The role of protease-activated receptor 2 in regulating ion transport in human middle ear epithelia

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The role of protease-activated receptor 2 in regulating ion transport in human middle ear epithelia

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I would like to dedicate this book in their honor.

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Protease-activated receptor 2 (PAR2) has diverse functions in airway epithelia. We investigated the role of PAR2 in regulating epithelial surface liquid volume in human middle ear epithelia. The presence of PAR2 in human middle ear epithelial cells (HMEEC) was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) and PAR2 expression was observed more prominently at the basal layer of inflamed middle ear mucosa by immunostaining. Hydrolysis of synthetic fluorescent PAR2 increased with the effusion of middle ear in dose-dependent manner and its activity decreased with trypin inhibitor, aprotinin. Furthermore known endogenous activator of PAR2, human airway tryptase (HAT) was identified in middle ear effusion. PAR2 was highly related with middle ear inflammation.

Epithelial surface liquid volume was evaluated with confocal microscopy using Z-stack analysis. The height of surface liquid layer was 35.5 ± 1.6 μm with the treatment of 10 μM PAR2-AP (activating peptide) on apical side and 36.3 ± 4.6 μm with basolateral treatment of PAR2-AP. The increased surface

liquid layer was smooth and evenly elevated compared with control (30.3 ± 2.4 μm).

We also checked whether PAR2-AP could induce intracellular Ca^{2+} increasing and transepithelial current using fluorescent dye and vibrating probe. PAR2-AP induced intracellular Ca^{2+} increasing response similar to the response with ATP. And short circuit current was noted in the middle ear mucosa with the treatment of PAR2-AP. The increase of current density was prominent in the pretreatment of amirolide though, however it was blocked with 4,4'-Diisothiocyano-2,2'-

stilbenedisulfonic acid (DIDS). Therefore the activation of PAR2 can induce fluid secretion in the middle ear by increasing intracellular Ca^{2+} and PAR2 may be related with middle ear epithelial ion channel.

These results imply that serine protease such as HAT in the middle ear effusion has a role in regulating epithelial surface liquid volume by controlling chloride secretion via PAR2.

Key words : protease-activated receptor (PAR), airway surface liquid, ion channel, human middle ear epithelia

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I. INTRODUCTION

Protease-activated receptors (PARs) play important biologic roles in inflammation, injury, pain, and other tissue responses.[1] In 1991, PARs were first identified as thrombin receptor in the mechanism of the coagulation effect on platelet activation. Later they were found to play a critical role in angiogenesis and fibrosis of wound healing.[1,2] Sequencing of human genome shows that more than 2% of all human genes are proteases or protease inhibitors, indicating the importance of proteolysis in human biology.[3] Additionally, proteases possess various regulatory functions that are mediated through intracellular signaling pathways, so it is now considered as multifunctional, hormone-like signaling molecules that play diverse roles in various physiological and pathological process.[4]

PARs are G protein-coupled receptors that are activated by proteolytic cleavage of the N-terminal extracellular domain.[5] This newly cleaved amino-terminus functions as a “tethered ligand” and binds to the second extracellular loop. In PAR2, activating proteases cleave PAR2 at Arg36 and Ser37 to expose the tethered ligand SLIGKV and activate intracellular signal transduction.[6] So far 4 PARs have been cloned and characterized and thrombin

activates PAR1, PAR3 and PAR4 and trypsin activates PAR2 and PAR4 mainly.[5,6]

In the airway, there has been currently a great interest in the role of PAR2.[1,5,7] PAR2 is expressed in the cardiovascular, gastrointestinal system, pancreas, liver, brain, kidney, prostate, ovary, eye, skin and airway.[1,2,6] PARs are expressed by airway epithelial and smooth muscle cells, as well as endothelial and vascular smooth muscle cells within respiratory tract.[5] PAR2 is also known to be expressed by human mast cells, macrophages, and neutrophils.[6,7] In some cells of respiratory tract, such as the airway smooth muscle (PAR1, PAR2, PAR3) and mast cells (PAR1, PAR2), co-express multiple PARs.[5] Effects on these cells trigger pathways related to inflammation, mucosal protection, healing and repair, and neurogenic inflammation.[1] Endogenous proteases such as trypsin, tryptase, matrix metalloproteases (MMP), and coagulation factors as well as proteolytic allergens such as the mite cysteine (Der p 1) and exogenous serine proteases (Der p 3, 6, and 9), have been shown to modulate respiratory epithelial cell function via PAR2.[5,6,8] In the airway epithelium, PAR2 agonists can cause intracellular Ca^{2+} increase, increase of ion transport, production of IL-6, IL-8, MMP-2 and 9, production of cyclooxygenase 2 (COX-2) and prostaglandin E_2 (PGE_2).[2,7] In addition, mast cell tryptase and human airway tryptase (HAT) which are known to activate PAR2 are readily detected in bronchoalveolar lavage fluid from patients with chronic inflammatory disease and this has led many to believe PAR2 has a pro-inflammatory role.[9,10] Furthermore, airway inflammation responses are associated with increase epithelial PAR2 expression and elevated concentrations of PAR-activating, and PAR-inactivating, proteases in the extracellular space.[5]

The middle ear mucosa is considered an extension of airway epithelia and its surface is covered with a thin airway surface liquid (ASL) layer.[11] Various ion

channels are also expressed in middle ear epithelia to control the volume and composition of ASL.[12] In the previous study, middle ear fluid was mainly absorbed by epithelial sodium channel (ENaC) and fluid secretion with chloride was controlled by Ca^{2+}-dependent Chloride Channel (CaCC).[13]

In this study we investigated the role of PAR2 in regulating epithelial surface liquid volume in human middle ear epithelium. First, the increase of the height of ASL of normal human middle ear epithelial cells by PAR2 activation with PAR2-activating peptide (PAR2-AP) need to be identified. Second, the correlation of PAR2 activation and Cl^- current is verified for the role of CaCC in fluid secretion in normal human middle ear epithelial cells. This step is confirmed with treatment of 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) blocking CaCC. Third, the increase of intracellular Ca^{2+} by PAR2 activation is verified to prove the effect of CaCC in ASL. This step will enrich the investigation of the pathogenesis of otitis media with effusion.

II. MATERIALS AND METHODS

1. Middle ear epithelial cell culture

Primary cultures of NHMEE cells were prepared as described previously.[14] 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 pore size of 0.45 μm (Transwell-clear, Costar Co., Cambridge, MA, USA) at a density of 1.0×10^4 cells/ cm^2. 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.[16]) Cultures were grown submerged for the first 9 days, at which time, the air-liquid interface was created by removing the apical medium and feeding the cultures from the basal compartment only and further cultured for 7 to 10 day for complete differentiation. Confluence of passage-2 NHMEE cells (8-9 days after seeding) was verified by measurement of transepithelial resistance (Rt. $>$ 1,000 Ω/cm^2 at room temperature) using endohm meter.

