

Mechanism of Periostin Production in Human Bronchial Smooth Muscle Cells

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Keywords

Periostin · Interleukin-13 · Bronchial smooth muscle cells · Airway remodeling · Asthma

Abstract

Background: Asthma is a chronic airway inflammatory disease characterized by airway remodeling, in which the bronchial smooth muscle (BSM) cells play an important role. Periostin, a biomarker that reflects Th2-driven inflammatory diseases such as asthma, may play an important role in the asthmatic airway. Although periostin is mainly produced in airway epithelial cells and fibroblasts after interleukin (IL)-13 stimulation, whether BSM cells produce periostin remains unclear. Therefore, we investigated periostin production in BSM cells and the mechanisms involved. **Methods:** Human BSM cells were cultured, and the effect of IL-13 stimulation on periostin production was evaluated using quantitative polymerase chain reaction and enzyme-linked immunosorbent assay (ELISA). We evaluated the phosphorylation of signal transducer and activator of transcription factor 6 (STAT6), extracellular signal-regulated kinase (ERK)1/2, and

Akt after IL-13 stimulation. Furthermore, using ELISA, we evaluated the influence of several phosphorylation inhibitors on periostin production. **Results:** Periostin mRNA expression increased in a dose- and time-dependent manner after IL-13 stimulation; periostin production was induced 24 and 48 h after stimulation. IL-13 stimulation induced the phosphorylation of STAT6, ERK1/2, and Akt. IL-13-induced periostin production was attenuated by inhibiting STAT6 phosphorylation and strongly suppressed by inhibiting mitogen-activated protein kinase kinase 1/2 phosphorylation or phosphatidylinositol 3-kinase (PI3K) phosphorylation. **Conclusions:** BSM cells produced periostin after IL-13 stimulation, via the JAK/STAT6, ERK1/2, and PI3K/Akt pathways. Understanding the mechanism of periostin production in BSM cells may help to clarify asthma pathogenesis.

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Introduction

Asthma is a chronic airway inflammatory disease characterized by clinically repetitive symptoms such as wheezing, cough, shortness of breath, and variable air-flow limitation [1]. In addition, asthmatic airway inflammation causes tissue injury, subsequently leading to structural changes, referred to as airway remodeling [2]. Typical pathological changes in airway remodeling are wall-thickening, subepithelial fibrosis, mucus metaplasia, myofibroblast hyperplasia, bronchial smooth muscle (BSM) hyperplasia, and hypertrophy [3–5]. BSM cells are considered to play an important role in asthma pathogenesis because an increase in BSM mass and in the distance from the epithelium to the smooth muscle are key structural changes that distinguish severe asthma from moderate asthma, with an increase in several chemokines, such as interleukin (IL)-8 and eotaxin, in the BSM mass [6].

Periostin is a 90-kDa secreted extracellular matrix protein of the fasciclin-containing family and is induced by Th2 cytokines such as IL-13 [7]. Moreover, it functions as a matricellular protein in cell activation by binding several integrin receptors [8, 9], and has recently been recognized as a biomarker that reflects Th2-driven inflammatory diseases [10]. High serum periostin levels are associated with frequent exacerbations in asthma as well as the number of peripheral eosinophils and increased fractional exhaled nitric oxide [11]. Furthermore, high serum periostin levels are good predictors of improvements in lung function after treatment with anti-IL-13 antibody in patients with severe asthma [12]. Thus, periostin is considered to be a promising asthma biomarker. Periostin can also be associated with asthma airway remodeling [7].

Airway epithelial cells and lung fibroblasts are reportedly main cellular sources of periostin production [10, 13]. Although both BSM cells and periostin are implicated in asthmatic airway remodeling, only a few reports have demonstrated that BSM cells have the potential to produce periostin. In addition, the intracellular signaling pathway of periostin production has not been completely evaluated in human BSM cells.

Therefore, we investigated periostin production in human BSM cells and its signaling pathway.

Materials and Methods

Reagents

Recombinant human IL-13 was purchased from R&D Systems (Minneapolis, MN, USA). The signal transducer and activator of transcription factor 6 (STAT6) inhibitor, AS1517499 (100 nM;

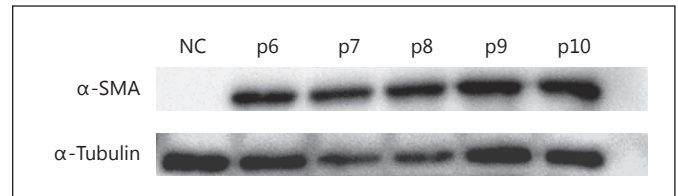


Fig. 1. Confirmation of α -smooth muscle actin (α -SMA) expression in BSM cells. BSM cells were cultured for passages 6–10 (p6–10). The expression of α -SMA was evaluated by Western blot analysis and was observed in passages 6–10. A549 cells were used as a negative control (NC).

