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The Administration of P2Y12 Antagonist (P113) Attenuates Airway hyperresponsiveness and Airway Inflammation in an Ovalbumin (OVA) Specific Allergic Asthma Model

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-ABSTRACT-

The Administration of P2Y12 Antagonist (P113) Attenuates Airway hyperresponsiveness and Airway Inflammation in an ovalbumin (OVA) Specific Allergic Asthma Model

Background and Objective: Leukotriene E4 contributes to airway inflammation by interacting with P2Y12 receptor as well as type 1 and 2 cysteinyl leukotriene receptors. We investigated the effects of P113, a P2Y12 receptor antagonist, on airway inflammation in a mouse model of allergic asthma.

Materials and Methods: Sensitized BALB/c mice were intragastrically treated with P113 (30 mg/kg) 30 minutes before OVA challenge. Airway responsiveness (AHR), cell composition and cytokine levels in bronchoalveolar lavage (BAL) fluid were evaluated. Human eosinophil EOL-1 cells were treated with LTE4 with or without pre-treatment of P113. Intracellular expression of ECP was evaluated by Western blot, secreted levels of ECP in cell culture supernatant were measured by ELISA.

Results: P113 treatment decreased AHR to methacholine and airway inflammatory cell numbers in BAL fluid following OVA challenge ($P<0.01$, respectively), attenuated the levels of Th2 cytokine such as Interleukin(IL) -4, IL-5 and IL-13, but not Th1 cytokine in the BAL fluid. The numbers of inflammatory cells in peribronchial and perivascular areas as well as mucus-containing goblet cells were
decreased in P113 treated mice compared to vehicle group ($P < 0.01$). LTE4 stimulation decreased intracellular expression but increased secretion of ECP from EOL-1 cells, which was abolished by P113 pre-treatment.

**Conclusion:** P113, a P2Y12 receptor antagonist could prevent the development of AHR, airway inflammation, and cytokine production in allergen challenged mice, affect the activation and function of eosinophils, suggesting that it may be a novel therapeutic target for asthma treatment.

**Key words:** Leukotriene E4, Asthma, Eosinophil, Purinergic P2Y12
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ABBREVIATIONS

**AHR:** airway hyperresponsiveness

**BAL:** bronchial alveolar lavage

**CCL5:** chemokine CC-ligand 5

**CysLTs:** cysteinylleukotrienes

**CysLTR:** cysteinylleukotrienes receptor

**ECP:** eosinophil cationic protein

**EDN:** eosinophil derived neurotoxin

**ELISA:** enzyme-linked immune sorbent assay

**EPO:** eosinophil peroxidase

**H&E:** hematoxylin & eosin

**ICAM-1:** intercellular Adhesion Molecule 1

**IHC:** immunohistochemistry

**LT:** leukotriene

**MCh:** methacholine

**OVA:** ovalbumin

**PAS:** periodic acid-Schiff

**P2Y12R:** purinergic P2Y12 receptor

**VCAM-1:** vascular cell adhesion protein 1
I. INTRODUCTION

Eosinophils are prominent leukocytes in allergic inflammation which contain abundance of preformed cytokines and mediators, including eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil derived neurotoxin (EDN) and growth factors, in their granules (Blanchard et al. 2009, Rothenberg et al. 2006, Gleich 2000). Human eosinophils not only produce and secrete cysteinyl leukotrienes (CysLTs) but also express both CysLT receptor 1 (CysLTR1) and CysLT receptor 2 (CysLTR2) on the cell surface, suggesting they have an autocrine mechanism. CysLTs, including leukotriene (LT) C4, D4 and E4, are well known proinflammatory lipid mediators which contribute to the pathogenesis of asthma by affecting leukocyte recruitment, enhancing activities and functions of eosinophils as well as mast cells (Peters-Golden et al. 2007). Among the three common CysLTs, LTE4 is the most stable form and most abundant in urine of asthmatics (Sala et al. 1990). LTE4 is known to have greater inflammatory effects compared to LTC4 and LTD4 on inducing bronchial constriction and increasing the infiltration of inflammatory cells (Laitinen et al. 1993, Gauvreau et al. 2001). However, while LTC4 and LTD4 act by binding to CysLTR1 and CysLTR2, LTE4 binds to those receptors with low affinities (Lynch et al. 1999, Heise et al. 2000, Woszczech et al. 2007, Woszczech et al. 2008).
Recently, P2Y12 receptor (P2Y12R) has been suggested as a novel potential receptor for LTE4(Foster et al. 2013, Paruchuri et al. 2009). P2Y12 is an adenosine diphosphate (ADP) chemoreceptor previously found on platelet membrane. Concurrently, P2Y12R also was found to be expressed on eosinophil granule membranes (Neves et al. 2010). This finding suggested the benefit of P2Y12R inhibition in attenuating inflammatory activity of eosinophils.

