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The Laryngoscope

Regulation of Interleukin-33 and Thymic Stromal Lymphopoietin in Human Nasal Fibroblasts by Proinflammatory Cytokines

Kazuaki Nomura, MD; Takashi Kojima, PhD; Jun Fuchimoto, MD; Kazufumi Obata, MD; Takashi Keira, MD; Tetsuo Himi, MD; Norimasa Sawada, MD

Objectives/Hypothesis: Epithelial-derived interleukin (IL)-33 and thymic stromal lymphopoietin (TSLP) are critical regulators of innate and adaptive immune responses associated with Th2 cytokine-mediated inflammation, including allergic rhinitis. IL-33 and TSLP are expressed not only in epithelial cells but also fibroblasts, endothelial cells, and smooth muscle cells at nasal mucosal sites. However, the role and the regulation of IL-33 and TSLP in nasal fibroblasts remain unknown. We investigated the signal transduction regulation of IL-33 and TSLP induced by proinflammatory cytokines in nasal fibroblasts.

Study Design: In vitro, prospective study.

Methods: Nasal fibroblasts were derived from human nasal mucosa without allergic rhinitis. Expression of IL-33 and TSLP was examined in nasal fibroblasts treated with proinflammatory cytokines IL-1β or tumor necrosis factor (TNF)-α after pretreatment with or without various inhibitors of signal transduction pathways.

Results: In nasal fibroblasts, both Western blotting and reverse transcriptase-polymerase chain reaction demonstrated that expression of mRNAs and proteins of IL-33 and TSLP was increased by treatment with IL-1β or TNF-α. Immunostaining revealed that IL-33–positive nuclei were markedly increased by the treatment with IL-1β or TNF-α. Enzyme-linked immunosorbent assay showed that fibroblast-released TSLP was significantly increased by treatment with IL-1β or TNF-α. The upregulation of both IL-33 and TSLP proteins by treatment with IL-1β was prevented by inhibitors of pan- protein kinase C (PKC), p38 mitogen-activated protein kinase, and nuclear factor (NF)-κB. In the cells treated with TNF-α, upregulation of IL-33 protein was prevented by inhibitors of phosphoinositide 3-kinase (PI3K), c-Jun N-terminal kinase (JNK), and NF-κB, whereas upregulation of TSLP protein was prevented by inhibitors of pan-PKC, PI3K, JNK, and NF-κB.

Conclusions: Expression of IL-33 and TSLP in nasal fibroblasts was regulated via distinct signal transduction pathways including NF-κB.

Key Words: Interleukin-33, thymic stromal lymphopoietin, human nasal fibroblasts, signal transduction.

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INTRODUCTION

The nasal epithelium, which is the first site of exposure to inhaled antigens, may play an essential role in innate immunity to allergic rhinitis. It is thought that epithelial cell-derived cytokines, including thymic stromal lymphopoietin (TSLP), interleukin (IL)-25, and IL-33 are critical regulators of innate and adaptive immune responses associated with Th2 cytokine-mediated inflammation at nasal mucosal sites.[1] We previously reported high expression of TSLP and IL-33 in the epithelium from patients with allergic rhinitis with recruitment and infiltration of dendritic cells.[2,3] TSLP was produced by treatment with a TLR2 ligand, $Pam_3Cys\text{-}Ser\text{-}(Lys)_4$ and a mixture of IL-1β and tumor necrosis factor (TNF)-α in human nasal epithelial cells.[2] IL-33 was induced by treatment with the TLR9 ligand ODN2006 and IFN-γ in human nasal epithelial cells.[3] However, the detailed mechanisms of signal transduction in induction of IL-33 and TSLP remain unknown.

IL-33 is a newly identified member of the IL-1 family, which is a ligand for the orphan IL-1 family receptor ST2, and it is expressed in various types of cells including epithelial cells, endothelial cells, fibroblasts, and smooth muscle cells.[4,5] In bronchial epithelium and airway smooth muscle cells from patients with asthma and skin lesions of atopic dermatitis, high levels of IL-33 are observed compared with healthy controls.[6–9] IL-33 is a dual-function protein that may act as both an extracellular cytokine with allergic inflammatory diseases and an intracellular nuclear factor with transcriptional regulatory properties.[10] TSLP is an IL-7–like cytokine that potently induces deregulation of Th2 responses, a hallmark feature of allergic inflammatory diseases such as asthma, atopic dermatitis, and allergic rhinitis.[2,11–14] TSLP is produced by epithelial cells, skin keratinocytes, stromal cells, smooth muscle cells, lung fibroblasts, and mast cells.[12] Nasal polyp fibroblasts produce TSLP in response to stimulation by TNF-

From the Department of Otolaryngology (K.N., K.O., T.KEIRA, T.H.), and Department of Pathology (T.KOJIMA, J.F., N.S.), Sapporo Medical University School of Medicine, Sapporo, Japan.

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Send correspondence to Takashi Kojima, PhD, Department of Pathology, Sapporo Medical University School of Medicine, S1 W17, Sapporo 060-8556, Japan. E-mail: ktakashi@sapmed.ac.jp

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α.[13] In conjunctival fibroblasts, IL-33 mRNA was induced by treatment with IL-1β.[15] It is possible that IL-33 and TSLP derived from nasal fibroblasts may be closely associated with nasal allergic inflammation.

In the present study, to elucidate the signal transduction regulation of IL-33 and TSLP in nasal fibroblasts, fibroblasts derived from human nasal mucosa without allergic rhinitis were treated with the proinflammatory cytokines IL-1β and TNF-α after pretreatment with various signal transduction inhibitors. The upregulation of both IL-33 and TSLP by treatment with the proinflammatory cytokines in nasal fibroblasts was regulated via distinct signal transduction pathways including nuclear factor (NF)-κB.

MATERIALS AND METHODS

Reagents and Antibodies

Recombinant human IL-1β and TNF-α were purchased from PeproTech EC, Ltd. (London, UK). IMD-0354 (an NF-κB inhibior) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO). GF109203X (a pan-protein kinase C (PKC) inhibitor), PD98059 (an extracellular signal-regulated kinase [ERK] inhibitor), SB203580 (a p38 mitogen-activated protein kinase [MAPK] inhibitor), LY294002 (a phosphoinositide 3-kinase [PI3K] inhibitor), SP600125 (a c-Jun N-terminal kinase [JNK] inhibitor), and MG-132 (an NF-κB inhibitor) were purchased from Calbiochem Novabiochem Corp. (San Diego, CA). Salubrinal (an NF-κB inhibitor) was purchased from Tocris Bioscience (St. Louis, MO). A polyclonal rabbit anti-human IL-33 antibody was purchased from MBL, Co., Ltd. (Nagoya, Japan), and a polyclonal sheep anti-human TSLP antibody was purchased from R&D Systems, Inc. (Minneapolis, MN). Monoclonal rabbit anti–phospho-NF-κB p65 (ser536) and p105 (ser933) antibodies were purchased from Cell Signaling (Beverly, MA). Alexa 488 (green)-conjugated anti-rabbit immunoglobulin G (IgG) was purchased from Molecular Probes Inc. (Eugene, OR). The enhanced chemiluminescence (ECL) Western blotting system was obtained from GE Healthcare UK, Ltd. (Buckinghamshire, UK).

Isolation, Cell Culture, and Treatments

Nasal mucosal tissues were obtained from three patients without allergic rhinitis who underwent inferior turbinectomy at Sapporo Medical University, the Sapporo Hospital of Hokkaido Railway Company, or the KKR Sapporo Medical Center Tonan Hospital. Informed consent was obtained from all patients, and this study was approved by the ethics committees of the above institutions.

Primary cultured nasal fibroblasts were derived from human nasal mucosa tissues as described. The tissues were minced into pieces 2 to 3 mm^3 in volume and washed with phosphate-buffered saline (PBS) containing antibiotics four times. These tissue specimens were suspended in 10 mL of dispersing solution with 0.5 μg/mL DNase I (Sigma-Aldrich) and 0.08 mg/mL Liberase Blenzyme 3 (Roche, Basel, Switzerland) in PBS and then incubated at 37°C for 20 minutes. The dissociated specimens were subsequently filtrated with 300 μm mesh followed by filtration with 40 μm mesh. After centrifugation at 1,000*g* for 4 minutes, the supernatants were moved into another tube and then centrifuged at 1,400*g* for 10 minutes.

The isolated human nasal fibroblasts were plated on 35-mm or 60-mm culture dishes (Corning Glass Works, Corning, NY) coated with rat tail collagen (500 μg dried tendon/mL 0.1% acetic acid). The cells were then placed in a humidified, 5% CO_2:95% air incubator at 37°C and cultured with bronchial epithelial basic medium (BEBM; Lonza Walkersville, Inc., Walkersville, MD) containing 10% fetal bovine serum (FBS; CCB, Nichirei Bioscience, Tokyo, Japan) and supplemented with bovine pituitary extract (1% v/v), 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 50 μg/mL gentamycin, 50 μg/mL amphotericin B, 0.1 ng/mL retinoic acid, 10 μg/mL transferrin, 6.5 μg/mL triiodothyronine, 0.5 μg/mL epinephrine, 0.5 ng/mL epidermal growth factor (Lonza Walkersville, Inc.), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich). This BEBM medium with 10% FBS was very suitable to culture and passage of not only human nasal epithelial cells but also human nasal fibroblasts. In this experiment, second or third passaged cells were used.