Table 1. Primer sequences

Primers	Sequences
Periostin forward	5'-CCCCGTGACTGTCTATAAGCC-3'
Periostin reverse	5'-TGACCTTGGTGACCTCTTCTTG-3'
GAPDH forward	5'-CACCATCTTCCAGGAGCGAG-3'
GAPDH reverse	5'-CCTTCTCCATGGTGGTGAAGAC-3'

Axon Medchem, Groningen, The Netherlands), selective mitogen-activated protein kinase kinase (MEK) 1/2 inhibitor, U0126 (10 μ M; Cell Signaling Technology, Beverly, MA, USA), and phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002 (10 μ M; Cell Signaling Technology) were dissolved in dimethylsulfoxide. These inhibitors were added 1 h before IL-13 stimulation (10 ng/mL). The assay involved 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA).

Antibodies

The antibodies used were rabbit anti-STAT6 antibody (1:1,000), rabbit anti-phospho-STAT6 antibody (1:1,000), rabbit anti-extracellular signal-regulated kinase (ERK)1/2 antibody (1:1,000), rabbit anti-phospho-ERK1/2 antibody (1:1,000), rabbit anti-Akt antibody (1:1,000), rabbit anti-phospho-Akt antibody (1:1,000), anti-mouse immunoglobulin G (IgG), HRP-linked antibody (1:10,000), anti-rabbit IgG, HRP-linked antibody (1:10,000) (all purchased from Cell Signaling Technology), and rabbit anti- α -smooth muscle actin antibody (1:1,000) (Abcam, Cambridge, MA, USA). A mouse anti- α -tubulin antibody (1:5,000) (Sigma-Aldrich) was used as the internal control for protein loading.

Cells and Cell Cultures

Primary normal human BSM cells were obtained at passage 3 from a commercial supplier (Lonza, Walkersville, MD, USA) and were repeatedly cultured for passages 6–10. The cells were purchased multiple times and were used for experiments. The cells were cultured in smooth muscle cell basal medium (SmGM-2 BulletKit, Lonza) with growth factors, including 5% fetal bovine serum, 0.5 ng/mL human epidermal growth factor, 5 μ g/mL insulin, 2 ng/mL human fibroblast growth factor-basic, 50 μ g/mL gentamicin, and 50 ng/mL amphotericin B. Cells were maintained at 37°C in a humidified atmosphere (5% CO₂) and pas-

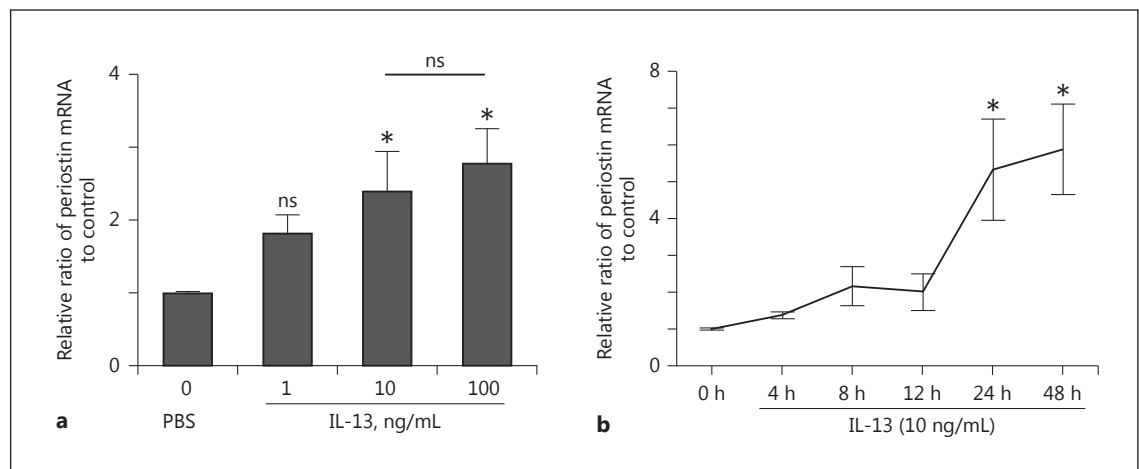


Fig. 2. Induction of periostin mRNA in a dose- and time-dependent manner after IL-13 stimulation in BSM cells. **a** BSM cells were treated with IL-13 (1, 10, or 100 ng/mL) for 24 h, and periostin mRNA expression was determined using quantitative (q)RT-PCR. **b** BSM cells were stimulated with an IL-13 concentration of 10 ng/mL for 48 h, and periostin mRNA expression was determined using qRT-PCR. Results are given as mean $\pm$ SEM from 3 samples. * $p < 0.05$, significant differences compared with control (**a**) or 0 h (**b**). ns, not significant.

saged when they reached 80–95% confluence. BSM cells were then seeded in 6- or 24-well plates (Nunc; Intermed, Roskilde, Denmark) at a density of 5×10^4 cells/cm², and when 80–85% confluence was observed, the cells were cultured without growth factors for 24 h before stimulation. All experiments in this study were performed using BSM cells purchased from Lonza, and no ethical issues exist.

