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Interferon-γ Suppresses Na^+–H^+ Exchanger in Cultured Human Endolymphatic Sac Epithelial Cells

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ABSTRACT

Adequate regulation of endolymphatic pH is essential for maintaining inner ear function. The Na^+–H^+ exchanger (NHE) is a major determinant of intracellular pH (pH_i), and facilitates Na^+ and fluid absorption in various epithelia. We determined the functional and molecular expression of NHEs in cultured human endolymphatic sac (ES) epithelial cells and examined the effect of IFN-γ on NHE function. Serial cultures of human ES epithelial cells were generated from tissue samples. The molecular expression of NHE1, -2, and -3 isoforms was determined by real-time RT-PCR. The functional activity of NHE isoforms was measured microfluorometrically using a pH-sensitive fluorescent dye, 2′,7′-bis(carbonylethyl)-5(6)-carboxyfluorescein (BCECF), and a NHE-inhibitor, 3-methylsulfonyl-4-piperidinobenzoyl guanidine methanesulfonate (HOE694). NHE1, -2, and -3 mRNAs were expressed in human ES epithelial cells. Functional activity of NHE1 and -2 was confirmed in the luminal membrane of ES epithelial cells by sequentially suppressing Na^+-dependent pH_i recovery from intracellular acidification using different concentrations of HOE694. Treatment with IFN-γ (50 nM for 24 h) suppressed mRNA expression of NHE1 and -2. IFN-γ also suppressed functional activity of both NHE1 and -2 in the luminal membrane of ES epithelial cells. This study shows that NHEs are expressed in cultured human ES epithelial cells and that treatment with IFN-γ suppresses the expression and functional activity of NHE1 and -2. J. Cell. Biochem. 107: 965–972, 2009.

KEY WORDS: ENDOLYMPHATIC SAC; ION CHANNEL; INTERFERON-γ; Na^+–H^+ EXCHANGER

The endolymphatic sac (ES) belongs to the nonsensory portion of the membranous labyrinth in the inner ear. ES epithelium, which is composed of heterogeneous cell type [Lundquist, 1965], plays an important role in regulating fluid volume of the endolymph. Surgical ablation of ES in experimental animals induces endolymphatic hydrops [Kimura and Schuknecht, 1965]. Maintaining pH homeostasis of endolymph is essential for the normal function of the inner ear [Couloigner et al., 2000]. Alteration of ES endolymph pH causes hearing loss in various situations [Wangemann et al., 2007]. ES epithelial cells are strong candidate for regulator of endolymphatic pH because of their strong immunoreactivity for the proteins such as H^+-ATPase and anion exchangers [Stanković et al., 1997]. Especially, the pH of the ES endolymph is relatively acidic than those of cochlear and vestibular endolymph [Tsujikawa et al., 1992], which indicates that ES epithelial cells have specific pH regulatory system. However, the exact mechanism of maintaining acidic pH of the endolymph in ES is unclear.

Na^+–H^+ exchanger (NHE) family exchanges Na^+ and H^+ according to their concentration gradients. The NHE gene family has been shown to include different isoforms (NHE1–NHE9), and NHE1, NHE2, and NHE3 isoforms are the most characterized members [Orlowski and Grinstein, 2004]. The NHE plays an essential role in intracellular pH (pH_i) homeostasis, cell volume regulation [Grinstein et al., 1992], transepithelial Na^+ absorption [Tse et al., 1993a]. Furthermore, NHEs regulate the pH of body fluid in kidney [Petrovic et al., 2004], airway surface liquid [Choi et al., 2006], intestines [Muller et al., 2000]. Previous studies have also suggested the presence of NHEs in outer hair cells [Ikeda et al., 1996] and vestibular dark cells [Wangemann et al., 1996]. Wu and Mori [1998] also showed the existence of Na^+-dependent H^+ efflux in the guinea pig ES. These findings suggest the possible role of NHEs in regulating the endolymph pH.

Interferon (IFN)-γ, which is produced by Th1 lymphocytes, has multiple effects including antiviral activity, augmentation of major

Eun Jin Son and In Seok Moon contributed equally to this work.

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histocompatibility complex expression, and stimulation of T and natural killer (NK) cells [Gattoni et al., 2006]. Accumulating evidence has implicated the role of IFN-γ in chronic inflammatory diseases including irritable bowl syndrome through the suppression of NHEs and the resultant failure of Na^+ absorption [Magro et al., 2005]. An immunologic mechanism for endolymphatic hydrops, which is the underlying pathophysiology of Meniere's disease, has also been suggested [Derebery, 1996; Keles et al., 2004]. IFN-γ is induced in the inner ear of immunized animals in response to secondary antigen challenge in the ES [Pawankar et al., 2004]. In addition, the Th1 cell subset is increased significantly in the serum of patients with acute, low-tone sensorineural hearing loss associated with Meniere's disease [Fuse et al., 2003].

