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Effect of secreted lymphocyte antigen-6/urokinase-type plasminogen activator receptor-related peptide-1 (SLURP-1) on airway epithelial cells

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ABSTRACT

Acetylcholine (ACh) exerts various anti-inflammatory effects through α7 nicotinic ACh receptors (nAChRs). We have previously shown that secreted lymphocyte antigen-6/urokinase-type plasminogen activator receptor-related peptide-1 (SLURP-1), a positive allosteric modulator of α7 nAChR signaling, is down-regulated both in an animal model of asthma and in human epithelial cells treated with an inflammatory cytokine related to asthma. Our aim of this study was to explore the effect of SLURP-1, signal through α7 nAChR, in the pathophysiology of airway inflammation. Cytokine production was examined using human epithelial cells. Ciliary beat frequency of murine trachea was measured using a high speed camera. The IL-6 and TNF-α production by human epithelial cells was augmented by siRNA of SLURP-1 and α7 nicotinic ACh receptor. The cytokine production was also dose-dependently suppressed by human recombinant SLURP-1 (rSLURP-1). The ciliary beat frequency and amplitude of murine epithelial cells were augmented by PNU282987, a selective α7 nAChR agonist. Those findings suggested that SLURP-1 and stimulus through α7 nicotinic ACh receptors actively controlled asthmatic condition by stimulating ciliary beating and also by suppressing airway inflammation.

1. Introduction

Acetylcholine (ACh) is released from postsynaptic parasympathetic cholinergic neurons innervating the airway smooth muscle and submucosal glands [1]. ACh is also synthesized and released by non-neural cells, such as epithelial and inflammatory cells [1–8]. Evidence suggests that this non-neural cholinergic system is involved in the regulation of a variety of cell functions, including proliferation, apoptosis and cytokine production [4,8,9]. However, the roles of non-neural ACh in the pathophysiology of the lung remain unclear.

ACh exerts its effects via muscarinic and nicotinic receptors (mAChRs and nAChRs, respectively), both of which are present within the airways [3]. Among the many nAChR subtypes, α7 nAChRs expressed in macrophages are involved in down-regulating the production and release of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and high mobility group box-1 protein (HMGB-1), thereby suppressing inflammatory responses [10–12]. Moreover, α7 nAChRs are key regulators of airway epithelial cells and appear to play a crucial role in the differentiation of bronchial epithelial cells [13,14].

Secreted lymphocyte antigen-6/urokinase-type plasminogen activator receptor-related peptide-1 (SLURP-1) was first identified in a urine peptide library and purified from human blood [15]. Subsequently, Chimienti et al. [16] used *Xenopus oocytes* expressing recombinant human α7 nAChRs to show that SLURP-1 binds to α7 nAChRs and exerts an allosteric effect. SLURP-1 is produced by keratinocyte and various epithelial cells. We recently showed that SLURP-1 is expressed in human and murine ciliated bronchial epithelial cells [17].

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In that context, the aim of the present study was to explore the involvement of SLURP-1 in the pathophysiological aspect of bronchial epithelial cells on asthma. We used siRNAs to inhibit SLURP-1 synthesis and signal through α7 nAChR. We studied the effects of α7 nAChR agonists on ciliary movement because airway ciliated epithelial cells play a crucial role in trapping and removing various inhaled stimulants and because they are also involved in allergic inflammation. The present study demonstrated knockdown of SLURP-1 mRNA augmented and recombinant human SLURP-1 (rSLURP-1) downregulated IL-6 and TNF-α production. Moreover signal through α7 nAChR augmented ciliary beat frequency (CBF) and ciliary beat amplitude (CBA) of airway epithelial cells. These results suggested that SLURP-1 produced by ciliated epithelial cells and signal through α7 nAChR increased CBF and CBA, and suppressed cytokine production resulting in downregulation of asthmatic phenotype.

