Interferon-γ suppresses Na^+-H^+ exchanger in cultured human endolymphatic sac epithelial cells

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Interferon-γ suppresses Na^+-H^+ exchanger in cultured human endolymphatic sac epithelial cells

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The Master's Thesis

submitted to the Department of Medicine,

the Graduate School of Yonsei University

in partial fulfillment of the requirements for

the degree of Doctor of Philosophy

Eun Jin Son

April 2006

This certifies that the Master's Thesis
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April 2006

ACKNOWLEDGEMENTS

I would like to acknowledge Professor Won-Sang Lee for inspiring me to give my best in all I do, for believing in my forts, and for guiding my stride in this very exciting field. As well, I thank Professor Myung-Hyun Chung for his guidance with improving this paper, and Professor Min-Goo Lee for his constructive criticism that has encouraged me greatly in my humble efforts. I would also like to acknowledge Jung Lim Kim; without her, my endeavors would have resulted in nothing meaningful. My family has unswervingly endorsed the time required for this work, so my love and thanks go to my husband and son. And I thank God for His everlasting love and patience.

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ABSTRACT

Interferon-ɣ suppresses Na^+-H^+ exchanger in cultured human endolymphatic sac epithelial cells

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Objective: Absorption of endolymphatic fluid may involve sodium transport in endolymphatic sac epithelium. The Na^+-H^+ exchanger (NHE) is a major determinant of intracellular pH (pH*i*) and facilitates sodium and fluid absorption in epithelial cells. The effect of IFN-γ on expression and function of NHE in cultured human endolymphatic sac epithelial cells was investigated. **Material and Methods**: Serial cultures of human endolymphatic sac epithelial cells were performed. RT-PCR for NHE1,-2,-3 isoforms was done and compared between control P-2 cultured human endolymphatic sac epithelial cells and IFN-γ treated cells. Functional activity of NHE isoforms in control and IFN-γ treated cells were measured microfluorometrically using a pH-sensitive fluorescent dye, 2'7'-bis(carbonylethyl)-5(6)-carnoxyfluorescein (BCECF) and compared. Na^+-dependent pH*i* recovery from intracellular acidification and its suppresion by a known NHE-inhibitor, HOE694, were examined to determine specific activity of NHE isoforms. **Results**: Treatment with IFN-γ suppressed mRNA expression of NHE1 and -2, but had minimal effect on NHE3 expression in the cultured human endolymphatic sac epithelial cells, confirmed by RT-PCR. Functional activities of NHE1 and -2 were measured by sequential suppression of Na^+-dependent pH*i* recovery form intracellular acidification by 2 different concentrations of HOE694 known to inhibit each NHE isoform selectively. The results show that treatment with IFN-γ suppressed functional activity of both NHE1 and -2 in

cultured human endolymphatic cells. **Conclusion**: This study showed treatment with IFN-γ suppresses the expression and functional activity of NHE1 and -2 expressed in cultured human endolymphatic sac epithelial cells.

Key words : endolymphatic sac epithelium, interferon-γ, Na^+-H^+ exchanger

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I. INTRODUCTION

The endolymphatic duct and sac belong to the nonsensory portion of the membranous labyrinth of the inner ear. Ion regulation, fluid regulation, phagocytosis and immune defense mechanisms are the generally accepted functions of the endolymphatic sac. The inner ear is a labyrinthine tubular system that contains two distinctly separate fluid compartments of defined, yet differing ionic composition. The perilymph is considered to be a filtrate of the cerebrospinal fluid or blood and consequently contains a high Na^+ concentration (about 145 mM), low K^+ (about 5 mM), and 1- 2 mM Ca^{2+}. The endolymph in contrast is unique as an extracellular fluid in that it contains only 1 mM Na^+, yet 155 mM K^+ and Ca^{2+} in the nanomolar range. Thus it very much mirrors the cytosolic ion composition.[1,2] For the production, absorption and regulation of the endolymph volume and composition predominantly two anatomically distant sites are discussed. The stria vascularis in the scala media is thought to be the tissue which extrudes K^+ in to the endolymph. Probably the stria vascularis is also involved in water transport. The endolymphatic sac is generally accepted as the absorption site of the endolymph.