Enzyme-Linked Immunosorbent Assay

The periostin concentration in the supernatant of 24-well plates was evaluated using a Periostin ELISA kit (Shino-Test Corp., Kanagawa, Japan) according to the manufacturer's instructions. The optical density (OD) at 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). The periostin concentrations were calculated using standard curves generated with the recombinant kit standards. Data were analyzed using Microplate Manager 6 software (Bio-Rad).

MTT Assay

After the collection of supernatants for each stimulation, cells seeded in 24-well plates were incubated with 100 μ L of MTT (5 mg/mL) for 2 h, and 100 μ L of DMSO was added to each well with shaking in the dark for 15 min. The absorbance value (OD) at 570 nm was measured using a microplate reader (Bio-Rad).

Quantitative Reverse Transcriptase Polymerase Chain Reaction

Total RNA from BSM cells was isolated using the TRIzol protocol (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was separated using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer's protocol. mRNA level was quantified using the Mx-3000P (Stratagene, La Jolla, CA, USA) and QuantiTect SYBR Green PCR (Qiagen, Tokyo, Japan) according to the manufacturers' instructions. Relative mRNA ex-

pression was calculated using the $\Delta\Delta C_t$ method. Individual data were normalized to glyceraldehyde-3-phosphate dehydrogenase. The primers used are listed in Table 1.

Western Blot Analysis

BSM cells were cultured in supplement-free medium for 24 h before exposing them to IL-13, to avoid the influence of growth factors.

Extraction of cell lysates and Western blot analysis were performed as previously described [14]. Cells were lysed using a lysis buffer solution (20 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, 1% Nonidet P-40, 150 mM NaCl, 0.1% sodium dodecyl sulfate, and 0.1% sodium deoxycholate), followed by sodium dodecyl sulfate gel electrophoresis and semi-dry transfer of proteins to polyvinylidene difluoride membranes. Protein concentrations in all samples were measured using a BCA protein assay kit (Thermo Scientific), and identical quantities of protein were applied to the gel. Nonspecific binding of proteins to the membranes was blocked by incubation in Tris-buffered saline and Tween 20 buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) with 2% ECL prime blocking reagent (GE Healthcare, Little Chalfont, UK). The membranes were then incubated with primary antibodies. Immunodetection was performed using an ECL prime Western blotting detection kit (GE Healthcare). Photographs were obtained using a cold CCD camera (EZ-Capture MG; ATTO, Tokyo, Japan).

Statistical Analysis

All experiments were repeated on at least 3 separate occasions. Values were expressed as means $\pm$ standard error of the mean. Analyses were performed using JMP Pro v11.2.0 (SAS Institute Japan Ltd., Tokyo, Japan). Differences between multiple groups were analyzed for significance by analysis of variance. p values of <0.05 were considered to be statistically significant.

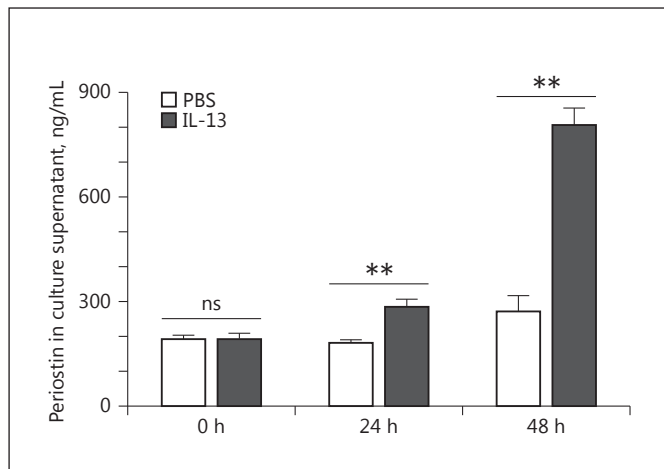


Fig. 3. Periostin protein induced by IL-13 stimulation in BSM cells. BSM cells were treated with PBS (vehicle) or IL-13 (10 ng/mL) for 0, 24, and 48 h. Periostin protein in culture supernatant was determined by ELISA and its value was corrected by MTT assay. Results are given as mean $\pm$ SEM from 5 samples. ** $p < 0.01$, significant differences compared with vehicle. ns, not significant.