2. Materials and methods

2.1. Reagents

Polyinosinic:polycystidylic acid (Poly (I:C)), N-(3R)-1-Azabicyclo[2.2.2]oct-3-yl-4-chloro-benzamide monohydrochloride hydrate (PNU282987, a selective α7 nAChR agonist) and methllycaconitine (MLA, a selective α7 nAChR antagonist) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Human recombinant SLURP-1 was purchased from Abnova Taipei City, Taiwan. Human recombinant SLURP-1 was purified following the methods shown below. The mature peptide coding region of human MBP rSLURP-1 was amplified by 5′ NdeI-SP hSLURP-1: 5′-GAGTTAcatatgCTCAAGTGCTACACCTGCAAG-3′ with NdeI site (underline), 3′ EcoRI hSLURP-1: 5′-gagttaGAATTCTCAGAGTTCCGAGTTGCAGAG-3′ with EcoRI site (underline) and subcloned into the *Eschericia coli* expression vector pMAL-c5x (New England BioLabs, Beverly, MA) in frame with N-terminal MBP tag and used to transform competent *E. coli* Shuffle T7 cells (New England Bio Labs). Recombinant fusion proteins were purified from bacterial lysate applying the method advocated by the supplier (New England BioLabs) using a column buffer containing 20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, and 10 mM 2-ME. The eluted fraction containing 10 mM maltose was dialyzed against PBS. Eodotoxin was removed from recombinant protein using detoxi-gel endotoxin removing gel (Thermo Scientific, Rockford, IL).

siRNAs for SLURP-1(s109), select siRNA for CHRNA-7(61873) and negative control siRNA were purchased from Applied Biosystems (Life technologies, Foster City, CA). Transfection was undertaken using Lipofectamine 2000 (Life technologies).

2.2. Cell culture and treatment

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza (Walkersville, MD, USA). The cells were cultured in bronchial epithelial growth medium (BEGM) supplemented with BulletKit (Lonza Walkersville, Inc., MD, USA) on collagen type I-coated dishes at 37 °C in a 5% CO_2/95% air incubator as previously reported[18]. Cells and supernatants were collected 18 h after treatment of Poly: IC at a concentration of 10 μmol/L.

For knock down of the specific gene, siRNAs were used. When the cell became 30% confluent, siRNAs were transfected using lipofectamine 2000. After 54 h, the cells were stimulated, followed by the harvest of cells and supernatants after 18 h.

For examining mRNA expression, real-time PCR method was used. Total RNA was extracted by using Trizol Reagent (Invitrogen Corporation, Carlsbad, CA, USA) and then reverse transcribed by using a cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). A Light Cycler-Fast Start DNA Master SYBR Green I kit (Roche diagnostics, Mannheim, Germany) was used for real-time PCR. The primers used were GAPDH 5′-TGCACCACCAACTGCTTAGC-3′, 5′-GGCATGGACTGTGGTCATGAG-3′, 87 bp, human SLURP-1, 5′-TTCCGAGACCTCTGCAACTC-3′ (forward) and 3′-ATAAGCGTGGGGTATGGAAG-5′ (reverse), 163 bp, IL-6 5′-CAGACAGCCACTCACCTCTTC-3′, 5′-CCTCTTTGCTGCTTTCACAC-3′, 122 bp.

Interleukin-6 (IL-6) and TNF-α levels were measured by using a Quantikine Immunoassay kit (R&D systems, Minneapolis, MN, USA).

2.3. CBF and CBA measurements

CBF and CBA of the tracheal surface were measured by videomicroscopy equipped with a high speed camera as previously reported [19–22]. Briefly, mice were anaesthetized by pentobarbital Na (70–80 mg/kg, ip). The trachea was removed from the animal. Thin slices of the trachea were made by using razor blades (300–500 μm). Experiments were approved by the animal research committee of Osaka Medical College and were in accordance with the NIH Guide for the care and use of laboratory animals. Specific-pathogen-free wild-type C57BL/6J mice were purchased from SLC (Shizuoka, Japan). Tracheal slices were placed on a coverslip precoated with Cell-Tak (Becton Dickinson Labware, Bedford, MA, USA). Coverslips were placed in a microperfusion chamber (30 μl) that was mounted on an inverted light microscope (T-2000, NIKON, Tokyo, Japan) and connected to a high-speed camera (FASTCAM-1024PCI, Photron Ltd., Tokyo, Japan). The stage of the microscope was heated to 37 °C. The chamber was perfused with the control solution (37 °C) at a constant rate (300 μl/min) and aerated with a gas mixture (95% O_2 and 5% CO_2). For the CBA and CBF measurements, video images were recorded for 2 s at 500 Hz. Before the experiments, the cells were perfused with the control solution for 5 min and then stimulated with various drugs. After the experiments, CBA and CBF were measured using an image analysis program (DippMotion 2D, Ditect, Tokyo, Japan) [22]. CBA and CBF ratios (CBA_t/CBA_0, CBF_t/CBF_0) was calculated to make comparisons across the experiments. The experiment was performed using five to eight coverslips from three to four animals.

