Effect of purinergic receptor agonists on mucin secretion in human middle ear epithelial cell

Jae Young Choi

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Joo-Heon Yoon

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Jae Young Choi

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This certifies that the
Doctoral Dissertation of Jae Young
Choi is approved.

Thesis Supervisor : Joo-Heon Yoon

Won Sang Lee: Thesis Committee Member#1

Dong Goo Kim: Thesis Committee Member#2

Eun Chang Choi:Thesis Committee Member#3

Min Goo Lee: Thesis Committee Member#4

The Graduate School
Yonsei University

June 2005

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ABSTRACT

Effect of purinergic receptor agonists on mucin secretion in human middle ear epithelial cell

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Department of Medicine

The Graduate School, Yonsei University

(*Directed by Professor* Joo-Heon Yoon)

Puringeric agonists regulate mucin secretion in the airway epithelial cells. This study examined the effects of the purinergic agonists on Ca^{2+} influx *($[Ca^{2+}]_i$)*, and mucin secretion along with their underlying signaling pathway in normal human middle ear epithelial (NHMEE) cells. The effects of caffeine, an inositol 1,4,5-triphosphate (IP_3) receptor inhibitor, on the UTP induced $[Ca^{2+}]_i$ and mucin secretion in NHMEE cells were also examined. The NHMEE cells were stimulated

with various purinergic agonists, including UTP, and the $[Ca^{2+}]_i$ was measured using a miniature Ussing double perfusion chamber. $P2Y_2$ receptor in NHMEE cells was also localized by immunohistochemistry. UTP-induced mucin secretion was quantified by an immunoblotting assay. The order of the purinergic agonist potency with respect to $[Ca^{2+}]_i$ determined in this study was ATP=UTP>2-MeSATP> ADP>>adenosine which is consistent with that obtained from $P2Y_2$ receptor activation. The $P2Y_2$ receptor is expressed in the apical and basal cell layers of cultured NHMEE cells. UTP-induced $[Ca^{2+}]_i$ was inhibited by 2-aminoethoxydiphenyl borate(2-APB 100μM/ml) but not by ryanodine(10μM). UTP-induced mucin secretion was inhibited by a Ca^{2+} chelating agent, BAPTA-AM, and was stimulated by inomycin. UTP-induced mucin secretion was also suppressed by U73122 and 2-APB while Calphostin C suppressed it to a small extent and PD98059 was ineffective. Caffeine also inhibited the UTP-induced $[Ca^{2+}]_i$ and mucin secretion. These results suggest that the $P2Y_2$ receptor is expressed in NHMEE cells, and plays a major role in modulating the $[Ca^{2+}]_i$. from the IP_3-sensitive intracellular Ca^{2+}store. UTP-induced mucin secretion in NHMEE cells is strongly dependent on Ca^{2+}- and IP_3.

Key words: **mucin, caffeine, signaling transduction, IP_3, Ca^{2+}**

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I. INTRODUCTION

Otitis media with effusion is the most common cause of hearing impairment in children. Moreover, the results of conservative treatment are disappointing, when the effusion changes into mucoid fluid and surgical intervention is needed. The hallmark of mucoid otitis media is mucus hypersecretion from middle ear mucosa[1]. However, the underlying mechanisms of mucus hypersecretion are poorly understood.

Nucleotides such as uridine-5-triphosphate (UTP) and adenosine triphosphate (ATP) regulate mucin secretion by activating the

purinergic receptors in the airway epithelial cells[2,3]. Recently, we showed that purinergic receptors are expressed in the cultured normal human middle ear epithelial (NHMEE) cells and that the agonist, UTP, stimulates mucin secretion[4]. However, it is unclear what purinergic receptor subtypes are functionally active in terms of the intracellular calcium level in NHMEE cells.