2. RT-PCR for PAR2 in human middle ear epithelial cells

Total cellular RNA was extracted from passage-2 normal human middle ear epithelial cells (NHMEE cells) after treatments using RNA TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The extracted RNA was treated with amplification grade deoxyribonuclease I (Invitrogen) prior to cDNA synthesis. The DNase I treated RNA (3 μg) was converted to cDNA by adding GeneAmp® RNA PCR Kit components (MMLV-reverse transcriptase, RNase Inhibitor and Random Hexamers, Applied Biosystems Inc., Hammonton, NJ, USA) to a total volume of 25 μl. After reverse transcription, RT-PCR was performed to amplify DNA fragments. All amplification reactions were performed in a MyCycler™ thermal cycler system

(Bio-Rad Laboratories, Inc., CA, USA). PCR products were run for 34 cycles using the amplification conditions. PCR products were visualized by 2% agarose gel electrophoresis and ethidium bromide staining. The used primer sequences were forward primer: 5'-GACTTTCTCTCGGTGCGTCC-3', and reverse primer: 5'-CGTTACTTGGCAAACCCACC-3'.

3. Immunohistochemical staining for PAR2 protein

Human middle ear mucosa harvested during tympanomastoidectomy surgery in patients with chronic otitis media was fixed with 10% formaldehyde solution for 24 hr and then dehydrated and embedded in paraffin. Paraffin blocks were sectioned into 4-mm-thick slices. After deparaffinizing and rehydrating, slides were incubated in antigen retrieval solution (Tris-EDTA, pH 9.0) for 20 min at 95–100 ℃. To block endogenous peroxidase, slides were treated with 0.3% H_2O_2 for 15 min at room temperature. Slides were blocked in 10% normal serum with 1% bovine serum albumin in TBS for 2 h at room temperature and then incubated overnight at 4 ℃ with a monoclonal mouse antibody against human PAR2 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The slides were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in antibody diluent solution (DAKO, Glostrup, Denmark) for 1 hr at room temperature. Slides were developed with DAB (DAKO) at room temperature and counterstained with hematoxylin (Merck KGaA, Darmstadt, Germany).

4. Western blot analysis for human airway tryptase

The middle ear fluid was collected by myringotomy in 6 patients with serous

otitis media during ventilation tube insertion procedure. The sample fluid was frozen immediately after collection and stored at -20 ℃. The protein amount of middle ear effusion was quantified using the Pierce BCA Protein Assay Kit (Thermo Rad Laboratories, Hercules, CA, USA). Equal amounts of total protein (25 μg) were resolved by 10% SDS-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (PVDF, Immobilon; Millipore Corp, Bedford, MA, USA) electrophoretically at 250mA for 90 min at 4℃. The PVDF membrane was blocked with 5% skim milk in Tris-buffered saline (TBS, 50 mM Tris-Cl, pH 7.5 and 150 mM NaCl) for 2 hr at room temperature, followed by overnight incubation with primary antibodies in TBS-T (0.5% Tween 20 in TBS). Primary antibodies were used for HAT(human airway tryptase) (polyclonal antibody, Santa Cruz, Dallas, Texas, USA). The membrane was washed with 1 X TBST for 10 min and then probed for 1 hr with secondary antibodies conjugated with horseradish peroxidase. The membrane was exposed to a high performance autoradiography film (Fuji XR film, Fuji film Corporation, Tokyo, Japan) after visualization using the ECL system (Santa Cruz).

5. Real-time PCR of PAR2 with IL-1 β

After treatment of 5ng/ml IL-1β 12 hr, the expression level of PAR2 is compared with control. NHMEE cells were harvested for real time-PCR. Real-time PCR was performed in a 96–well format using the PE Biosystems ABI PRISM® 7300 sequence detection system (Foster City, CA, USA). The results were normalized against GAPDH as an internal control. The standard curve used for quantification was generated with serial 10-fold dilutions of each mRNA. The thermocycler parameters were 50℃ for 2 min and 95℃ for 10 sec, followed by 40 cycles of 95℃ for 15 sec and 60℃ for 1 min. All reactions were

performed in duplicate. Relative mRNA expression was obtained using the comparative threshold method. Each reaction consisted of 10μl of 2xTaqMan PCR Universal PCR master mix (Applied Biosystems), 3 μg of cDNA, o.5 μl of primers (10p), and 6 μl of distilled water in 20 μl. The used primer sequences were forward primer: 5'-CATACATGGCAACAACTGGA-3', and reverse primer: 5'-TTCACGATGACCCAATACCT-3'.

6. Hydrolysis of PAR2 synthetic peptides with effusion of otitis media

The fluorescence-quenched synthetic substrates PAR2 [5-FAM]-Ser-Lys-Gly -Arg-Ser-Leu-Ile-Gly-Lys(Dabcyl)-Asp} were synthesized as described previously[15]. Middle ear effusion was collected during ventilation tube insertion procedure. All experiments were performed in physiologic salt solution (PSS; 140mM NaCl, 5mM KCl, 1mM MgCl2, 1mM CaCl2, 10mM HEPES, and 10mM glucose) at 37.8℃. The PSS (1 ml) was equilibrated at 37.8℃ for 30 min before adding synthetic substrate and the enzyme solution. The hydrolysis of PAR2 was analyzed according to the amount of applied effusion. The cells were illuminated at 490nm and proteolytic enzyme activities were continuously measuring emitted fluorescence at 520nm using a PMT chamber system (Photon Technology International Inc., Birmingham, NJ). Fluorescence of peptide product after exhaustive cleavage by trypsin was found to be proportional to concentration. Accordingly, an increase in fluorescence corresponds to an increase in concentration of the cleaved peptide, allowing the kinetic parameters Km and Kcat to be determined from an analysis of initial velocities of product formation obtained at different substrate concentrations.

7. Measurement of $[Ca^{2+}]i$ with Fluorescence microscope

To measure the intracellular Ca^{2+} level, passage-2 NHMEE cells were loaded with 5 μM Fluo-3 AM (Invitrogen Co., USA) for 30 min at 37 ℃. For Ca^{2+}

detection, the cells were illuminated with the light of wavelengths 488 nm, and the emitted fluorescence was collected with a 525 nm emission filter. The fluorescence intensity was quantitatively analyzed by using Meta Morph software (Version 7.1, Universal Imaging Corp., USA). PAR2-AP (10 μM) treatment and compared with the treatment of UTP (100 μM).