2.4. Statistical analysis

Data were statistically analyzed using the Wilcoxon/Kruskal–Wallis test. Values of $P < 0.05$ were considered to be significant.

3. Results

3.1. Effect of knockdown of SLURP-1 and α7-nAChR

To examine the role of SLURP-1on airway inflammation, we first examined the effect of SLURP-1 mRNA using siRNA specific for SLURP1. SLURP-1 specific siRNA inhibited SLURP-1 mRNA expression to 33.5 ± 2.5% and augmented IL-6 mRNA expression to 188.8 ± 10.6%. As shown in Fig. 1A, inhibition of SLURP-1 mRNA augmented inflammatory cytokine production of IL-6 and TNF-α. In contrast, control siRNA did not have any effect on control and Poly (I:C) simulated epithelial cells. The data indicated endogenous SLURP-1 modified cytokine production. When we examined the effect of siRNA specific for α7 nAChR, cytokine production was also increased, indicating that stimulus through α7 nAChR possessed inhibitory effects on inflammatory cytokine production (Fig. 1B).

Next, we examined the effects of two types of rSLURP-1. As shown in Fig. 2, both IL-6 and TNF-α production was inhibited by

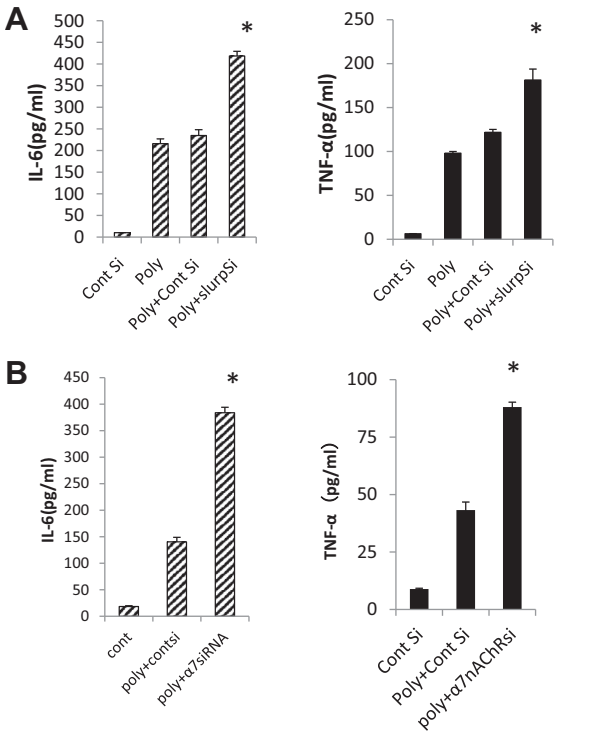

Fig. 1. Effect of is SLURP-1 and is α7 nAChR on cytokine production. NHBE cells become 30% confluent, siRNA(specific for SLURP-1 and α7 nAChR, and control) were transfected using lipofectamine 2000. After 54 h, the cell were stimulated with Poly (I:C), following the harvest of cells and supernatants after 18 h. Data represent means ± SEM, $^{*}P < 0.05$ compared with Poly (I:C), $n = 3$ for each group. Similar experiments were conducted 3 times, and one representative experiment is shown.