In contrast to the apparently universal activation of the exocytotic mechanism by Ca^{2+}, the role of $Ca^{2+}$and the underlying signaling pathways in nucleotide-induced mucin secretion are unclear. UTP up-regulate mucin gene expression and stimulate mucin secretion via Ca^{2+}-independent, diacylglycerol (DAG)-Protein Kinase C (PKC) and extracellular signal regulated kinase (ERK) Mitogen-activated protein kinase (MAPK) pathway in hamster tracheal epithelial cells[5,6]. However, depletion of intracellular Ca^{2+} strongly suppressed the UTP-induced mucin secretion in our preliminary study. This result is consistent with the reports on conjunctival epithelium[7], cholangial epithelium[8] where nucleotide-induced mucin secretion was reported to be Ca^{2+}-dependent. These results suggest that the Ca^{2+}-dependency of mucin secretion by nucleotides might depend on the cell type or species. In addition, Ca^{2+} release from the endoplasmic reticulum (ER) is endowed with two types of Ca^{2+} release channels, the inositol 1,4,5-triphosphate (IP_3) sensitive channel and the ryanodine-sensitive,

Ca^{2+} induced Ca^{2+} release (CICR) [9].

This study first examined the effects of the purinergic agonists on Ca^{2+} influx *($[Ca^{2+}]_i$)* and the underlying release mechanism from the ER in NHMEE cells. Secondly, Ca^{2+}-dependency of UTP-induced mucin secretion and the involvement of signal molecules such as IP_3 and PKC in UTP-induced mucin secretion were investigated. Lastly, this study aimed to confirm the effect of IP_3 inhibition using caffeine, an another inhibitor of IP_3 on UTP induced $[Ca^{2+}]_i$ and mucin secretion in NHMEE cells.

II. MATERIALS AND METHODS

1. Cell culture

The isolation, expansion, cryopreservation, and the serial passaged culture of NHMEE cells were performed as described previously (5, 22). Briefly, the passage-2 NHMEE cells (10^5 cells/well) were seeded in Transwell clear culture inserts (Costar, Cambridge, MA), and cultured in a 1:1 mixture of the bronchial epithelial cell basal medium and Dulbecco's modified Eagle's medium containing all the supplements [10]. Cells were maintained in an incubator in a humidified 95% air/5% CO_2 atmosphere for 7 days until confluent. An air-liquid interface was then created by removing the apical medium and feeding cells basolaterally.

2. Chemicals and Solutions

Fura-2-AM was purchased from Molecular Probes (Eugene, OR). Calphostin C, and heparin were obtained from Calbiochem (La Jolla, CA). All other chemicals including UTP, ATP, UDP, 2-methylthioadenosine 5-triphosphate (2MeS-ATP), adenosine, 2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) and caffeine were acquired from Sigma chemical (St. Louis, MO). The standard perfusion solution contained (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 D-Glucose, and 10 HEPES (pH 7.4 with NaOH).

3. Measurement of $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ measurements in monolayers of the NHMEE cells were performed using previously reported protocols with slight modification [11]. Briefly, cells were loaded with Fura-2 by incubating them for 30 min in a medium containing 3 M Fura-2-AM. A membrane bearing Fura-2-loaded NHMEE cells was then mounted in a miniature Ussing chamber (AKI Institute, Uni. of Copenhagen, Denmark) attached to the stage of an inverted microscope. A transparent coverslip was placed at the bottom of the perfusion chamber, which allowed fluorescence measurements from the dye-loaded monolayer using objective lenses having a long working distance (more than 2 mm). Both luminal and basolateral perfusates were heated to 37C and delivered to the chamber by gravity flow (rate = 35 ml/min). Fura-2 fluorescence was recorded (PTI Delta Ram, Photon Technology International, NJ)at excitation wavelengths of 350 nm and 380 nm, and the 350/380 fluorescence ratioswere calibrated by exposing the luminal surface of the cells to solutions containing various purinergic agonists and blockers. When we examined the effect of caffeine on $[Ca^{2+}]_i$, the cells were pretreated with caffeine for 1 min, and then stimulated with UTP.