The cells were treated with 10 ng/mL IL-1β or 20 ng/mL TNF-α for 24 hours after pretreatment with or without 10 μM GF109203X, 10 μM PD98059, 10 μM SB203580, 10 μM LY294002, 10 μM SP600125, 0.1 μM IMD-0354, 10 μM salubrinal, or 10 μM MG-132 for 30 minutes.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction Analysis

Total RNA was extracted and purified from human nasal fibroblasts using TRIzol reagent (Invitrogen, Carlsbad, CA). There was 1 μg of total RNA reverse transcribed into cDNA using a mixture of oligo (dT) and Superscript II RTase under the recommended conditions (Invitrogen). Each cDNA synthesis was performed in a total volume of 20 μL for 50 minutes at 42°C and terminated by incubation for 15 minutes at 70°C. Polymerase chain reaction (PCR) containing 100 pM primer pairs and 1.0 μL of the 20 μL total reverse transcriptase (RT) reaction was performed in 20 μL of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, and 0.5 U of Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), employing 30 cycles with cycle times of 15 seconds at 96°C, 30 seconds at 55°C, and 60 seconds at 72°C. Final elongation time was 7 minutes at 72°C. A total of 9 μL of the 20-μL total PCR reaction was analyzed in 1% agarose gel after staining with ethidium bromide. To provide a quantitative control for reaction efficiency, PCR reactions were performed with primers coding for the housekeeping gene glucose-3-phosphate dehydrogenase (G3PDH).

Primers used to detect G3PDH, IL-33, and TSLP by RT-PCR had the following sequences: G3PDH (sense 5′-ACCAC AGTCCATGCCATCAC-3′ and antisense 5′-TCCACCACCCTGT TGCTGTA-3′, amplicon length: 452 bp), IL-33 (sense 5′-GTGGA AGAACACAGCAAGCA-3′ and antisense 5′-AAGGCAAAGCACT CCACAGT-3′, amplicon length: 240 bp), TSLP (5′-CATGGAAGT GCTGTCGAAGA-3′ and antisense 5′-TTTCCGTGACCAAT CCTTTC-3′, amplicon length 231 bp). We performed triplicate experiments and with triplicate measurements of each sample from t different donors.

Western Blot Analysis

The cells were scraped from a 60-mm dish containing 300 μl of buffer (1 mM $NaHCO_3$ and 2 mM phenylmethylsulfonyl fluoride), collected in microcentrifuge tubes, and then sonicated for 10 seconds. The protein concentrations of the samples were determined using a bicinchoninic acid protein assay reagent kit (Pierce Chemical Co., Rockford, IL). Aliquots of 15 μL of protein/lane for each sample were separated by electrophoresis in 4% to 20% sodium dodecyl sulfate-polyacrylamide gels (Daiichi Pure Chemicals Co., Tokyo, Japan), and electrophoretically transferred to a nitrocellulose membrane (Immobilon; Millipore Co., Bedford, UK). The membrane was saturated for 30 minutes at room temperature with blocking buffer (25 mM Tris, pH 8.0, 125 mM

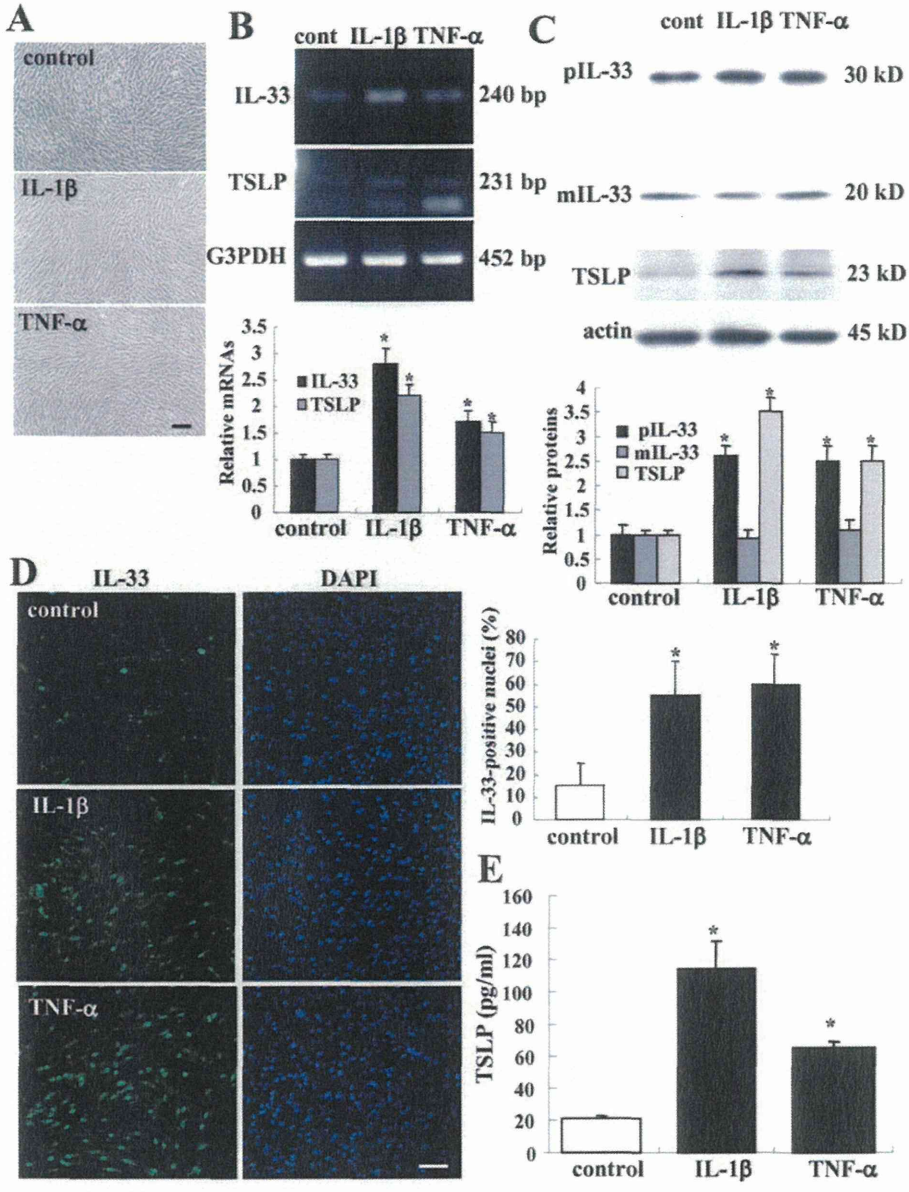

Fig. 1. Phase-contrast images (A), reverse transcriptase-polymerase chain reaction (RT-PCR) for interleukin (IL)-33 and thymic stromal lymphopoietin (TSLP) (B), Western blotting for IL-33 and TSLP (C), immunostaining for IL-33 (D), and enzyme-linked immunosorbent assay (E) for TSLP in human nasal fibroblasts treated with or without 10 ng/mL IL-1β or 20 ng/mL tumor necrosis factor (TNF-α). (B) Lower bands were dimmers of PCR primers of TSLP and nonspecific reaction. The bar graphs of (B), (C), and (D). pIL-33 = proform IL-33; mIL-33 = mature IL-33. G3PDH = glucose-3-phosphate dehydrogenase. Bars: 80 μm. *$P < .01$ versus controls.

NaCl, 0.1% Tween 20, and 4% skim milk) and incubated with anti–IL-33, anti-TSLP, and anti–phospho-NF-κB p65 and p105 antibodies overnight at 4°C. Then it was incubated with horseradish peroxidase-conjugated anti-rabbit and anti-sheep IgG antibodies at room temperature for 1 hour. The immunoreactive bands were detected using an ECL Western blotting system. We performed triplicate experiments and with triplicate measurements of each sample from three different donors.

Immunocytochemistry

The cells in 35-mm glass-coated wells (Iwaki, Chiba, Japan), were fixed with cold acetone and ethanol (1:1) at −20°C for 10 minutes. After rinsing in PBS, the cells were incubated with an anti–IL-33 antibody overnight at 4°C. Alexa Fluor 488 (green)-conjugated anti-rabbit IgG was used as a secondary antibody. The cell nuclei were stained with DAPI, and examined and photographed with an Olympus IX 71 inverted microscope (Olympus Co., Tokyo, Japan). We performed triplicate experiments and with triplicate measurements of each sample from three different donors.

Enzyme-Linked Immunosorbent Assay

The concentrations of human IL-33 and TSLP in cell culture supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits for human IL-33 and TSLP (R&D Systems) according to the manufacturer's instructions. We performed triplicate experiments and with triplicate measurements of each sample from three different donors.

