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ORIGINAL ARTICLE

Histamine induces human lung fibroblast-mediated collagen gel contraction via histamine H1 receptor

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ABSTRACT

Background: Airway remodeling is implicated in irreversible airflow limitation of refractory asthma, which includes increased smooth muscle mass and subepithelial fibrosis. Activated fibroblasts acquire contractile phenotype to participate in tissue contraction and structural alteration of extracellular matrices. Histamine is a potent mediator of allergic inflammation, substantially involved in asthmatic pathophysiology. *Objective*: We hypothesized that histamine might play a role in airway remodeling, and investigated its effect on fibroblast-mediated collagen gel contraction. *Methods*: Fibroblast-mediated collagen gel contraction was studied. Histamine's regulation of collagen gel contraction was characterized by using specific histamine-receptor antagonists, an IP_3 receptor antagonist and a PKC inhibitor. *Results*: Histamine induced contraction of collagen gels embedded with human lung fibroblasts, in a time-dependent manner, and at the concentration more than 10^{-6} M, both in four primary cultured adult lung fibroblasts and three fetal lung fibroblast cell lines. This effect was attenuated by H1 receptor antagonist, whereas those for H2 to H4 receptors failed to show an inhibitory effect. Furthermore, IP_3 receptor-mediated Ca^{2+} mobilization was implicated in histamine's action on collagen gel contraction. *Conclusions*: Our results suggest that histamine is involved in airway remodeling through its action on lung fibroblasts, and antihistamine drugs, especially H1 receptor antagonists, might be potentially beneficial for a subset of asthmatic patients.

KEYWORDS 2-APB, diphenhydramine, gel contraction assay, histamine, lung fibroblast

INTRODUCTION

Asthma is a chronic airway disorder that diminishes quality of life and is sometimes fatal. The prevalence of asthma is increasing throughout the world, especially among children, which is now recognized as a serious global health problem, with an estimated 300 million patients [1–3]. Asthma is characterized by allergic chronic inflammation of the airways, and multifaceted analyses have enlightened the complex web of inflammatory responses underlying the asthmatic pathophysiology, which involves the release of mediators, such as histamine, eicosanoids, cytokines, chemokines, and proteases. Inhaled corticosteroids are the most effective anti-inflammatory medications for persistent asthma, and have drastically improved its management; however, about 10% of patients with severe asthma remain poorly controlled despite optimal therapy and have higher mortality [4]. In such cases, irreversible airflow limitation and alterations in airway structure have been described, which is termed as airway remodeling [5].

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Pathological features of airway remodeling include increased smooth muscle mass, activated fibroblasts, and subepithelial fibrosis [6]. Fibroblast numbers and smooth muscle cell size are reported to be negatively associated with prebronchodilator and postbronchodilator forced expiratory volume in 1 second (FEV1) in asthmatic patients [7]. Activated lung fibroblast converts its phenotype into myofibroblast to acquire contractile property which generates traction force, and further participates in tissue remodeling via degradation/production of extracellular matrices [8]. It is speculated that activated fibroblasts participate in bronchoconstriction, augmenting the action of bronchial smooth muscle cells to cause airflow limitation. Thus lung fibroblast is a key therapeutic target for asthma treatment.

The conventional view of airway narrowing in asthmatics involves the early-phase response to allergen inhalation followed by the late-phase reaction characterized by immune cell infiltration and cellular inflammation. In this process, histamine is released from antigen-sensitized mast cells or basophils, and has a substantial role in allergic inflammation through its ability to produce smooth muscle contraction and promote vascular permeability [9]. Histamine binds to four types of G protein-coupled receptors (GPCRs), i.e., H1 to H4 receptors, which are differentially expressed in various cell types. The recent discovery of histamine H4 receptor and its regulatory role in the immune system revived attention on this classical chemical mediator [10]. Histamine H1 receptor antagonists are widely used in the management of asthma especially in cases complicated with atopic diseases, such as allergic rhinitis, urticaria, and atopic dermatitis. In a recent review on clinical trials it has been demonstrated that H1 receptor antagonists are beneficial to alleviation of asthma symptoms in the patients of allergic rhinitis [11], while in the "Global Initiative for Asthma (GINA)" guideline antihistamines are not recommended as a first-line medication of asthma [2]. It remains elusive whether antihistamines can be beneficial for asthmatics comorbid with or without other atopic diseases. Thus elucidation of its effect on airway remodeling may help infer the role of antihistamines in the treatment of asthma.

Our previous study demonstrated that histamine stimulates human lung fibroblast migration, suggesting that fibroblasts might be recruited into asthmatic airways triggered by the release of histamine [12]. In the present study, we further explored their participation in tissue contraction as well as structural alteration in extracellular matrices. We utilized three-dimensional gel contraction system, where fibroblasts were cultured in type I collagen gel, as a model of airway remodeling. Sizes of collagen gels were measured to assess the effect of antihistamines. Signaling pathways involved in histamine's action in human lung fibroblasts were further investigated, using pharmacological inhibitors.

MATERIALS AND METHODS

Reagent

Histamine H1, H2, H3, and H4 receptor antagonists, i.e., diphenhydramine, ranitidine, clobenpropitand, and JNJ7777120, were purchased from Sigma (St. Louis, MO), and were used at the concentration of 10^{-4}, 5×10^{-6}, 10^{-6}, and 10^{-6} M, respectively, according to the previous research [12]. IP_3 receptor antagonist, 2-amino-ethoxydiphenyl borate (2-APB), and protein kinase C inhibitor, bisindolylmaleimide, were purchased from Merck (Darmstadt, Germany), and were used at the concentration of 7.5×10^{-5} M and 10^{-7} M, respectively, according to previous reports [13, 14]. Pan-MMP inhibitor, GM6001, was purchased from Calbiochem (EMD Biosciences, Darmstadt, Germany). The effect of these reagents on cell viability was evaluated by MTT assay, and no overt cytotoxic effect was observed in the preliminary experiments at these concentrations in HFL-1 cells (Figure S1).

Cell Culture

Tissue culture media and supplements were purchased from GIBCO (Life Technologies, Grand Island, NY). We have previously established four primary cultured lung fibroblasts using "outgrowth method," from lung tissues of subjects who underwent lung tumor resection [15]. Clinical features of the subjects are shown in Table 1. Harvested tissues were minced into small pieces and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). The outgrowing cells were collected by trypsinization and they showed uniformly spindle-like morphology following cell passages. To exclude the possible contamination of epithelial cells, we performed immunostaining of these cells. The obtained cells were positively stained for vimentin whereas we could not find any cell positively stained for pan-cytokeratin. Human fetal lung fibroblast cell lines (HFL-1, WI-38, and IMR-90), HUVEC (human umbilical vein endothelial cells), and THP-1 (Human acute monocytic leukemia cells) were obtained from American Type Culture Collection (ATCC). BSMC (normal human bronchial smooth muscle cells), PASMC (human pulmonary artery smooth muscle cells), NHBE (normal human

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TABLE 1. Clinical Features of the Subjects ($N = 4$)

Case	Age	Gender	Location	Histology	Staging	Pack-year	Comorbidities
#1	67	Male	LLL	SCC	T1aN0M0	150	IP
#2	82	Female	LLL	Ad	T1bN0M0	0	
#3	82	Male	RLL	SCC	T2aN0M0	30	COPD
#4	75	Male	LLL	Ad	T2bN0M0	62.5	COPD, IP

BI = Brinkman index (pack-year); LLL = left lower lobe; RLL = right lower lobe; SCC = squamous cell carcinoma; IP = interstitial pneumonia; Ad = adenocarcinoma; COPD = chronic obstructive pulmonary disease.

bronchial epithelial cells), and SAEC (human small airway epithelial cells) were purchased from Lonza (Basel, Switzerland). All the lung fibroblasts were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Primary lung fibroblasts were used at 3 to 7 passages following primary culture, and the cell lines were used at 18 to 23 passages. The study was approved by the Ethics Committee at the University of Tokyo. All subjects granted written informed consent for the acquisition of material for research.

Cell Viability Examination

MTT assay was performed to test the toxicity of reagents and diluents. Briefly, after being cultured for 6 hour with each reagent in 96-well culture plate, the medium were discarded and 100 μL MTT solution (200 μg/mL in DMEM) per well was added. After another 4 hour incubation, MTT solution was discarded and 100 μL of DMEM per well was added to melt formazan crystal. The OD values were read at 540 nm wavelength using microplate reader (Bio-Rad, Hercules, CA).