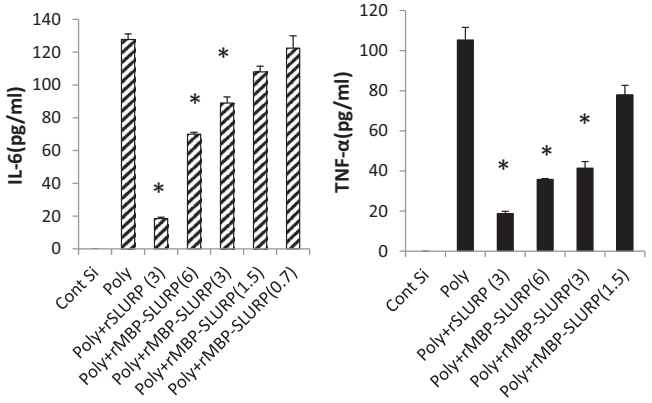

Fig. 2. Effect of is rSLURP-1 on cytokine production When NHBE cells become confluent, the cell were stimulated with Poly (I:C), following the harvest of cells and supernatants after 18 h. rSLURP-1 was human rSLURP-1 purchased from ABNOVA. MBP-rSLURP-1 was made as described in the methods. The concentration of rSLURP-1 is shown in the parenthesis as protein of SLURP-1 (μg/mL). Data represent means ± SEM, $^{*}P < 0.05$, $n = 4$ for each group. Similar experiments were conducted 5 times, and one representative experiment is shown.

rSLURP-1s. Exogenous addition of SLURP-1 possessed suppressive effects on inflammatory cytokine production.

3.2. Effects of PNU-282987 (an α7-nAChR agonist) on CBF and CBA

As previously reported, the ciliated epithelial cells produce prominent amount of SLURP-1[18]. We examined the role of a selective α7-nAChR agonist on ciliary movement. The addition of 1 μM PNU282987 (α7-nAChR agonist) increased the CBF ratio rapidly and then decreased gradually (Fig. 3A). The CBF ratio at 30 s after the PNU282987 addition was 1.17 ± 0.01 ($n = 11$), whereas the addition of the vehicle (DMSO) alone decreased the CBF ratio slightly. Prior treatment of MLA (50 nM) abolished the CBF increase stimulated by 1 μM PNU282987 (Fig. 3B).

Furthermore, we examined the effects of Ca^{2+} influx via voltage-activated Ca^{2+} channels on CBF during α7-nAChR stimulation. The effects of PNU282987 (1 μM) were completely suppressed in the presence of nifedipine (50 μM, a Ca^{2+} channel blocker) and in a Ca^{2+}-free solution (Fig. 3C and D). We also examined the effects of a high K^{+} solution (30 mM), to depolarize the membrane potential of tracheal ciliary cells. The switch from the control solution to the high K^{+} solution increased CBF ratio transiently, which was not noted in the presence of nifedipine, as previously reported [20] (data not shown). Thus, the activation of α7-nAChR stimulated CBF via Ca^{2+} influx via voltage-activated Ca^{2+} channels.

A previous report showed that a β2-agonist, procaterol, increases CBA, which is an important parameter to increase the rate of ciliary transport [22]. We measured CBA in tracheal ciliary cells upon stimulation with PNU-282987. The addition of PNU-282987 increased CBA ratio by 23% within 30 s and then decreased gradually ($n = 4$) (Fig. 3E). The increases in CBA ratio stimulated by 1 μM PNU-282987 were not observed in the presence of 50 μM nifedipine (data not shown). Thus, the increase of CBA in tracheal ciliary cells by α7-nAChR agonist was mediated through an increase in $[Ca^{2+}]i$.

4. Discussion

In this study, we found that the IL-6 and TNF-α production by human epithelial cells was augmented by siRNA of SLURP-1 and that of α7 nAChR. The cytokine production was also dose-dependently suppressed by human rSLURP-1. The ciliary beat frequency and amplitude of murine epithelial cells were augmented by PNU282987, a selective α7 nAChR agonist. Those findings suggested that SLURP-1 and stimulus through α7 nicotinic ACh receptors actively controlled asthmatic condition by stimulating ciliary beating and also by suppressing airway inflammation. .

Signal through α7 nAChR exerts various pharmacological effects and is one of the promising targets for neurogenic diseases and inflammatory diseases[3,23–25] [26]. Normal human bronchial epithelial cells express α7, α9, α10-nAChR[27]. α7 nAChR plays a role in the regulation of cystic fibrosis transmembrane conductance regulator (CFTR)[28]. The signal through α7 nAChR reduces TNF-α expression as in the case of our results (Fig. 1C), which is accompanied with upregulation of NF-κB [29,30]. We found SLURP-1, a positive allosteric modulator of α7 nAChR signaling and the products of epithelial cells, possessed the inhibitory effects. SLURP-1 is also produced by epithelial cells and keratinocytes. Gene defects of SLURP-1 results Mal de Meleda, characterized by keratoderma in human [31]. Recombinant SLURP-1 activates NF-κB and Raf-1/mek1/ERK1/2 cascade[29]. We found suppressive effects of SLURP-1 using recombinant protein and gene knockdown by siRNA.