Data Analysis

All data are expressed as means ± standard error. Differences between groups were tested by analysis of variance and

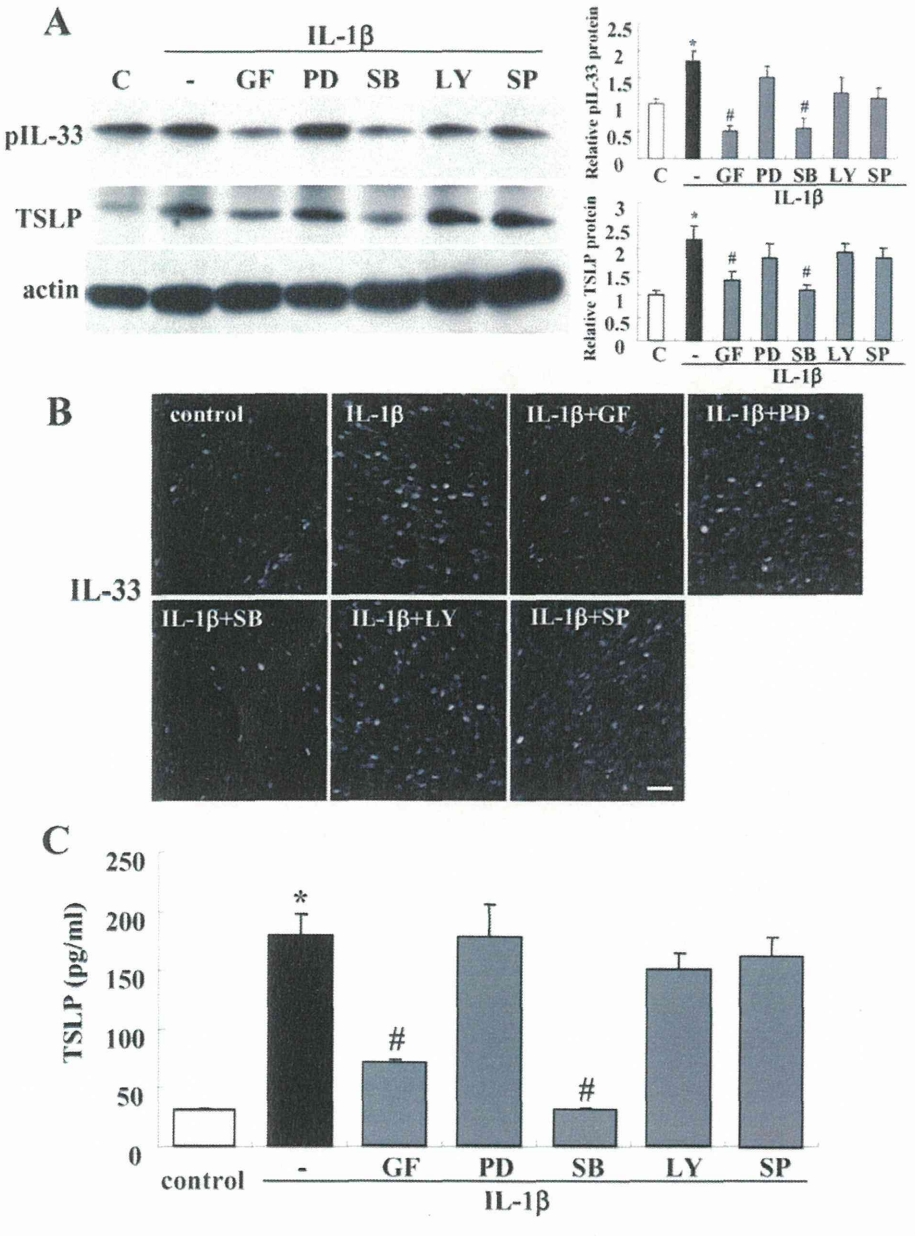

Fig. 2. Western blotting for interleukin (IL)-33 and thymic stromal lymphopoietin (TSLP) (A), immunostaining for IL-33 (B), and enzyme-linked immunosorbent assay (C) for TSLP in human nasal fibroblasts treated with 10 ng/mL IL-1β after pretreatment with 10 μM GF109203X (GF), 10 μM PD98059 (PD), 10 μM SB203580 (SB), 10 μM LY294002 (LY), and 10 μM SP600125 (SP). The bar graphs of (A). pIL-33 = proform IL-33. Bar: 40 μm. *$P < .01$ versus controls. #$P < .01$ versus IL-1β. C = control.

an unpaired two-tailed Student t test was considered to be significant when $P < .05$.

RESULTS

Induction of IL-33 and TSLP in Human Nasal Fibroblasts

In primary human fibroblasts, mRNAs and proteins of IL-33 and TSLP were detected (Fig. 1B,C). In Western blotting, the molecular weight of mature IL-33 protein was approximately 20 kDa, whereas that for the proform of IL-33 (pro–IL-33) protein it was approximately 30 kDa as previously reported.[15]

To investigate whether proinflammatory cytokines induced IL-33 and TSLP in human nasal fibroblasts, the cells were treated with 10 ng/mL IL-1β and 20 ng/mL TNF-α. Expression of mRNAs of IL-33 and TSLP was significantly increased by treatment with IL-1β or TNF-α (Fig. 1B). Expression of proteins of pro–IL-33 and TSLP, but not mature IL-33, was significantly increased by treatment with IL-1β or TNF-α (Fig. 1C). In the treatment with IL-1β or TNF-α, IL-33–positive nuclei were significantly increased compared to the control (Fig. 1D). In ELISA, the fibroblast-released TSLP was significantly increased by treatment with IL-1β or TNF-α (Fig. 1E). However, in the present study, fibroblast-released IL-33 protein was not detected in cell culture supernatants of the control and pretreated group (data not shown).

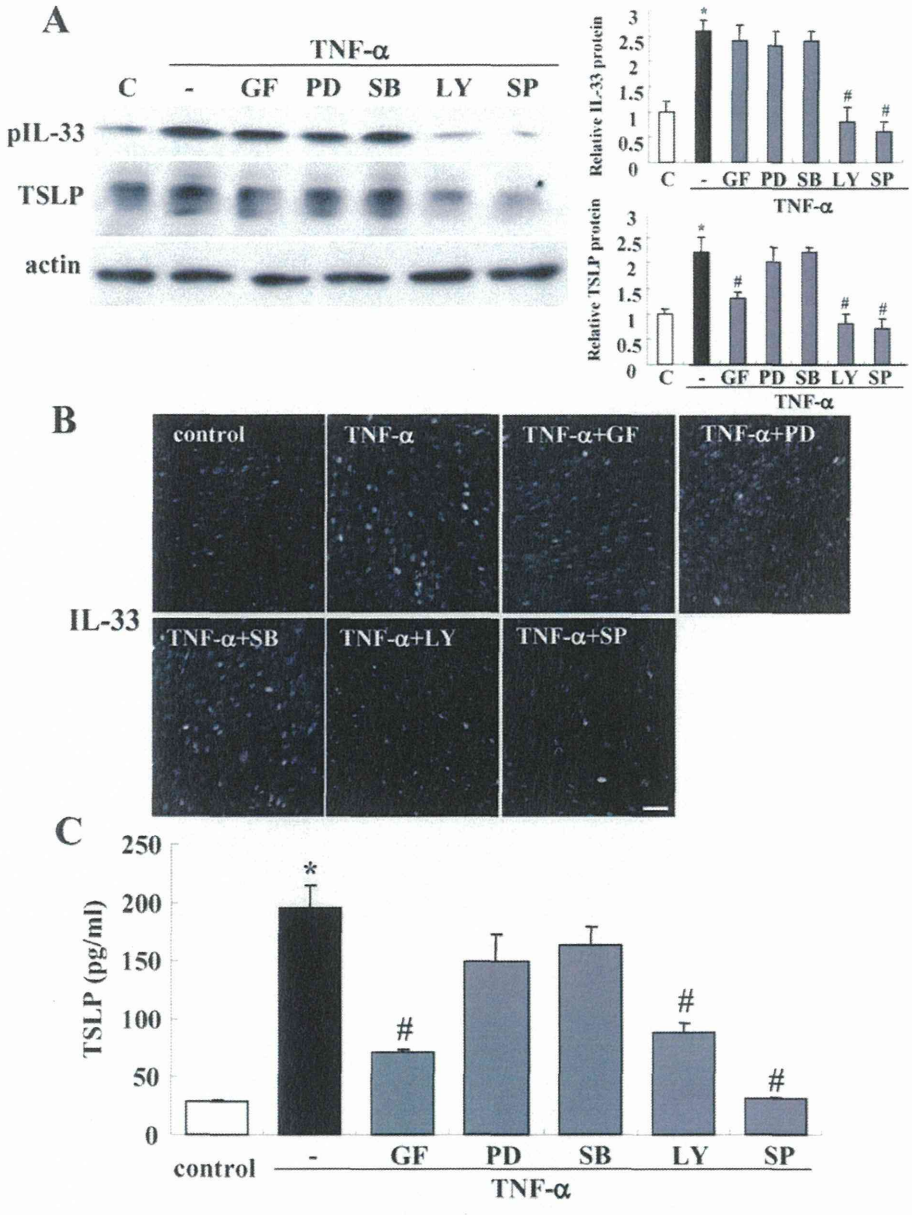

Fig. 3. Western blotting for interleukin (IL)-33 and thymic stromal lymphopoietin (TSLP) (A), immunostaining for IL-33 (B) and enzyme-linked immunosorbent assay (C) for TSLP in human nasal fibroblasts treated with 20 ng/mL tumor necrosis factor (TNF-α) after pretreatment with 10 μM GF109203X (GF), 10 μM PD98059 (PD), 10 μM SB203580 (SB), 10 μM LY294002 (LY), and 10 μM SP600125 (SP). The bar graphs of (A). pIL-33 = proform IL-33. Bar: 40 μm. *$P < .01$ versus controls. #$P < 0.01$ versus TNFa. C = control.