Collagen Gel Contraction Assay

Collagen gel (Cellmatrix type I-A; Nitta Gelatin Inc., Osaka, Japan), distilled water, 4× concentrated DMEM and suspended fibroblasts were gently mixed. Volumes were adjusted so that the collagen concentration was 1.75 mg/mL and the cell density was 3×10^5 cells/mL, with physiological ionic strength equaling 1× DMEM. The mixture (0.5 mL) was dispensed into each well of 24-well tissue culture plates. Gelation occurred in 20 minute at room temperature. Gel contraction was initiated by detaching the perimeter of collagen gels from the side of the wells. Gels were then transferred to 60-mm tissue culture dishes containing 5 mL of medium containing the designated concentration of reagent and placed in the incubator. The surface area of collagen gels was determined daily for 3 days using the Densitograph image analysis system (ATTO, Tokyo, Japan).

Western Blotting

HFL-1 cells were serum-starved and stimulated with histamine. Cells were lysed in cold lysis buffer solution (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40), followed by SDS gel-electrophoresis and semi-dry transfer of the proteins to polyvinylidene difluoride membrane. Nonspecific binding of proteins to the membrane was blocked by incubation in TBS-T buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween-20) supplemented with 2% ECL Prime Blocking Reagent (GE Healthcare, Buckinghamshire, UK) and the membrane was incubated with primary antibodies to extracellular signal-regulated kinase1/2 (Erk1/2) antibody (Cell signaling, Danvers, MA), phosphorylated Erk1/2 antibody (Cell signaling), α-smooth muscle actin (α-SMA) antibody (Sigma), and α-tubulin antibody (Sigma). After incubation with the secondary anti-rabbit horseradish peroxidase (HRP)-linked antibody (Sigma), immunodetection was performed with ECL Prime Western Blotting Detection Kit (GE Healthcare). Pictures were taken by the cold CCD camera (EZ-Capture MG; ATTO, Tokyo, Japan). The density of band was measured by densitometry.

Intracellular Ca^{2+} Concentration

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using the fura-2 fluorescence method as described previously [16]. Fura-2 acetoxymethylester (fura-2 AM) was obtained from Dojin Chemicals (Kumamoto, Japan). Cells were trypsinized, washed twice with the standard solution, adjusted to cell density of 10^6 cells/mL in extracellular fluid (1× Tyrode buffer, 1 μM Nicardipine plus 1.8 mM $CaCl_2$ or Ca-chelator ethyleneglycol-*bis*-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA; 0.5 mM), pH 7.4), and loaded with 1 μM fura-2 AM for 30 minute at 37°C. Diphenhydramine (10^{-4} M) and 2-APB (75×10^{-6} M) were added 30 minute before measurements. The Ca^{2+} fura-2 fluorescence of the suspended cells was measured by a spectrofluorometer (CAF-110; JASCO Co. Ltd., Tokyo, Japan). The excitation

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TABLE 2. RT-PCR Primers Used in This Study

Gene	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)
GAPDH	GGTGAAGGTCGGAGTCAACGGA	GAGGGATCTCGCTCCTGGAAGA
COL1A1	AGGACAAGAGGCATGTCTGGTT	TTGCAGTGGTAGGTGATGTTCTG
ACTA2 (α-SMA)	GCACCCAGCACCATGAAGA	ACCGATCCAGACAGAGTATTT
HRH1	TGGTTTGGATTCCAGAAAGC	CACACTGAACCCCCTCATCT
HRH2	CAGTTCGGGTCGCCATCTC	CTGGTCTCGTTCCTGCTGTTC
HRH3	TCCACTGCTATGCCGAGTT	TGCGCCTCTGGATGTTCAG
HRH4	CCGTTTGGGTGCTGGCCTTCTTAG	GATCACGCTTCCACAGGCTCCAAT

wavelengths were 340 and 380 nm, and emission was measured at 500 nm. The intensity of 500 nm fluorescence (F340 and F380) was measured and the ratio of F340 to F380 was calculated as an indicator of $[Ca^{2+}]_i$.

RNA Isolation and RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The synthesis of cDNA was performed using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Quantitative RT-PCR analysis was performed using Mx-3000P (Stratagene, La Jolla, CA) and QuantiTect SYBR Green PCR (Qiagen). Expression level was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). RT-PCR was performed using PC818 thermal cycler (ASTEC, Fukuoka, Japan), and AmpliTAQ Gold (Applied Biosystems, Foster City, CA). After 30–35 cycles of amplification, PCR products were electrophorated using 2% agarose gel. Primer sequences are shown in Table 2. RNA of Human Pulmonary Alveolar Epithelial Cell (3205) and Human Brain (636530) were obtained from ScienCell Research Laboratories (Carlsbad, CA) and Clontech (Palo Alto, CA), respectively.

Gelatin Zymography

Conditioned media without FBS after stimulation with histamine at the concentration of 10^{-4} M for 48 hour were collected and equal amounts of protein were mixed with 4× non-reducing SDS-PAGE sample buffer. The samples were applied to a 10% (w/v) polyacrylamide gel impregnated with 1 mg/mL gelatin (Sigma). After electrophoresis, SDS was removed from the gel by washing 3 times for 20 minute in 2.5% Triton X-100 solution. Then the gels were incubated overnight with gentle shaking at 37 °C in buffer (50 mM Tris-HCl, pH7.6, 5 mM $CaCl_2$, 200 mM NaCl, 0.02% Brij35). The gel was stained with 0.5% Coomassie blue R250 in 50% methanol and 5% acetic acid for 2 hour at room temperature, and subsequently destained with 40% methanol–10% acetic acid solution until the bands became clear.

Hematoxylin and Eosin Staining and Immunostaining of Collagen Gel

Collagen gels were fixed in formalin solution and embedded in paraffin, and vertical sections (4 μm) were stained with hematoxylin and eosin. Immunostaining for Ki67 was performed using the streptavidin–biotin-peroxidase method (LSAB2 Kit/HRP; DAKO, Kyoto, Japan) and rabbit monoclonal anti-Ki67 antibody (abcam, Cambridge, UK).

Statistics

Results were confirmed by repeating experiments on at least three separate occasions, each performed in triplicate. Analyses were performed using PASW Statistics 18 (IBM SPSS inc., Chicago, USA). For sequential observation, repeated measures analysis of variance (ANOVA) was used to analyze the differences between individual groups. For non-sequential observation, Dunnett's test was applied for comparisons with control group, and Tukey-Kramer's HSD was applied for multiple comparisons. $P < .05$ was considered to be significant.

RESULTS

Histamine Induces Collagen Gel Contraction of Primary Cultured Lung Fibroblasts

We have previously established four primary cultured human lung fibroblasts from resected lung tissues in the peripheral portion of the lungs [15]. They showed uniformly spindle-like appearance and were positively stained for vimentin, supporting that they are lung fibroblasts derived from bronchioles or alveoli. Basal expression of α-SMA and α1(I) collagen (COL1A1) in these primary cultured lung fibroblasts and fibroblast cell lines (HFL-1, WI-38, and IMR-90) was variable (Figure 1A). To investigate the effect of histamine on collagen gel contraction of lung fibroblasts,