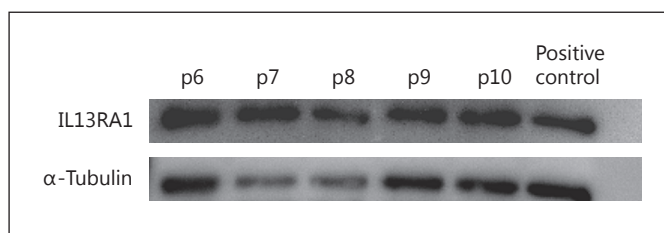


Fig. 4. Confirmation of the IL-13 receptor α 1 (IL13RA1) in BSM cells. BSM cells were cultured for passages 6–10. IL13RA1 was evaluated by Western blot analysis. Expression of IL13RA1 is observed in passages 6–10 (p6–p10). A549 cells were used as a positive control.

Results

Confirmation of α -Smooth Muscle Actin Expression

In order to confirm that the BSM cells used in this study (passages 6–10) maintained the smooth muscle phenotype as BSM cells, we demonstrated α -smooth muscle actin expression by Western blot analysis (Fig. 1).

IL-13-Induced Periostin Production in BSM Cells

To assess periostin production in BSM cells, we first evaluated periostin mRNA expression after 24 h of exposure to IL-13 at concentrations of 1, 10, or 100 ng/mL using quantitative reverse transcriptase polymerase chain

reaction. Periostin mRNA expression after IL-13 stimulation (10 ng/mL) increased 2.4-fold, which was significantly higher than that after IL-13 without stimulation. IL-13 induces periostin mRNA production in a dose-dependent manner (Fig. 2a). Besides this, BSM cells were stimulated with 10 ng/mL IL-13 and cultured for 0, 4, 8, 12, 24, and 48 h. Periostin mRNA expression increased 5.3-fold, which was significantly higher after IL-13 stimulation (10 ng/mL) for 24 h than for 0 h (Fig. 2b). Periostin production increased in a time-dependent manner. Next, we validated the effects of IL-13 stimulation on periostin production using enzyme-linked immunosorbent assay for 24 and 48 h. Periostin production was detected in the culture supernatant from BSM cells. Periostin concentration increased to 288 ± 19 ng/mL at 24 h and to 808 ± 46 ng/mL at 48 h in a time-dependent manner (Fig. 3).

Intracellular Signaling Pathway of Periostin Production in BSM Cells

IL-13 activates several cell signaling pathways, including the JAK/STAT6, ERK1/2, and PI3K/Akt pathways via IL-13 receptor α 1 (IL13RA1) [15]. Thus, we investigated the effect of the above 3 intracellular signaling pathways on periostin production.

Confirmation of IL13RA1

We performed Western blot analysis and checked IL-13RA1 expression in order to confirm the presence of this receptor in the BSM cells used in this study (passages 6–10). The expression of IL13RA1 was observed in passages 6–10 (Fig. 4).

Effect of AS1517499 on IL-13-Induced Periostin via the JAK/STAT6 Pathway

First, we investigated STAT6 phosphorylation. In BSM cells, IL-13 stimulation induced STAT6 phosphorylation peaking after 30 min (Fig. 5a). We then investigated the inhibitory effects on periostin production using AS1517499, a STAT6 inhibitor.

We performed Western blot analysis after IL-13 stimulation with or without AS1517499 (100 nM) for 2 h. A decrease in STAT6 phosphorylation by AS1517499 was confirmed; however, phosphorylation did not completely disappear (Fig. 5b). We then investigated periostin protein at various concentrations of AS1517499 (20, 100, 200, and 500 nM) by ELISA. AS1517499 significantly inhibited IL-13-induced periostin production at 100, 200, and 500 nM ($p < 0.01$); however, it could not suppress IL-13-induced periostin production to the control level (Fig. 5c). Thus, IL-13 induces periostin production