Disruption of fluid homeostasis in the inner ear causes sensory dysfunctions, such as well-known, but little-understood Meniere's disease. Here the underlying

overproduction or reduced absorption of the endolymph may have severe pathophysiological consequences, such as endolymphatic hydrops, or a permeability increase of tight junctions resulting in vertigo attacks, hearing losses, tinnitus, and a sensation of fullness in the inner ear. The mechanisms that lead to endolymphatic hydrops are not known. Allergic autoimmune reaction is one possible hypothesis as well as a neural or viral etiology.

Various mechanisms that regulate Na^+ transport across luminal cell membranes have been identified. These include the electrogenic process of Na^+ absorption via an epithelial Na+ channels[3] and the electroneutral process of Na^+-H^+ exchanger (NHE)s.[4] The NHE gene family is comprised of nine members that are categorized by cellular localization. There are resident plasma membrane isoforms including NHE1 (basolateral) and NHE2 (luminal), recycling isoforms (NHE3, -4, -5), as well as intracellular isoforms (NHE6, -7, -9).[5] Among them, the NHE1, -2 and -3 isoforms are the most characterized members of this gene family. The NHEs are the major determinants of intracellular pH (pH*i*), and also facilitate NaCl absorption in epithelial cells.[6] Previous studies also reported that the NHEs play an important role in fluid absorption in various tissues including the intestine and the bile duct.[3,7] The NHEs are also expressed in proximal and distal human airways, and they are involved in the regulation of pH*i* in human airway epithelial cells.[8,9] NHE was identified in guinea pig endolymphatic sac epithelium.[10] Because transepithelial absorption of Na^+ is closely related to the fluid absorption in many epithelial tissues, alterations in NHE activity may underlie ion transport dysfunction and fluid absorption by the endolymphatic sac epithelium. However, it is unclear whether the NHEs have a regulatory function in fluid absorption in endolymphatic sac epithelium.

Endolymphatic hydrops, which is known as the underlying pathophysiology of Meniere's disease, is also suspected to cause autoimmune sensorineural hearing loss.[11] Immunologic basis for Meniere's disease has also been shown.[12-15] Thus, some immunologic component may be a common contributor to both diseases with

characteristic deterioration of hearing, probably secondary to the disturbance of inner ear fluid homeostasis.

Interferon- γ (IFN- γ), a cytokine produced by Th1 lymphocytes, is induced in the inner ear in response to secondary antigen challenge in the endolymphatic sacs of immunized animals.[16] Also, the Th1 subset is increased significantly in the serum of patients with acute low-tone sensorineural hearing loss associated with Meniere's disease.[17] IFN- γ has been shown to downregulate the expression of NHE2 and NHE3 in rat intestine and human Caco-2/bbe cells.[18]

Here, we evaluated the effect of IFN- γ, a cytokine produced by Th1 lymphocytes that mediate inflammation and immune reactions, on the expression and function of NHE in cultured human endolymphatic sac epithelial cells.

II. MATERIALS AND METHODS

1. Cell culture

Human endolymphatic sacs are excised during translabyrinthine acoustic neuroma surgery. The sac is separated from the posterior bony surface with a mucosa knife and cut with a pair of scissors at the external aperture of the vestibular aqueduct. Tissue specimen is cut into fine pieces and placed in culture medium, then treated with 1% Pronase (type 14 protease, Sigma, St. Louis, MO, USA) for 18 hours at 4 ℃. The serial culture of NHESE cells is performed by the same methods described by Choi *et al*.[19] for normal human middle ear epithelium. Briefly, the cultures were maintained at 37℃ in a humidified atmosphere of 5% CO_2. The culture medium is a 1:1 mixture of bronchial epithelial growth media and DMEM, hydrocortisone 0.5ug/mL, insulin 5ug/mL, transferrin 10ug/ml, epinephrine 0.5ug/ml, triiodothyronine 6.5ng/ml, gentamycin 50ug/ml, amphotericin B 50ng/mL, EGF 25ng.ml, all-trans retinoic acid $5x10^{-8}$ M, bovine serum albumin 1.5 ug/ml, bovine pituitary extract 1%v/v. The culture medium is changed every other day after the explants established attachment. The culture medium is changed twice a week. The cells were dissociated when they reached 50-60% confluence with 0.24% trypsin/EDTA (Clonetics) and seeded for subsequent passage. The passage-2 human endolymphatic sac epithelial cells are seeded in Transwell clear culture inserts (Costar, Cambridge, MA, USA), and cultured in the same culture media for 7 days until confluent. An air-liquid interface is then created by removing the apical medium and feeding cells basolaterally.