Previous study demonstrated that the metabolite of P113 competes with ADP to bind to P2Y12R. Consequently, P113 is considered as a novel antagonist of LTE4. P113 is a thienopyridine class antiplatelet agent which has been used to inhibit blood clots in treatment of various cardiovascular diseases (Laidlaw et al. 2012). The drug has also been proposed as a novel medication for inflammatory disease, including asthma, due to its anti-inflammatory and immunomodulatory effects observed in cardiovascular diseases (Foster et al. 2001). However, their anti-inflammatory effects in allergic disease such as asthma have not been completely understood.

Based on those findings, we aimed to investigate the inhibition effect of P113 as well as Montelukast on airway inflammation and CysLT-elicited eosinophil degranulation by \textit{in vivo} and \textit{in vitro} systems.
II. MATERIALS AND METHODS

1. Animals

Female BALB/c mice (6-8 weeks of age) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed under specific pathogen- and OVA-free conditions and maintained on a 12-hour light-dark cycle with food and water ad libitum. All animal experiments performed in the present study were approved by the Institutional Animal Care and Use Committee of Ajou University. (IACUC 2013-0068).

2. OVA sensitization, challenge and P113 treatment

The experimental protocol for allergen sensitization and challenge was modified from described procedures (Takeda et al. 2005). Briefly, mice in asthma group were sensitized with 10μg of OVA (Fisher Scientific, Pittsburgh, PA, USA) emulsified in 1mg of Aluminum hydroxide adjuvant (Sigma-Aldrich, St. Louis, Mo, USA) in a total volume of 100μl by intraperitoneal injection on days 0 and 14. For OVA challenge, mice were nebulized with OVA aerosols (1% OVA in saline) for 20 minutes on days 28, 29, and 30 (OVA/OVA vehicle group). For P113 treatment, some were administered by oral gavage with 30mg/kg P113 (Sigma-Aldrich, St. Louis, MO, USA) dissolved in phosphate buffer saline (PBS) for 30 minutes before each OVA challenge (OVA/OVA P113 treatment group). Control mice were sensitized by OVA and given PBS as sham challenge (OVA/Saline vehicle group).
3. Measurement of airway hyperresponsiveness (AHR)

The airway resistance to inhaled methacholine (MCh) (Sigma-Aldrich, St. Louis, MO) was measured using the flexiVent System (SCIREQ, Montreal, Canada) 48 hours after the last OVA challenge, as previously described (Tarkowski et al. 2007). Briefly, the mice were anesthetised with an intraperitoneal injection of pentobarbital sodium (100 mg/kg, JW pharmaceutical, Seocho-gu, Seoul, Korea); then, a tracheotomy was performed, and a cannula was inserted. The mice were connected to a computer-controlled small-animal ventilator and ventilated with a tidal volume of 10 mL/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of 2 cm H2O to achieve a mean lung volume close to that during spontaneous breathing. After baseline measurement, each mouse was challenged with MCh aerosol, with increasing concentrations (0, 1.56, 3.12, 6.25, and 12.50 mg/mL) and the peak airway responses to the inhaled MCh were recorded.