4. Fluorescent immunohistochemistry

Fluorescent immunohistochemistry was performed in the cultured NHMEE cells by using an anti-$P2Y_2$ receptor antibody. Briefly, cells were fixed with 4% paraformaldehyde for 24 hours, cryoprotected with sucrose and stored in a deep freezer until required. Frozen samples were then sectioned at 10m and stained by dropping 20 μL (10 ng/mL) of primary rabbit antibody directed against $P2Y_2$ (1:500, Alomone Labs, Jerusalem, Israel)onto a histologic section. After incubating for 1 hour, NHMEE cells were washed 3 times for 10 minutes with PBS. The section were then incubated with Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:200, Jackson Immunoresearch, PA) for 30 mim in a dark room, washed with PBS, and mounted with 10 μL glycerol. Sections so treated were then examined under a fluorescent microscope (high-performance cooled charge-coupled device (CCD) imaging systems Apogee Instruments Inc). Negative controls were performed routinely an antisense peptide.

5. Quantitation of mucin secretion

Before collecting "nucleotide-treated" samples and their corresponding "time-controls", accumulated secretion was removed from the apical surface of the cells by thoroughly washing cells with PBS. To collect "time-control" samples, NHMEE cells were incubated in medium alone for 30 min, and cellular secretionsin the apical medium

were collected and saved. These cells were then exposed to medium containing 100μM UTP and cellular secretions in medium werecollected as "treated" samples. The incubation times of these treated samples were the same as those of the "time-control" samples. In experiments designed to examine the effects of BAPTA-AM(50μM), caffeine and other inhibitors, they were added 10 min prior to UTP treatment thus extending the total treatment period to 40 min. To remove cellular debris, collected samples were centrifuged at 2,500 r.p.m. for 3 min and supernatants were assayed for mucin by dot-blotting method using 17Q2 mucin monoclonal antibody(Covance, CA)[10].

6. Statistical Analysis

The results are expressed as a mean ± standard deviation based on the triplicate experiments and at least two independent experiments from two different primary tissues. The data was analyzed using a Student's *t*-test for the paired and unpaired values. *P* value <0.05 was considered significant.

III. RESULTS

1. Regulation of $[Ca^{2+}]_i$ by purinergic agonists

The cultured NHMEE cells were stimulated with various purinergic agonists. Both ATP (100 μM) and UTP (100 μM) induced relatively large $[Ca^{2+}]_i$ responses. 2MeATP (100μM) also induced $[Ca^{2+}]_i$ but adenosine (100μM) did not (Fig. 1). The agonist profile obtained for the increase in $[Ca^{2+}]_i$,i.e., ATP>UTP>2MeATP>>adenosine is consistent with that obtained from $P2Y_2$ receptor activation[12]. In contrast, UDP (100μM), a $P2Y_6$ agonist, barely induced $[Ca^{2+}]_i$ (Fig. 1).

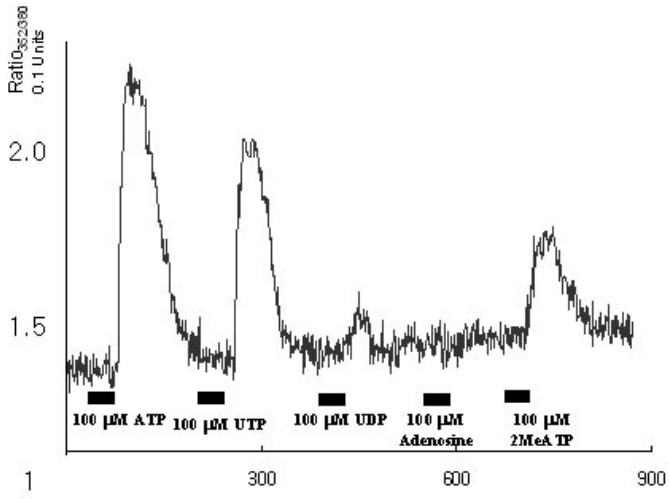

Fig. 1. Mobilization of the $[Ca^{2+}]i$ by the purinergic agonists in cultured normal human middle ear epithelial cells.

