to the basolateral side 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 the absorptive and secretory functions of 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-dependent fluid transport across NHMEE cells. When cells were pretreated with IL-1β basolaterally for 12 h (10 ng/ml), transepithelial fluid absorption was significantly reduced (Fig. 7A). Interestingly, the decreased fluid absorption after IL-1β treatment was almost completely reversed by bumetanide treatment (100 μM). Thus, bumetanide-sensitive fluid transport 24 h after fluid application, increased from 14.1 ± 1.8 to 28.3 ± 2.1 μL upon IL-1β treatment (Fig. 7B). These results indicate that IL-1β augments NKCC1-dependent fluid transport in NHMEE cells.

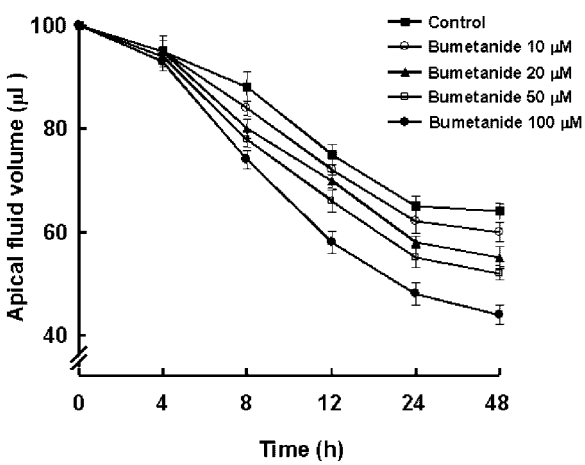

Fig. 6. Role of NKCC in the fluid transport of NHMEE cells. One hundred microliters of KBR solution with or without various concentrations of bumetanide was applied on the luminal surface and the remaining fluid volume was measured at the indicated time as described in Materials and Methods section. Bumetanide treatments inhibited fluid absorption in a dose dependant manner.

Upregulation of NKCC1 in the Middle Ear Epithelial Cells From Otitis Media Patients

Lastly, we investigated whether the level of NKCC1 expression in middle ear epithelial cells is altered in OME patients. The amount of middle ear epithelial cells from one patient is too small to perform immunoblotting. Therefore, we collected middle ear epithelial cells from 4 to 5 patients and mixed them as one pool. As shown in Figure 8, NKCC1 expression was elevated to an average of 2.2 ± 0.4 folds in the epithelial cell pools from OME patients compared with those from control subjects. These results suggest that middle ear inflammation indeed increases the expression of NKCC1 in epithelial cells.

DISCUSSION

OME, 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, and can lead to language deficits. Although multi-factorial pathogenic mechanisms such as negative middle ear pressures and bacterial infections have been suggested, the exact molecular mechanisms of fluid collection in the middle ear cavity are not well understood. Hypersecretion of periciliary fluid is one of the important mechanisms of fluid collection [Lim et al., 2000]. It is generally assumed that the fluid secretion in respiratory-type epithelia is driven by the Cl^- transport from the basolateral to apical sides [Furukawa et al., 1997]. However, the specific electrolyte transport mechanisms that generate the driving force for the fluid transport, and the location of the associated transporters in the polarized middle ear epithelia, remain to be elucidated.

Our investigation addressed the question of whether NKCCs are expressed in middle ear epithelial cells. By measuring pH_i changes due to NH_4^+ uptake, we demonstrated the

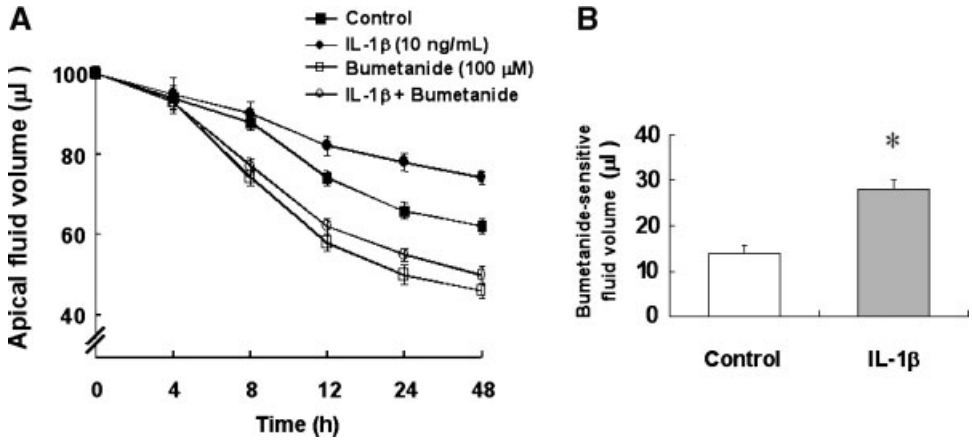

Fig. 7. Augmentation of bumetanide-sensitive fluid transport by IL-1β. **A**: After application of 100 μL of KBR solution on control and IL-1β-treated cells with or without bumetanide, the remaining fluid volumes were measured at the indicated time. **B**: Bumetanide-sensitive fluid volume 24 h after the luminal fluid application was calculated (n = 5).

presence of a bumetanide-sensitive NH_4^+ uptake in the basolateral membrane of cultured NHMEE cells. The bulk of the NH_4^+ 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 NHMEE cells by immunoblotting. These data are similar to the results from nasal [Shin et al., 2004] and tracheobronchial [Zeitlin et al., 1988] epithelia where NKCC1 plays a major role in $[Cl]_i$ uptake by 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 bumetanide in the presence of $BaCl_2$ and ouabain, and was more dependent on Cl^- than on Na^+ (Figs. 2 and 3). These data indicate that 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, electrochemical gradients of KCCs are set to exit K^+ and Cl^- ions from the cells. Therefore, basolateral KCCs would not contribute to the transepithelial Cl^- and fluid secretion toward the luminal side.