Ciliary movement is not only a key defense mechanism but also related to pathophysiology of respiratory diseases such as asthma and COPD. β-agonist, which is used for treatment of asthma and COPD, can stimulate ciliary movements [22,32]. Regarding on acetylcholine, it has been reported that signals through M3 receptor augment ciliary beat and those through M2 receptor inhibit ciliary beats [33]. We found that signal through α7-nAChR increased ciliary beats and amplitude, suggesting that this signal induced more increased clearance of mucus and stimulating agents. Although nicotine is highly toxic to humans, it suppresses certain aspects of the asthmatic phenotype and acute lung injury [11,14,34]. Thus

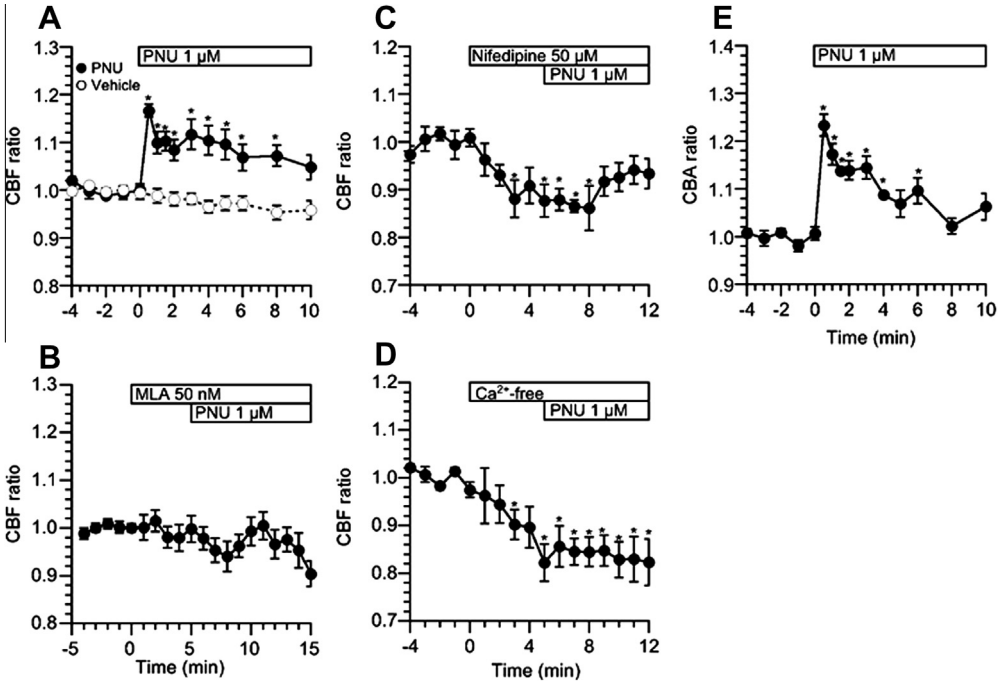

Fig. 3. Effects of an α7-nAChR agonist (1 μM PNU-282987) on CBF and CBA in tracheal ciliary cells. (A) The addition of PNU-282987(1 μM, PNU) increased CBF transiently, while the addition of DMSO (vehicle) did not. (B) The prior addition of MLA (50 nM, an antagonist of α7-nAChR) did not induce any increase in the CBF ratio during stimulation with 1 μM PNU-282987. (C) Nifedipine. The addition of nifedipine decreased CBF by 10%, and the further addition of 1 μM PNU-282987 (PNU) did not increase CBF ratios. (D) Ca^{2+}-free solution. The switch to a Ca^{2+}-free solution decreased CBF ratio by 10% and the further addition of PNU (1 μM) had no effects of the CBF ratios. (E) Changes in CBA ratio induced by PNU-282987. The addition of 1 μM PNU-282987 (PNU) increased the CBA ratios transiently ($n = 4$ each points). Data represent means ± SEM *significantly different compared with the values before experiments ($P < 0.05$).

various agonists which stimulate α7-nAChR have been investigated instead of nicotine. Our results of pathophysiological role of endogenous protein SLURP-1 on epithelial cells provided promise of treatments through α7-nAChR.

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