2. Localization of the $P2Y_2$ receptor

The $P2Y_2$ receptor was immunolocalized using confocal fluorescence microscopy, and was observed in the apical and basal cell layers of the cultured NHMEE cells, but not in the intermediate cell layers. No signals were observed in the samples treated with the anti-sense peptide (Fig 2).

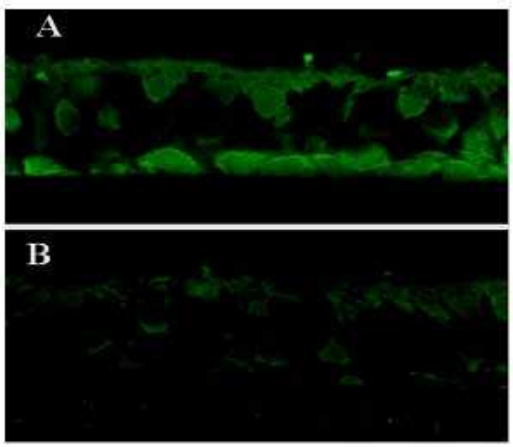

Fig. 2 Immunohistochemical analysis of the normal human middle ear epithelial (NHMEE) cells using antibody against the $P2Y_2$ receptor. The NHMEE cells showed positive immunofluorescent reactions in the apical and basal cell layers (A), whereas, the negative controls, which were treated with the anti-sense peptides, showed no immunoreactivity (B)

3. Contribution of intra- and extra-cellular stores to UTP-induced calcium mobilization

This study further investigated the pattern of UTP-induced $[Ca^{2+}]_i$ in NHMEE cells. The increase in $[Ca^{2+}]_i$ evoked by UTP persisted in the Ca^{2+}-free external solutions, suggesting that the increase in $[Ca^{2+}]_i$ was due to its release from the intracellular Ca^{2+}store (Fig. 3A). The application of 10 μM ryanodine, a CICC antagonist, did not inhibit UTP-induced $[Ca^{2+}]_i$. In contrast, 2-aminoethoxydiphenyl borate (100μM), an IP_3 receptor antagonist significantly inhibited the UTP induced $[Ca^{2+}]_i$. (Fig. 3B and C) This suggests that UTP stimulates $[Ca^{2+}]_i$ from the internal stores, presumably via the IP_3-sensitive Ca^{2+} channels but not through the ryanodine-sensitive CICC.

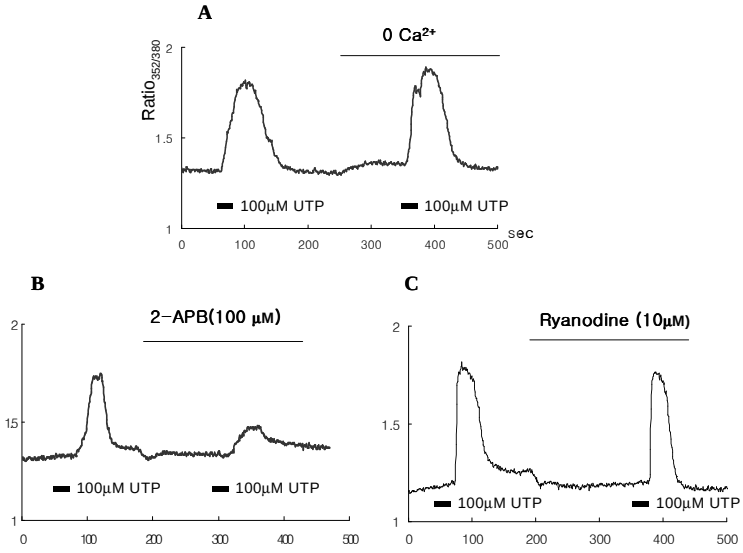

Fig. 3. Effect of the inhibition of the Ca^{2+} uptake from external and internal stores on the UPT-induced Ca^{2+}influx *[*Ca^{2+}*]i* in NHMEE cells. The increase in *[Ca2+]i* evoked by UTP persists in a Ca2+ free solution (A). 2ABP (100μM/ml) inhibits the UTP induced *[*Ca^{2+}*]i* but ryanodine fail to inhibit. (B & C)