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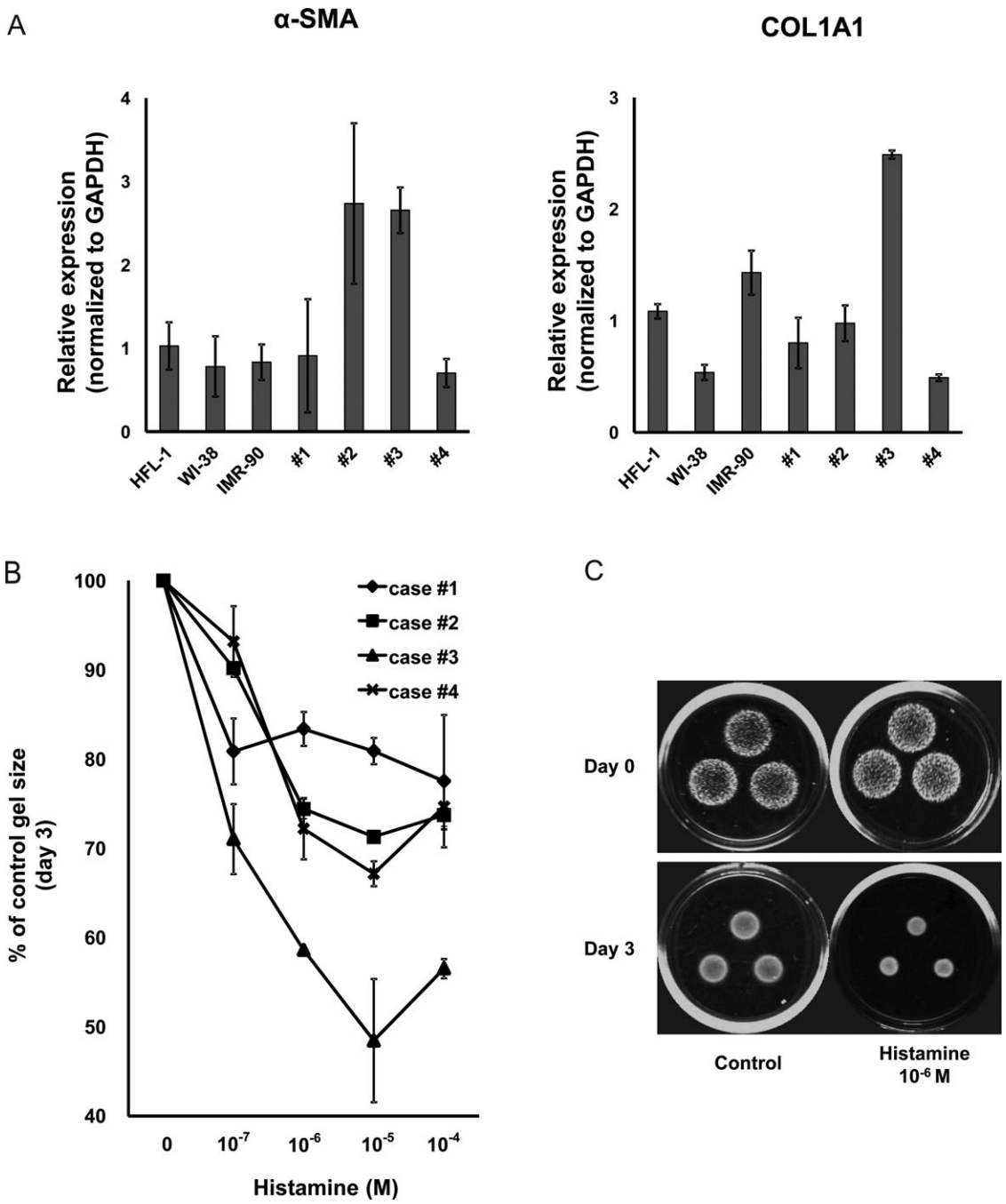


FIGURE 1. Histamine induces collagen gel contraction of primary cultured human lung fibroblasts. (A) Basal expression of α-SMA and COL1A1 in lung fibroblasts. Quantitative RT-PCR was performed for α-SMA and COL1A1 in HFL-1, WI-38, IMR-90, and four primary cultured lung fibroblasts. Expression was normalized to that of GAPDH. Error bars: SD. Left: α-SMA. Right: COL1A1. (B) Dose-dependent effect of histamine on primary cultured human lung fibroblasts. The surface area of the gels on day 3 was measured, and the value was normalized to that of the unstimulated control gels. Data are the mean $\pm$ SEM for three separate experiments, each performed in triplicate study. (C) The representative picture of collagen gels on day 0 and day 3 (case #3). Left: control gels. Right: histamine-stimulated gels.

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we first used these primary cultured fibroblasts to obtain results in the cells derived from different individuals. In all these cases, histamine strongly induced collagen gel contraction as compared to the unstimulated control (Figure 1B and 1C). This effect was seen at the concentration of 10^{-7} to 10^{-4} M. This result showed that histamine could elicit collagen gel contraction in human lung fibroblasts, and the action of histamine to induce gel contraction was commonly shared among individuals with different clinical backgrounds, albeit to a variable degree.

Histamine Induces Collagen Gel Contraction of Lung Fibroblasts in a Time-Dependent Manner

To test the above-observed effect of histamine in commercially available human lung fibroblasts, we used three fetal fibroblast cell lines (HFL-1, WI-38 and IMR-90 cells). Consistent with the result of primary cultured fibroblasts, histamine stimulated gel contraction of these cells in a time-dependent manner at the concentration of 10^{-6} M, though their effects were variable among the cell lines (Figure 2A). The effect of histamine was the most prominent in IMR-90 cells whereas WI-38 cells showed comparatively mild response to histamine. Since HFL-1 cells have been extensively characterized in our previous studies [12], we used this cell line in the following experiments.

Next we examined the dose-dependent effect of histamine on lung fibroblast-mediated gel contraction. At the concentration of 10^{-7} M, histamine failed to show any effect, though the effect was observed at the concentration of 10^{-7} to 10^{-4} M in primary cultured adult lung fibroblasts. In HFL-1 cells, its significant effect was seen at the concentration of 10^{-6} to 10^{-4} M (Figure 2B). These results were similar to those observed in primary cultured lung fibroblasts.

Histamine-Induced Collagen Gel Contraction of Lung Fibroblasts is Inhibited by Histamine H1 Receptor Antagonist

Four types of histamine receptors are differentially expressed in a variety of cell types [10]. To investigate which type of receptor is involved in histamine-mediated collagen gel contraction, we utilized histamine receptor antagonists. Diphenhydramine, ranitidine, clobenpropitand, and JNJ7777120 are selective histamine receptor antagonists for H1 to H4 subtypes, respectively. When used at the concentration shown to be effective in previous studies, diphenhydramine (H1 receptor antagonist) was the only agent which could inhibit the effect of histamine at the concentration of 10^{-6} M. (Figure 3A).

With regard to the action on gel contraction, the other histamine receptor antagonists had no effect in the presence (Figure 3B, left) or absence (Figure 3B, right) of histamine at the concentration of 10^{-6} M. We confirmed that H1 receptor is predominantly expressed in human lung fibroblasts by RT-PCR (Figure S2), and these results were consistent with the previous reports [17].

Histamine Transduces Intracellular Signaling via H1 Receptor

Upon ligand binding on histamine receptors, conformational change allows interaction with heterotrimeric G proteins, consisting of α-β-γ subunits, leading to activation of classical and non-classical signaling cascades [18]. The classical pathway requires association with specific Gα subunits (Gαq, Gαs, and Gαi/o), and each Gα subunit activates distinct molecular signaling cascades. Histamine H1 receptor binding leads to activation of Gαq, which results in the activation of phospholipase C (PLC), which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Increased intracellular IP_3 binds to IP_3 receptors, including calcium channels, on the endoplasmic reticulum, resulting in increased cytosolic calcium levels. In addition, DAG activates protein kinase C (PKC), which can phosphorylate other molecules, thus altering their activity. The non-classical signaling by the G protein $\beta\gamma$ subunits includes mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways [10].

To confirm that histamine transduces intracellular signaling in lung fibroblasts, we examined phosphorylation of Erk1/2, one of the components of MAPK cascades, since its specific detection is widely utilized as a downstream signaling component of GPCR. Histamine treatment elicited rapid phosphorylation of Erk1/2 in lung fibroblasts, peaking at 5–10 min after stimulation (Figure 4A, left, $n = 3$). Histamine failed to elicit Erk1/2 phosphorylation when used at the concentration of 10^{-7} M, whereas 10^{-6} to 10^{-4} M of histamine clearly phosphorylated Erk1/2, suggesting the effective intracellular signal transduction (Figure 4A, right, $n = 3$). These results were in accordance with the result of gel contraction (Figure 2B).