4. Bronchoalveolar lavage (BAL) and lung histology

BAL fluids were collected immediately after AHR measurements by lung lavage with 1 ml of Hank’s balanced salt solution (HBSS). The BAL fluids were centrifuged at 1200 rpm the cell pellets were resuspended in HBSS. The number of total leukocyte was counted with a hemocytometer, and cell differentiation was performed on cytospin slides using Wright-Giemsa staining. After BAL fluid was
collected, the lungs were fixed in 13% formalin, embedded in paraffin, and cut into 5μm sections. The numbers of inflammatory and mucus-containing cells were quantified as previously described (Tomkinson et al. 2001). Tissue sections were visualized using the imageJ software program (National Institutes of Health, USA). For detection of inflammatory cells, lung sections were stained with hematoxylin and eosin (H&E) and the numbers of inflammatory cells per micrometer square of perivascular and peribronchial area were determined. In addition, numbers of mucus-containing cells per micrometer of basement membrane were counted after staining the lung sections with periodic acid-Schiff (PAS) and counterstained with hematoxylin.

5. Enzyme-linked immunosorbent assay (ELISA)

The BAL fluid was centrifuged and the supernatant was frozen until further analysis. The levels of IL-4, IL-5, IL-13, and IFN-γ in the BAL fluid were measured using a sandwich ELISA, according to the manufacturer’s instructions (eBioscience, San Diego, CA, USA). The lower limits of detection were 4pg/mL for IL-4, IL-13 and IL-5, and 15pg/mL for IFN-γ.

Eosinophil cationic protein (ECP) levels in human eosinophil cells (EOL-1) granule supernatants were analyzed by a quantitative ECP ELISA kit (Medical & Biological Labs, Nakaku Nagoya, Japan) according to the manufacturer’s instructions. The lower limits of detection were 0.125ng/ml for ECP.
Chemokine CC-ligand 5 (CCL5) levels in BAL fluid were measured by a quantitative CCL5 ELISA kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

6. In vitro cell culture

EOL-1 cells were purchased from Sigma Aldrich (St. Louis). Cells (1 x 10^6/ml) were seeded into each well of 6-well plates, grown in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin under a 5% CO₂ humidified atmosphere at 37°C. After reaching the appropriate cell confluence, the cells were pretreated with P113 (10 μg/ml) or montelukast (10 μM) in serum-free media for 30 minutes when indicated. Then the cells were stimulated with 300 nM of LTE4 for further 30 minutes. After treating, the cells were lysed in RIPA buffer containing protease inhibitor cocktail (Fisher Scientific, Pittsburgh, PA). The expression of the ECP was then determined by Western blot.

7. Western blot analysis

Cell lysates were prepared from EOL-1 cells. Total 30 μg of protein from each cell lysate were loaded onto a 15% SDS-PAGE gel, subjected to electrophoresis, and transferred to PVDF membranes. The membranes were blocked by 5% skim milk in PBS containing 0.05% Tween-20 and then incubated with the appropriate
antibodies. Antibodies used against human target proteins were, ECP (Santa cruz Biotechnology, sc-135469, 1:1000), and β-actin (Santa cruz Biotechnology, sc-1616, 1:1000).

8. Immunohistochemistry (IHC)

For immunofluorescence staining, tissue sections were preincubated in blocking buffer (PBS containing 1% BSA) for 1 hr at room temperature (RT) and then incubated 16-20 hr at 4°C with primary antibodies. Biotinylated secondary antibody (Vector, Burlingame, CA, USA) was used (2 hr at room temperature) and finally avidin-biotin horseradish peroxidase complex (Vectastain ABC Elite kit) for 60 min at room temperature. The reaction product was developed with the diaminobenzidine (DAB) (Vector, Burlingame, CA, USA). Cell nuclei were counterstained with Hematoxylin. Sections were dehydrated in ascending alcohol concentrations and Xylene and mounted using permount. Stained slides were analyzed with a Scan scope (Aperio, San Diego, CA, USA).