The molecular mechanisms responsible for the function of the ES are complicated to study because of the difficulties in approaching the tissue in vivo. Recently, we developed a reliable culture system for human ES epithelial (HESE) cells [Kim et al., 2008], which enable us to differentiate cultured endolymphatic epithelial cells into both mitochondrial-rich cell and ribosomal rich cells. Here, we demonstrate the molecular and functional expression of NHEs in cultured HESE cells. We also show that IFN-γ suppresses functional and molecular expression of NHE1 and -2. These results support an immunologic mechanism in Meniere's disease and autoimmune hearing loss.

MATERIALS AND METHODS

CELL CULTURE

Human ESs were excised during acoustic neuroma surgery (n = 18) using a translabyrinthine approach. All procedures were approved by the Institutional Review Board of Yonsei Medical Center. The sac is separated from the posterior bony surface with a mucosal knife and cut with a pair of microscissors at the external aperture of the vestibular aqueduct. Primary cultures of HESE cells were performed as described previously [Kim et al., 2008]. Epithelial cells were isolated from the tissue by treatment with 1% Pronase (type XIV protease; Sigma, St. Louis, MO) for 18 h at 4°C. To remove fibroblasts and endothelial cells, isolated cells were placed in a plastic dish and cultured for 30 min at 37°C. Epithelial cells were subcultured in a 1:1 mixture of bronchial epithelial cell basal medium and Dulbecco's modified Eagle's medium containing insulin (5.0 μg/ml), hydrocortisone (0.5 μg/ml), epinephrine (0.5 μg/ml), triiodothyronine (6.5 μg/ml), transferrin (10 ng/ml), gentamicin (50 μg/ml), and amphotericin (50 ng/ml), all supplied by Clonetics Corp. and further supplemented with epidermal growth factor (EGF, 25 ng/ml; Collaborative Res., Bedford, MA), all-*trans*-retinoic acid (50 nM; Sigma), and bovine pituitary extract (1%, v/v, Pel Freez). The culture medium was changed every other day. Cells were dissociated when they reached 50–60% confluence with 0.25% trypsin:EDTA (Clonetics Corp.) and 2,000 cells/cm^2 were seeded for subsequent passage. For differentiation, Passage-2 HESE cells (10^5 cells per culture) were seeded onto semi-permeable membranes in each well. The medium used for differentiation was the same as that used for the subculture with the exception of a lower concentration of EGF (0.5 ng/ml). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2.

MEASUREMENT OF INTRACELLULAR pH (pH_i) AND NHE ACTIVITY

Measurement of pH_i in culture monolayers was performed based on previously reported protocols with a slight modification [Namkung et al., 2003]. Passage-2 HESE cells loaded with the pH-sensitive fluorescent probe bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM, 2.5 μM) for 10 min at room temperature and mounted in a miniature Ussing chamber attached to the stage of an inverted microscope. The miniature Ussing chamber consisted of top (mucosal) and bottom (serosal) half-chambers (volume = 250 μL each) made from light-absorbing polyacetal. A Transwell wafer containing the polarized epithelial monolayer is mounted between the two half-chambers with the mucosal surface up, and the two half-chambers are tightly sealed together. A glass coverslip was affixed to the bottom of the serosal chamber with dental sticking wax (model Deiberit-502; Ludwig Bohme). The mucosal chamber is open to the atmosphere, and both half-chambers have inlet and outlet ports to allow solution to flow. Serosal and mucosal perfusates are heated to 37°C and delivered to the chamber by gravity flow (rate = 3–5 ml/min). BCECF fluorescence was calibrated and recorded using a previously described protocol [Namkung et al., 2003]. Briefly, fluorescence at excitation wavelengths of 490 and 440 nm was recorded using a recording setup (Delta Ram; PTI, Inc.), and 490/440 ratios were calibrated intracellularly by perfusing the cells with solutions containing 145 mM KCl, 10 mM HEPES, and 5 μM nigericin (pH 6.0–7.6).

REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION

NHE1, -2, -3, and β2 microglobulin (β2-M) mRNA was quantified using TagMan probes. One microgram of cDNA, oligonucleotides at a final concentration of 800 nM of the primers and TaqMan hybridization probe (200 nM) were used in a total volume of 25 μl. The probe used for real-time polymerase chain reaction (RT-PCR) was labeled with carboxyfluoroscein (FAM) at the 5′-end and with a quencher carboxytetramethylrhodamine (TAMRA) at the 3′-end. PCR primer used for human NHE1, -2, and -3 and TaqMan probes (P) were as follows: *NHE1*: F: 5′-ATG ATG CGG AGC AAG GAG ACT-3′, R: 5′-GTC ACT GAG GCA GCG CTG TAT-3′, P: 5′-TCT TCA CCC CCG CGC CCA T-3′; *NHE2*: F: 5′-TGG AGG GCA TCG GAA CCT-3′, R: 5′-TCG CTG CTT CTC TTA AGG CTT CT-3′, P: 5′-CCC AAA TCG GGC TTT CCG GCT TTA-3′; *NHE3L*: F: 5′-GAG GAC ATA TCC CAG-3′, R: 5′-CCT TCA GGT TCA GCT CGT AGG-3′, P: 5′-TGG TTT GGT TTA TTC CAA GTT TGG TAA-3'; *β2-M*: F: 5′-CGCTCCGTGGCCTTAGC-3′, R: 5′-GAGTACGCTGGATAGCCTCCA-3′, P: 5′-TGCTCGCGCTACT-CTCTCTTTC-TGGC-3′. Real-time RT-PCR was performed using a PE Biosystems ABI PRISM 7700 Sequence Detection System. The thermocycler parameters were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate.

SOLUTIONS AND CHEMICALS

The HEPES-buffered solution contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). The Na^+-free solution contained 140 mM *N*-methyl-D-glucamine chloride (NMDG-Cl), 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.4 in Tris-base). The osmolarity of all solutions was adjusted to 310 mOsm with

the major salt prior to use. BCECF-AM was purchased from Molecular Probes (Eugene, OR). 3-Methylsulfonyl-4-piperidino-benzoyl guanidine methanesulfonate (HOE694) was a generous gift form Dr. O.W. Moe (University of Texas Southwestern Medical Center, Dallas, TX). The specific NHE3 inhibitor, S3226 (3-[2-(3-guanidino-2-methyl-3-oxopropenyl)-5-methyl-phenyl]-*N*-isopropylidene-2-methylacrylamide dihydrochloride), was kindly provided by Aventis Pharma (Frankfurt, Germany). All other chemicals, including IFN-γ, were purchased from Sigma.

STATISTICAL ANALYSIS

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

RESULTS

MORPHOLOGY OF CULTURED HUMAN ES EPITHELIAL CELLS

Cultures from 4 specimens (out of 18) failed due to the fibroblast contamination. Cells formed a confluent sheet 9 days after being seeded and differentiated. SEM showed polygonal shaped cells with abundant microvilli, indicating polarized epithelial cells (Fig. 1A). As reported previously, 5–10% of the cultured cell differentiated into mitochondria-rich cell with long microvilli on their apical surfaces (Fig. 1B). More than 40% of the cultured cell differentiated into intermediated cells having both characteristics of mitochondrial-rich cell and ribosomal-rich cells (Fig. 1C). We assumed that these two types of the cultured cell involved in ion transport and fluid absorption in cultured HESE cells.

STEADY-STATE pH_i AND INTRINSIC BUFFER CAPACITY (β_i) IN HEPES-BUFFERED SOLUTIONS

The steady-state pH_i of HESE cells bathed in HEPES-buffered solution was 7.09 ± 0.04 (mean ± SD, n = 11). In each experiment, the buffer capacity (β_i) was calculated in Na^+-free, HEPES-buffered solution. BaCl (1 mM) was included to prevent the movement of NH_4^+ through K^+ channels. β_i was calculated at the mid-point of each resulting step change in pH_i (ΔpH_i) as $\beta_i = \Delta[NH_4^+]_j/\Delta pH_i$, where $\Delta[NH_4^+]_j$ is the change in the intracellular NH_4^+ concentration. Assuming that $[NH_3]_i$ is equal to the extracellular NH_3 concentration, and that pK_a of NH_4Cl is 8.9 at 37°C, $[NH_4^+]_i$ was calculated from the known pH_i using the Henderson–Hasselbalch equation. The data for β_i against pH_i were fitted with a second-order polynomial expression: $\beta_i = 78.502\ (pH_i)^2 - 1{,}137.492\ (pH_i) + 4{,}127.061$. Figure 2 shows that β_i was very low at this average resting pH_i and increased steeply at acidic pH_i.