2. RT-PCR

The gene-specific PCR primer sets for the human NHE1, NHE2 and NHE3 were designed to detect the isoform-specific mRNA in the human airways (**Table 1**). The

sequence information of some isoforms was retrieved from the GeneBank. The oligonucleotide amplimers for β2- microglobulin were used as the control gene for RT-PCR. They generated a 266bp PCR fragment. Briefly, the total RNA is isolated from cultured cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH). One μg of total RNA per 20μL reaction volume is reverse transcribed(RT) in first strand cDNA using random hexamenucleotide primer and murine leukemia virus reverse transcriptase (Perkin Elmer, Foster, CA, USA) at 42℃ for 50 min. The resulting first-strand cDNA is directly used for polymerase chain reaction (PCR) amplification. The annealing temperatures were 58℃, 65℃ and 58℃ for NHE1, -2 and -3, respectively. PCR products are separated by electrophoresis on a 2% agarose gel (FMC BioProducts, Rockland, ME, USA) containing 50 ng/mL ethidium bromide, and the image is captured using a CSC Chemoluminescense Detection Module (Raytest, Straubenhardt, Germay). Negative controls were performed by omitting the reverse transcriptase from the RT reaction in order to verify that the amplified products were from the mRNA and did not originate from a genomic DNA contamination. No PCR products were observed in the absence of reverse transcriptase.

Table 1. PCR primer sequences specific to the target genes.

Primers	Sequences
NHE1	Sense: 5'- CCA GCT CAT TGC CTT C-3'
(245 bp)	Antisense:5'- TGT GTC TGT TGT AGG ACC GC-3'
NHE2	Sense: 5'- ATC CGT CAG CGA ACT TTA TCC TAC AAC A-3'
(276 bp)	Antisense:5'- GGTGGT CCC GGC ATC TGC GTC TGA G-3'
NHE3	Sense: 5'- GCA GAC CTG GCT TCT GAA CC-3'
(365 bp)	Antisense:5'- GGA ACT TCC TGT CGA AGT GG-3'
β2M	Sense: 5'- CTC GCG CTA CTC TCT CTT TCT GG -3'
(266 bp)	Antisense:5'- GCT TAC ATC TCT CCA TCC CAC TTA A-3’

3. Measurements of intracellular pH (pH*i*) and NHE activity

pH*i* was measured in the monolayers using the pH-sensitive fluorescent probe bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-AM. Cells were loaded with BCECF-AM for 10 min at room temperature in solution containing 2.5 μM BCECF-AM and mounted in the miniature Ussing chamber attached to the stage of an inverted microscope (**Fig. 1**). The miniature Ussing chamber consisted of a top (mucosal) and bottom (serosal) half-chambers (volume = 250 μL each), which were made from light-absorbing polyacetal. The Transwell wafer containing the polarized epithelial monolayer is mounted between the two half-chambers with the mucosal surface up. Effective sealing is achieved using rubber O-rings embedded in the grooves of the two half-chambers, which were screwed together tightly. A glass coverslip was affixed to the bottom of the serosal chamber with a dental sticking wax (model Deiberit-502; Ludwig Bohme). The mucosal chamber is open to the atmosphere. Both half-chambers have inlet and outlet ports to allow the solution to flow. The serosal and mucosal perfusate are heated to 37 °C and delivered to the chamber by gravity flow (rate = 3~5 ml/min). BCECF fluorescence was recorded and calibrated using a previously described protocol.[20] Briefly, the fluorescence at excitation wave-lengths of 490 and 440 nm was recorded using a recording setup (Delta Ram; PTI Inc.), 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. NHE activity was determined 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[21] with modification. **Fig. 2** shows a typical pH-recovery experiment for determination of NHE activity. The addition of NH_4^+ to the luminal membrane produced rapid intracellular alkalinization due to the influx of NH_3 into the cells. 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 can rapidly leave the cytoplasm. This caused a large undershoot of the pH*i*, significantly below the starting value. When Na^+-containing solution was applied on the luminal