4. Ca^{2+} dependency of mucin secretion

The role of intracellular Ca^{2+} on mucin release in NHMEE cells was investigated. The UTP-induced mucin secretion (408± 16 % of control) was suppressed by the depletion of intracellular Ca^{2+} with 50μM BAPTA-AM (176± 19 % of the control level.) and increasing the intracellular Ca^{2+} level with 1μM inomycin, a Ca^{2+} ionophore, enhanced the UTP-induced mucin secretion (528± 21% of the control). Furthermore, the BAPTA-AM suppressed the constitutional mucin secretion (73± 9% of the control) and inomycin increased constitutional secretion (163± 12 %) (Fig. 4). Therefore, it appears likely that both UTP-induced and constitutional mucin secretion are Ca^{2+}-dependent in NHMEE cells.

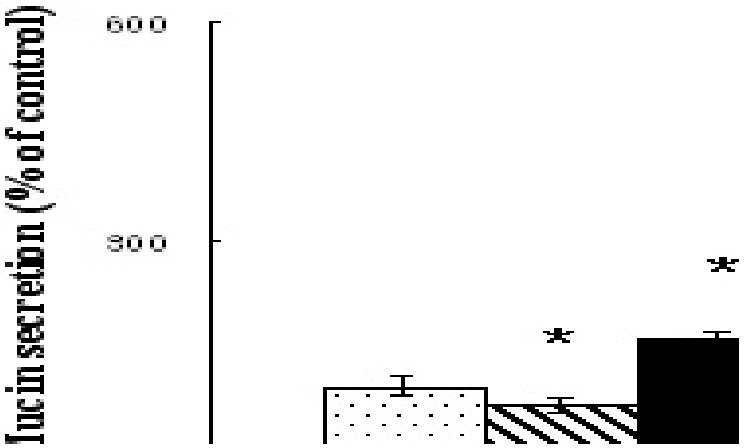

Fig. 4 Effect of intracellular calcium on mucin secretion in NHMEE cells. The depletion of intracellular Ca^{2+} by adding 50 μM 2-bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) suppresses both the UTP-induced mucin secretion and constitutional mucin secretion and inomycin (1 μM) enhanced the constitutional and UTP-induced mucin secretion. BAPTA, BAPTA-AM; Ino, inomycin. * indicates significant difference from the control and ** indicates significant difference from the UTP treated-control.

5. Involvement of PLC and IP_3 in the signaling pathway of the UTP-induced mucin secretion.

UTP-induced mucin secretion (378± 19% of the control) was strongly suppressed by blocking the PLC with 10 μM of U73122 (118± 25% of the control). 2-ABP(100μM), an IP_3 inhibitor, also suppressed the UTP-induced mucin secretion (165± 19% of the control). Calphostin C (0.1μM), a PKC inhibitor, partially inhibited the UTP-induced mucin

secretion (285± 12% of the control), but was less potent than U73122 and heparin. However, PD98059, an ERK MAPK inhibitor, did not suppress the UTP-induced mucin secretion (352± 15% of the control). (Fig. 5) This suggests that the PLC and IP_3 pathways are essential for UTP-induced mucin secretion in NHMEE cells.