8. Measurement of transepithelial current by Scanning Vibrating Electrode Technique (SVET)

Vibrating probe was used to measure the trans-epithelial current of middle ear mucosa under short circuit condition. Harvested middle ear mucosa during surgery was modified of previous report.[16] Briefly, the short circuit current (Isc) was measured with vibrating a platinum-iridium wire microelectrode insulated with parlene-C (Micro Electrodes, Gaithersburg, MD, USA) and coated with Pt black on the exposed tip. The electrode tip of the probe was vibrated at two frequencies between 400 and 700 Hz along a horizontal (*x*) and vertical (*z*) axis by piezo-electric bimorph elements (Applicable Electronics, Forestdale, MA) and was positioned 4 ± 2 μm from the apical surface of middle ear mucosa. The *x*-axis was perpendicular to the face of the epithelium. A platinum-black electrode served as reference in the bath chamber. The signals from the oscillators driving the probe, which were connected to a dual-channel phase-sensitive detector (Applicable Electronics), were digitized (16 bit) at a rate of 0.5 Hz. A perilymph-like physiological saline solution [150 mM NaCl, 3.6 mM KCl, 1 mM $MgCl_2$, 0.7 mM $CaCl_2$, 5 mM glucose, and 10 mM HEPES (pH 7.4)] was used for perfusion. The electrode was positioned where current density showed a maximum *x* value and minimum *z* value; data derived from the x direction current density were plotted with Origin software, version 8.0. (OriginLab Software, Northampton, MA,USA).

9. Chemicals

PAR2-AP (PAR2-AP, SLIGRL-NH_2) was purchased from the Korean Basic Science Institute (Seoul, Korea). PAR2-AP directly interacts with the second extracellular loop of the receptor without proteolytic cleavage of N-terminal. Other PAR2-AP (2-Furoyl-LIGRLO-NH2 from Tocris, Minneapolis, MN, USA) was used in the measurements of change of $[Ca^{2+}]_i$ and chloride current density with vibrating probe.

III. RESULTS

1. Expression of PAR2 in human middle ear epithelial cell

First of all, we checked whether PAR2 is expressed in cultured NHMEE cells. mRNA transcript of *PAR2 i*s well expressed in expected size in two separated cultures of middle ear epithelia (M1, M2) (Fig.1). The expression of PAR2 is also noted in inflamed thick middle ear mucosa harvested from patient with chronic otitis media (Fig. 2A). Immunoractivity is detected in the apical and basolateral cell membrane. Especially the PAR2 expression is prominent along the basement membrane (Fig. 2B).

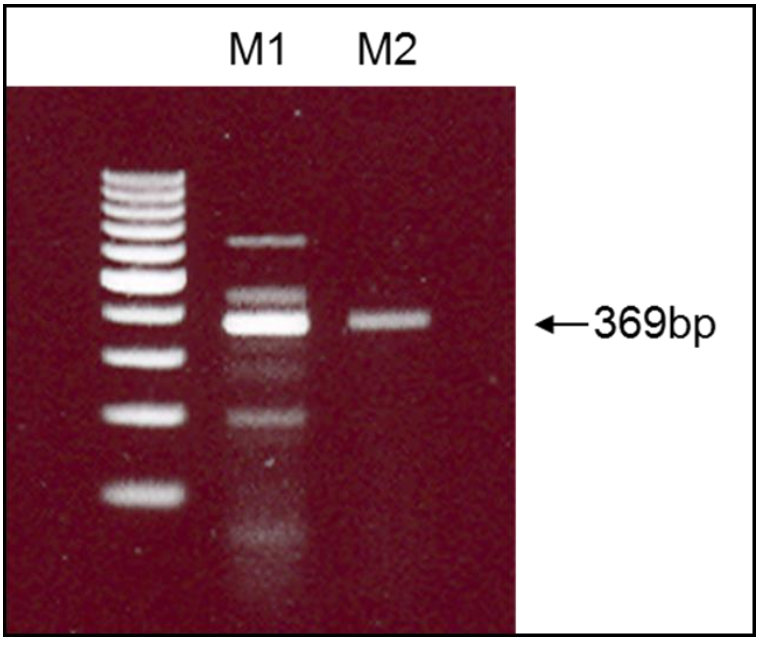


Fig. 1. PAR2 expression in the NHMEE cells with RT-PCR. In two different cultured middle ear epithelial cell lines (M1 and M2), mRNA for *PAR2* was expressed in expected size, 369 bp.

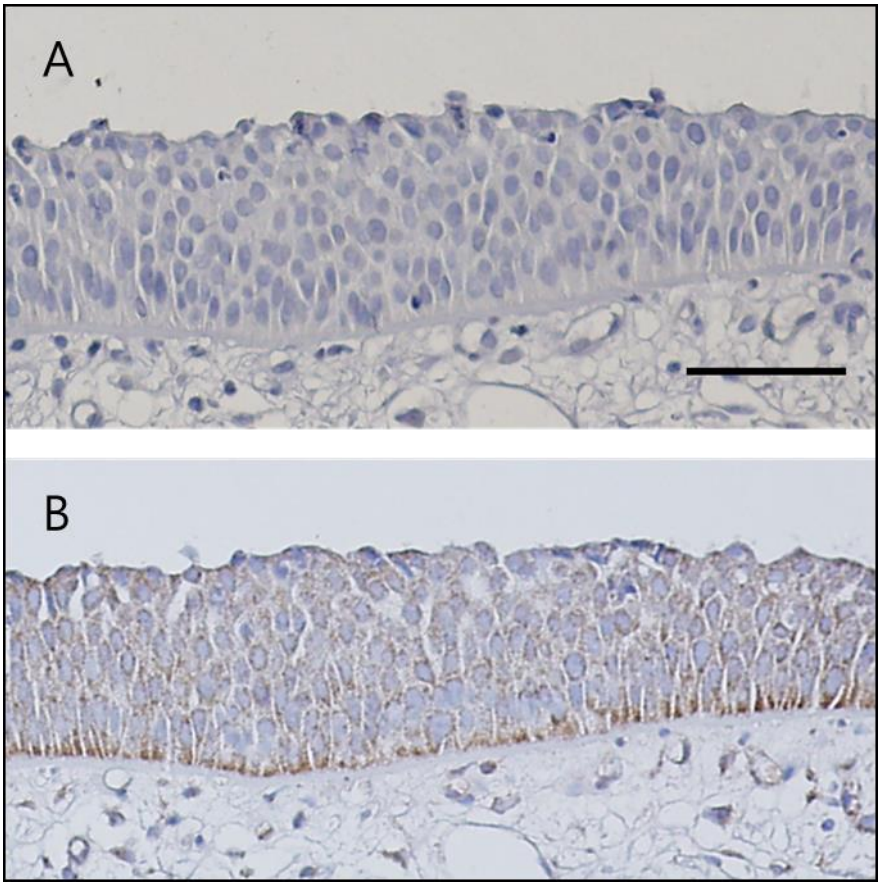


Fig. 2. Expression of PAR2 in middle ear mucosa from the patient of chronic otitis media. **A:** The thick epithelial layer was stained with hematoxylin for contrast . **B**: Immunoreactivity for PAR2 is noted and prominent along with the basement membrane of middle ear mucosa from the patients with chronic otitis media. (scale bar = 20 ㎛)

2. PAR2 in middle ear inflammation

HAT, known endogenous PAR2 activator, was identified by Western blot in effusion of inflamed middle ear (Fig. 3). PAR2 expression level is upregulated by 1.57 times than vehicle alone after treatment with IL-1β, inflammatory cytokine in HMEE cells (Fig. 4).