9. Statistical analysis

The results were presented as the mean ± SEM. Comparisons among the study groups were performed by one-way ANOVA followed by Tukey’s post hoc test. All statistical analysis were complemented using SPSS version 19.0 (SPSS Inc,
Chicago, IL, USA) and a $P$-value of less than 0.05 was considered statistical significant. The graphs were processed using Graph Pad Prism version 5.0 (GraphPad software Inc., San Diego, California, USA).
III. RESULTS

1. P113 treatment prevents the development of AHR and airway inflammation

To determine the effects of P113 treatment on allergen-induced AHR and airway inflammation, mice were treated with 30mg/Kg P113 during the OVA challenge phase. Following OVA sensitisation and challenge, OVA/OVA vehicle mice had significantly higher lung resistance responded to MCh (Fig 1A) as well as greater numbers of inflammatory cells (Fig 1B), including eosinophil, in BAL fluid compared to sham-sensitised, OVA challenged mice ($P < 0.01$). Mice treated with P113 developed significantly lower airway responsiveness to inhaled MCh and eosinophil counts in BAL fluid compared to the vehicle mice ($P < 0.01$).

As shown in Fig. 2, P113 treatment reduced the levels of IL-4, IL-5, and IL-13 in BAL fluid of sensitised and challenged mice, but we did not find a significant difference in the IFN-γ level compared to the vehicle-treated mice.

Histopathological analysis of lung tissue sections revealed that the numbers of inflammatory cells, including eosinophils, in the peribronchial and perivascular areas increased in mice after OVA sensitisation and challenge compared to sham-sensitised, OVA-challenged mice (Fig.3A). Similarly, the numbers of PAS$^+$ mucus-containing goblet cells increased in the sensitised and challenged mice (Fig.3B). However, the administration of the P113 significantly decreased the numbers of
inflammatory cells and PAS$^+$ mucus-containing goblet cells in lung tissue (Fig. 3A and B).
Fig. 1. Effect of P113 treatment on airway responses in an OVA specific asthma model

(A) Changes in lung resistance changes in response to increasing doses of methacholine at doses of 0, 1.56, 3.12, 6.25 and 12.5 mg/kg were assessed 48 hours after the final challenge, and expressed as a percent of baseline (saline) values. (B) Cell composition in BAL fluid. OVA/Saline vehicle, mice sensitized by OVA and challenged with saline; OVA/OVA vehicle, mice sensitized and challenged with OVA; OVA/OVA P113 treatment, OVA challenged mice treated with P113. The data expressed as the mean±SEM. n=15 for each group. **P<0.01 and *P<0.05 are vs OVA/Saline vehicle group. **P<0.01 and *P<0.05 vs OVA/OVA vehicle groups. RL, resistance of lung; OVA, ovalbumin;
Fig. 2. Effect of P113 treatment on Th2 cytokine levels

The levels of IL-4, IL-5, IL-13 and IFN-γ were determined by ELISA in BAL fluid. OVA/Saline vehicle, mice sensitized by OVA and challenged with saline; OVA/OVA vehicle, mice sensitized and challenged with OVA; OVA/OVA P113 treatment, OVA challenged mice treated with P113. Data are expressed as the means±SEM (n=5). **P<0.01 and *P<0.05 vs OVA/OVA vehicle groups. IL, interleukin; IFN, interferon;
Fig. 3. Effect of P113 treatment on lung histology

(A) Lung tissue histology with hematoxylin and eosin (H&E) and (B) periodic acid-Schiff (PAS) staining. Quantitative analysis of inflammatory and PAS$^+$ cells in lung tissue was performed as described in Materials and Methods. i, mice sensitized by OVA and challenged with saline (OVA/Saline vehicle); ii, mice sensitized and challenged with OVA (OVA/OVA vehicle); iii, OVA challenged mice treated with P113 (OVA/OVA P113 treatment). **$P<0.01$ vs the OVA/OVA group,
2. P113 treatment decreased the secretion of chemokine CC-ligand 5 (CCL5) into BAL fluid but did not affect the expression of adherent molecules in lung tissue

To determine the mechanisms by that P113 treatment could attenuate the infiltration of inflammatory cells into lung tissue, we investigated the secretion of CCL5 in BAL fluids and the expressions of two adherent molecules, vascular cell adhesion protein 1 (VCAM-1) and intercellular Adhesion Molecule 1 (ICAM-1) in lung homogenates from P113 treated and non-treated mice.