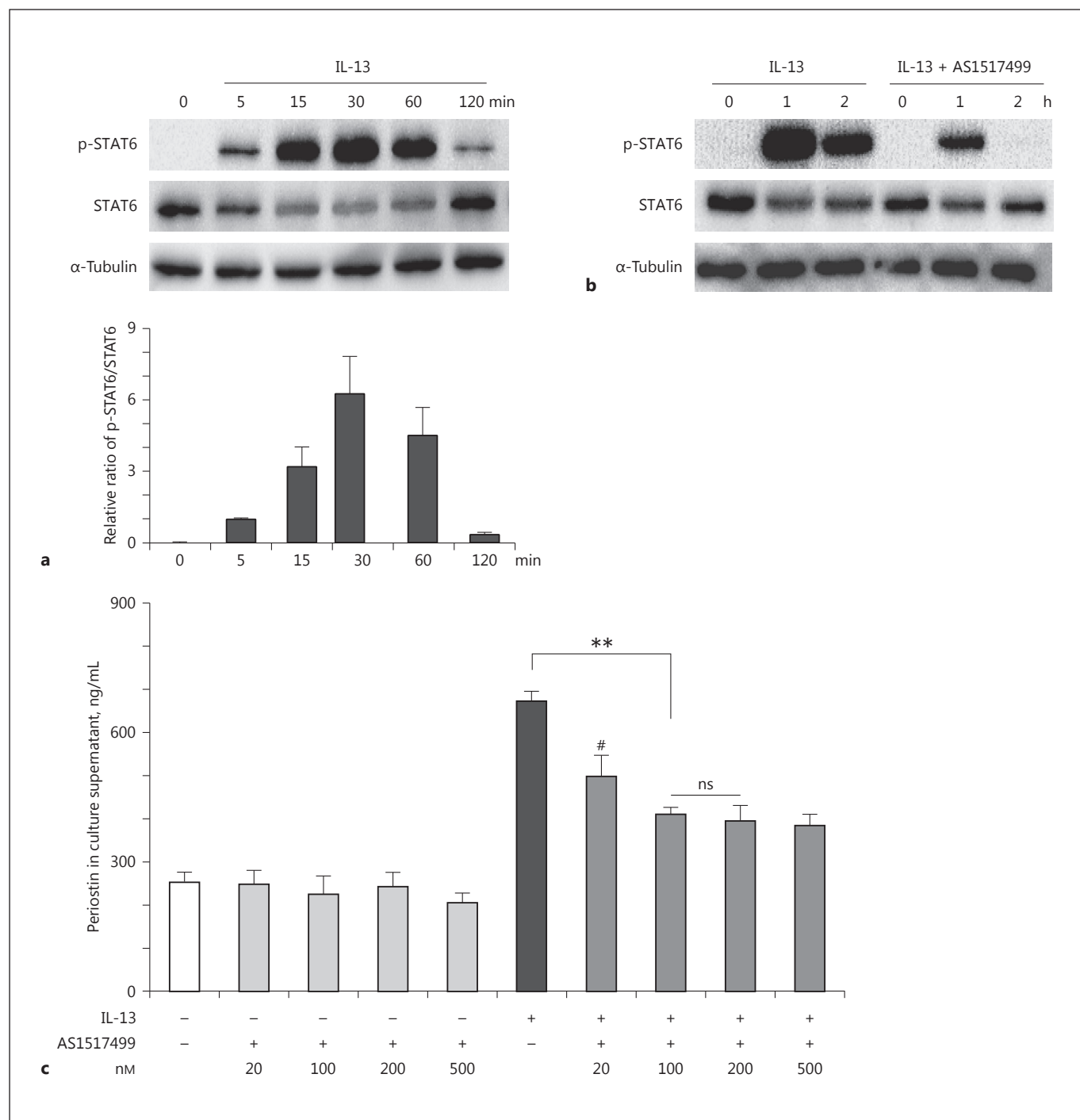


Fig. 5. Effect of STAT6 inhibitor on IL-13-induced periostin. **a** BSM cells were treated with PBS or IL-13 (10 ng/mL) for 120 min. Total cell protein was extracted and analyzed by Western blot analysis. IL-13 induced the phosphorylation of STAT6. **b** The degree of STAT6 phosphorylation in the presence of AS1517499 was assessed. The left 3 lanes represent IL-13-stimulated STAT6 phosphorylation. The right 3 lanes represent IL-13-stimulated STAT6 phosphorylation in the presence of AS1517499. Decreased STAT6 phosphorylation within 1 h after IL-13 stimulation was confirmed.

c BSM cells were treated with PBS or IL-13 (10 ng/mL) for 48 h. The periostin protein in the culture supernatant was assessed by ELISA and its value was corrected by cell number using the MTT assay. DMSO was used as a control and solvent for inhibitors. IL-13-induced periostin production was attenuated by AS1517499. Data are expressed as the mean $\pm$ SEM from at least 3 independent experiments. # $p < 0.05$ and ** $p < 0.01$, significant differences compared with BSM cells stimulated by IL-13. p-STAT6, phosphorylated STAT6. ns, not significant.