RECOVERY FROM ACID LOAD IN HEPES-BUFFERED SOLUTIONS

NHE activity was interpreted as Na^+-induced pH_i recovery from an acid load achieved by exposure to 40 mmol/L NH_4Cl using the methods of Roos and Boron [1981] with modification. Figure 3 shows a typical pH-recovery experiment for the determination of NHE activity. Removal of extracellular Na^+ significantly acidified the cells by 0.18 ± 0.06 pH units in 33 ± 4 min (n = 7, $P < 0.05$), suggesting that an Na^+-dependent process was maintaining steady-state pH_i. Addition of NH_4^+ to the luminal membrane produced rapid intracellular alkalinization (7.81 ± 0.16) due to the influx of NH_3. Subsequent removal of extracellular NH_4^+ caused a rapid fall in pH_i due to dissociation of NH_4^+ into H^+ (which remains in the cells) and NH_3 (which rapidly leaves the cytoplasm). This caused a large shift in the pH_i to 6.51 ± 0.08, significantly below the starting value. When Na^+-containing solution was applied to the apical cell surface, the pH_i subsequently recovered (at an average rate of 0.368 ± 0.091 ΔpH_i/min), as a result of NHE activity. Typically, the data from first 10–40 s of initial pH_i recovery were fitted to a linear equation using Felix software (version 1.4; PTI, Inc.). Treatment with IFN-γ did not significantly change β_i. Therefore, all NHE activity measurements are expressed as ΔpH_i/min, and this value was directly analyzed without compensating for β_i.

FUNCTIONAL ACTIVITY OF NHE ISOFORMS IN HUMAN ES EPITHELIAL CELLS

RT-PCR was performed to detect NHE1, -2, and -3 in cultured HESE cells. As shown in Figure 4, NHE1 (245 bp), NHE2 (276 bp), and NHE3 (365 bp) were all expressed in the cultured HESE cells. To

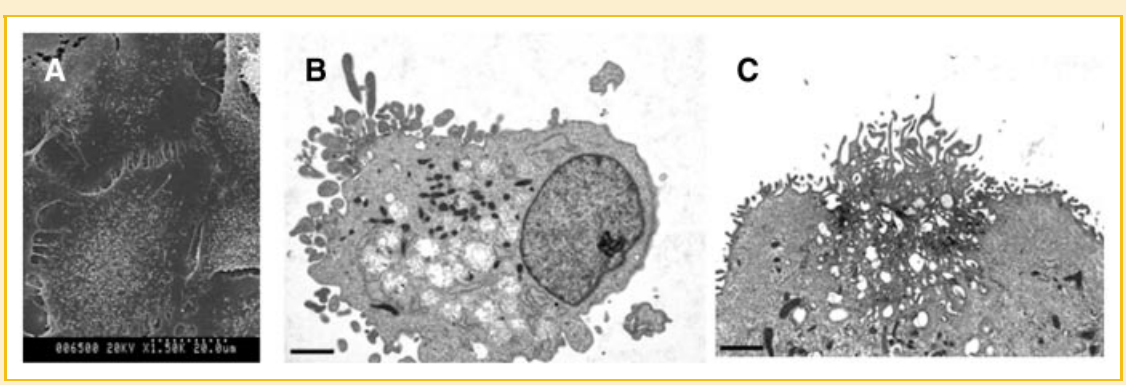

Fig. 1. Appearance of passage-2 human endolymphatic sac (ES) epithelial cells. A: SEM image of cultured cells 14 days after being seeded. Polygonal shaped cells with abundant microvilli are observed. B: Mitochondria-rich cell which has long microvilli on their apical surface and numerous mitochondria in their cytoplasm. C: Intermediated cells had some mitochondria and ER, and especially well-developed cytoplasmic projections, such as irregular-shaped microvilli and long filopodia on the apical cell surface.

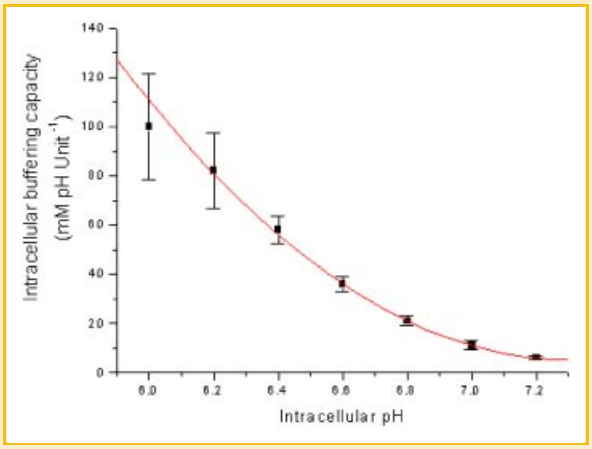

Fig. 2. The relationship between pH_i and intrinsic buffer capacity (β_i). Data gathered in intervals of 0.2 pH units are shown as mean ± SE (n = 11). The continuous line was fitted using a second-order polynomial function.

distinguish the role of NHE isoforms in Na^+-induced pH_i recovery, an NHE-specific inhibitor (HOE694) was used. Prior studies have established the following inhibitor constant (K_i) values for the NHE1, -2, and -3 isoforms: 0.16, 5.0, and 650 μM, respectively [Counillon et al., 1993]. As previously mentioned, the initial rate of luminal NHE activity was 0.368 ± 0.091 ΔpH_i/min. The recovery rate in the presence of 1 μM HOE694 decreased to 0.269 ± 0.087 ΔpH_i/min. Na^+-induced pH_i recovery was almost completely suppressed to 0.087 ± 0.012 ΔpH_i/min by 5 μM HOE694 (Fig. 5A,B). Addition of 650 μM HOE694 showed no further suppression of the recovery rate (0.069 ± 0.019 ΔpH_i/min). These results suggest that NHE1 and