In OVA-sensitized and challenged mice treated with P113, level of CCL5 in BAL fluid was significantly lower compared to OVA/OVA vehicle group (Fig.4.). However, P113 treatment did not affect the expression of VCAM-1 as well as ICAM-1 in lung homogenates (Fig.5.)
**Fig. 4** Effects of P113 treatment on the secretion of CCL5 in BAL fluids

### P < 0.01 vs OVA/Saline vehicle group. **P < 0.01 vs OVA/OVA vehicle groups.**

BAL, bronchial alveolar lavage; CCL5, chemokine CC-ligand 5;
Fig. 5. Effects of P113 treatment on the expression of adhesion molecules in mice lung homogenates

(A) VCAM-1 and (B) ICAM-1. Data are expressed as the means±SEM. ## $P< 0.01$ versus the OVA/Saline vehicle group. NS, not significant;
3. P113 inhibits eosinophil cationic protein (ECP) secretion and expression from LTE4 stimulated eosinophil granules

As shown in Fig 6, the expression of ECP in the peribronchial and perivascular areas increased in mice after OVA sensitization and challenge compared to sham-sensitized, OVA-challenged mice. However, the administration of the P113 significantly decreased the expression of ECP in lung tissue.

Next, we quantified intracellular expression levels of ECP in EOL-1 cells stimulated with LTE4 followed by P113 treatment. The intracellular expression of ECP was decreased by LTE4 treatment, while the P113 treatment inhibited LTE4-reduced ECP expression (Fig. 7A).

We also investigated whether LTE4 induce the secretion of ECP from human eosinophils. As shown in Fig 7B. The levels of ECP in culture supernatant were significantly increased by LTE4 ($P<0.01$). In addition, montelukast as well as P113 attenuated the secretion of ECP from EOL-1 cells induced by LTE4 ($P<0.01$ for both). Notably, inhibiting effect of p113 on LTE4-induced eosinophil, ECP secretion was significantly higher than that of montelukast ($P<0.01$).
Fig. 6. Effect of P113 treatment on ECP expression in lung tissue

(A) mice sensitized by OVA and challenged with saline (OVA/Saline vehicle); (B) mice sensitized and challenged with OVA (OVA/OVA vehicle); (C) OVA challenged mice treated with P113 (OVA/OVA P113 treatment).
Fig. 7. Effects of P113 and montelukast on ECP expression in human eosinophil, EOL-1

Cells were pretreated with P113 (10μg/ml) or montelukast (10 μM) in serum-free media for 30 minutes then were stimulated with 300nM of LTE4 for further 30 minutes. (A) Intracellular expression of ECP in EOL-1 cell investigated by Western Blot. (B) Levels of secreted ECP in cell culture supernatants evaluated by ELISA. ##, P < 0.01 compared to negative control group; **, P < 0.01 compared to LTE4 treated group. P-values were obtained by one way ANOVA.
IV. DISCUSSION

Asthmatic airway inflammation is characterized by increased infiltration of inflammatory cells including eosinophils and, mucus secretion along with with bronchoconstriction and lung tissue remodeling, which could be enhanced by cysteinyleukotrienes including LTC4, LTD4 and LTE4 (Kanaoka et al. 2004, Barnes et al. 1998). There are at least three G-protein coupled receptors for cysLTs, including CysLT1R and CysLT2R (Laidlaw et al. 2012), and the newly discovered CysLT3R (Foster et al. 2013, Paruchuri et al. 2009). LTD4 and LTC4 strongly bind to CysLT1R and CysLT2R, which were found to be expressed by various cells of immune system (Kanaoka et al. 2004). Leukotriene receptor antagonist such as montelukast is a well-known CysLTR1 antagonist that competes with LTD4 to bind to its receptor, and thereby attenuates the bronchoconstriction and airway inflammation induced by the CysLT (Takeda et al. 2010). Additionally, montelukast could exhibit its anti-inflammatory effects by inhibiting the production of Th2 cytokines (Wu et al. 2003).