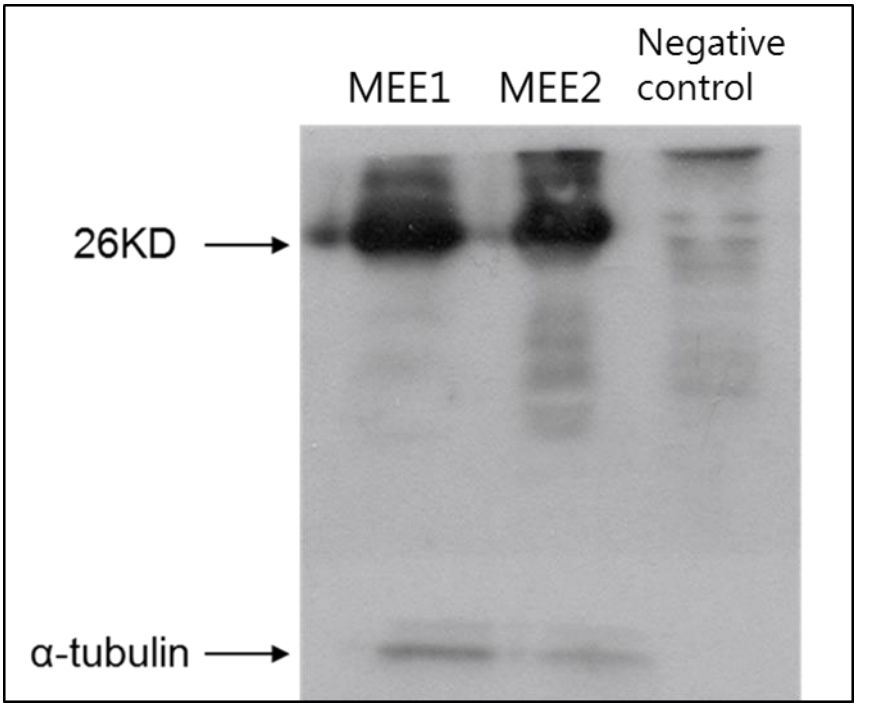


Fig. 3. Expression of human airway tryptase in middle ear effusion.
A molecular weight of 27 kDa of human airway tryptase was identified in desiccated middle ear effusion from two patients with serous otitis media by Western blot analysis.

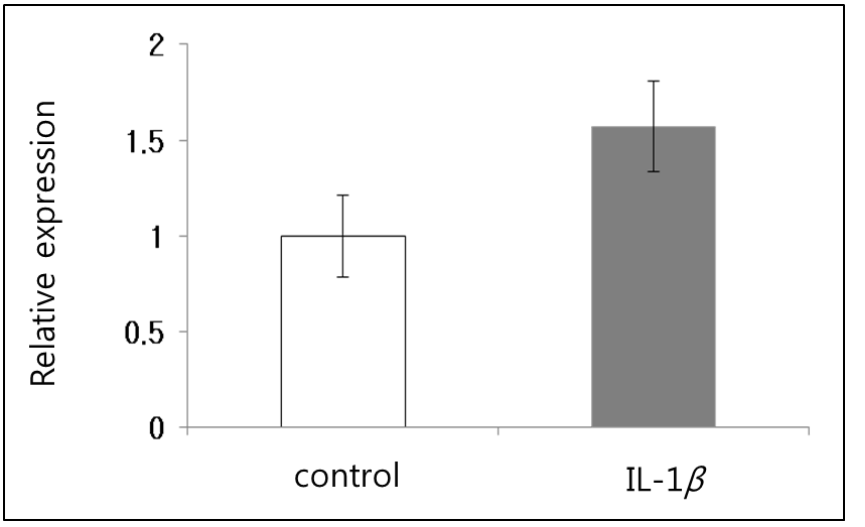


Fig. 4. Change of *PAR 2* expression after IL-1*β* treatment. mRNA of *PAR2* was increased 1.57 fold after treatment of IL-1*β*, inflammatory cytokine in real time PCR.

3. Hydrolysis of PAR2 with effusion of otitis media

The fluorescence-quenched synthetic PAR2 peptides were treated with effusion fluid to monitoring the manifestation of fluorescence increase for PAR2 activation. PAR2 cleavage increases with 10 ㎕ effusion. It is partially blocked with trypsin inhibitor, aprotinin (Fig. 5A). The cleavage of synthetic PAR2 peptides increased in proportion to the amount of applied effusion fluid (Fig. 5B). The result of 2 ㎕ treatment of effusion is approximately same as that of 10 ㎕ treatment of effusion mixed with trypsin inhibitor. It implies that there is a certain protease that can activate PAR2 in middle ear effusion. The increase of hydrolysis of synthetic peptide is partially blocked by trypsin inhibitor (aprotinin) and the protease may be trypsin- like protease.

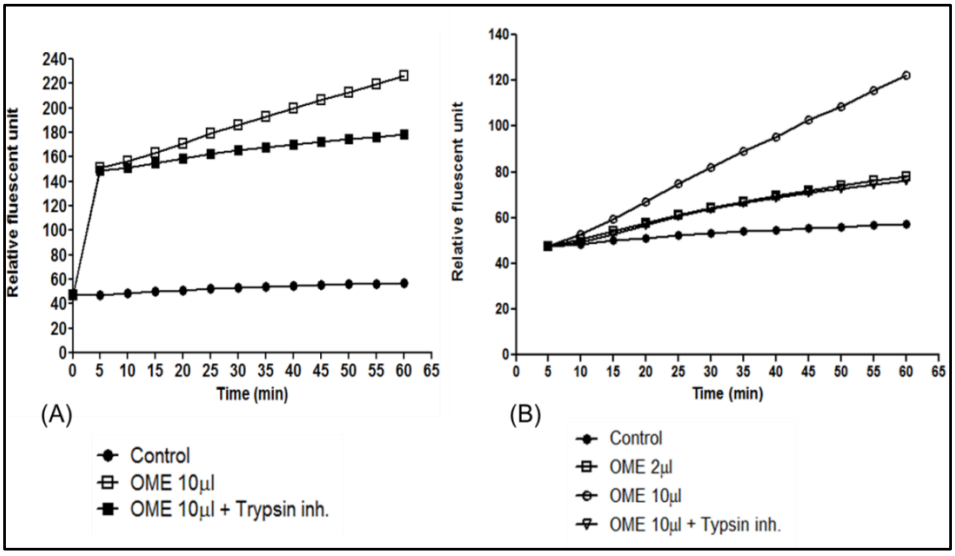


Fig. 5. PAR2 cleavage test with effusion of otitis media. A: Hydrolysis of synthetic PAR2 increased with application of effusion of middle ear and it was partially blocked with trypsin inhibitor, aprotinin. **B:** PAR2 cleavage test with effusion of otitis media. PAR2 cleavage increased in a dose-dependent manner and partially decreased with trypsin inhibitor.