cell surface, the pH*i* subsequently recovered, as a result of NHE activity present in the cultured human endolymphatic sac epithelial cells. Typically, the first 10– 40 seconds of the initial linear portion of the pH*i* recovery was fitted to a linear equation using the Felix software (version 1.4; PTI Inc.).

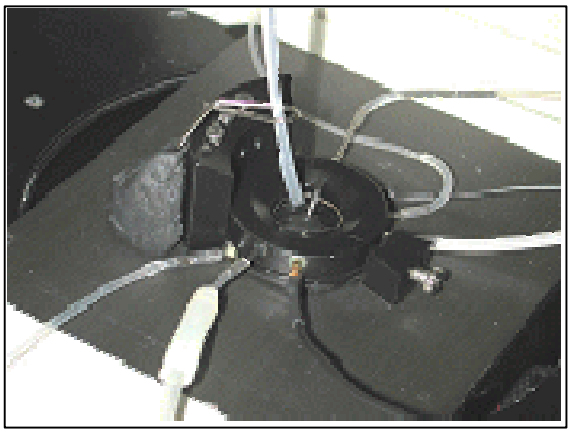

Figure 1. Miniature Ussing chamber attached to the stage of an inverted microscope.

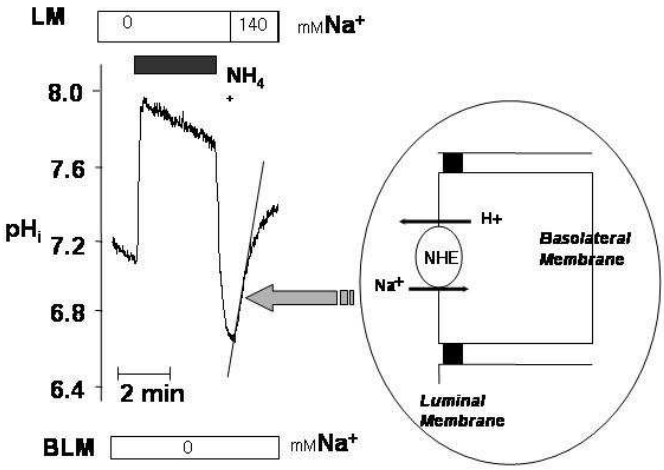

Figure 2. Na^+/H^+ exchanger (NHE) activity in cultured human endolymphatic sac epithelial cells.

The pH*i* of cultured human endolymphatic sac epithelial cells was measured using BCECF as described in the Materials and Methods section. The membrane-specific NHE activities of cultured human endolymphatic sac epithelial cells were analyzed using a double perfusion chamber with separate applications of 140mM Na^+. The application of Na^+ to the luminal side evoked an increase in pH*i*. *LM*, luminal membrane; *BLM*, basolateral membrane.

4. 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 with Tris-base). The osmolarity of all solutions was adjusted to 310 mOsm with the major salt prior to use. The BCECF-AM was purchased from Molecular Probes (Eugene, OR, USA). All other chemicals, including 3-methylsulphonyl-4-piperidinobenzoyl guanidine methanesulfonate (HOE694),[22] were purchased from Sigma.

5. 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. The effect of IFN-γ on NHE expression

We determined the effect of IFN-γ on the expression of NHE isoforms in cultured human endolymphatic sac epithelial cells by RT-PCR. Of the three isoforms NHE1 (245 bp), NHE2 (276 bp) and NHE3 (365 bp), IFN-γ suppressed NHE1 and -2 expression. However, NHE3 expression was not changed after IFN-γ treatment (**Fig. 3**).