However, the last and the most stable metabolite of LTs is LTE4, which was found to be increased more highly in the urine of asthmatics compared to healthy subjects (Sampson et al. 1995). Urinary LTE4 was particularly high in patients with AERD (6-fold greater on average than in aspirin-intolerant asthmatics) and further increased by as much as 10-fold from baseline after oral aspirin challenge (Christie et al. 2000).
In addition, nasal administration of LTE4 increased the infiltration and accumulation of various inflammatory cells in murine lung tissue. LTE4 was found to bind weakly to CysLT1R as well as CysLT2R; therefore, many scientists tried to identify the high affinity receptors for LTE4. Recently, several novel candidates for LTE4 receptors, including GPR99 as well as P2Y12, were suggested; however, the most potential LTE4 receptor has not yet been identified (Laitinen et al. 1993, Foster et al. 2013, Kanaoka et al. 2013). Recently, several sporadic reports demonstrating the effect of P2Y12 receptor antagonist on inhibiting inflammatory response have been published (Laidlaw et al. 2012). In addition, P2Y12 antagonist has been proposed as a novel treatment for inflammatory disease, including asthma, due to its anti-inflammatory and immunomodulatory effects observed in cardiovascular diseases (Foster et al. 2001).

Based on those findings, we investigated the protective role of a P2Y12 receptor antagonist, P113, on asthma pathogenesis by using a mouse asthma model and *in vitro* experiments. Previous study reported that treatment with P2Y12 antagonist attenuated the mucus secretion and inflammatory cell infiltration into the airways of asthmatic mice (Paruchuri et al. 2009), which is consistent with our findings in the present study. In addition, we provided the evidence to show that P113 treated mice exhibited the decrease of AHR to methacholine and the inhibited secretion of Th2 cytokines in BAL fluid compared to those of control mice (without treatment).
Paruchuri et al. demonstrated that knocking out of P2Y12, but not of CysLT1R and CysLT2R, attenuated the effect of LTE4, suggesting that P2Y12 is a newly potential receptor for LTE4. Taken together, these findings indicated a crucial role of LTE4 in asthma pathogenesis and inhibition of P2Y12 receptor may be a new therapeutic target of asthma.

Eosinophil is the most important cell in allergic airway inflammation and increased number of eosinophils in airway is one of the characteristics of asthma (Fahy 2009). Eosinophils that infiltrated to asthmatic airways could undergo primary lysis and release free eosinophil granules which contain most of the important effecting mediators of esoniophils including ECP. Additionally, the mediators released from eosinophil granules were known to play crucial role in tissue repair/remodeling in asthma pathogenesis (Persson 2014). In this study, we found that the attenuation of eosinophilia after treating with P113 was striking in BAL cell differential count compared to another study using montelukast which did not significantly affect the recruitment of eosinophils into the asthmatic airways (Wu et al. 2003). This finding suggested that P113 may have important effect on eosinophil activation and function.

The fact that LTE4 increased the infiltration of eosinophils in asthmatic lung, induced the release of ECP from isolated human eosinophil granules by binding to P2Y12 receptor suggested that LTE4 could affect the activation and function of
eosinophils (Paruchuri et al. 2009, Neves et al. 2010). We further investigated the influence of LTE4 as well as P2Y12 antagonist, P113, on the activation and function of eosinophil *in vitro*. Expectedly, we found that LTE4 stimulation caused the decreased intracellular expression and increased extracellular secretion of ECP from human eosinophils, which indicates that LTE4 could trigger the degranulation of eosinophils. Moreover, P113 treatment suppressed the LTE4-induced decrease of intracellular expression and increased extracellular secretion of ECP. We also found that P113 treatment significantly inhibited the expression of ECP in lung tissue sections from asthmatic mice. Those findings suggested that P113, possibly via binding to P2Y12 receptor, may abolish LTE4-induced degranulation of eosinophils.