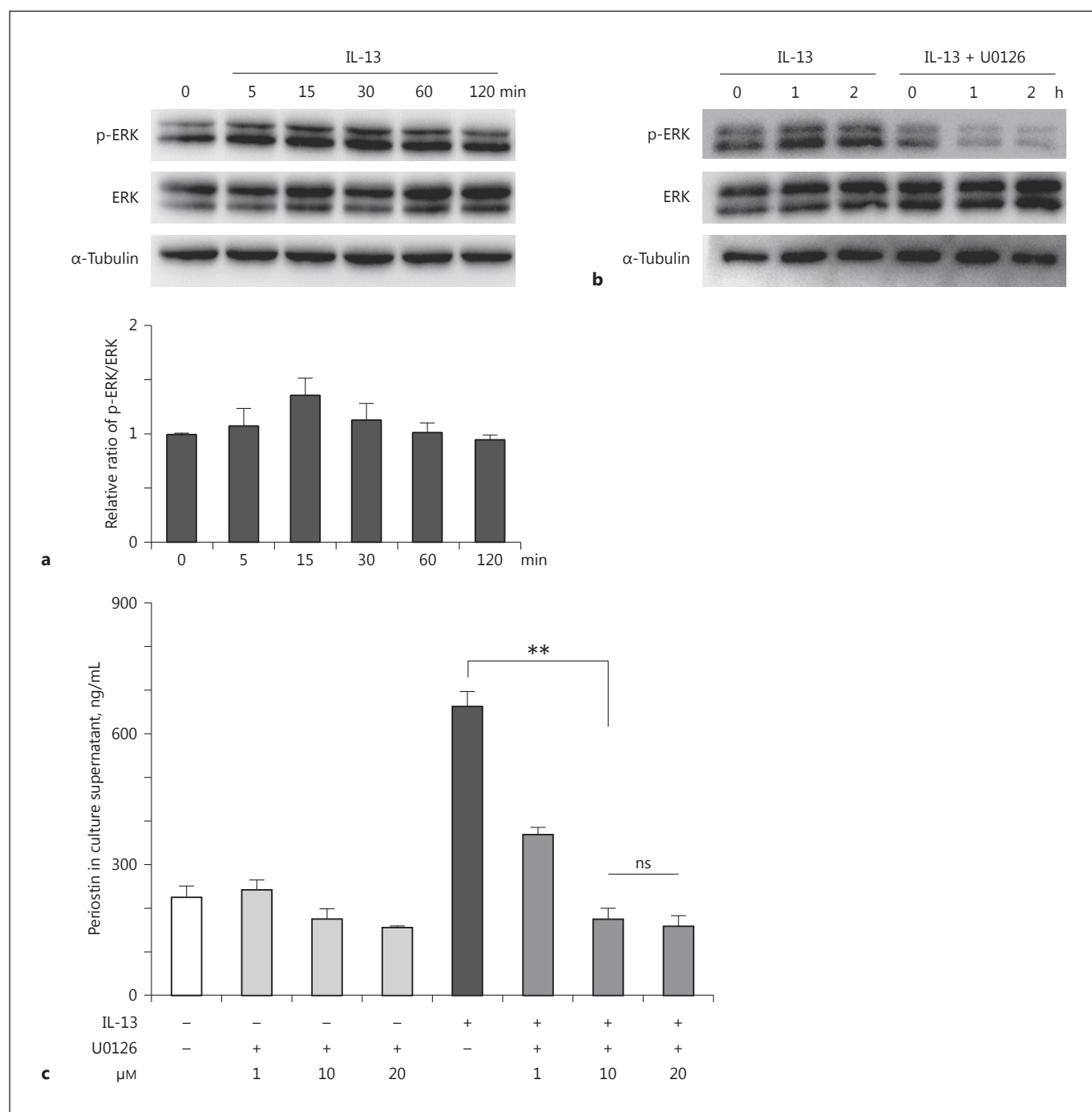


Fig. 6. Effect of MEK inhibitor on IL-13-induced periostin. **a** BSM cells were treated with PBS or IL-13 (10 ng/mL) for 120 min. Total cell protein was extracted and analyzed by Western blot analysis. IL-13 induced the phosphorylation of ERK1/2. **b** The degree of ERK phosphorylation in the presence of U0126 was assessed. The left 3 lanes represent IL-13-stimulated ERK1/2 phosphorylation. The right 3 lanes represent IL-13-stimulated ERK1/2 phosphorylation in the presence of U0126. A lack of ERK phosphorylation by

U0126 (10 μM) was confirmed. **c** BSM cells were treated with PBS or IL-13 (10 ng/mL) for 48 h. Periostin protein in culture supernatant was determined by ELISA. IL-13-induced periostin production was suppressed by U0126. Data are expressed as the mean ± SEM from at least 3 independent experiments. ** $p < 0.01$, significant differences compared with BSM cells stimulated by IL-13. p-ERK, phosphorylated ERK; ns, not significant.

through the JAK/STAT6 pathway. We also investigated other intracellular signaling pathways.

Effect of U0126 on IL-13-Induced Periostin via the ERK1/2 Pathway

Next, we investigated ERK1/2 phosphorylation. IL-13 increased ERK1/2 phosphorylation 5 min after stimulation, and ERK1/2 phosphorylation was stable for 120 min (Fig. 6a). We then investigated the effects of inhibiting the ERK1/2 pathway on periostin production using U0126, an MEK inhibitor. Cells were treated as previously described. We performed Western blot analysis after IL-13 stimulation with or without U0126 (10 μ M) for 2 h. The lack of ERK phosphorylation by U0126 (10 μ M) was confirmed (Fig. 6b). We then investigated periostin protein at various concentrations of U0126 (1, 10, and 20 μ M) by ELISA. U0126 suppressed IL-13-induced periostin production to the control level at 10 and 20 μ M in BSM cells ($p < 0.01$) (Fig. 6c). Thus, the ERK1/2 pathway seems to be important in periostin production.