4. PAR2-AP increased intracellular Ca^{2+}.

We examined changes in $[Ca^{2+}]_i$ after PAR2 activation. Intracellular Ca^{2+} increased with PAR2-AP (2-Furoyl-LIGRLO-NH2, 1 μM) treatment (Fig. 7). The peak manifestation of fluorescence was nearly similar as ATP stimulation. An immediate increase of $[Ca^{2+}]_i$ was evoked by PAR2-AP treatment and followed by a sustained plateau. Although the response was not uniform among the cells, there were similar trends in each cells and the peak response was nearly as much as the response of ATP (100 μM) treatment (Fig. 8). Although this result was not statistically verified, $[Ca^{2+}]_i$ increased with PAR2-AP notably similar with the response of ATP.

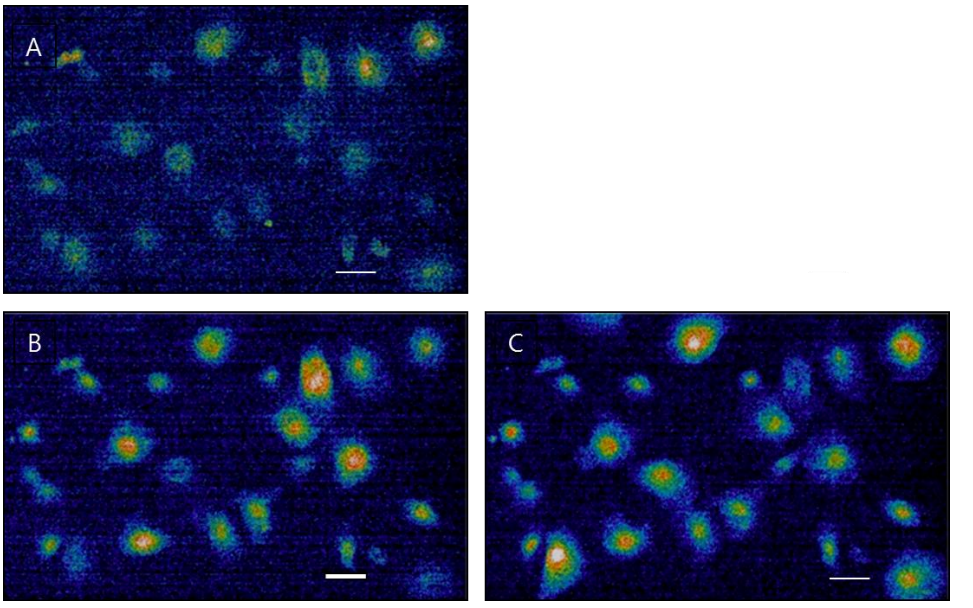


Fig. 6. Fluorescent manifestation of $[Ca^{2+}]_i$ after PAR2-AP treatment in passage 2 HMEE cells.

A : After 30 min incubation of Fluo-3 (5 uM), free Ca^{2+} manifestation at about 50 sec. **B**: In the case of PAR2-AP (2-Furoyl-LIGRLO-NH2, 1 μM) application, fluorescent manifestation is approximately same as the response of ATP (100 μM) . **C**: In the case of ATP treatment, fluorescent manifestation at 50 seconds. (scale bar = 20 ㎛)

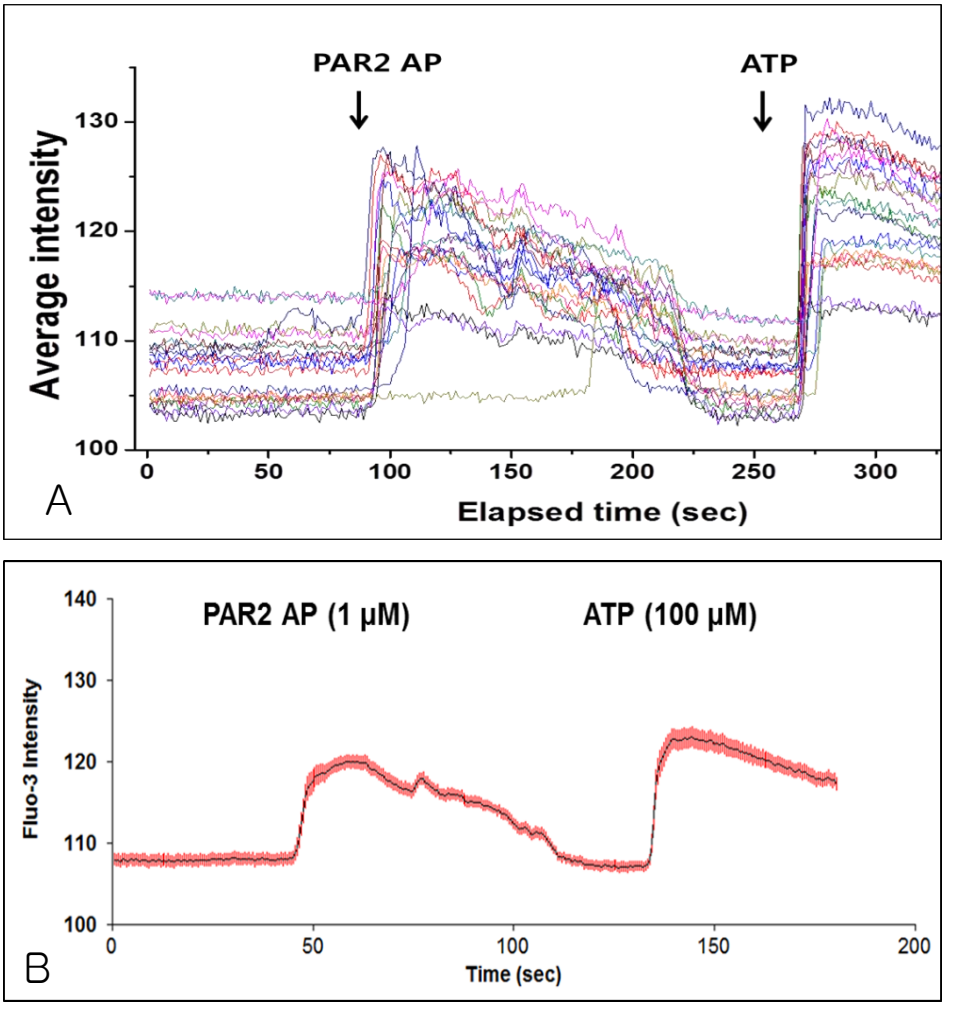
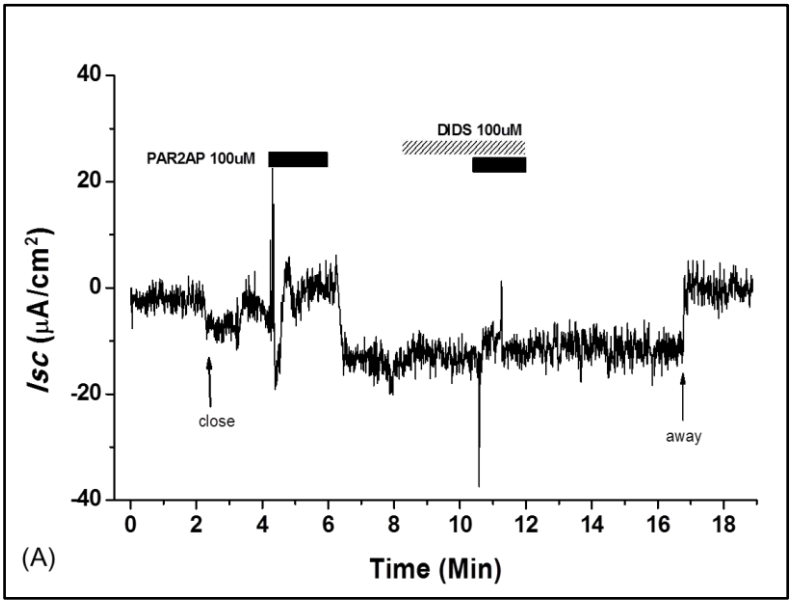