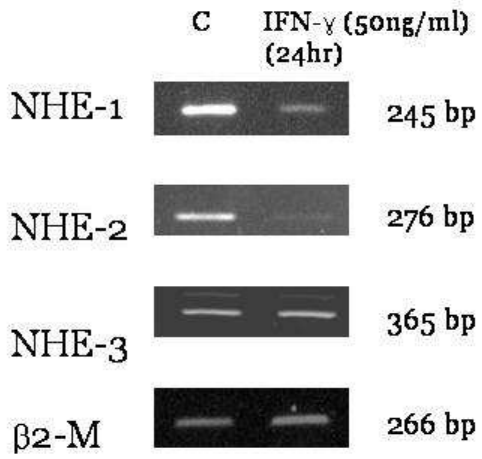

Figure 3. RT-PCR for Na^+/H^+ exchanger (NHE) isoforms in cultured human endolymphatic sac epithelial cells.

NHE1 (245 bp), NHE2 (276 bp) and NHE3 (365 bp) were all expressed in the NHMEE cells. IFN-γ treatment for 24 hrs increases NHE1 and -2 expression. NHE3 expression was unchanged after IFN-γ treatment.

2. The effect of IFN-γ on functional activity of NHE

The effect of IFN-γ on the Na^+-induced pH*i* recovery was then evaluated. To 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 (*Ki*) values for the NHE1, -2 and -3 isoforms: 0.16, 5, and 650 μM, respectively.[23] As previously mentioned, the initial rate of apical NHE activity was 0.702±0.08 Δ ratio/min. The recovery rate in the presence of 1 μM HOE694 was decreased to 0.198±0.12 Δ ratio/min. Na^+-induced pH*i* recovery was sensitive to HOE694 at 5 μM. The pHi recovery rate was decreased to 0.048± 0.01 Δ ratio/min by 5 μM HOE694 (**Fig. 4A & C**). These results suggest that NHE1 and -2 regulate pH*i* in cultured human endolymphatic sac epithelial cells.

The recovery rate was greatly decreased after exposure to IFN-γ (control: 0.702±0.08 Δ ratio/min, IFN-γ treated: 0.396±0.07 Δ ratio/min). Interestingly, treatment with IFN-γ suppressed Na^+-induced pH*i* recovery that was sensitive to 1 and 5 μM of HOE694 (**Fig. 4 B & C**). We interpreted the HOE694 (1 μM)-sensitive Na^+-induced pH*i* recovery rate as NHE1 activity, which was greatly decreased after IFN-γ treatment (control: 0.198±0.12 Δ ratio/min, IFN-γ treated: 0.084±0.05 Δ ratio/min, $P < 0.05$) (**Fig. 4C**). Also, the HOE694 (5 μM)-sensitive Na^+-induced pH*i* recovery rate, representing NHE2 activity, was decreased after IFN-γ treatment (control: 0.048± 0.01 Δ ratio/min, IFN-γ treated: 0.003±0.002 Δ ratio/min, $P < 0.05$) (**Fig. 4C**). These results suggest that IFN-γ suppresses NHE1 and -2 activity in cultured human endolymphatic sac epithelial cells.