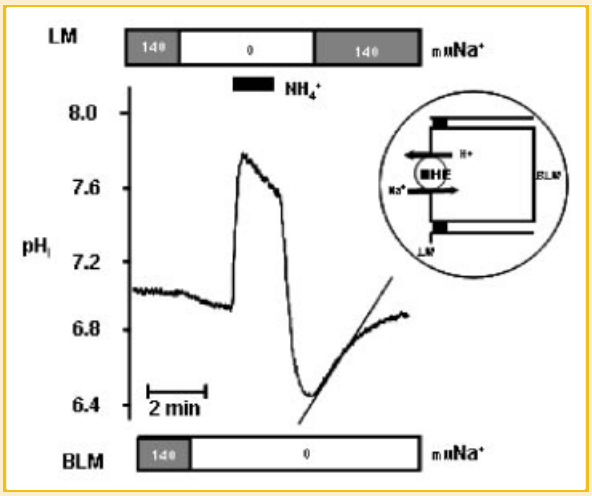

Fig. 3. Na^+/H^+ exchanger (NHE) activity in cultured HESE cells. The pH_i was measured using BCECF as described in Materials and Methods Section. Membrane-specific NHE activity was analyzed using a double perfusion chamber and separate applications of 140 mM Na^+. Application of Na^+ to the luminal membrane evoked an increase in pH_i. LM, luminal membrane; BLM, basolateral membrane.

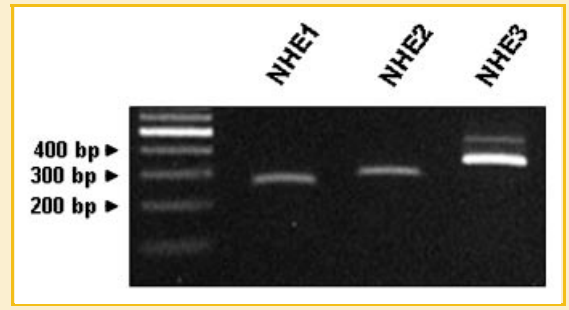

Fig. 4. RT-PCR of Na^+/H^+ exchanger (NHE) isoforms in cultured human ES epithelial cells. NHE1 (245 bp), NHE2 (276 bp), and NHE3 (365 bp) were all expressed.

-2 (NHE1 ≪ NHE2) regulate pH_i in the luminal membrane of ES epithelial cells. Although NHE3 mRNA and proteins are expressed, it seems not to play a major role in regulating NHE activity. We further confirmed that S3226 (20 μM), NHE-3-specific inhibitor, does not affect the Na^+-induced pH_i recovery. However Na^+-dependent pH_i recovery was further inhibited by 25 μM 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), which blocks all NHE isoforms at this concentration (Fig. 5C). These data indicate that the remainder of the Na^+-dependent pH recovery after treatment of 650 μM HOE694 could be due to another NHE isoforms besides NHE1, NHE2, or NHE3. The recovery of pH_i from an acid loading still occurs very slowly with Na^+-free solution, which is not sensitive to NHE inhibitors (Fig. 5C). Our data indicate that Na^+-independent H^+ extrusion system also exists in cultured HESE cells.

THE EFFECT OF IFN-γ ON FUNCTIONAL ACTIVITY OF NHEs

The effect of IFN-γ on Na^+-induced pH_i recovery was then evaluated. Na^+-induced pH_i recovery rate was gradually decreased with IFN-γ (50 nM) until 24 h and unchanged thereafter (Fig. 6B). Na^+-indepedent pH_i was not affected by IFN-γ treatment (data not shown). Therefore, we performed the following experiments after 24 h INF-γ treatment. After exposure to 50 nM IFN-γ for 24 h, the recovery rate was significantly decreased: to 0.198 ± 0.122 ΔpH_i/min in 140 mM Na^+; to 0.129 ± 0.051 ΔpH_i/min in 1 μM HOE694; and to 0.031 ± 0.081 ΔpH_i/min in 50 μM HOE694 (Fig. 6A). More specifically, Na^+-induced pH_i recovery rate in 1 μM HOE694 decreased about 30% after 24 h IFN-γ treatment (to 0.069 ± 0.012 ΔpH_i/min, compared to 0.099 ± 0.019 ΔpH_i/min in control cells; $P < 0.05$). In contrast, the recovery rate in 5 μM HOE694 decreased about 50% after IFN-γ treatment (to 0.098 ± 0.020 ΔpH_i/min, compared to 0.182 ± 0.038 ΔpH_i/min in control cells; n = 7, $P < 0.01$, Fig. 6C). These results suggest that IFN-γ treatment mainly affects NHE2 activity in the luminal membrane of HESE cells.