Distinct Signal Transduction Regulation of IL-33 and TSLP Induced by Proinflammatory Cytokines in Human Fibroblasts

To evaluate which signal transduction pathways regulate IL-33- and TSLP-induced proinflammatory cytokines in human nasal fibroblasts, the cells were treated with 10 ng/mL IL-1β or 20 ng/mL TNF-α after pretreatment with 10 μM GF109203X (a pan-PKC inhibitor), 10 μM PD98059 (an ERK inhibitor), 10 αM SB203580 (a p38 MAPK inhibitor), 10 μM LY294002 (a PI3K inhibitor), and 10 μM SP600125 (a JNK inhibitor).

In treatment with IL-1β, upregulation of IL-33 and TSLP proteins was prevented by pretreatment with GF109203X or SB203580 in Western blotting (Fig. 2A). The numbers of IL-33–positive nuclei induced by treatment with IL-1β were inhibited by pretreatment with GF109203X or SB203580 (Fig. 2B). The increase of TSLP released from the fibroblasts by treatment with IL-1β was inhibited by pretreatments with GF109203X and SB203580 (Fig. 2C).

In the cells treated with TNF-α, upregulation of IL-33 protein was prevented by pretreatment with LY294002 or SP600125, whereas upregulation of TSLP protein was prevented by pretreatment with GF109203X, LY294002, or SP600125 (Fig. 3A). The numbers of IL-33–positive nuclei induced by treatment with TNF-α were inhibited by pretreatment with LY294002 or SP600125 (Fig. 3B). The increase of TSLP released from the fibroblasts by treatment with TNF-α was inhibited by pretreatment with GF109203X, LY294002, or SP600125 (Fig. 3C).

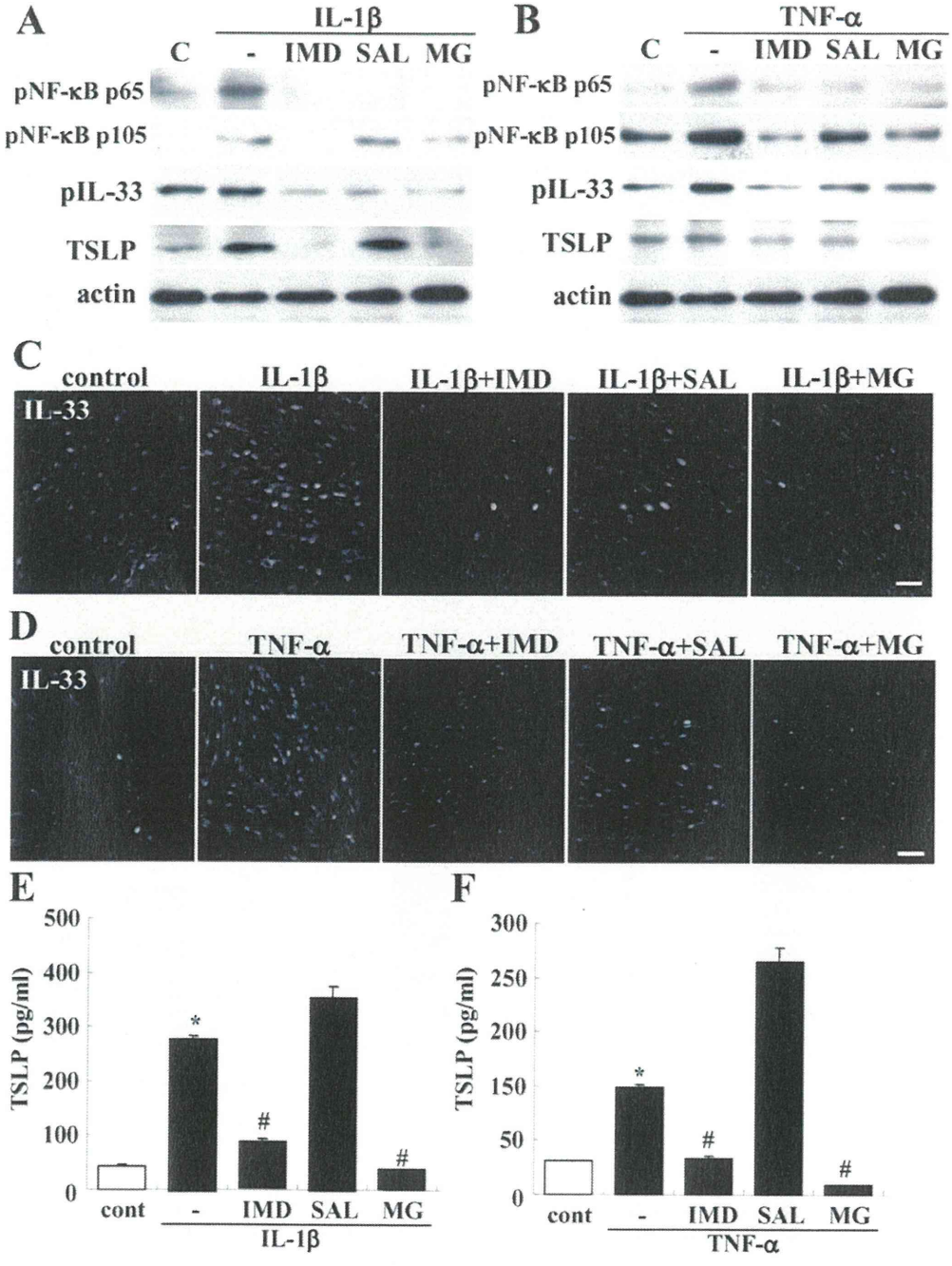

Fig. 4. Western blotting for phospho-NF-κB p65 and p105, interleukin (IL)-33 and thymic stromal lymphopoietin (TSLP) (A,B), immunostaining for IL-33 (C,D), and enzyme-linked immunosorbent assay (E,F) for TSLP in human nasal fibroblasts treated with or without 10 ng/mL IL-1β (A,C,E) or 20 ng/mL tumor necrosis factor (TNF)-α (B,D,F) after pretreatment with 0.1 μM IMD-0354 (IMD), 10 μM salubrinal (SAL), and 10 μM MG-132 (MG). pIL-33 = proform IL-33. Bar: 40 μm. *$P < 0.01$ versus controls. #$P < .01$ versus IL-1β or TNF-α.

Upregulation of IL-33 and TSLP Induced by Proinflammatory Cytokines via an NF-κB Pathway in Human Fibroblasts

IL-33 and TSLP may be regulated via an NF-κB pathway.[16,17] To investigate whether IL-33 and TSLP induced by proinflammatory cytokines in human nasal fibroblasts were regulated via an NF-κB pathway, the cells were treated with 10 ng/mL IL-1β or 20 ng/mL TNF-α after pretreatments with NF-κB inhibitors 0.1 μM IMD-0354, 10 μM salubrinal, and 10 μM MG-132.

In the fibroblasts treated with IL-1β or TNF-α, upregulation of phospho-NF-κB p65 and p105 proteins were observed (Fig. 4A,B). The upregulation of phospho-NF-κB p65 protein was prevented by all NF-κB inhibitors, whereas the upregulation of phospho-NF-κB p105 protein was inhibited by pretreatment with IMD-0354 or

MG-132 but not by salubrinal (Fig. 4A,B). Upregulation of IL-33 protein by treatment with IL-1β or TNF-α was inhibited by all NF-κB inhibitors (Fig. 4A,B). Upregulation of TSLP protein by treatment with IL-1β or TNF-α was inhibited by pretreatment with IMD-0354 or MG-132 but not by salubrinal (Fig. 4A,B). The numbers of IL-33-positive nuclei induced by treatment with IL-1β or TNF-α were inhibited by pretreatments with all NF-κB inhibitors (Fig. 4C,D). The increase of TSLP released from the fibroblasts by treatment with IL-1β or TNF-α was inhibited by pretreatment with IMD-0354 or MG-132 but not salubrinal (Fig. 4E,F).

DISCUSSION

In the present study, we demonstrated that in human nasal fibroblasts, IL-33 was present in the nuclei and TSLP was secreted. They were induced by proinflammatory cytokines IL-1β and TNF-α via distinct signal transduction pathways including NF-κB.

It is thought that production of IL-33 and TSLP may be regulated via distinct signal transduction pathways. Primary human airway smooth muscle cells pretreated with inhibitors of p38 MPAK or p42/44 MAPK, but not PI3K, show a significant decrease in TSLP release after IL-1β and TNF-α treatments.[18] IL-1β- and TNF-α-induced IL-33 mRNA expression is mediated by a p42/44 MAPK pathway in human colonic subepithelial myofibroblasts.[17] In the present study, upregulation of IL-33 and TSLP proteins by treatment with IL-1β was prevented by inhibitors of panPKC and p38 MAPK. In treatment with TNF-α, upregulation of IL-33 protein was prevented by inhibitors of PI3K and JNK, whereas upregulation of TSLP protein was prevented by inhibitors of pan-PKC, PI3K and JNK. These findings suggested that, in human nasal fibroblasts, there was differential signal transduction regulation of IL-33 and TSLP induced by different proinflammatory cytokines.