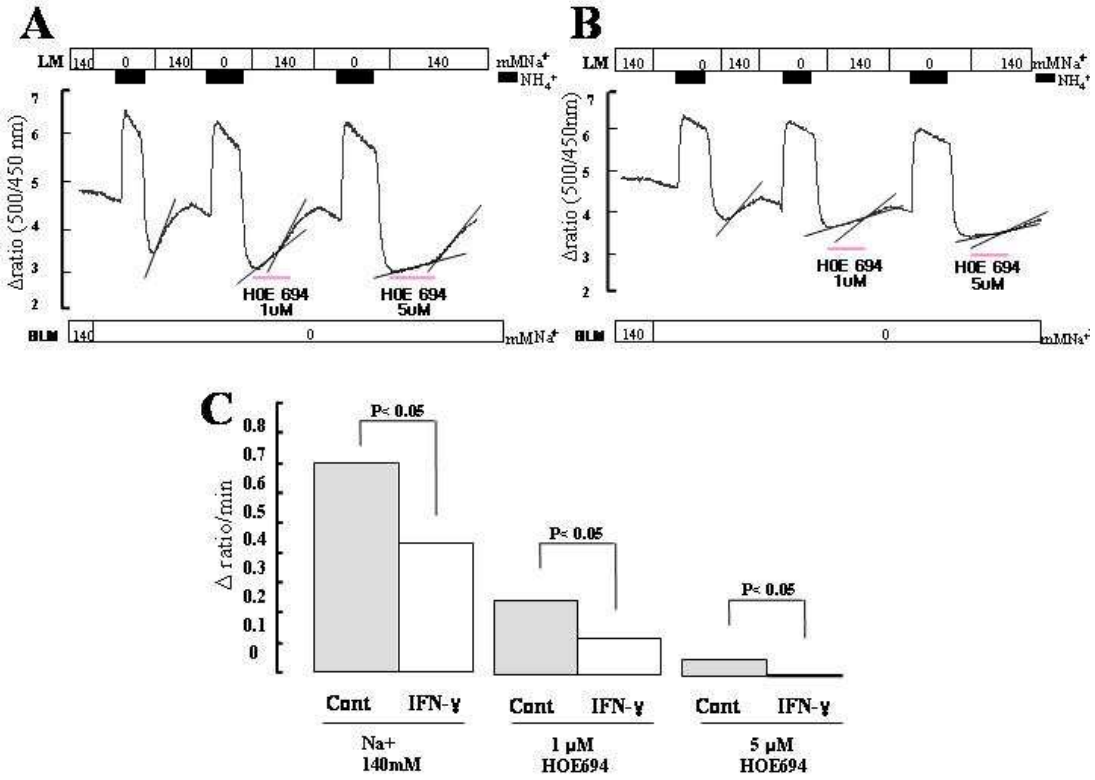

Figure 4. Suppression of Na^+-induced pH*i* recovery by IFN-γ in cultured human endolymphatic sac epithelial cells.

The Na^+-induced pH*i* recovery rate was measured after treatment with IFN-γ (50ng/mL, 24 h). Representative traces of control and IFN-γ -treated samples are presented in *A* and *B*. *LM*, luminal membrane; *BLM*, basolateral membrane. *C*. Effects of IFN-γ suppression on NHE1 and -2 were determined by comparing the HOE694 (1 and 5 μM, respectively)-sensitive Δ ratio/min. Summarized results of three experiments. * indicates P< 0.05, difference from control.

IV. DISCUSSION

The NHE gene family mediates electroneutral transepithelial Na^+ absorption and plays an important role in fluid absorption in various tissues.[3,7] However its role in the endolymphatic sac epithelium, which is considered to function in the regulation of endolymphatic fluid volume and composition, is still unclear. NHEs have been identified in guinea pig endolymphatic sac epithelium,[10] but there has been no reports concerning humans. In our study, NHE1, -2 and -3 were expressed in cultured human endolymphatic sac epithelial cells, and NHE1 and -2 were functionally active in pH*i* regulation in these cells. Also, previous reports have suggested that NHEs function in fluid absorption in fluid absorption in various epithelia including those of the kidney and intestine.[24-27] Also, the vectorial Na^+ absorption has important implications in airway surface liquid volume and composition, and NHE is thought to play a role as well as other sodium channels such as epithelial Na^+ channels and paracellular sodium transport in fluid absorption in airway epithelium.[28] In our preliminary experiments, epithelial Na^+ channels were also identified in cultured human endolymphatic sac epithelial cells (data not shown). Thus, sodium transport mediated by NHEs as well as other sodium channels such as epithelial Na^+ channels may contribute to fluid absorption in endolymphatic sac epithelium.

NHE1 and -2 are most well known isoforms of the NHE family, and are located in the plasma membrane.[5] Our experiments identified functionally active NHE1 and -2, but other isoforms may contribute to ion transport and pH*i* regulation in endolymphatic sac epithelium. NHE1 is considered constitutively expressed in basolateral membranes, and NHE2 in luminal membranes of polarized epithelial cells.[6] Inhibition of Na^+- sensitive pH*i* recovery by 1 uM of HOE694 is significant in luminal stimulation in cultured endolymphatic sac epithelial cells, and suggest of luminal presence of NHE1. Although further studies are needed, such unique expression may be related to the fact that the epithelial cells are exposed to the very distinct composition of endolymphatic fluid which is closer to intracellular fluid.