We also determined the effect of IFN-γ treatment on NHE isoform expression using real-time RT-PCR. The treatment with 50 nM IFN-γ produced a marked reduction in NHE1 and -2 expression (NHE1 by 0.44 ± 0.09-fold, NHE2 by 0.35 ± 0.06-fold, n = 3, $P < 0.05$), while NHE3 expression was unchanged after IFN-γ treatment (0.97 ± 0.10-fold) (Fig. 7).

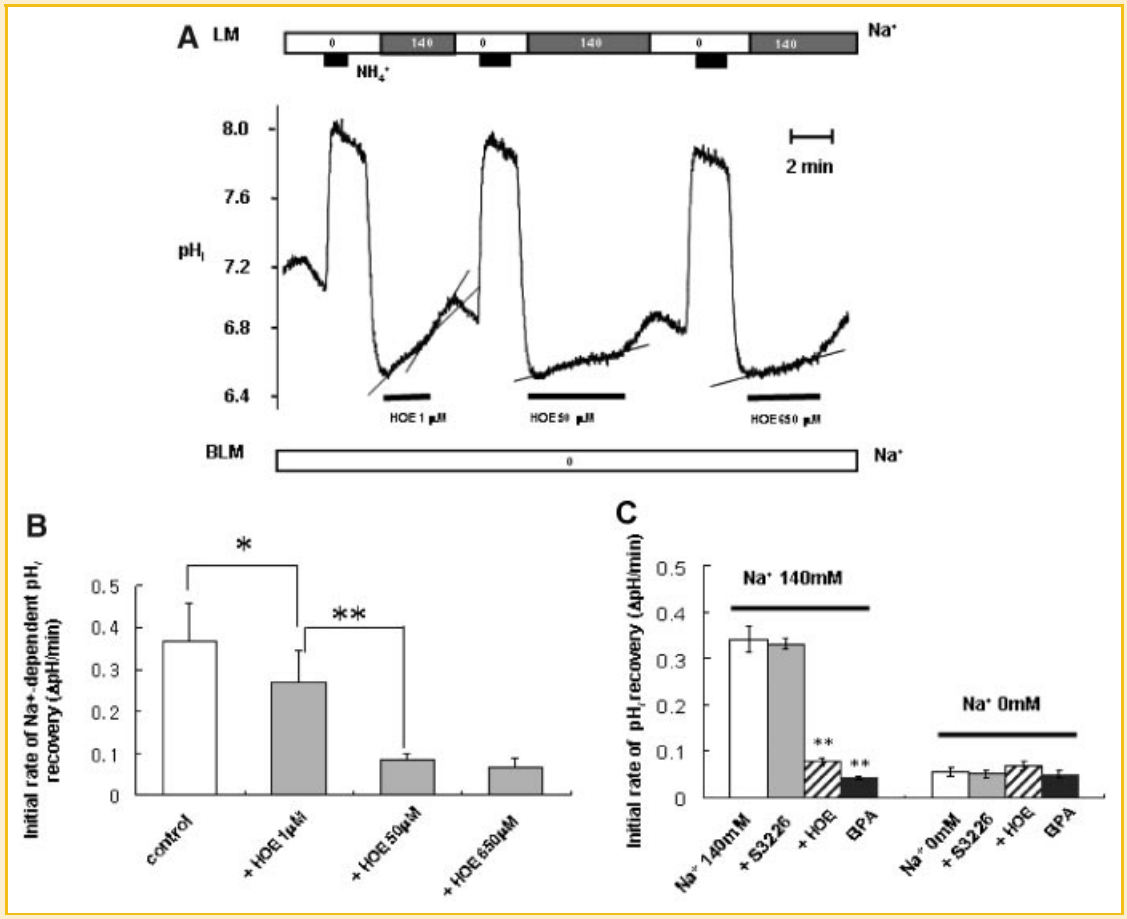

Fig. 5. The role of Na^+/H^+ exchanger (NHE) isoforms in pH_i regulation in the luminal membrane of cultured human ES epithelial cells. The Na^+-induced pH_i recovery rate was measured in the presence of different concentrations of HOE694. A: A representative trace showing the effects of HOE694. LM, luminal membrane; BLM, basolateral membrane. B: Summarized results of seven experiments. C: Effects of NHE inhibitors on Na^+-dependent and Na^+-independent pH_i recovery rate. $^*P < 0.05$, $^{**}P < 0.01$ compared to the control.