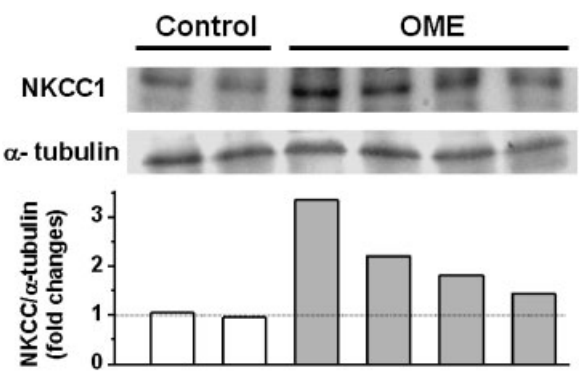

Fig. 8. Upregulation of NKCC1 in middle ear epithelial cells from otitis media patients. Immunoblotting of NKCC1 was performed with protein extracts of middle ear epithelial cells obtained from OME patients (4 pools from 21 patients) and was compared with samples from control subjects (2 pools form 9 patients). Equal amounts of protein (50 μg) were loaded in each lane. The staining intensities of immunoblots were calibrated and the NKCC1/α-tubulin ratios versus the average of two control samples are presented in the **lower panel**.

In a wide variety of secretory epithelia, including those of the intestines [Grubb et al., 2000] and salivary glands [Evans et al., 2000], the activity of basolateral NKCC1 is the rate-limiting step of transepithelial Cl^- fluxes when apical Cl^- channels are activated. Previous animal studies have also shown the importance of NKCC1 in the fluid transport of airway epithelia. In fetal sheep lung, the addition of the loop diuretics bumetanide or furosemide to lung liquid reduced luminal liquid production [Cassin et al., 1986; Carlton et al., 1992]. 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 μL 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 the bulk of fluids by a Cl^--driven mechanism. In general, cultured airway epithelia show both absorptive and secretory activities depending on the situation. 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 for fluid transport. In our experimental conditions, NHMEE cells at resting state dominantly absorb luminal fluid in a time-dependent manner for up to 24 h. Interestingly fluid absorption was substantially increased by basolateral NKCC1 inhibition (Fig. 7). These data strongly suggest that NKCC1 significantly contributes to electrolyte and fluid transport across middle ear epithelia.

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 [Dharmsathaphorn et al., 1985; Lytle and Forbush, 1996; Shin et al., 2004]. Recently, there has been an increasing body of evidence supporting the important role of cytokines in the modulation of ion channels and transporters. IL-1β is one of the major pro-inflammatory cytokines produced by macrophages. It has been shown that IL-1β alters Na^+ absorption and fluid transport in the airway epithelia [Dagenais et al., 2004]. Moreover, previous reports demonstrated that the effusion of otitis media contains high levels of IL-1β [Yellon et al., 1991]. These findings prompted us to investigate the effects of IL-1β on NKCC activity in middle ear cells.

As shown in Figures 4 and 5, IL-1β increased the bumetanide-sensitive NH_4^+ transport and NKCC1 expression in the basolateral membrane of NHMEE cells. The activation of NKCC1 by IL-1β would lead to an increased $[Cl]_i$, which would increase the driving force for fluid secretion in epithelial cells. These results are consistent to the results from endothelial cells where cytokines including IL-1β upregulated NKCC [Topper et al., 1997]. Our results also suggest activation of NKCC1 activation by cytokines in middle ear effusions is possibly associated with otitis media. Accordingly, we confirmed that NKCC1 is upregulated in middle ear epithelial cells from OME patients. The above results imply that cytokines can upregulate NKCC1 in middle ear epithelia, which may in turn offer a major driving force for fluid hypersecretion in OME patients. NKCC activity can be regulated by various factors such including vasoactive intestinal polypeptide and ATP. However, such upregulation of NKCC1 by pro-inflammatory cytokines might be involved in hypersecretions observed in other inflammatory airway diseases 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 activate NKCC1 in middle ear inflammation need to be determined in future studies.

In conclusion, we found that NKCC is present in the basolateral membrane of and contributes to fluid transport across middle ear epithelial cells. More importantly, the inflammatory cytokine IL-1β increased NKCC1 expression and augmented NKCC-mediated fluid transport in middle ear epithelia. In addition, upregulated NKCC1 expression was demonstrated in middle ear cells from OME patients. The above results suggest that upregulation of NKCC1 by inflammatory cytokines could be an important underlying mechanism of excess fluid collection in OME.

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