We further tested whether the observed effect of diphenhydramine on gel contraction was associated with blockade of receptor-mediated signal transduction. Histamine-induced phosphorylation of Erk1/2 was clearly inhibited by diphenhydramine at the concentration of 10^{-4} M (Figure 4B, $n = 3$). This result confirmed that diphenhydramine indeed blocked

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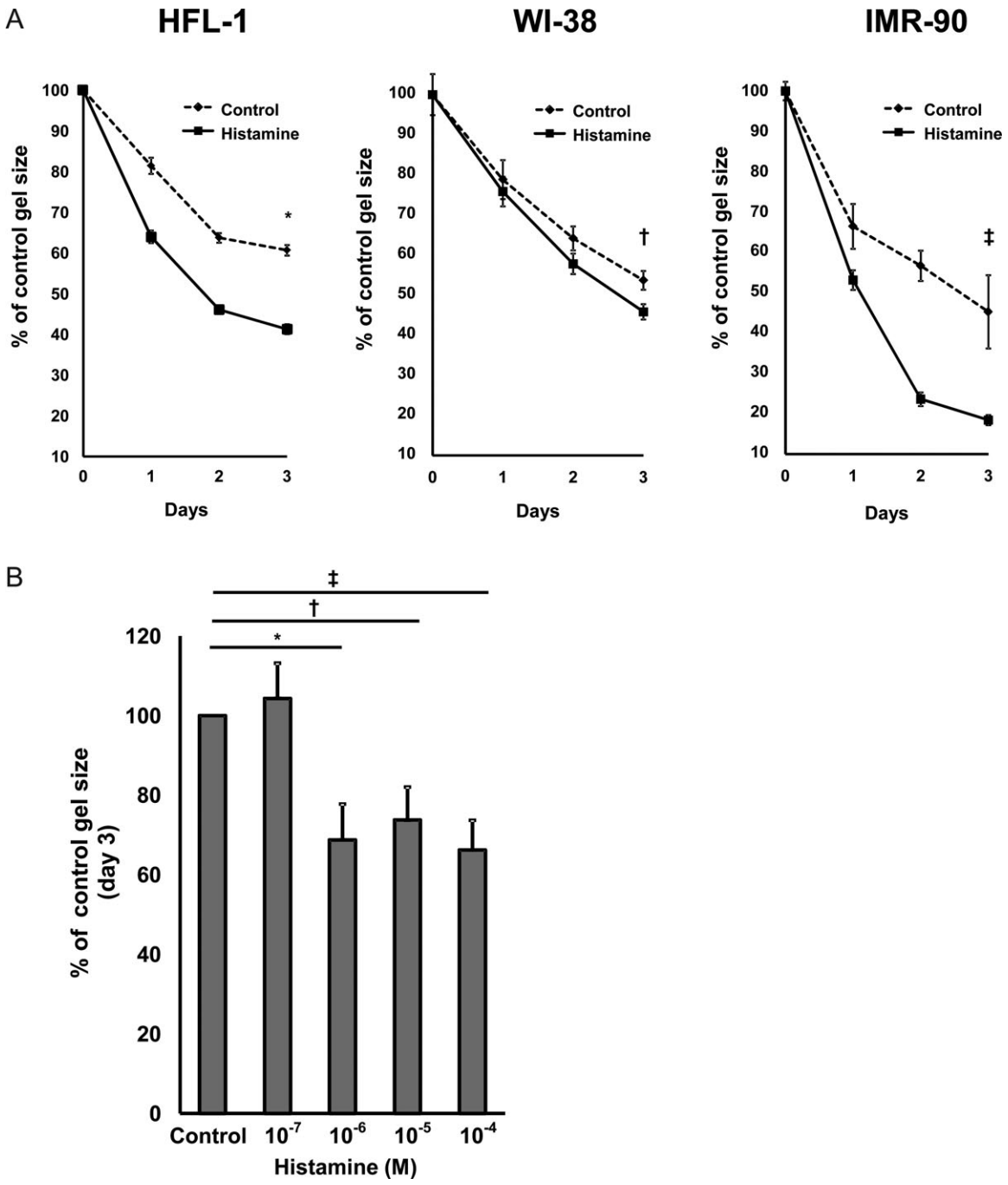


FIGURE 2. Histamine induces collagen gel contraction of human lung fibroblasts. (A) Time-course effect of histamine on HFL-1, WI-38, and IMR-90 human fetal lung fibroblasts. The surface area of the gels was measured daily for 3 days, and the value was normalized to that on day 0. Histamine was used at the concentration of 10^{-6} M. Data are the mean ± SEM for three separate experiments, each performed in triplicate study. Two-way repeated measures ANOVA was used to analyze the difference between groups. $^{*}P < .001$, $^{\dagger}P = .048$, $^{\ddagger}P = .002$. (B) Effect of histamine on HFL-1. The surface area of the gels on day 3 was measured, and the value was normalized to that of the unstimulated control gels. Data are the mean + SEM for four separate experiments, each performed in triplicate. Dunnett's test was applied for comparisons with control group. $^{*}P = .048$, $^{\dagger}P = .026$, $^{\ddagger}P = .016$.

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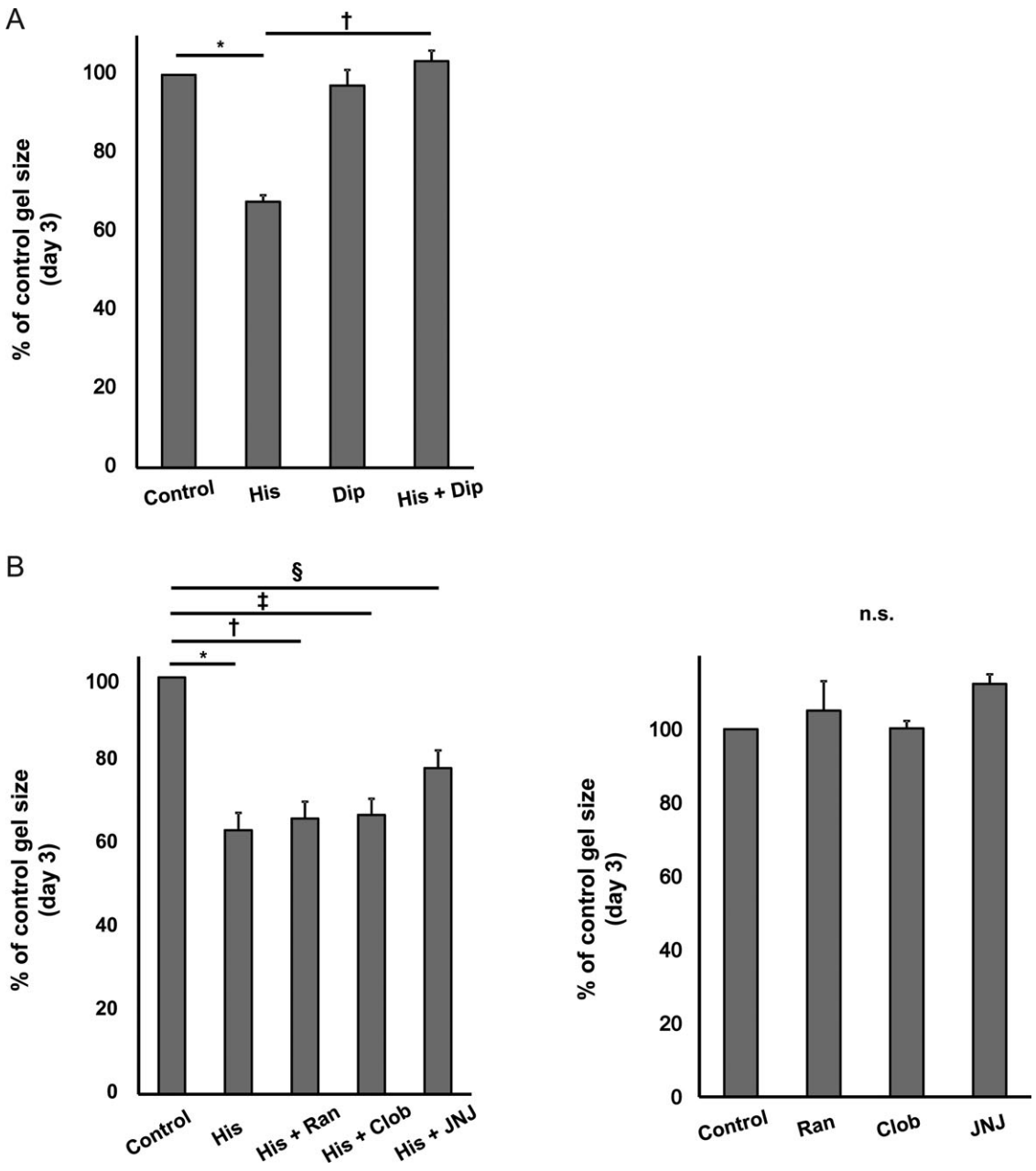