DISCUSSION

The ES is covered with a monolayer of heterogeneous epithelial cells such as mitochondrial-rich cells and ribosomal-rich cells [Peters et al., 2002]. Among them, mitochondrial-rich cells are thought to play an important role in ion transport and fluid absorption. As we previously reported [Kim et al., 2008], 5–10% cultured cells have morphological characteristics of mitochondrial cells. In addition, 40–45% of cells feature characteristics of absorptive epithelia such as long microvilli, mitochondria, and pilopodia. We assumed that these two cell types are responsible for ion transport and fluid absorption in ES.

Maintaining adequate pH of endolymph is essential for inner ear function. Wangemann et al. [2007] presented strong evidence that hearing loss of pendrin knockout mice results from the acidification of the endolymph. Interestingly, the pH of endolymph in ES is more acidic than that of cochlear suggesting that H^+ is being secreted into the endolymph. ES epithelial cells express multiple proteins maintaining optimal acid–base balance. Vacuolar-type H^+-ATPase, which secretes H^+, is expressed in ES [Dou et al., 2004], and its inhibitor, Bafilomycin, causes alkalization of endolymph in guinea pigs [Couloigner et al., 2000]. Pendrin, which secretes HCO_3^-, is also expressed in ES epithelia [Royaux et al., 2003]. However, the exact mechanism of pH homeostasis of ES endolymph is still not fully understood in human. A previous study suggests a molecular expression of NHEs in the inner ear [Ikeda et al., 1996; Wangemann et al., 1996]. Furthermore, Wu and Mori [1998] found a Na^+-dependent pH_i regulating mechanism sensitive to EIPA in guinea pig ES tissue. Limitations of using ES tissue specimens were that neither the localization of NHE expression to either luminal or basolateral cell membranes nor confirmation of the activity of different NHE isoforms were possible. In this study, we demonstrated the molecular expression of NHEs. Functional activity of NHE as measured by Na^+-induced pH_i recovery form an acid load was sensitive to specific concentration of HOE694 to suggest that NHE2 mediates most of the NHE activity in the luminal membrane of the HESE cells. NHE2 is the predominant epithelial isoform in the luminal membrane of numerous epithelia and participates in transepithelial Na^+ transport [Tse et al., 1993b; Hoogerwerf et al., 1996]. It is noteworthy to mention that NHE1 also has a minor but significant