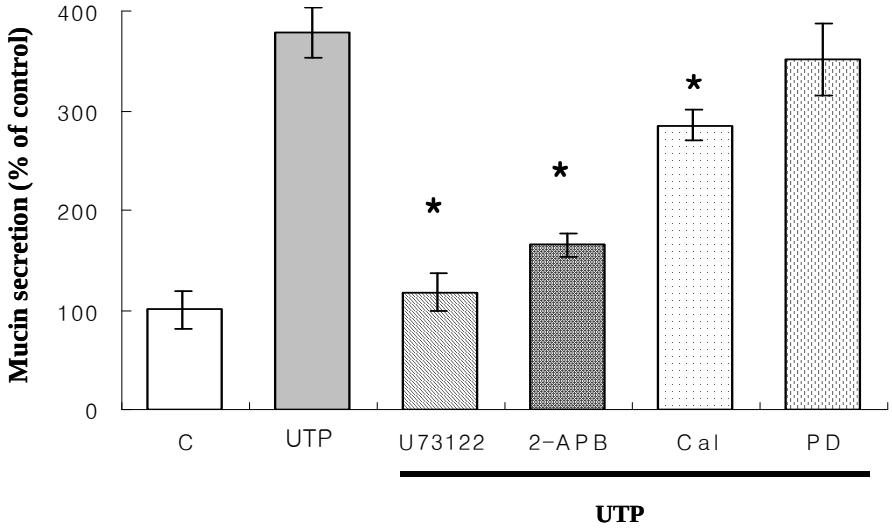

Fig. 5 Effect of the inhibitors on the UTP-induced mucin secretion in human middle ear epithelial cells. C, control; Cal, Calphostin C, PD, PD98059;2-APB, 2-aminoethoxydiphenyl borate. * indicates significant difference from UTP treated-sample.

6. Effect of caffeine, an another IP_3 inhibitor, on UTP-induced $[Ca^{2+}]_i$ and mucin secretion

Caffeine is widely used as a blocker of the IP_3-dependent response [13]. Because IP_3 is essential in UTP-induced mucin secretion, we next wanted to confirm the effect of caffeine on UTP induced $[Ca^{2+}]_i$ in NHMEE cells. UTP-induced $[Ca^{2+}]_i$ is partially suppressed by 5mM caffeine. Fig 6 shows that the inhibitory effect of caffeine gradually increased in a dose-dependent manner.

Fig.6. The Effect of caffeine on the UTP-induced Ca2+ mobilization in NHMEE cells. Caffeine suppressed UTP-induced mucin Ca^{2+} influx in a dose-dependant manner.

As a next, the effect of caffeine on UTP-induced mucin secretion was also evaluated. 100M of UTP promptly induced mucin secretion in NHMEE cells and caffeine suppressed this UTP-induced mucin secretion in a dose-dependent manner.(Fig 7A). More than 10mM of caffeine also suppressed constitutional mucin secretion in a dose-dependent manner (Fig 7B).