FIGURE 3. Histamine-induced collagen gel contraction is inhibited by histamine H1 receptor antagonist. (A) Effect of H1 receptor antagonist, diphenhydramine (Dip) on HFL-1 gel contraction induced by histamine (His). Histamine was used at the concentration of 10^{-6} M. Data are the mean+ SEM for 3 separate experiments, each performed in triplicate. Tukey-Kramer's HSD was applied for multiple comparisons. $^{*}P < .001$, $^{\dagger}P = .002$. (B) Effects of histamine H2 to H4 receptor antagonists in the presence (left) or absence (right) of histamine (His) at the concentration of 10^{-6} M, on HFL-1 gel contraction. Ran, Clob, and JNJ stand for ranitidine, clobenpropitand, and JNJ7777120, which are selective histamine H2 to H4 receptor antagonists, respectively. The surface area of the gels on day 3 was measured, and the value was normalized to that of the unstimulated control gels. Data are the mean + SEM for 4 (left) and 3 (right) separate experiments, each performed in triplicate. Tukey-Kramer's HSD was applied for multiple comparisons (left), and Dunnett's test was applied for comparisons with control group (right). $^{*}P < .001$, $^{\dagger}P < .001$, $^{\ddagger}P < .001$, $^{\S}P = .005$. n.s. = not significant.

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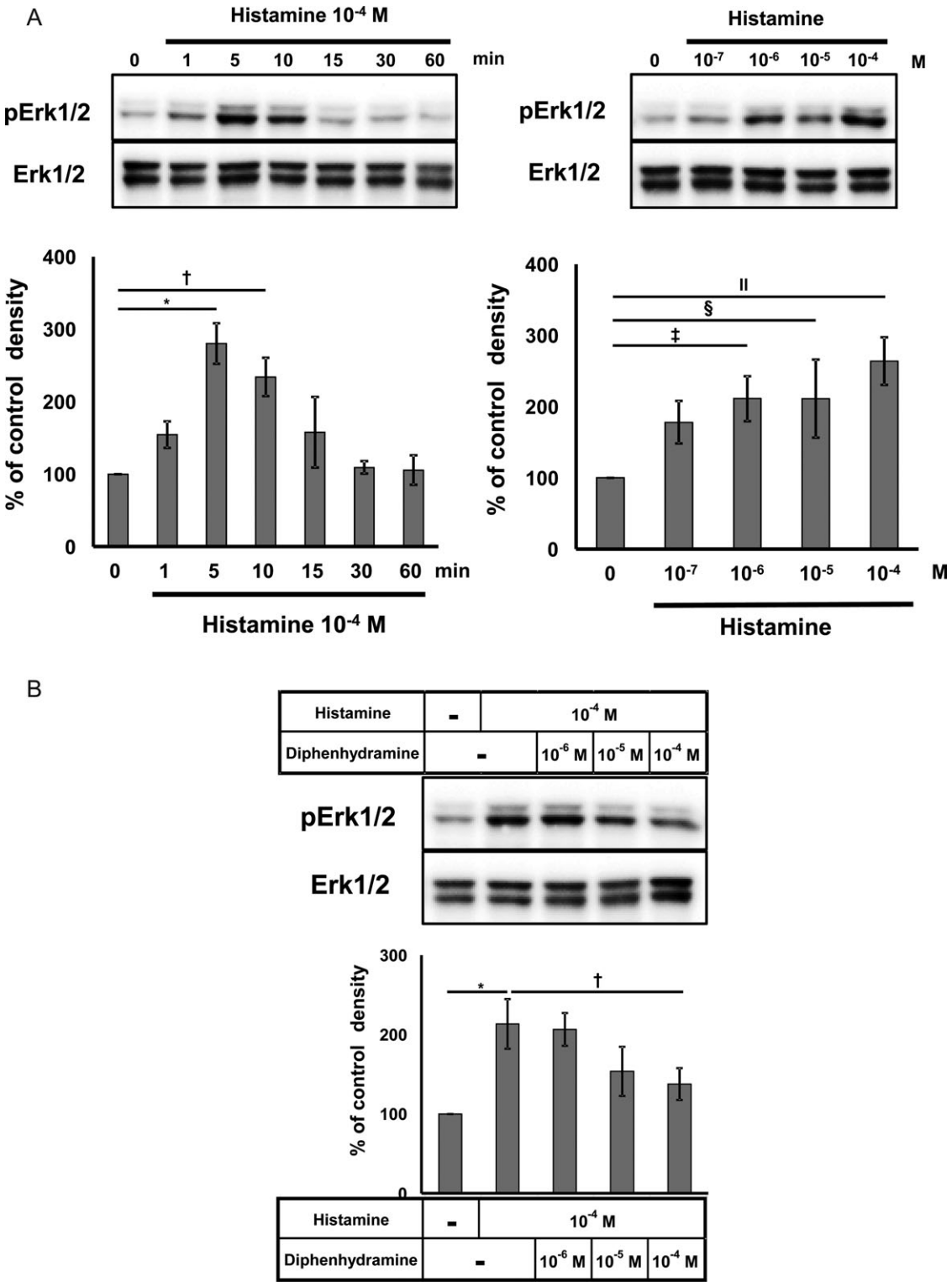


FIGURE 4. Histamine transduces intracellular signaling via H1 receptor. Immunoblotting for total and phosphorylated Erk1/2 (pErk1/2). Each picture of immunoblotting shows representative data from three separate experiments. (A) Left: Time-course effect of histamine (10^{-4} M) on HFL-1. Right: Dose-dependent response of histamine (10 minute) on HFL-1. Dunnett's test was applied for comparisons with control group. $^{*}P = .023$, $^{\dagger}P = .037$, $^{\ddagger}P = .040$, $^{\S}P = .026$. $^{\text{II}}P = .008$. (B) Effect of diphenhydramine on phosphorylation of Erk1/2 induced by the histamine (10^{-4} M) 10 minute after stimulation in HFL-1. The density of the bands of pErk1/2 was quantified by densitometry analysis. Data are the mean ± SEM for three separate experiments. Tukey-Kramer's HSD was applied for multiple comparisons.$^{*}P = .016$, $^{\dagger}P = .016$.

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histamine-elicited intracellular signaling at the concentration used for gel contraction assay (10^{-4} M).

Histamine-Induced Collagen Gel Contraction of Lung Fibroblasts is Inhibited by 2-APB

Previous reports indicate that histamine-induced smooth muscle cell contraction is mediated through classical signaling pathways including histamine H1 receptor and PLC that leads to the generation of IP_3 and DAG [19]. Therefore we explored the involvement of these pathways in the regulation of collagen gel contraction of lung fibroblasts.

Since PKC is the central effector downstream of DAG, we first tested the effect of bisindolylmaleimide (PKC inhibitor) on gel contraction. Bisindolylmaleimide alone had no effect, and it failed to attenuate histamine-induced gel contraction of lung fibroblasts, suggestive of its little role in this experimental model (Figure 5A).

To prove the involvement of IP_3 receptor in Ca^{2+} mobilization from the endoplasmic reticulum, 2-aminoethoxydiphenyl borate (2-APB) has been widely used in many studies [20]. When lung fibroblasts were treated with 2-APB, histamine-mediated gel contraction was significantly inhibited while 2-APB alone did not significantly affect gel contraction (Figure 5B). These results suggested that the signals which mediate histamine-induced gel contraction are mostly counteracted by 2-APB, thus implying the critical role of Ca^{2+} signaling.

Effect of Histamine on $[Ca^{2+}]_i$ of Lung Fibroblasts

The contractile model of smooth muscle cells is delineated as a sliding-filament mechanism in which actomyosin cross-bridges, powered by ATP hydrolysis catalyzed by myosin ATPase, leading to tension development. IP3 binds to its receptor on the endoplasmic reticulum, thereby releasing Ca^{2+} that activates Ca^{2+}-calmodulin-dependent myosin light chain kinase (MLCK). MLCK phosphorylates myosin light chain (MLC), which triggers cross-bridge cycling, i.e., the movement of myosin heads along actin filaments [21].