Adhesion molecules were known to mediate for the migration of intravascular immune cells into the surrounding tissues during inflammatory response (Stanciu et al. 1998). In the present study, we found increased expression of VCAM and ICAM in lung tissue homogenates from OVA-sensitized and challenged mice; however, those expression were not suppressed by treating with P113 (Fig. 6). Consequently, we speculated that P2Y12 antagonist could inhibit the migration of inflammatory cells via attenuating the production and/or secretion of several chemokines. The dramatically decreased airway eosinophilia in P113 treated mice urged us to investigate the secretion of CCL5, a potent eosinophil attraction (Fuentes-Beltran et
al. 2009), in BAL fluid. Expectedly, CCL5 secretion was significantly decreased by the treatment of P113 (Fig. 5). This finding suggested that the inhibition of P2Y12R by its antagonist could decrease the recruitment of eosinophils into the asthmatic airways.

Further studies are needed to elucidate the effects of P2Y12 antagonist, such as P113, on eosinophil activation and function as well as on asthmatic inflammation. In addition, the combinations with CysLTR1 antagonist may be more beneficial in the management of asthma and need to be further investigated.

Overall, our results provide compelling evidence showing that P113 could attenuate allergic inflammation and airway hyper-responsiveness in asthma probably by inhibiting the secretion of Th2 cytokines as well as attenuating the activation of eosinophils. Those findings suggested a novel therapeutic treatment for asthma by using P2Y12 antagonists.
V. CONCLUSION

P113, a P2Y12 receptor antagonist could prevent the development of AHR, airway inflammation, and cytokine production in allergen challenged mice, affect the activation and function of eosinophils, suggesting that it may be a novel therapeutic target for asthma treatment.
REFERENCES


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국문요약

Ovalbumin (OVA) specific 알레르기천식모델에서 P2Y12 길항제(P113)투여에따른기도과민성과기도염증의감소

서 동 현
(지도교수: 박해심)

연구배경 및 목적 : 류코트리엔 (LT) E4 는 P2Y12 수용체와 상호작용을 할 뿐만 아니라 제 1 형과 2 형 시스테이닐 류코트리엔 수용체에 의해기도 염증에 기여한다. 우리는 알레르기 천식 마우스 모델의 기도 염증에 P2Y12 수용체 길항제인 P113 의 효과를 조사하였다.

재료 및 방법 : Ovalbumin (OVA)으로 감작시킨 마우스에 P113 을 30mg/kg 의 농도로 OVA 를 흡입시키기 30 분 전에 처리하였다. 기도과민성과 기관지 폐포 세척액에서 세포를 구분하고 사이토 카인의 양을 측정하였다. 사람의 호산구 세포인 EOL-1 에 P113 을 전처리 한군과 아닌 군으로 나누어 각각 LTE4 를 처리하였다. Western blot 분석법을 통하여 세포 내 호산구 양이온 단백질 (ECP)의 발현을 측정하였고, 효소 면역 측정법 (ELISA)을 이용하여 세포 배양 상층액에서 분비된 ECP의 양을 측정하였다.
결과: P113 처리군에서 methacholine에 의한 기도과민성과 기관지 폐포 세척액내의 기관지 염증 세포의 수가 OVA 단독 흡입군과 비교하여 통계적으로 유의하게 감소하였고 ($P<0.01$), Th2 사이토카인인 인터루킨 (IL) -4, IL-5, IL-13이 현저하게 감소하였지만 Th1 사이토카인은 차이가 없었다. 또한 P113 처리군에서 기관지와 혈관 주변의 염증 세포의 수와 배상 세포 (goblet cell)에서 분비되는 점막의 양이 현저하게 감소하였다 ($P<0.01$). EOL-1 세포에서 P113을 전 처리한 군에서 LTE4의 자극으로 인해 분비되는 ECP의 양을 LTE4 단독 자극을 준 군과 비교하였을 때 현저하게 감소시켰다.

결론: P113은 P2Y12 수용체의 길항제로써 기도과민성, 기도 염증, 사이토카인의 생산등을 감소시켰고, 또한 호산구의 기능과 활성을 감소시키므로 천식을 치료하는데 있어서 새로운 치료 약물로 제안될 수 있다고 생각한다.