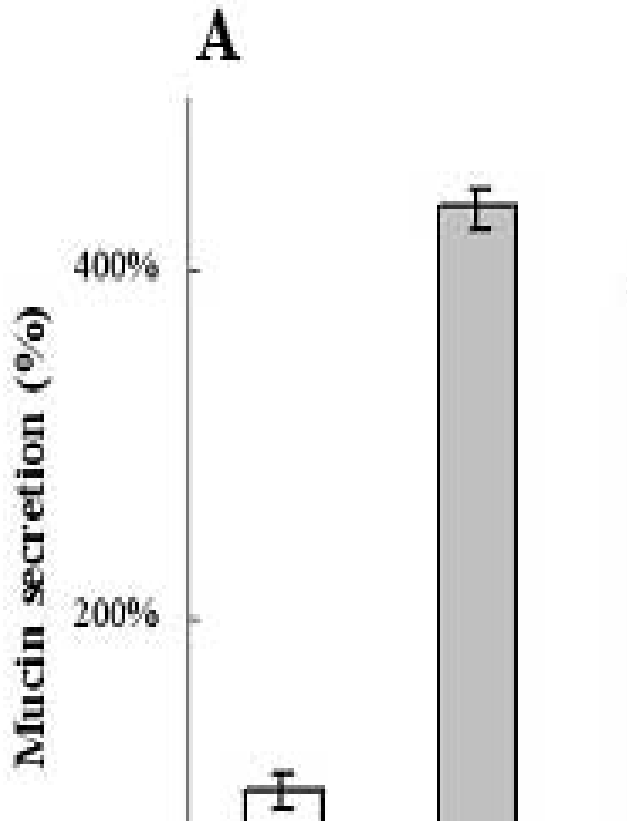

Fig. 7 Effect of caffeine on mucin secretion in NHMEE cells. Caffeine suppressed the UTP-induced mucin secretion in NHMEE cells in a dose- dependent manner.(A) More than 10mM of caffeine also suppressed the constitutional mucin secretion. (B)

IV. DISCUSSION

Extracellular purinergic agonists play significant regulatory roles in various biological responses. They also regulate the mucociliary clearance of the airway epithelium via uracil-sensitive receptors such as $P2Y_2$, $P2Y_4$ and $P2Y_6$.[2,14,15] We previously reported that P2Y2 and $P2Y_6$ mRNA transcripts are present in middle ear epithelial cells both *in vivo* and *in vitro* [4]. However, in this study, $P2Y_6$ could not modulate $[Ca^{2+}]_i$ in NHMEE cells because its potent agonist, UDP, did not activate $[Ca^{2+}]_i$. $P2Y_6$ inactivation in NHMEE cell is a unique finding. $P2Y_6$ was found to be as potent as $P2Y_2$ in the nasal and tracheobronchial epithelium[2,15]. The order of potency (ATP>UTP>2MeATP>>adenosine) described earlier suggests that $P2Y_2$ acts on $[Ca^{2+}]_i$ [12]. The activation of the $P2Y_2$ receptors in the middle ear epithelium by purinergic stimulation resembles findings in the tracheobronchial[3] and nasal[2] epithelium, and is also consistent with a result in the middle ear cell line [16]. It was also confirmed that the $P2Y_2$ receptor is localized in the basal and apical layers of cultured NHMEE cells, but not in the intermediate layers. It is unclear why the $P2Y_2$ receptors are expressed only in these layers.

Nucleotides have previously been shown to stimulate the release of

Ca^{2+} from the ER in mammalian cells via the $P2Y_2$ receptor.[17] The ERis endowed with two different types of Ca^{2+} release channels, i.e. IP_3 and ryanodine receptors.[9] Our data suggest that the intracellular Ca^{2+} level is increased through the IP_3 receptors in NHMEE cells, but not through the ryanodine receptors. These results are consistent with the report by Yang et al.[18] where UTP directly stimulate PLC-mediated IP_3 accumulation and Ca^{2+} mobilization in canine tracheal epithelial cells.

There are conflicting reports about the role of Ca^{2+} in nucleotide-induced mucin secretion in airway epithelial cells. Mucin release by ATP does not require an increase in the intracellular Ca^{2+} level in primary hamster tracheal epithelial cells [6]. On the other hand, nucleotide-induced mucin secretion is regulated independently with Ca^{2+} and the PKC pathways in human nasal epithelial cells[19], the spontaneously transformedrat goblet cell line, SPOC1 cells[20]. These discrepancies suggest that the Ca^{2+}-dependency of the nucleotide-induced mucin secretion differs according to the cell type. In this study, the removal of the intracellular Ca^{2+} with BAPTA-AM strongly blocked the UTP-induced mucin secretion, and inomycin enhanced the UTP-induced mucin secretion in NHMEE cells. These results suggest that UTP-induced mucin secretion is more Ca^{2+}-dependent in NHMEE cells than in the other airway epithelial cells.