NF-κB is a transcription factor that regulates production of cytokines and chemokines in various inflammatory diseases. Induction of mRNAs of IL-33 and TSLP by IL-1β and TNF-α is dependent on an NF-κB/AP-1 pathway.[16,17] In the present study, when the nasal fibroblasts were pretreated with three NF-κB inhibitors, IMD-0354 (IκB kinase β inhibitor effect), salubrinal (eIF2a dephosphorylation inhibitor effect), and MG-132 (proteasome inhibitor effect), before treatment with IL-1β or TNF-α, upregulation of IL-33 protein by treatment with IL-1β or TNF-α was inhibited by all NF-κB inhibitors, whereas upregulation of TSLP protein by treatment with IL-1β or TNF-α was inhibited by pretreatment with IMD-0354 or MG-132 but not salubrinal. Upregulation of phospho-NF-κB p65 and p105 proteins by treatment with IL-1β or TNF-α was observed in the nasal fibroblasts. The upregulation of phospho-NF-κB p65 protein was prevented by all NF-κB inhibitors, whereas the upregulation of phospho-NF-κB p105 protein was inhibited by pretreatment with IMD-0354 or MG-132 but not by salubrinal. These results indicated that IL-33 and TSLP induced by proinflammatory cytokines in the nasal fibroblasts were dependent on phosphorylation of different NF-κB isoforms.

IL-33 is a dual function protein that may act as both an extracellular cytokine in allergic inflammatory diseases and an intracellular nuclear factor with transcriptional regulatory properties.[10] The sequence of IL-33 predicts a nuclear localization signal, and IL-33 is found in the nucleus when expressed ectopically.[19,20] However, the precise role of IL-33 within the nucleus is still unclear. In the present study, IL-33 was localized in the nuclei of nasal fibroblasts. The IL-33-positive nuclei were markedly increased together with an increase of the protein and mRNA by treatments with proinflammatory cytokines. However, IL-33 release from the nasal fibroblasts was not detected by ELISA. It is thought that full-length biologically active IL-33 may be released during necrosis as a danger signal or alarmin.[21,22]

CONCLUSION

In human nasal mucosa, IL-33 and TSLP from not only epithelial cells but also fibroblasts and endothelial cells may lead to allergic disease. We speculate that IL-33 in nuclei of the fibroblasts may also be released during necrosis. Because IL-33 and TSLP from nasal fibroblasts are sensitive to stimulation by cytokines and are regulated via distinct signal transduction pathways, this culture system provides us with an indispensable and stable model for studying the regulation of IL-33 and TSLP and finding potential inhibitors to prevent and provide therapy for allergic disease.

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REVIEW ARTICLE

JSA Best Presentation Award 2010

Aspirin-Intolerant Asthma (AIA) Assessment Using the Urinary Biomarkers, Leukotriene E_4 (LTE_4) and Prostaglandin D_2 (PGD_2) Metabolites

Noritaka Higashi[1], Masami Taniguchi[1], Haruhisa Mita[1], Hiromichi Yamaguchi[1], Emiko Ono[1] and Kazuo Akiyama[1]

ABSTRACT

The clinical syndrome of aspirin-intolerant asthma (AIA) is characterized by aspirin/nonsteroidal anti-inflammatory drug intolerance, bronchial asthma, and chronic rhinosinusitis with nasal polyposis. AIA reactions are evidently triggered by pharmacological effect of cyclooxygenase-1 inhibitors. Urine sampling is a non-invasive research tool for time-course measurements in clinical investigations. The urinary stable metabolite concentration of arachidonic acid products provides a time-integrated estimate of the production of the parent compounds *in vivo*. AIA patients exhibits significantly higher urinary concentrations of leukotriene E_4 (LTE_4) and 1,15-dioxo-9α-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid (tetranor-PGDM), a newly identified metabolite of PGD_2, at baseline. This finding suggests the possibility that increased mast cell activation is involved in the pathophysiology of AIA even in a clinically stable condition. In addition, lower urinary concentrations of primary prostaglandin E_2 and 15-epimer of lipoxin A_4 at baseline in the AIA patients suggest that the impaired anti-inflammatory elements may also contribute to the severe clinical outcome of AIA. During the AIA reaction, the urinary concentrations of LTE_4 and PGD_2 metabolites, including tetranor-PGDM significantly and correlatively increase. It is considered that mast cell activation probably is a pathophysiologic hallmark of AIA. However, despite the fact that cyclooxygenease-1 is the dominant *in vivo* PGD_2 biosynthetic pathway, the precise mechanism underlying the PGD_2 overproduction resulting from the pharmacological effect of cyclooxygenease-1 inhibitors in AIA remains unknown. A comprehensive analysis of the urinary concentration of inflammatory mediators may afford a new research target in elucidating the pathophysiology of AIA.

KEY WORDS

aspirin, asthma, biomarker, cysteinyl-leukotriene, mast cell, prostaglandin D_2, urine

ABBREVIATIONS

AIA, Aspirin-intolerant asthma; NSAID, Nonsteroidal anti-inflammatory drug; CRSwNP, Chronic rhinosinusitis with nasal polyposis; COX, Cyclooxygenase; CysLT, Cysteinyl-leukotriene; PGD_2, Prostaglandin D_2; LTE_4, Leukotriene E_4; HPLC, High-performance liquid chromatography; ATA, Aspirin-tolerant asthma; 15-epi-LXA_4, 15-Epimer of lipoxin A_4; IgE, Immunoglobulin E; LXA_4, Lipoxin A_4; 9α,11β-PGF_2, 9α,11β-Prostaglandin F_2; 2,3-Dinor-9α,11β-PGF_2, 2,3-Dinor-9α,11β-prostaglandin F_2; Tetranor-PGDM, 11,15-Dioxo-9α-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid; EIA, Enzyme immunoassay; *ent*-$PGF_{2\alpha}$, Prostaglandin $F_{2\alpha}$ enantiomer;

[1]Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagamihara National Hospital, Kanagawa, Japan.
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Correspondence: Noritaka Higashi, MD, PhD, Clinical Research Center for Allergy and Rheumatology, Sagamihara National Hospital, 18-1 Sakuradai, Minami-ku, Sagamihara, Kanagawa 252-0392, Japan.
Email: n-higashi@sagamihara-hosp.gr.jp
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GPCR, G-protein coupled receptors; PPAR-γ, Peroxisome proliferator activated receptor γ; BMMC, Bone marrow-derived mast cell; 15R-PGD$_2$, 15R-methyl-prostaglandin D$_2$; DP2, Prostaglandin D$_2$ receptor 2; GSH, Glutathione.

INTRODUCTION

The clinical syndrome of aspirin-intolerant athma (AIA) is characterized by aspirin/nonsteroidal anti-inflammatory drug (NSAID) intolerance, bronchial asthma and chronic rhinosinusitis with nasal polyposis (CRSwNP).[1,2] Aspirin/NSAID-induced asthma reactions are triggered by pharmacological effect of cyclooxygenease-1 (COX-1) inhibitors, whereas COX-2-specific inhibitors (coxibs) are tolerated in the vast majority of cases.[3,4] Several clinicoepidemiologic studies[5-9] have demonstrated that AIA is one of the common risk factors for the development of refractory asthma. Previous *in vitro* studies demonstrated the release of chemical mediators from leukocytes in AIA patients and these *in vitro* tests may be applicable to a diagnosis of aspirin/NSAIDs intolerance.[10-13] However, there is no experimental evidence which directly supports this hypothesis.[14,15] That is, there is neither an appropriate *in vitro* test to diagnose AIA nor an animal model to help fully elucidate the pathogenesis of AIA. Thus, biological fluid samples from AIA subjects are the only research tools available. Urine samples constitute a non-invasive research tool for time-course measurements in the clinical setting, although such data does not provide any information on the sites of the production. The urinary concentration of chemical mediator metabolites is remarkably

Table 1 Inflammatory mediators in biological samples

Invasive ----------→ Non-invasive						
BALF	ELF	Induced sputum	Plasma	EBC	Saliva	Urine
unmetabolized compounds			metabolites	unmetabolized compounds		urinary metabolites
local production			whole body production	Local production		whole body production
instantaneous measure of endogenous production of the compounds						time-integrated measure of endogenous production of the compounds
	ext. high levels		ext. Low levels	Low levels		relatively high levels

Abbreviations: BALF, bronchoalveolar lavage fluid; ELF, epithelial lining fluid; EBC, exhaled breath condensate; ext., extremely.