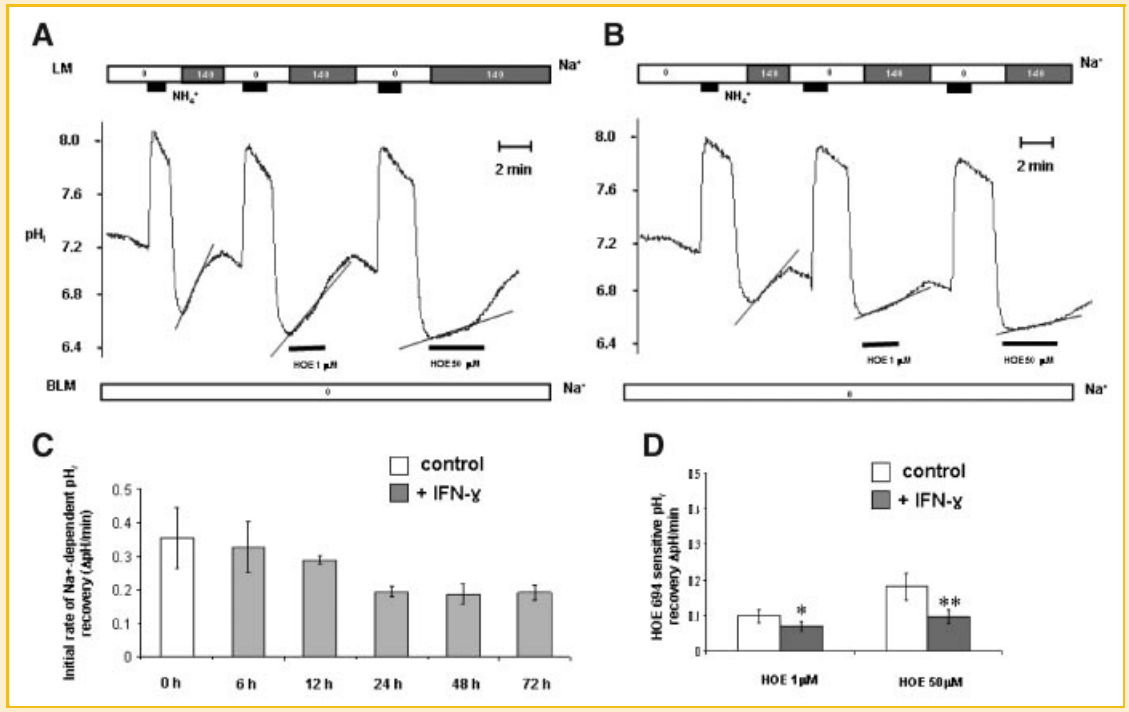

Fig. 6. Suppression of Na^+-induced pH_i recovery by IFN-γ. The Na^+-induced pH_i recovery rate was measured in the luminal membrane of cultured human ES epithelial cells after treatment with IFN-γ. Representative traces of control and IFN-γ-treated samples (50 ng/ml, 24 h) are presented in (A–C). Time course of suppression of Na^+-induced pH_i recovery by IFN-γ (50 ng/ml, n = 7). D: Effects of IFN-γ on NHE1 and -2 were determined by comparing to the HOE694 (1 and 5 μM, respectively)-sensitive ΔpH/min. LM, luminal membrane; BLM, basolateral membrane. $^*P < 0.05$; $^{**}P < 0.01$ compared to control.

role in pH_i regulation in the luminal membrane of cultured human ES cells. NHE1 is proposed to regulate pH_i and is thought to be constitutively expressed in basolateral membranes [Ritter et al., 2001]. The exact functional role of NHE1 in ES is also needed to be elucidated with further studies. Another critical observation in the work by Wu and Mori [1998] is that the Na^+-dependent pH_i regulating mechanism was not active in the range of steady-state pH_i, implying that the role of NHE in the inner ear fluid homeostasis in vivo may be limited. However, our results show that in cultured HESE cells such Na^+-dependent pH_i regulating mechanism does function in the steady state. We believe that vectorial H^+ transport by NHE2 plays an important role in regulating pH of endolymph in human ES.

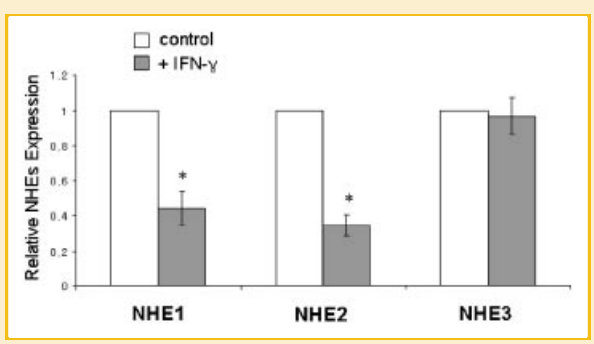

Fig. 7. Effect of IFN-γ treatment (50 nM, 24 h) on NHEs expression. Real-time RT-PCR shows decreased NHE1 and -2 expressions after IFN-γ treatment. Data were normalized to β2-microglobin expression. n = 3, $^*P < 0.05$ compared to the control.

Our preliminary data also showed that H^+-ATPase and pendrin involved in regulating pH_i in cultured HESE cells. Now we are performing experiments about the interaction of these ion transporters in acid–base balance of cultured HESE cells. In addition to pH regulation, Na^+ transport coupled to H^+ secretion by NHE could be a mechanism underlying fluid absorption in several absorptive epithelia, including the intestines and the kidney [Petrovic et al., 2004; Muller et al., 2000]. The molecular and functional expression of NHEs suggests their role in Na^+ and fluid absorption in human ES.

The pathogenesis of the endolymphatic hydrops underlying Meniere's disease is still unclear, although an autoimmune basis has been suggested [Bouman et al., 1998]. The Th1 subset of lymphocytes, in particular, seems to be increased in Meniere's disease [Fuse et al., 2003], and IFN-γ production by Th1 cells is elevated in animal models [Tomiyama, 2001; Pawankar et al., 2004]. IFN-γ has been shown to downregulate expression of NHEs in Caco-2 cells and rat intestinal brush-border membranes by direct suppression of transcription or by a nongenomic pathway [Rocha et al., 2001; Magro et al., 2005]. Such downregulation of NHE by IFN-γ leads to the disruption of pH homeostasis and fluid absorption, which is considered part of the pathogenesis for irritable bowl syndrome. We tested the effect of IFN-γ in cultured ES epithelial cells. IFN-γ suppressed both expression and functional

activity of NHE1 and -2. Downregulation of NHEs and subsequent disruption of pH homeostasis and fluid absorption by cytokines produced during immune reactions may contribute to the development of hydrops in the inner ear. Further experiments are in progress using ES specimens from known Meniere's disease patients.

In summary, we have demonstrated that NHEs are expressed in cultured HESE cells and that IFN-γ suppresses the expression and functional activity of NHE1 and -2 in these cells. NHE in the luminal membrane may function to maintain acidic endolymphatic pH and fluid homeostasis. Our results also support the hypothesis that dysfunction of NHEs by inflammatory cytokine could cause pH changes and fluid accumulation in inner ear.

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