There is some controversy regarding the signaling downstream of nucleotide-induced mucin secretion. The activation of the $P2Y_2$ receptor by nucleotide has previously been shown to trigger the activation of PLC, and subsequently PKC. Both of these enzymes have been linked to mucin secretion in hamster[6], human[14] treacheonchial cultures or SPOC1 cells[20]. However, the stimulatory effect of PKC varies according to the cell type.[2] This study found that U73122 and 2-ABP almost completely inhibited the UTP-induced mucin secretion, while Calphostin C suppressed it slightly and PD98059 was ineffective, suggesting that PLC and IP_3 are important signaling molecules in the UTP-induced mucin secretion in NHMEE cells. The crucial role of IP_3 in nucleotide-induced mucin secretion has not yet been reported in other airway epithelial cells, and a more detailed role of IP_3 needs to be elucidated.

An additional aim of this study was to confirm the effect of caffeine on the UTP induced $[Ca^{2+}]_i$ and mucin secretion in NHMEE cells. Caffeine, although best known as an activator of the ryanodine receptor, also inhibits the opening of the IP_3 receptor.[12] In this study, caffeine partially inhibited the UTP-induced $[Ca^{2+}]_i$ and mucin secretion in NHMEE cells, which confirming that NHMEE cells have a caffeine-sensitive IP_3 receptor, and IP_3 plays an important role in the

UTP-induced mucin secretion in NHMEE cells.

V. CONCLUSION

The $P2Y_2$ receptor was found to be expressed in NHMEE cells, and play a major role in modulating the $[Ca^{2+}]_i$. from the ER. UTP-induced mucin secretion in NHMEE cells is strongly Ca^{2+}- and IP_3-dependent.

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국문 요약

인체중이점막 상피세표에서 퓨린수용체 길항제가 점액분비에 미치는 영향

<지도교수 윤 주헌>

연세대학교 대학원 의학과

최 재영

퓨린수용체는 기도상피세포에서 점액분비를 조절하는 것으로 알려져 있다. 본 연구에서는 인체중이점막상피세포에서 퓨린수용체의 자극제가 Ca2+의 세포내 유입*([Ca^{2+}]i)* 과 점액 분비에 미치는 영향을 그 작용기전과 함께 알아보고자 한다. 또한 연구자는 inositol 1,4,5-triphosphate (IP_3)의 길항제인 caffeine이 UTP에 의한 *[Ca^{2+}]i 와 점액분비에 미치는 영향을 알아보았다. 인체중이점막 상피세포를 UTP를 포함한 여러 퓨린수용체 자극제로 자극한수* miniature Ussing double perfusion chamber를 이용하여 *[Ca^{2+}]i를* 측정하였다. 또한 $P2Y_2$ 수용체 항체를 이용하여 인체중이점막상피세포에서 이 수용체의 분포양상을 확인하였다. UTP에 의한 점액분비는 immunoblotting assay를 통하여 정량하였다. 여러 수용체 자극제

의 자극 강도는 ATP=UTP>2-MeSATP>ADP>>adenosine순이었으며, 이는 $P2Y_2$ 수용체의 자극과 일치한다. $P2Y_2$ 수용체는 중이점막상피세포의 첨부와 기저부에서 발현되었다. UTP 에 의한 $[Ca^{2+}]i$ 는 2-APB(100μM)에 의해 억제되었으나, ryanodine(10μM)에 의해서는 억제되지 않았다.UTP에 의한 점액분비는 Ca2+chelating agent인 BAPTA-AM에 의해 억제되고, inomycin에 의해 촉진되었다. UTP에 의한 점액분비는 U73122 와 2-APB에 의해서는 억제되었으나, Calphostin C와 PD98059에 의해서는 억제되지 않았다. Caffeine은 UTP에 의한 $[Ca^{2+}]i$ 와 점액분비를 억제하였다. 이상과 같은 결과는 $P2Y_2$ 수용체가 인체중이점막상피세포에 발현하며, IP_3-sensitive intracellular Ca^{2+} store를 자극하여 세포내 $[Ca^{2+}]i$.을 유도함을 알 수 있었다.

핵심 되는 말: 점액, 카페인, 신호전달, IP_3, Ca^{2+}