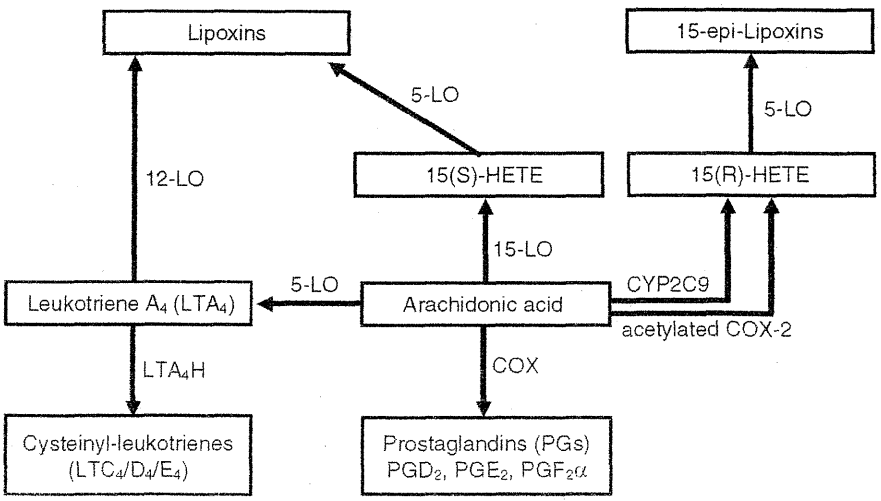

Fig. 1 Arachidonic acid cascade (Adapted from Yamaguchi *et al.*[49])

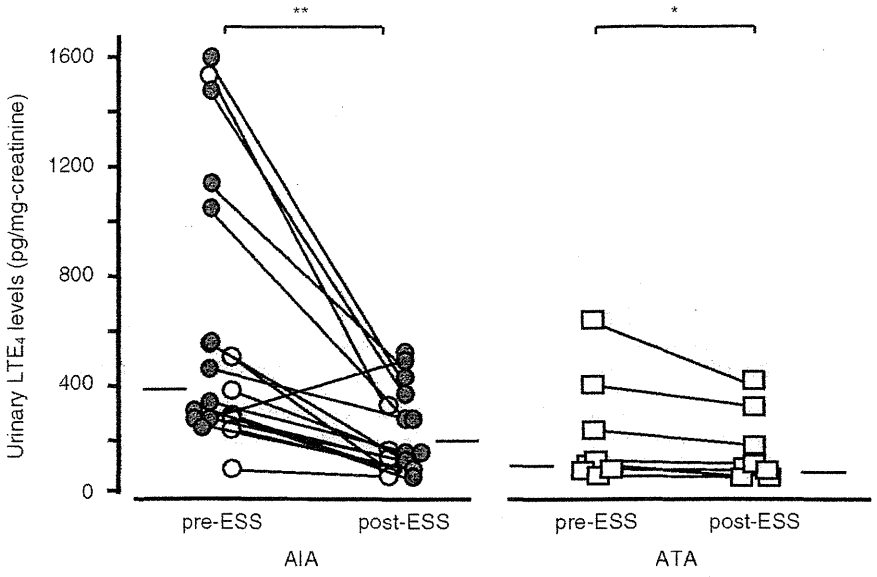

Fig. 2 Changes in the urinary LTE_4 concentrations following endoscopic sinus surgery (ESS). *Horizontal bars* indicate medians. Patients with aspirin-intolerant asthma (AIA, n = 19) and patients with aspirin tolerant asthma (ATA, n = 8) are denoted by *circles* and *squares*, respectively. Additional urinary data (12 AIA patients, closed *circles*) were included in the original figure published previously.[26] The asterisks (*, **) indicate $p < 0.05$ and $p < 0.01$, respectively, in comparison with baseline values (pre-ESS).

higher than the plasma concentration, which allows us to correlate the mediator metabolite levels with clinical symptoms. The characteristics of the biological fluid samples are shown in Table 1. It is important to note that because various arachidonic acid metabolites (Fig. 1) are rapidly metabolized ***in vivo***, the urinary concentration of their stable metabolites provides a time-integrated estimate of the production of the parent compounds, allowing a detection of their generation ***in vivo***.[16] In this review article, we focus on the clinical implications of urinary biomarkers such as cysteinyl-leukotrienes (CysLTs) and prostaglandin D_2 (PGD_2) metabolites in AIA.

URINARY LEUKOTRIENE (LT) E_4 IN AIA AT BASELINE

Since the leukotriene (LT) C_4 is easily metabolized in the lungs and/or the liver, and then disappears into the body fluid, LTE_4 is the predominant metabolite among the CysLTs of defined structure.[17,18] It is reported that 4-13% of the intravenous or inhaled dose of LTC_4 is excreted in the urine.[16,19,20] Arachidonic acid metabolites are present in extremely small quantities in biological fluids (on an order ranging from pg/ml to ng/ml). When assayed by enzyme immunoassay (EIA), biological samples should be purified in order to eliminate any interfering substances. Thin-layer chromatography has been commonly employed to remove such interfering substances. We believe that purification using high-performance liquid chromatography (HPLC) is also convenient and provides a suitable quantification procedure. Recently it has been emphasized that additional chromatographic steps are required for obtaining reliable data on the urinary LTE_4 concentrations.[21,22] There is increasing evidence that the AIA group exhibits a significantly higher urinary LTE_4 excretion level at baseline than the aspirin-tolerant asthma (ATA) group, even in a clinically stable condition.[23-31] Of late it appears that the baseline urinary LTE_4 concentrations in the AIA group are on a decline compared with original data by Christie *et al.*[23] and Kumlin *et al.*,[29] perhaps because of a stabilization of asthma symptoms by inhaled corticosteroids. Considering the distinct evidence from several immunohistochemical studies,[32-34] LTC_4 producing cells such as eosinophils and mast cells seem to contribute to increased baseline concentrations of urinary LTE_4 in subjects with AIA. Interestingly, we have demonstrated that a severe ATA group with chronic rhinosinusitis with nasal polyposis (CRSwNP) also exhibited a significantly higher urinary LTE_4 concentration at baseline.[26] That is, CysLT overproduction is associated with the clinical features of severe asthma with CRSwNP, that is, the so-called "aspirin triad" in AIA. Taken together with the evidence that there is a close relationship between CRSwNP and CysLT overproduction in asthmatic subjects, we have proposed the concept of "hyper-leukotrienuria".[26,35] The preliminary data indicates that aspirin intolerance seldom develops in pa-

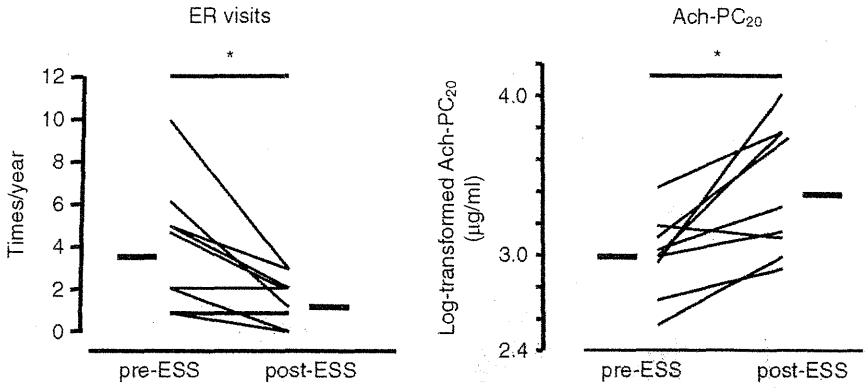

Fig. 3 Evidence of clinical improvement of asthma symptoms after endoscopic sinus surgery (ESS). *Horizontal bars* indicate means. The asterisk (*) indicates $p < 0.05$ in comparison with baseline values (pre-ESS, $n = 9$).

Table 2 Analytical significance of urinary eicosanoid concentrations

Urinary eicosanoids	Metabolites compounds				Unmetabolites compounds	
	LTE_4	D-ring PGDM	F-ring PGDM	PGEM and tetranor-PGEM	primary PGE_2	*ent*-$PGF_2\alpha$
Derived from	5-LO	COX (COX-1 dominant)		COX (COX-2 dominant)		non-enzymatic
As an index of	whole body production				local kidney production	free radical-mediated production
	LTC_4	PGD_2		PGE_2		

Abbreviations: LTE_4, leukotriene E_4; 5-LO, 5-lipoxygenase; PGDM, prostaglandin D_2 metabolite; F-ring PGDM, 9α,11β-prostaglandin F_2 and 2,3-Dinor-9α,11β-prostaglandin F_2; D-ring PGDM, tetranor-PGDM; PGE_2, prostaglandin E_2; PGEM, 13,14-dihydro-15keto-prostaglandin E_2; tetranor-PGEM, 9,15-dioxo-11α-hydroxy-2,3,4,5-tetranor-prostane-1,20-dioic acid; COX, cyclooxygenase; *ent*-$PGF_2\alpha$, prostaglandin $F_2\alpha$ *enantiomer*.