Fig. 7. $[Ca^{2+}]_i$ after PAR2-AP treatment in passage 2 HMEE cells.

A: Average intensity of Fluo-3 after PAR2-AP (2-Furoyl-LIGRLO-NH2, 1 μM) treatment. The intensity increased rapidly and sustained plateau was maintained in some degree**. B:** The mean value of 15 cell lines was presented as this. The size of response was as similar as the reponse of ATP apply.

5. PAR2-AP induces transepithelial current in HMEE cells.

When the vibrating probe was positioned toward middle ear mucosa, short current can be identified by the transepithelial current. The response was considered as proper when the initial current response was upward and upward currents usually show intracellular cation absorption and anion secretion. After PAR2-AP (2-Furoyl-LIGRLO-NH2, 1 μM) treatment, increased current density was noted after 5 sec and it maintained about 1 min (Fig. 9A). The mean value of current density was about 7.8 μA/cm^2. And after 2 min, DIDS (100 μM) was applied to block CaCC. In this, PAR2-AP didn't induce transepithelial current. So, it was thought that PAR2-AP may induce chloride current via CaCC. To exclude the possibility of this increased current density was made by sodium absorption, current density was measured with amirolide pretreatment. In condition of amirolide (10 μM) pretreatment, PAR2-AP induced short circuit current as expected but a little sharp compared to the response without pretreatment (Fig. 9B).



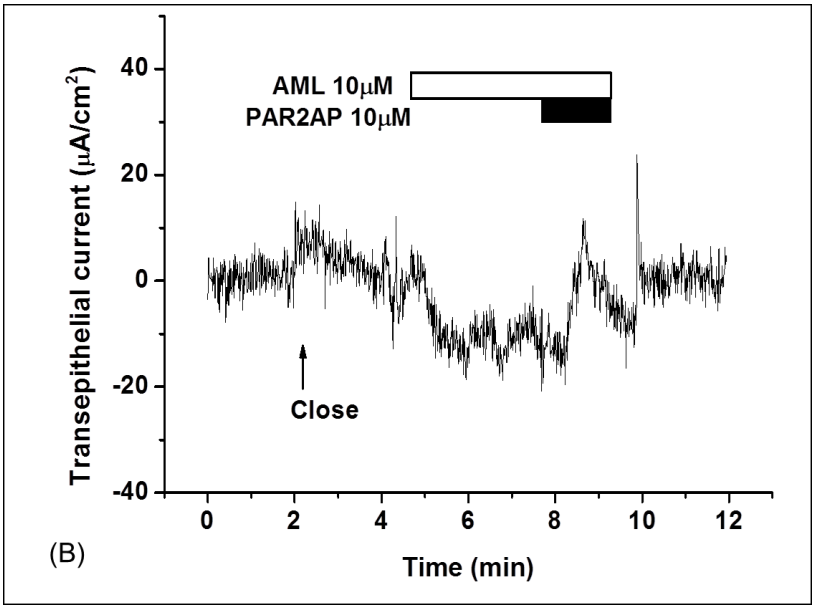


Fig. 8. Transepithelial current by PAR2 activation A: PAR2-AP (2-Furoyl-LIGRLO-NH2, 1 μM) induced upward current density and it was blocked with DIDS (100 μM), known blocker of CaCC. **B:** In case of blocking of ENaC with amirolide (10 μM), PAR2-AP induced short circuit current notably.

IV. DISCUSSION

A fluid-free and air-filled middle ear cavity is required for effective sound transmission. Middle ear effusion is a common disorder affecting approximately 80% of children.[14] Although generally transient, some of children have persistent middle ear effusion and resultant conductive hearing loss and speech delay. The natural mechanism for clearing middle ear effusion includes mucociliary transport, pumping action via the Eustachian tube, and transepithelial fluid transport. [16-18] So, the development of otitis media with effusion was explained by infection, dysfunction of the Eustachian tube, allergy, sinusitis, adenoid vegetation, barotrauma and immune reaction, in the hydrops ex vacuo theory.[19]

NHMEE is an extension of the respiratory tract covered with ASL. Optimal volume of ASL has a critical role.[20] The role of ASL for mucociliary clearance was evaluated in many airway epithelia such as trachea, bronchus, and nasal mucosa. Ion and water transport through the epithelium in disease condition may be greatly distorted and exudates such as middle ear effusion caused by inflammation may alter and disturb various ion channel activities in the middle ear.[20,21]

The middle ear epithelium, an extension of airway epithelia, can absorb and secrete electrolytes and maintain ASL by various ion channels. In the previous study, this was demonstrated that ion and fluid in human middle ear epithelial cells were mainly transported by ENaC and CaCC.[14] Increased secretion by ion channel dysfunction was suggested as the possible main pathophysiology of otitis media. However, a thorough evaluation of such ion and water transport channels in the middle ear has not been conducted.