Effect of LY294002 on IL-13-Induced Periostin via the PI3K/Akt Pathway

Finally, we investigated Akt phosphorylation. IL-13 induced Akt phosphorylation (Fig. 7a). We then investigated periostin production using LY294002, a PI3K inhibitor. Cells were treated as previously described. We performed Western blot analysis after IL-13 stimulation with or without LY294002 (10 μ M) for 2 h. The lack of Akt phosphorylation by LY294002 (10 μ M) was confirmed (Fig. 7b). We then investigated periostin protein at various concentrations of LY294002 (1, 10, and 20 μ M) by ELISA. LY294002 suppressed IL-13-induced periostin production to the control level at 10 and 20 μ M in BSM cells ($p < 0.01$) (Fig. 7c). Thus, the PI3K/Akt pathway seems to also be important, similar to the ERK1/2 pathway.

Discussion

Human BSM cells produced and secreted periostin after IL-13 stimulation in a dose- and time-dependent manner. IL-13 induced periostin production via the JAK/STAT6, ERK1/2, and PI3K/Akt pathways. This is the first study to describe the production and signaling pathway of periostin in human BSM cells.

The role of periostin in Th2-derived inflammatory diseases, including asthma, has recently been actively researched. Periostin interacts with collagen, fibronectin,

and integrins within the extracellular matrix [16]. Some studies have explored the role of periostin in binding to α M β 2 and α 4 β 1 integrins on eosinophils, leading to eosinophil localization, adhesion, and recruitment to the airways [17–19]. Another study showed that periostin is a critical mediator for the amplification and persistence of allergic inflammation [20], suggesting that it also functions as a matricellular protein, accelerating airway inflammation and remodeling. Therapeutic drugs such as lebrikizumab that target IL-13 have been developed as a treatment for bronchial asthma, showing significant improvement in respiratory function in patients with higher serum periostin levels [12]. A recent review of periostin in airway diseases suggested that periostin is implicated in asthmatic airway remodeling [7]. Therefore, evaluating periostin production would be meaningful for understanding severe asthma, and could lead to the discovery of a new treatment for managing asthma.

Airway epithelial cells and lung fibroblasts were previously demonstrated to be major cellular sources of periostin [10, 13]. However, to our knowledge, no previous study has demonstrated the mechanisms of periostin production in human BSM cells, although there is one that showed that periostin and α -smooth muscle actin are co-expressed in the airway smooth muscle layer in mice exposed to house dust mites [21]. So this is the first study to clarify the mechanisms of periostin production and secretion after IL-13 stimulation in BSM cells.

IL-13 activates several cell signaling pathways, including the JAK/STAT6, ERK1/2, and PI3K/Akt pathways, in epithelial cells, fibroblasts, and vascular smooth muscle cells, but not in BSM cells, via IL13RA1 [7, 15, 22, 23]. Among them, in the early stages of research into periostin production, the JAK/STAT6 signaling pathway was considered to be the main pathway for IL-13-induced periostin production. Using STAT6 knockout mice that were sensitized with house dust mites, a study demonstrated that STAT6 was a major transcription factor for producing periostin [20]. Furthermore, STAT6 siRNA decreased periostin expression in lung fibroblasts [24]. Subsequently, the ERK1/2 pathway, as well as the JAK/STAT6 pathway, has also been shown to be important for periostin production in human airway epithelial cells cultured at an air-liquid interface [25] and in human lung fibroblasts [26]. Furthermore, other studies demonstrated that in vascular smooth muscle cells, the PI3K/Akt and ERK1/2 pathways, rather than the JAK/STAT6 pathway, are important for producing periostin [27, 28]. These previous reports demonstrated that these pathways would be involved in periostin production; however, details of cross-