The original table has been published previously with some modification.[25]

tients with ATA, even with hyper-leukotrienuria, throughout the course of a 5-year follow-up period (unpublished data). This clinical finding leads us to the hypothesis that CysLT overproduction ***in vivo*** does not promote aspirin intolerance by itself. Interestingly, it was demonstrated for the first time that there is a significant decrease in the urinary LTE_4 concentrations after endoscopic sinus surgery in both the AIA and ATA groups. We carried out further investigation in 12 additional AIA patients, and found further evidence which suggests that CRSwNP is involved in CysLT overproduction in asthmatic subjects, as shown in Figure 2. Furthermore, we have preliminarily determined the clinical improvement in asthma-related emergency room visits and bronchial hyperresponsiveness after endoscopic sinus surgery. Unexpectedly, sinus surgery resulted in significantly fewer asthma-related emergency room visits. In addition, there were significant increases in the Ach-PC_{20} values after endoscopic sinus surgery. (unpublished data, Fig. 3) These data typically support the concept of "one airway, one disease".[36] Recent research suggests a close relationship between LT biosynthesis and vascular events such as arteriosclerosis.[37,38] We have reported significantly increased urinary LTE_4 concentrations in patients with acute exacerbated vasculitides.[39] It is also reported that the urinary LTE_4 concentration is slightly increased in current smokers[40] and obese subjects,[41] suggesting that the urinary LTE_4 concentration may be useful as a non-invasive biomarker of oxidative tissue inflammation and related pathophysiologic events.[42]

URINARY PGs CONCENTRATIONS IN AIA AT BASELINE

Recently we reported that the urinary PGE_2 concentrations at baseline in the AIA group are significantly lower than the ATA group.[25] Since PGE_2 in urine is an unmetabolized compound, the primary PGE_2 concentrations in urine have been considered to predominantly reflect local renal production[43] (Table 2). Interestingly, lower spontaneous production of PGE_2 has been reported in epithelial cells from nasal polyps[44] and sinonasal tissue[45] in subjects with AIA,

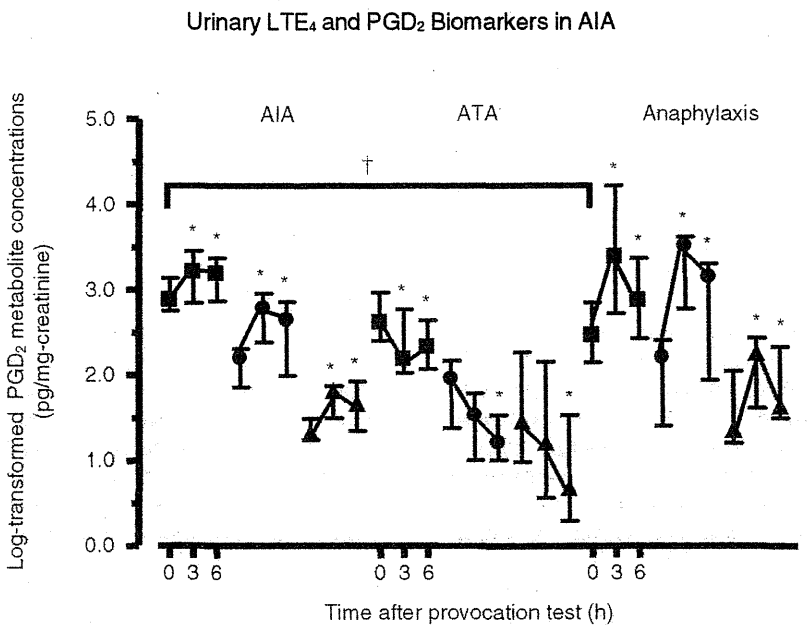

Fig. 4 Urinary PGD_2 metabolites following AIA and anaphylaxis reactions. The log-transformed urinary concentrations of PGD_2 metabolites are expressed as medians and interquartile ranges in the AIA (n = 10 or 8§), ATA (n = 7) and anaphylaxis (n = 8) groups, respectively. § In the case of tetranor-PGDM concentrations. The urinary tetranor-PGDM, 2,3-dinor-9α,11β-prostaglandin F_2 and 9α,11β-prostaglandin F_2 are denoted by *squares*, *circles* and *triangles*, respectively. The asterisk (*) indicates $p < 0.05$ compared with baseline values. A dagger (†) indicates $p < 0.05$ for the comparison between the AIA and anaphylaxis groups. Urine samples were collected at baseline and following the reactions.

which is associated with diminished COX-2 expression in these tissues.[46,47] Inhaled PGE_2 protects against both aspirin-induced bronchoconstriction and the massive release of urinary LTE_4,[48] so a critical deficiency in PGE_2 "braking" has been postulated as one possible mechanism for the AIA reaction. Similarly we have demonstrated a decreased urinary concentration of 15-epimer of lipoxin A_4 (15-epi-LXA_4),[49] which is also produced by cell-to-cell interaction involving acetylated COX-2 and 5-lipoxygenase.[50] In allergic airway inflammation, not only LXA_4 but also 15-epi-LXA_4 block both bronchial hyperresponsiveness and pulmonary inflammation induced by eosinophils via the LXA_4 receptor, leading to decreases in the numbers of eosinophils and T-lymphocytes and decreases in the concentrations of interleukin-5, interleukin-13, eotaxin, immunoglobulin E (IgE), PGs, and CysLTs.[51] Thus, it is a plausible explanation that the low COX-2 expression *in vivo* may result in the lower urinary concentrations of anti-inflammatory PGE_2 and 15-epi-LXA_4 at baseline in the AIA group, suggesting the deficiency of additional anti-inflammatory elements in AIA. Since the AIA subjects excreted significantly higher urinary LTE_4 concentration at baseline even in a clinically stable condition,[23-29] as described above, an imbalance between the local production of pro-inflammatory CysLTs and anti-inflammatory 15-epi-LXA_4 and PGE_2 at baseline, may play an important role in development of refractory asthma in AIA. Unexpectedly, urinary lipoxin A_4 (LXA_4) was significantly lower than 15-epi-LXA_4 because 15-epi-LXA_4 shows a two-fold longer half-life *in vivo*,[52] as calculated by conversion rate of 15-hydroxyprostaglandin dehydrogenase.

URINARY CONCENTRATIONS OF PG METABOLITES IN AIA AT BASELINE

The major urinary metabolites of PGE_2 and PGD_2 are shown in Table 2. Among LTC_4 producing cells such as eosinophils, basophils and mast cells, it is only the mast cells that produce significant quantities of PGD_2 in human.[53] Although there is evidence of some PGD_2 formation by eosinophils, platelets, macrophages and certain T lymphocytes, the reported amounts are 100 to 1000 times lower than those produced during IgE dependent activation of mast cells.[54-56] So the urinary PGD_2 metabolites are useful biomarkers of mast cell activation.[55] Although it is indeed also true that tryptase in the biological fluid samples is a mast cell-specific biomarker, it is impossible to detect tryptase in urine. Furthermore, it was reported that serum tryptase concentration was increased in only 6% of the patients who developed anaphylaxis.[57] Similarly, Bochenek *et al.* demonstrated that there was no change in serum tryptase despite the five-fold increase in plasma 9α,11β-PGF_2 follow-

ing the early phase of allergen-induced airway obstruction.[58] Thus, we concluded that the determination of the urinary PGD_2 metabolites is more sensitive and practical than serum tryptase for monitoring mast cell activation.[59] "F-ring" urinary PGD_2 metabolites, 9α,11β-prostaglandin F_2 (9α,11β-PGF_2) and 2,3-dinor-9α,11β-prostaglandin F_2 (2,3-dinor-9α, 11β-PGF_2), has been frequently used in the clinical studies.[25,55,60,61] Recently, Song *et al.* newly identified the most abundant "D-ring" PGD_2 metabolite in urine, 11,15-dioxo-9α-hydroxy-2,3,4,5-tetranorprostan-1,20-dioic acid (tetranor-PGDM).[61] We also examined the urinary tetranor-PGDM concentration with a newly commercially available enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA). Our data demonstrated that the urinary tetranor-PGDM concentrations were 4.8-fold (median) higher than 2,3-dinor-9α,11β-PGF_2 or 11.7-fold higher than 9α,11β-PGF_2 at baseline.[62] Interestingly, the urinary concentrations of tetranor-PGDM and LTE_4 at baseline in the AIA group was significantly higher than the anaphylaxis group[62] (Fig. 4). To our knowledge, this is the first report of a significantly higher baseline concentration of PGD_2 metabolites in urine in AIA patients, except for patients with mastocytosis.[63] Thus, the quantification of tetranor-PGDM may be an attractive strategy for further investigation into mast cell activation. This finding is consistent with the previous reports of higher baseline concentration of sputum PGD_2,[27] plasma 9α,11β-PGF_2 and serum tryptase in the AIA patients.[64] Furthermore, it is reported that there was a significant increase in the number of submucosal mast cell in the bronchial biopsy obtained from AIA versus ATA patients.[65,66] It is important to note that the purification of urine by HPLC is crucial for the precise quantification of tetranor-PGDM. One limitation of this biomarker of tetranor-PGDM is that it is generally unstable when not stored at -80℃. By contrast, there is no significant difference in the major urinary PGE_2 metabolites, 13,14-dihydro-15keto-PGE_2 (PGEM) and 9,15-dioxo-11α-hydroxy-2,3,4,5-tetranor-prostane-1,20-dioic acid (tetranor-PGEM), at baseline between AIA and ATA groups,[67] in spite of the fact that COX-2 contributes substantially to the biosynthesis of PGE_2[68] and that lower COX-2 expression is one of the unique characteristics of AIA.[46,47] Interestingly, aspirin provocation decreases the urinary concentrations of PGE_2 metabolites in only the ATA group, not the AIA group.[67]