To our knowledge, there has been no report regarding the role of PAR2 in

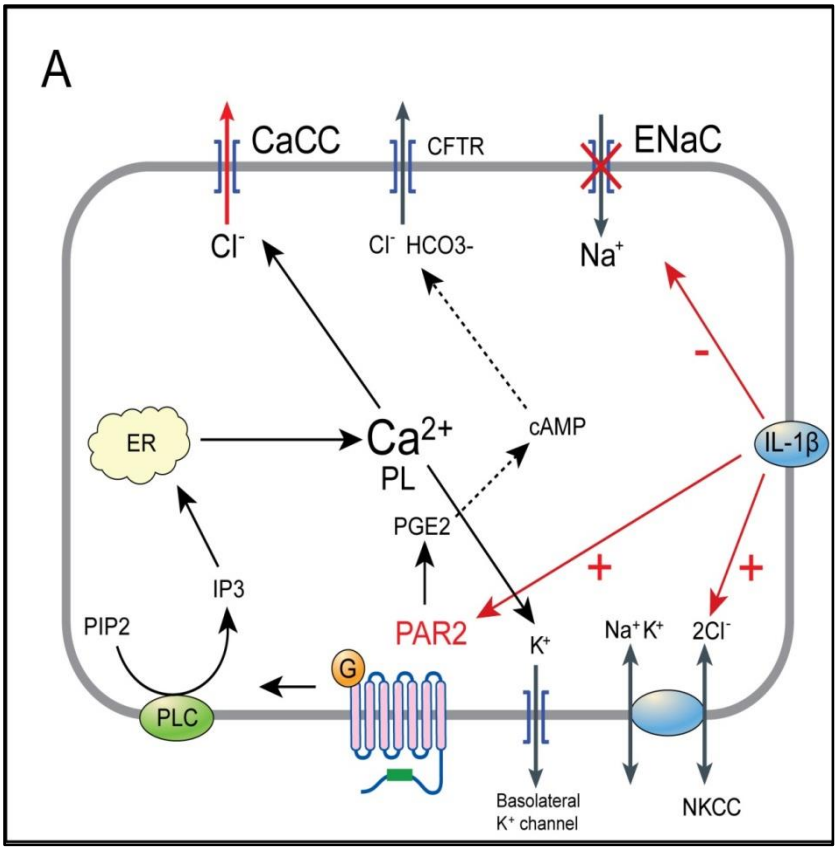
middle ear epithelia and the role of PAR2 in middle ear is not clear. We noted that PAR2 was upregulated in airway inflammation [2,7,9,10] and it was related fluid[22] and mucus[23] secretion. So in the middle ear, an extended space of airway mucosa, PAR2 may have a role related with surface liquid and we focused on the ion channels of HMEE cells with PAR2.

We found that PAR2 expressed in middle ear epithelia. Furthermore, its endogenous activator, human airway tryptase was also found in middle ear effusion, and the fluid can cleavage the PAR2 peptide in dose-dependent manner. Our data strongly indicate that PAR2 and its activation system play a physiologic role in middle ear system.

PAR2 activation increases intracellular Ca^{2+} level in HMEE cell as expected . We also identified that PAR2 induced DIDS- sensitive short circuit current in middle ear mucosa. These data all together suggest that PAR2 activation induced Cl^- secretion through CaCC. Furthermore, activation of apical and baselateral PAR2 increased surface liquid volume in cultured middle ear epithelia. These data were consistent with those from airway epithelia and submucosal gland, where PAR2 induced fluid secretion. Therefore, PAR2 activation can induce fluid overaccumulation, which is main pathology of serous otitis media.

Recent data indicates that ion transporters are differentially expressed in middle ear mucosa during inflammatory condition, which can be a possible pathogenesis of middle ear effusion. Interestingly, PAR2 expression is upregulated by inflammatory cyotokine, IL-1β beta, which was reported to suppress absorptive function of ENaC.[24] Therefore, upregulated PAR2 may disturb the function of ion channels and reinforce the effect of many inflammatory cytokines. This may be related with the pathophysiology of inflammatory middle ear disease such as chronic and serous otitis media.

In addition, middle ear mucosa is known to be an allergic target and PAR2 is reported to enhance airway hyperresponsiveness.[25] In condition of damaged epithelial tight junctions, mast cell activated by allergen releases tryptase which is activator of PAR2. Therefore PAR2 activation can be a possible mechanism of allergen-induced middle ear inflammation and aggravate the inflammatory responses in damaged middle ear epithelia.[26]



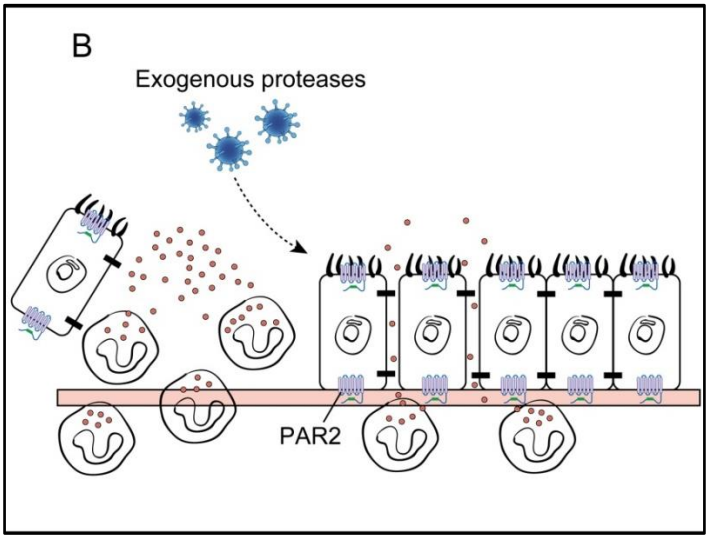


Fig. 9. Epithelial ion transport by PAR2 activation. A: In middle ear epithelial cells, PAR2 activation induces water secretion by CaCC and absorptive function of ENaC is suppressed by IL-1β beta, which aggravates fluid overacculumation.

B: Proteases such as mast cell tryptase or exngenous protease can damage epithelial tight junction and water secretion is aggravated in damaged epithelia , especially in inflammatory condition.

V. CONCLUSIONS

In summary, we showed that PAR2 and its activator are also expressed in middle ear system and its activation induces CaCC current and fluid secretion. The exact role of PAR2 in middle ear inflammation is not exactly understood and need to be more elucidated. Maybe initial increase of chloride current and fluid secretion by activation of PAR2 protects middle ear from external inflammatory stimuli. However middle ear inflammation may be aggravated by inflammatory cytokines, external pathogens to break epithelial tight junctions, and the disturbance of epithelial ion channels. In this aggravated inflammation process, PAR2 is certainly activated and related especially with epithelial ion channel function. So we suggest PAR2 regulates epithelial surface liquid volume via CaCC with increase of intracellular Ca^{2+}.

Further investigation in needed to elucidate the role of PAR2 in middle ear inflammation and how different it is in acute otitis media and chronic otitis media.

REFERENCES

1. Coelho AM, Ossovskaya V, Bunnett NW. Protease-activated receptor-2 : physiological and pathophysiological roles. Curr Med Chem Cardiovas Hematol Agents. 2003;1:61-72.
2. Reed CE. Inflammatory effect of environmental proteases on airway mucosa. Curr Allergy Asthma Rep. 2007;7:368-74.
3. Puente XS, Sanchez LM, Gutierrez-Fernandez A, Velasco G, Lopez-Otin C. A genomic view of the complexity of mammalian proteolytic systems. Biochem Soc Trans. 2005;33:331-4.
4. Ramachandran R, Hollenberg MD. Proteases and signaling: pathophysiological and therapeutic implications via PARs and more. Br J Pharmacol. 2008;153(Suppl 1): 263-82.
5. Lan RS, Stewart GA, Henry PJ. Role of protease-activated receptors in airway function: a target for therapeutic intervention? Pharmacol Ther.2002;95: 239-57.
6. Schmidlin F, Bunnett NW. Protease-activated receptors: how proteases signal to cells. Curr Opin Pharmacol. 2001;1:575-82.
7. Cocks TM, Moffatt JD. Protease-activated receptor-2 (PAR2) in the airways. Pulm Pharmacol Ther. 2001;14:183-91.
8. Knight DA, Lim S, Scaffidi AK, Roche N, Chung KF, Stewart GA, et al. Protease-acitivated receptors in human airways; upregulation of PAR-2 in respiratory epithelium from patients with asthma. J Allergy Clin Immunol. 2001;108(5):797-803.
9.Jarjour NN, Calhoun WJ, Schwartz LB, Busse WW. Elevated bronchoalveolar lavage fluid histamine levels in allergic asthmatics are

associated with increased airway obstruction. Am Rev Respir Dis. 1991;144: 83-7.