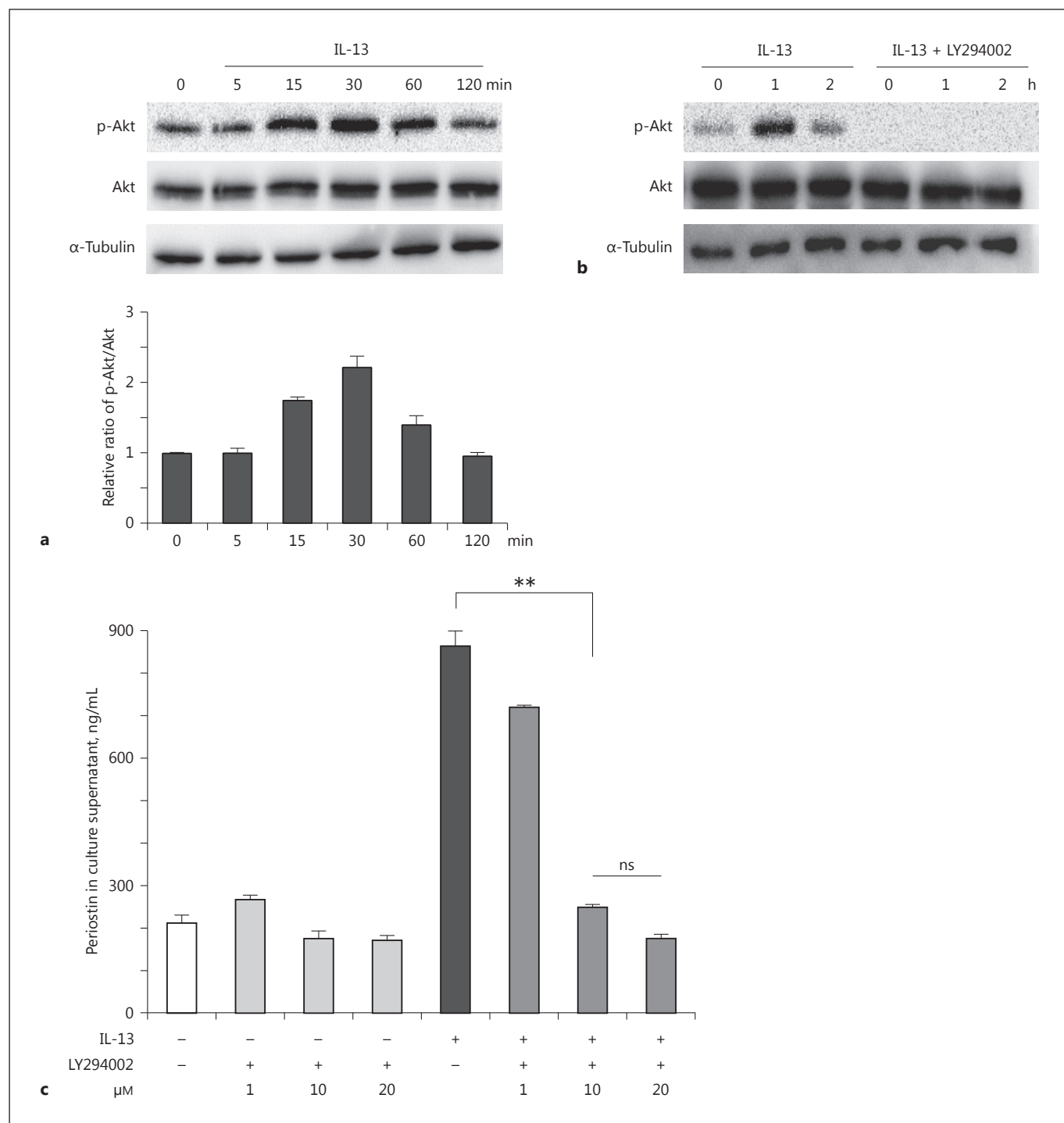


Fig. 7. Effect of PI3K inhibitor on IL-13-induced periostin. **a** BSM cells were treated with PBS or IL-13 (10 ng/mL) for 120 min. Total cell protein was extracted and analyzed by Western blot analysis. IL-13 induced the phosphorylation of Akt. **b** The degree of Akt phosphorylation in the presence of LY294002 was assessed. The left 3 lanes represent IL-13-stimulated Akt phosphorylation. The right 3 lanes represent IL-13-stimulated Akt phosphorylation in the presence of LY294002. A lack of Akt phosphorylation by

LY294002 (10 μM) was confirmed. **c** BSM cells were treated with PBS or IL-13 (10 ng/mL) for 48 h. Periostin protein in culture supernatant was determined by ELISA. IL-13-induced periostin production was suppressed by LY294002. Data are expressed as the mean ± SEM from at least 3 independent experiments. are indicated by ** $p < 0.01$, significant differences compared with BSM cells stimulated by IL-13. p-Akt, phosphorylated Akt; ns, not significant.

talk were not shown. This study demonstrated that periostin production is induced by IL-13 via the JAK/STAT6, ERK1/2, and PI3K/Akt pathways in human BSM cells. The results of our study are consistent with those previously reported.

With regard to the limitations of our study, we did not use BSM cells from asthma patients, so the periostin production in such cells from asthma patients might be enhanced compared to our results, with reference to a previous report demonstrating that periostin staining in epithelial cells was more intense in asthma patients than in controls [13]. However, we have demonstrated that human BSM cells have the potential to produce periostin after IL-13 stimulation; hence, BSM cells from patients with asthma might also produce periostin.

In conclusion, our study demonstrates that BSM cells produce and secrete periostin after IL-13 stimulation via the JAK/STAT6, ERK1/2, and PI3K/Akt pathways. This

suggests that BSM cells are an important cell source of periostin production in Th2-derived inflammatory diseases. Understanding the mechanism of periostin production in BSM cells might help clarify asthma pathogenesis and discover new therapeutic targets for managing asthma.

Acknowledgments

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Disclosure Statement

The authors have no conflicts of interest to declare.

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