We investigated whether histamine elicits increase of intracellular Ca^{2+} concentration, which is referred as $[Ca^{2+}]_i$, using the fura-2 fluorescence method. Histamine stimulation rapidly increased $[Ca^{2+}]_i$ in extracellular fluid with both nicardipine plus $CaCl_2$ (Figure 6, upper left) and EGTA (data not shown), suggesting the release of Ca^{2+} from the endoplasmic reticulum (Figure 6, upper left). This increase was inhibited by diphenhydramine, indicating that

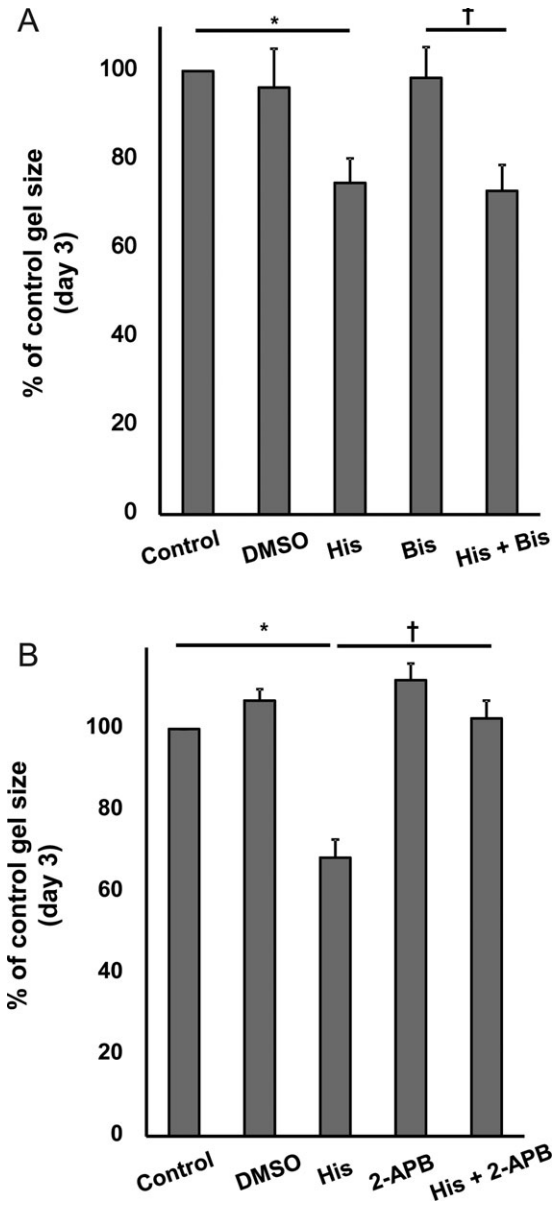


FIGURE 5. Histamine-induced collagen gel contraction of lung fibroblasts is inhibited by 2-APB. (A) Effect of PKC inhibitor, bisindolylmaleimide (Bis) on HFL-1 gel contraction induced by histamine (His) at the concentration of 10^{-6} M. Data are the mean+ SEM for four separate experiments, each performed in triplicate. Tukey-Kramer's HSD was applied for multiple comparisons.$^{*}P = .031$, $^{\dagger}P = .029$. (B) Effect of 2-APB on HFL-1 gel contraction induced by histamine (His) at the concentration of 10^{-6} M. The surface area of the gels on day 3 was measured, and the value was normalized to that of the unstimulated control gels. Data are the mean+ SEM for three separate experiments, each performed in triplicate. Tukey-Kramer's HSD was applied for multiple comparison.$^{*}P < .001$, $^{\dagger}P < .001$.

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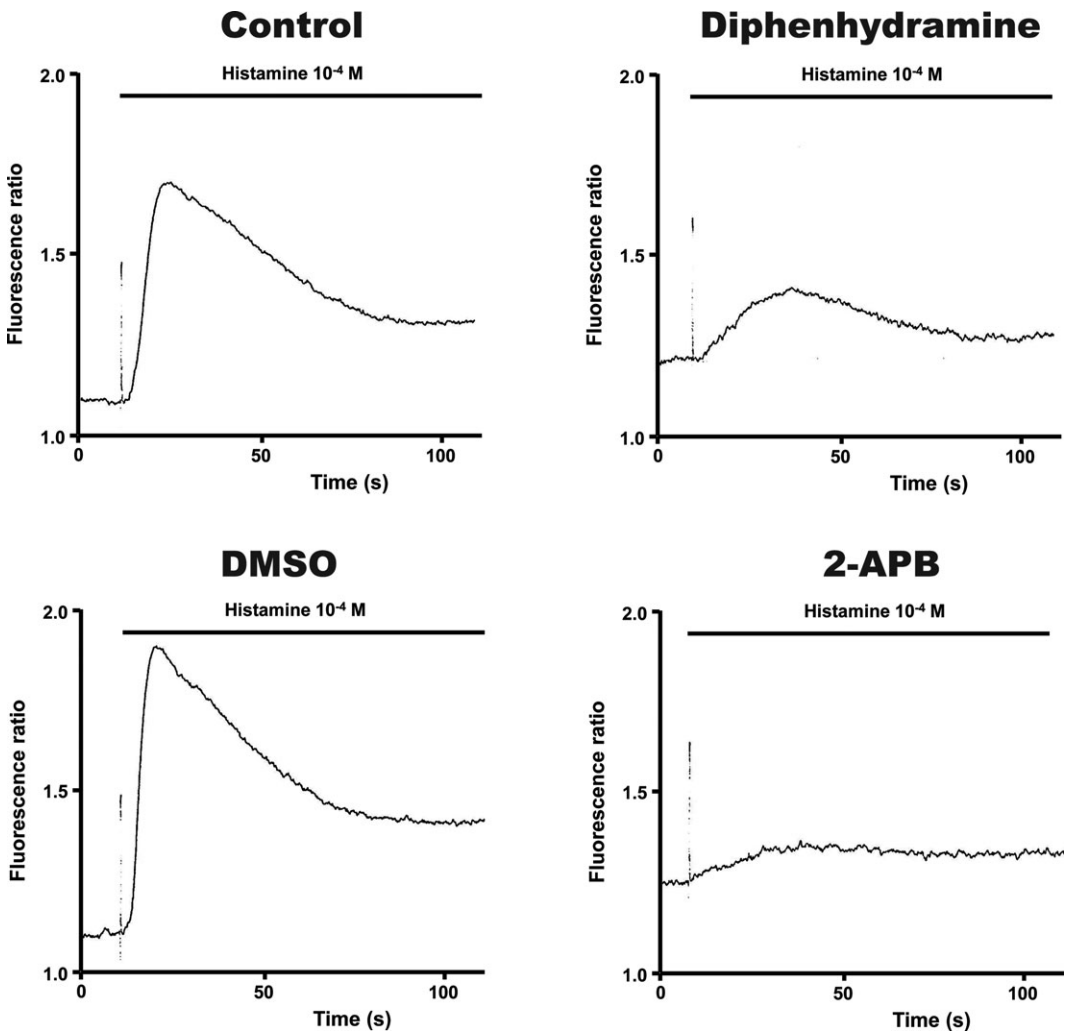


FIGURE 6. Effect of histamine on $[Ca^{2+}]_i$ of lung fibroblasts. Kinetics of $[Ca^{2+}]_i$ following histamine stimulation. Each figure shows representative data from three separate experiments. **Upper left:** untreated before stimulation. **Upper right:** Treated with diphenhydramine 30 minute before stimulation. **Lower left:** Treated with control DMSO 30 minute before stimulation. **Lower right:** Treated with 2-APB 30 minute before stimulation.

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histamine-elicited $[Ca^{2+}]_i$ increase is mediated by histamine H1 receptor (Figure 6, upper right). Furthermore, histamine-elicited $[Ca^{2+}]_i$ increase was abrogated by 2-APB (Figure 6, lower right) compared to the control (Figure 6, lower left) in extracellular fluid with both nicardipine plus $CaCl_2$ and EGTA (data not shown). These observations suggested that histamine triggers $[Ca^{2+}]_i$ increase through H1 receptor, and this effect is mediated by IP3-elicited Ca^{2+} mobilization from endoplasmic reticulum.