LTE_4 AND PG METABOLITES DURING ASPIRIN/NSAIDs-INTOLERANT ASTHMA REACTION

During systemic aspirin provocation, a runny nose and nasal congestion (>90%) are the first symptoms, and then acute bronchoconstriction develops more than 30 min after the administration of threshold dose of aspirin. This aspirin-induced bronchoconstriction almost never lasts beyond 24 hours. Extrapulmonary reactions such as skin reactions and gastrointestinal symptoms are minor complications.[69] The urinary LTE_4 concentrations after the systemic provocation test with aspirin in the AIA group significantly increased above the basal concentrations (approximately a 3-30-fold increase).[25,28,30,70,71] There is evidence that shows 1) excessive CysLTs overproduction *in vivo* during aspirin-induced bronchoconstriction is a pathophysiologic hallmark of AIA without exception, 2) an increase in urinary LTE_4 concentration after aspirin challenge, the most dramatic event in AIA, correlates with the severity of the aspirin-induced reaction and 3) there is no significant increase in the urinary LTE_4 concentration in ATA patients. Although CysLTs play a key role in the pathophysiology of aspirin/NSAID intolerance, LT receptor antagonists can attenuate but cannot completely block aspirin-induced asthmatic reactions.[72] One question that needs to be answered is which cells produce CysLTs during provocation test. Our study demonstrated the urinary concentrations of LTE_4 and PGD_2 metabolites (2,3-dinor-9α,11β-PGF_2,[25] 9α,11β-PGF_2[25,28] and tetranor-PGDM[62]) were significantly increased and correlated with each other after the aspirin provocation test in the AIA group. Bochenek *et al.*[64] have demonstrated an increase in urinary 9α,11β-PGF_2 and serum tryptase after aspirin provocation in AIA patients. Increased concentrations of histamine and N^{τ}-methyhistamine, a urinary metabolite of histamine, have also observed after aspirin provocation in AIA patients.[28,73] Taken together, it is clear that mast cell activation is closely associated with aspirin-induced bronchoconstriction. However, markedly different patterns of CysLTs and PGD_2 release in the aspirin-intolerant asthma group and the IgE-mediated anaphylaxis group,[25] respectively, leads us to speculate that there may be other, unknown cellular sources besides mast cells for CysLTs synthesis during acute AIA reactions. Because eosinophils also have the capacity to generate both LTC_4 in large quantity and PGD_2 in much smaller quantity,[74-77] eosinophils may be responsible for the production of some of these mediators. Concomitantly with LTC_4, the urinary concentration of LTB_4 glucuronide, a urinary metabolite of LTB_4, increased in the AIA group after aspirin provocation. Thus, there may be a possibility that these mediators were partly generated from eosinophils by cell-to-cell interaction.[70] However, considering that there was no significant change in the serum level of eosinophil cationic protein,[78] urinary eosinophil-derived neurotoxin or 3-bromotyrosine,[71] which are biomarkers of eosinophil activation, during AIA acute reaction, this hypothesis of activated eosinophils involvement remains a matter of speculation. More importantly, basophils also may be partly responsible for the production of LTC_4 and histamine, but not PGD_2.[55,79]

Song *et al.* demonstrated that the urinary tetranor-PGDM concentrations were suppressed by inhibition with aspirin, but not by a selective inhibition of COX-2.[61] Interestingly, Daham *et al.* recently demonstrated that the urinary tetranor-PGDM concentration remains unchanged in both the AIA and ATA groups following the administration of the selective COX-2 inhibitor celecoxib.[68] Taken together, despite the fact that COX-1 is the dominant *in vivo* PGD_2 biosynthesis pathway, the precise mechanism underlying the PGD_2 overproduction through the pharmacological effect of COX-1 inhibitors in the AIA group remains unknown.

PG PRODUCTION VIA THE FREE RADICAL-MEDIATED "ISOPROSTANE PATHWAY"

Recently, it was reported that there is another pathway in which arachidonic acid is metabolized *in vivo* by a free radical-mediated mechanism to yield a series of PG-like compounds termed isoprostanes that is independent on the catalytic activity of the COX enzyme.[80] In contrast with COX-derived PGs, which is an optically pure form, the radical-mediated peroxidation of arachidonic acid generates a racemic mixture of PGs (Fig. 5). Thus, the presence of the enantiomer to COX-derived PG indicates that the PG is generated via a free radical-mediated mechanism.[81] The concentration of the PG enantiomer in urine is a reliable index of systemic isoprostane and lipid oxidation.[81,82] In particular, quantification of the urinary $PGF_{2\alpha}$ enantiomer (*ent*-$PGF_{2\alpha}$) constitues a valuable tool for assessing oxidant stress *in vivo*.[82] As judged by the urinary *ent*-$PGF_{2\alpha}$ concentration, free radical-mediated PG generation is also involved in the pathophysiology of IgE-mediated anaphylaxis.[25] Therefore, we hypothesized that a free radical-mediated mechanism might be also responsible for the PGD_2 production which occurs during the AIA reaction. However, unexpectedly, there was no change in the urinary *ent*-$PGF_{2\alpha}$ concentrations after aspirin provocation in the AIA group.[25] It seems that PGD_2 overproduction *in vivo* in the AIA group after the aspirin provocation test is independent of the isoprostane pathway. More interestingly, recent studies on PG biosynthesis have demonstrated that nitric oxide nitrosylates cytosolic phospholipase A_2 ($cPLA_2$)[83] and COX-2,[84] resulting in the activation of these two enzymes and an increase in PG synthesis. These findings suggest that oxidative stress induces the post-translational modification of enzymes associated with eicosanoid biosynthesis.[85]

CONCLUSION AND CLINICAL RELEVANCE

This review article focuses on clinical significance of urinary biomarkers in AIA. We have analyzed a variety of biological samples, including serum, saliva,[24,86] sputum,[27] exhaled breath condensate[87,88] and bronchoalveolar lavage fluid[87,89] to assess the pathophysiology of allergic diseases such as AIA. To our knowledge, the biomarker of urinary LTE_4 is the only appropriately sensitive biomarker for the aspirin-induced asthma/sinus reaction. Christie *et al.*[90] have reported that AIA subjects exhibit selective hyperresponsiveness to LTE_4, but not to LTC_4, relative to that seen in ATA subjects. Interestingly, P2Y12, the adenosine diphosphate receptor, is responsible for the LTE_4-mediated activation and proliferation of mast cells, as well as amplification of allergic pulmonary inflammation.[91,92] Taken these findings together, it is suggested that a mechanism underlying AIA is that LTE_4-mediated signaling pathway may play an important role for the development of refractory asthma. In addition, new receptors for LTE_4 have recently been discovered. Their roles in CysLT signaling and related diseases, in particular AIA, need to be elucidated.[93,94] Single measurements of urinary LTE_4 concentrations do not allow a demonstration of the cellular source and target organ of CysLT production. We preliminarily compared the urinary LTE_4 concentrations in the same patients ($n = 3$) exhibiting the same extent of bronchoconstriction upon systemic challenge and inhalation challenge. The duration between the two different challenges was more than 2 weeks to avoid the refractory period in AIA. The extent of increase in the urinary LTE_4 concentration after systemic aspirin challenge was 3 to 10-fold higher than aspirin inhalation challenge (data not shown). This finding suggests that the site of CysLT production is not only the bronchi and lungs. Therefore, further quantification of the urinary PGD_2 metabolite concentrations are helping to elucidate how

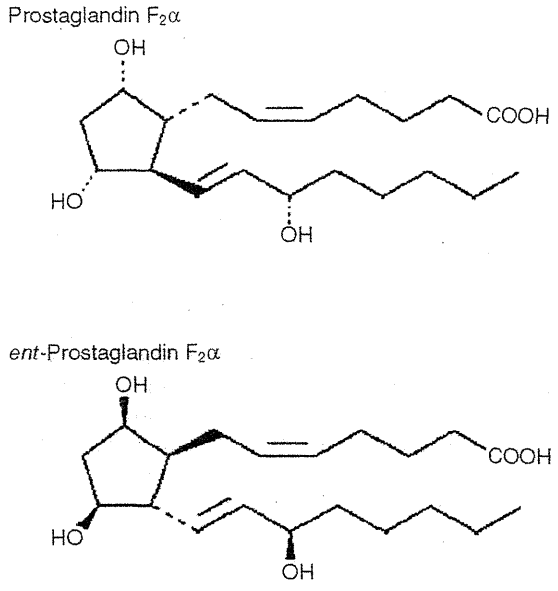

Fig. 5 Chemical structures of prostaglandin $F_{2\alpha}$ and *ent*-prostaglandin $F_{2\alpha}$.

mast cell activation is involved in the pathophysiology of AIA.[24,25,28,64,95] However, the precise mechanisms underlying the PGD_2 overproduction in the AIA group, which occurred despite the administration of a COX inhibitor, remains unknown. Paruchuri *et al.* have reported that LTE_4 activates human mast cells by a pathway that involves a cooperation between MK571-sensitive G protein coupled receptors (GPCRs) and peroxisome proliferator activated receptor γ (PPARγ), a nuclear receptor for dietary lipids. LTE_4 possesses a capacity for upregulating COX-2 expression and causing PGD_2 generation.[96] Furthermore, He *et al.*[85] have demonstrated that treatment of bone marrow-derived mast cells (BMMC) with PGD_2 reduces the ability of BMMC to generate LTC_4 upon calcium ionophore stimulation, but has little effect on LTB_4 generation. This effect can be reproduced by a selective agonist of the DP2 receptor, 15R-methyl-PGD_2 (15R-PGD_2). 15R-PGD_2 exerts its suppressive effect via a reduction in intracellular glutathione (GSH), a mechanism that involves the conjugation of its non-enzymatic breakdown product with GSH. Quantification of the new biomarker PGD_2 metabolite tetranor-PGDM provides a clinically useful tool for assessing mast cell activation *in vivo*. Urine samples afford a most non-invasive research tool for time-course measurements in a clinical study, and a lipidmics approach using biological fluid samples such as urine will provide further clinical data targeting mast cell activation, not only in allergic diseases, but also infectious diseases and cancer.

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