Meniere's disease is characterized by several typical clinical presentations including recurrent vestibular symptoms and fluctuation of hearing, but the diagnosis is considered definite when endolymphatic hydrops is confirmed. Pathogenesis of endolymphatic hydrops underlying this disease is still unclear and autoimmunologic responses are implicated.[11] Especially Th1 subset of lymphocytes seem to be increase in Meniere's disease,[17] and levels of IFN- γ produced by such Th1 cells is elevated in animal experimental models of autoimmune inner ear diseases.[15-16] IFN- γ has been shown to downregulate the expression of NHE2 and NHE3 in other epithelia by direct suppression of transcription or nongenomic pathway.[18] In cultured endolymphatic sac epithelial cells, both expression and functional activities of NHE1 and -2 were downregulated by IFN- γ. Such suppression of transepithelial sodium transport and subsequent fluid absorption by cytokines produced during immune reactions may contribute to the development of hydrops in the inner ear.

V. CONCLUSION

In summary, we have demonstrated that IFN-γ suppresses the expression and functional activity of NHE1 and -2 in cultured human endolymphatic sac epithelial cells. These findings support the hypothesis of impaired or down-regulated function of epithelial Na^+ and fluid transport via NHE may be associated with endolymphatic hydrops, and IFN-γ may be a possible mediator of these effects.

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국문요약

사람내림프낭상피세포에서 인터페론 감마에 의한 Na^+-H^+ Exchanger 의 억제

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손 은 진

목적: 사람 내림프낭상피세포에서 내림프액의 흡수와 생성 과정에는 이온의 이동이 관여한다. Na^+-H^+ exchanger (NHE)는 상피세포에서 세포 내로 Na^+ 이온과 물의 흡수를 담당하며 세포내 pH 조절에 중요한 역할을 한다. 본 연구에서는 배양된 사람 내림프낭상피세포에서 IFN-γ 가 NHE 의 발현과 기능에 미치는 영향을 알아보고자 한다.

재료 및 방법: 사람 내림프낭상피세포를 채취하여 계대 배양한 후, 2 계대 세포에서 RT-PCR 를 시행하여 대조군과 IFN-γ 처리한 세포에서 NHE1, -2, -3 의 분자학적 발현을 비교한다. NHE 의 기능을 보기 위해 형광색소인 2'7'-bis(carbonylethyl)-5(6)-carnoxyfluorescein (BCECF)를 이용하여 세포내 pH 를 측정하였다. 세포내 산성화를 유도한 후 NHE 의 기능에 의해 pH 가 회복되는 속도를 측정하고, NHE 억제제인 HOE694 를 첨가하여 이러한 세포내 pH 회복 속도가 감소되는 것을 이용하여 대조군과 IFN-γ 처리한 세포에서 NHE isoform 의 기능을 비교하였다.

결과: 배양된 내림프낭상피세포에서 IFN-γ 를 처치하는 경우, NHE1 과 -2 의 mRNA 발현은 감소하는 반면, NHE3 은 영향 받지 않는 것을 RT-PCR 결과에서 확인 하였다. NHE 억제제인 HOE694 는 농도에 따라 NHE isoform 을 각각 선택적으로 억제하는데, 이를 이용하여 세포내 산성화 상태에서 세포내 pH 가 회복되는 속도를 비교한 결과, 사람 내림프낭상피

배양세포에서 NHE1 과 -2 가 기능을 하는 것을 알 수 있었고 IFN-γ 를 처치하면 NHE1 과 -2 의 기능이 모두 억제되었다.

결론: 본 연구를 통해 배양된 사람내림프낭상피 세포에서 IFN-γ 에 의해 NHE1 과 -2 의 분자학적 발현과 기능이 모두 억제되는 것을 알 수 있었다.

핵심되는 말: 내림프낭상피세포, 인터페론 감마, Na^+-H^+ exchanger