Histamine Can Enhance Expression Levels of Active MMP-2 and GM6001 Attenuates Histamine-Induced Collagen Gel Contraction

Recently it has been reported that endothelin-induced contraction of collagen gels in which airway smooth muscle cells are embedded is not due to cell contraction but reflects collagen remodeling [22]. Collagen remodeling can result from the processes of degradation and production, which is regulated by fibrogenic cytokines as well as proteases such as matrix metalloproteinases (MMPs). We found that histamine modestly enhanced expression levels of active MMP-2 in gelatin zymography by stimulation of histamine for 48 hour (Figure 7A). Then we evaluated the effect of GM6001, pan-MMP inhibitor, which is widely used to block MMP activitity. GM6001 attenuated fibroblast-mediated collagen gel contraction in the basal level as previously reported [23], and GM6001 also significantly attenuated histamine-induced collagen gel contraction (Figure 7B). Collectively, MMPs might play an important role in histamine-induced collagen gel contraction.

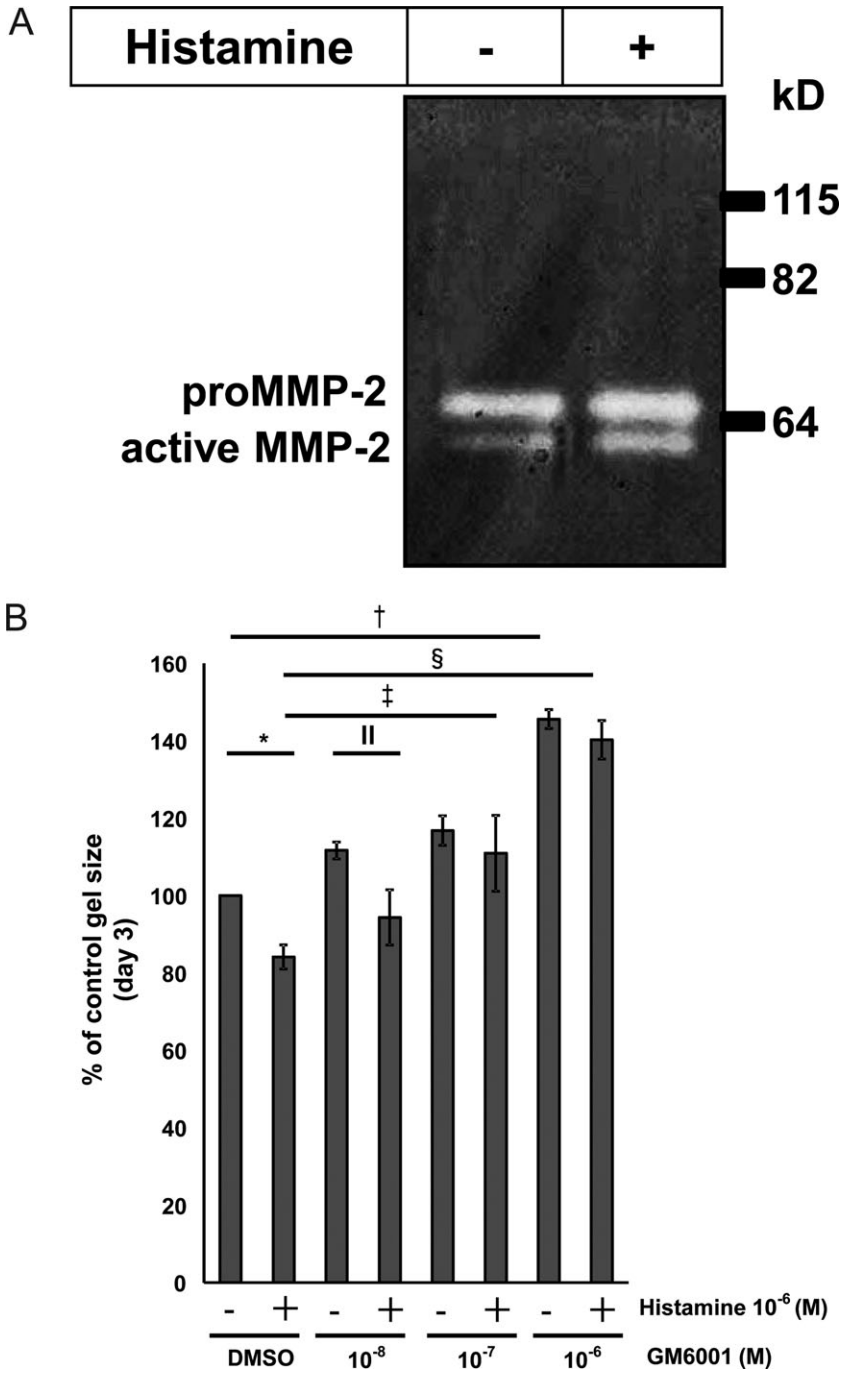


FIGURE 7. Histamine enhances expression levels of active MMP-2 and GM6001 attenuates histamine-induced collagen gel contraction. (A) Gelatin zymography. HFL-1 cells treated with histamine at the concentration of 10^{-4} M were cultured with serum free media for 48 hour. The conditioned media were collected and the same amount of protein was electrophoresed. Gelatin digestion by activated MMP-2 and MMP-9 was visualized by Coomassie blue staining. B: Effect of pan-MMP inhibitor, GM6001, on HFL-1 gel contraction induced by histamine at the concentration of 10^{-6} M. Data are the mean + SEM for three separate experiments, each performed in triplicate. Tukey-Kramer's HSD was applied for multiple comparisons. $^{*}P = .042$, $^{\dagger}P = .002$, $^{\ddagger}P = .0301$, $^{\S}P < .001$, $^{\|}P = .049$.

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DISCUSSION

In cases of steroid-resistant refractory asthma, airway remodeling is involved in the pathophysiological process, where lung fibroblasts play critical roles. Histamine is a causative mediator of allergic inflammation, released by mast cells and basophils. Mast cells are involved in activation of lung fibroblasts [24], and histamine has been implicated in airway remodeling [25]. However, there have been quite few reports describing the effect of histamine on lung fibroblasts [12, 17] while a number of studies describe its action on smooth muscle cells, despite the potential contribution of fibroblasts to airway narrowing when converted into myofibroblasts [8].

In the present study, we found that histamine induces collagen gel contraction of lung fibroblasts. This effect was confirmed in four human primary cultured adult lung fibroblasts and three human fetal lung fibroblast cell lines. Histamine-mediated collagen gel contraction was observed at the concentration of 10^{-7} to 10^{-4} M in primary cultured adult lung fibroblasts while its significant effect was seen at the concentration more than 10^{-6} M in HFL-1 cells. Histamine phosphorylated Erk1/2 at the concentration of $10^{-6}\sim10^{-4}$ M while it failed to do so at the concentration of 10^{-7} M. Therefore, we have chosen these concentrations of histamine to demonstrate dose-dependent effects on lung fibroblasts. The effect of histamine was mediated by histamine H1 receptor and the pathways antagonized by 2-APB, i.e., IP_3 signaling. In addition, the effects of H1 receptor antagonist and 2-APB were associated with inhibition of $[Ca^{2+}]_i$ increase elicited by histamine. Thus intracellular Ca^{2+} signaling was implicated in collagen gel contraction, which is well recognized as the pathway leading to cell contraction, especially in smooth muscle cells. Although we did not investigate single cell contraction of fibroblast in this study, enhanced fibroblast contractility could contribute to collagen gel contraction, at least in the initial phase.

Using three fetal lung fibroblast cell lines and four primary human adult fibroblasts obtained from lung parenchyma, we showed the effect of histamine on lung fibroblasts, but we did not use lung fibroblasts from the airways of patients with asthma or healthy control. This is limitation of the present study because it seems favorable to use fibroblasts of asthmatics when we discuss the pathogenesis of asthma and airway remodeling in asthmatic patients.

Collagen gel contraction assay is widely used as a reproducible model of tissue contraction, although the precise mechanisms of gel contraction remain largely unknown. The presumed cellular events include cell contraction, proliferation, migration, myofibroblastic differentiation, and collagen remodeling. The persistent effect of histamine on gel contraction for days suggests mechanisms other than cell contraction. It would be of interest to explore to what extent the aforementioned mechanisms contribute to the reduction of gel size.