10. Yasuoka S, Ohnishi T, Kawano S, Tsuchihashi S, Ogawara M, Masuda K, et al. Purification, characterization, and localization of a novel trypsin-like protease found in the human airway. Am J Respir Cell Mol Biol Chem. 1992;267:13573-9.

11. Son EJ, Gee HY, Lee MG, Lee WS, Choi JY. Uridine-5'-Triphosphate Stimulates Chloride Secretion via Cystic Fibrosis Transmembrane Conductance Regulator and Ca2+-Activated Chloride Channels in Cultured Human Middle Ear Epithelial Cells. Korean J Otorhinolaryngol Head Neck Surg. 2011; 54: 840-6.

12. Herman P, Tan CT, Portier F, Clerici C, Escoubet B, et al. Ion transports in the middle ear epithelium. Kidney Int Suppl.1998; 65: S94–7.

13. Choi JY, Son EJ, Kim JL, Lee JH, Park HY, et al. ENaC- and CFTR-dependent ion and fluid transport in human middle ear epithelial cells. Hear Res. 2006; 211: 26–32.

14. Choi JY. The pathogenesis of Otitis media with effusion. Korean J Otorhinolaryngol Head Neck Surg. 2008; 51: 2-8.

15. Hong JH, Hong JY, Park B, Lee SI, Seo JT, Kim KE, et al. Chitinase activates protease-activated receptor 2 in human airway epithelial cells. Am J Respir Cell Mol Biol. 2008;39:530-5.

16. Tonder O, Gundersen T. Nature of the fluid in serous otitis media. Arch Otolaryngol 1971;93(5):473-8.

17. Honjo I, Hayashi M, Ito S, Takahashi H. Pumping and clearance function of the eustachian tube. Am J Otolaryngol.1985; 6: 241–4.

18. Sade J, Meyer FA, King M, Silberberg A. Clearance of middle ear effusions by the mucociliary system. Acta Otolaryngol.1975; 79: 277–82.

19. Politzer A. Diseases of the ear. 4th ed ed. Philadelphia: Lea Brothers & Co; 1903

20. Boucher RC. Regulation of airway surface liquid volume by human airway epithelia. Pflugers Arch 2003;445:495-8.

21. Widdicombe JH. Regulation of the depth and composition of airway surface liquid. J Anat 2002;201:313-8.

22. Cho HJ, Choi JY, Yang YM, Hone JH, Kim CH, Gee HY et al. House dust mite extract activates apical Cl- channels through protease-activated receptor 2 in human airway epithelia. J Cell Biochem. 2010; 109: 1254-63.

23. Lee HJ, Yang YM, Kim K, Shin DM, Yoon JH, Cho HJ, et al. Protease-activated receptor 2 mediates mucus secretion in the airway submucosal gland. PLoS One 2012;7(8):e43188.

24. Choi JY, Choi YS, Kim SJ, Son EJ, Choi HS, et al. Interleukin-1beta suppresses epithelial sodium channel beta-subunit expression and ENaC-dependent fluid absorption in human middle ear epithelial cells. Eur J Pharmacol. 2007; 567: 19–25.

25. Ebiling C, Forsythe P, Ng J, Gordon JR, Hollenberg M, Vliagoftis H. Protease-activated receptor 2 activation in the airways enhances antigen-mediated airway inflammation and airway hyperresponsiveness through different pathway. J Allergy Clin Immunol. 2005;115: 623-30.

26. Reed CE, Kita H. The role of protease activation of inflammation in allergic respiratory disease. J Allergy Clin Immunol. 2004;114:997-1008.

ABSTRACT (IN KOREAN)

사람 중이 점막 상피 세포에서 이온 이동에 있어 Protease-activated receptor 2 의 역할

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한 수 진

중이점막은 상기도 점막의 연장선으로 기도 표면액의 적절한 유지는 상기도의 보호 기능 유지에 중요한 의미가 있으며 이는 상피 세포의 이온채널에 작용에 의하여 국소적인 반응으로 이루어 진다. 이온 채널의 기능장애에 따른 기도 표면액의 과다 및 중이 저류액의 발생이 중이염 발병할 수 있다는 가설이 제시된 바 있고, PAR2 는 상기도 상피세포에서 염증반응에 활성화 되며 이온 채널에 작용하여 chloride 이온의 분비와 점액 분비에 관여한다고 알려져 있다.

Protease-activated receptor 2 (PAR2) 는 인체의 여러 곳에 분포하며 다양한 조직 반응에 관여한다고 알려져 있다. 본 연구는 상기도 감염에 관여한다고 알려진 PAR2의 사람 중이 점막 세포의 기도 표면액 조절 기능 역할에 대해 알아보고자 하였다.

본 연구에서 중이 점막 세포를 계대 배양하여 중이내 PAR2 가 존재하는 것을 역전사-중합효소연쇄반응으로 확인하였으며, 중이 저류액 내에 PAR2를 활성화시키는 사람 기도 tryptase 가 존재함을

Western blot 법으로 확인하였다. 또한 PAR2 가 중이 저류액을 같이 처리하면 활성화되며, 이 반응이 저류액의 양에 비례하고 중이 저류액에 존재하는 활성화 물질이 trypsin 임을 간접적으로 확인하였다. PAR2-AP 를 이용하여 중이 점막세포에서 세포내 칼슘이 증가하는 것을 확인하였고, CaCC 에 의한 것으로 생각되는 단락전류를 관찰한 바 있다.

이러한 결과로 미루어 보아 중이 저류액 내에 PAR2 활성화 물질이 존재하며 PAR2 가 중이 점막 상피세포에서 기도 표면액 조절에 관여하는 것을 알 수 있었다. 중이내 PAR2 의 활성화로 인한 분비 반응이 중이 저류액을 유발한다고 단정 지을 수는 없으나, 여러 염증 매개 물질에 의한 중이 점막의 손상 및 중이 점막 이온채널의 기능 저하를 유발하여 중이 저류액을 악화시킬 수도 있을 것이다.

핵심되는 말 : Protease-activated receptor 2, 기도표면액, 중이 이온 채널, 사람 중이점막상피세포