Previous studies demonstrated that histamine stimulates lung fibroblast proliferation [17, 24], and it could be the case in our culture system. To evaluate cell proliferation of fibroblasts in the collagen gel, we performed H&E staining (Figure S3A) and immunostaining for Ki-67 which can detect proliferating cells in late G1, S, G2, and M phases, but not in resting cells in G0 (Figure S3B). The number of fibroblasts in a microscopic field was higher in histamine-treated gel (data not shown), but it was attributable to the enhanced contraction of collagen gel and subsequently increased cell density. Immunostaining for Ki-67 showed few Ki-67 positive cells found in collagen gels in both control and histamine-treated gels (Figure S3B). Thus, cell proliferation of lung fibroblasts in collagen gel did not appear to be enhanced by histamine, and cell proliferation might not play a role in collagen gel contraction.

Histamine stimulates lung fibroblast migration, and this effect could facilitate other mechanisms [12]. It is unlikely that histamine is involved in phenotypic differentiation into myofibroblasts as we could not find either any morphological change or increased expression of α-SMA determined by Western blotting following histamine stimulation for 48 hour (Figure S4).

In addition, condensation of collagen fibrils to the vicinity of fibroblasts has been suggested to be another mode of collagen remodeling, which can be a mechanistic explanation for gel contraction [22, 26]. The cells adhere to collagen fibrils through integrins, and protruded cell processes attached to collagen are withdrawn resulting in formation of adhesion complexes of collagen–integrin–cytoskelton at the membrane. Repeated protrusion and withdrawal of cell processes may lead to the condensation of collagen fibrils into the vicinity of the cells. These mechanisms employ the cell machineries similar to those required for cell migration. Considering the pro-migratory effect of histamine, this "collagen condensation" model seems a plausible explanation for collagen gel contraction. Further studies are necessary to delineate and interpret our findings in the pathophysiological context.

In previous reports, inhibition of MMP-2 and MMP-3 blocked the contractile response of human lung fibroblasts in a three-dimensional collagen lattice system [27], and histamine could upregulate MMP-9 in keratinocytes [28]. Taken together, it is

possible that histamine modulates the turnover of collagen and other extracellular matrices produced by fibroblasts inside the gels. In the present study, GM6001 attenuated histamine-induced fibroblast-mediated collagen gel contraction. Although the total amount of collagen in the collagen gels was not altered by histamine (data not shown), MMPs might play an important role in histamine-induced collagen gel contraction.

In conclusion, histamine could induce collagen gel contraction of human lung fibroblasts. Our findings suggest that histamine is involved in airway remodeling through its action on lung fibroblasts. Thus the present study provides evidence for the potential therapeutic efficacy of antihistamines, especially selective H1 receptor antagonists, in the management of refractory asthma.

***Declaration of interest*:** The authors report no conflict of interest. The authors are responsible for the content and writing of the paper.

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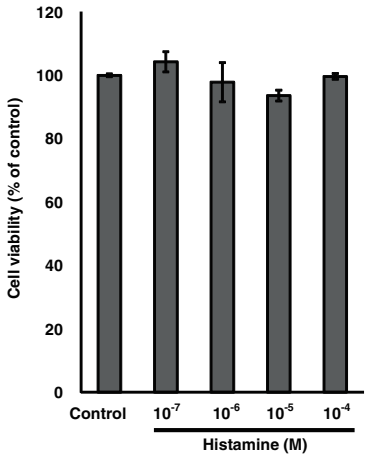
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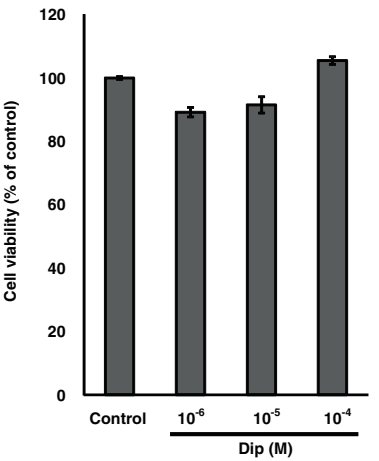
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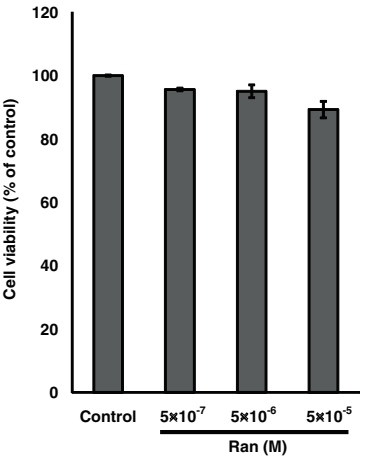
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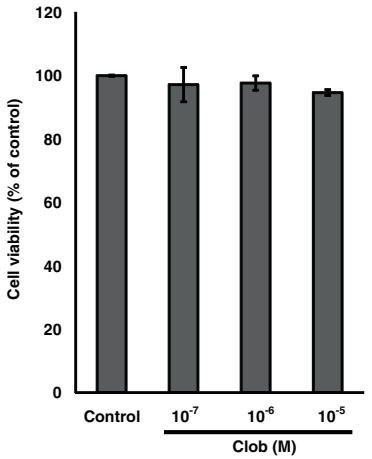
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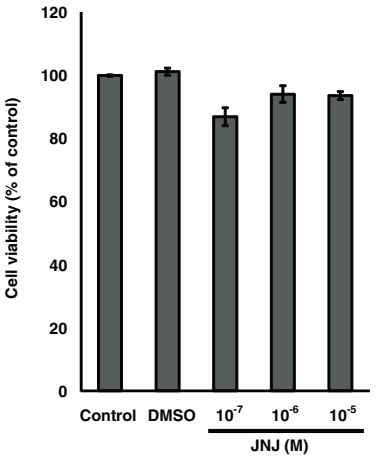
Supplementary Fig. 1. MTT assay in the cells treated with histamine and each inhibitor. No overt cytotoxic effect of histamine or inhibitors in HFL-1 cells was observed at the concentration used in this study. The OD values were normalized to that of control, and data are the mean ± SD, each performed in triplicate study. Dip, Ran, Clob, JNJ, and Bis stand for diphenhydramine, ranitidine, clobenpropitand, JNJ7777120, and bisindolylmaleimide, respectively.

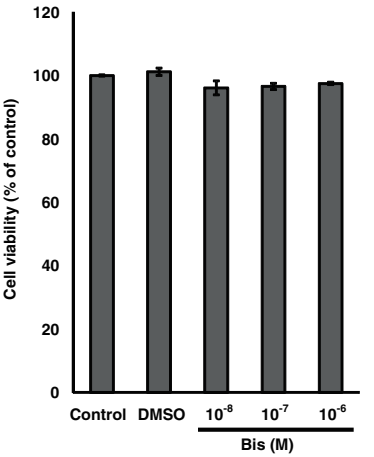


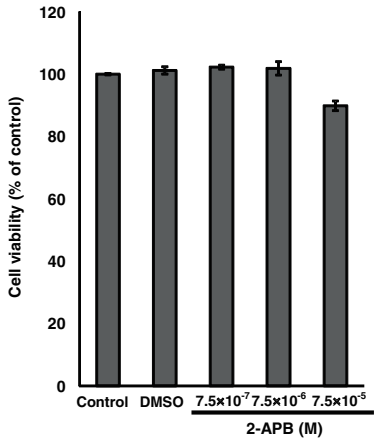


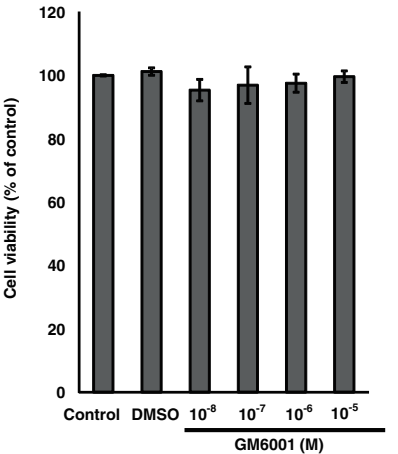




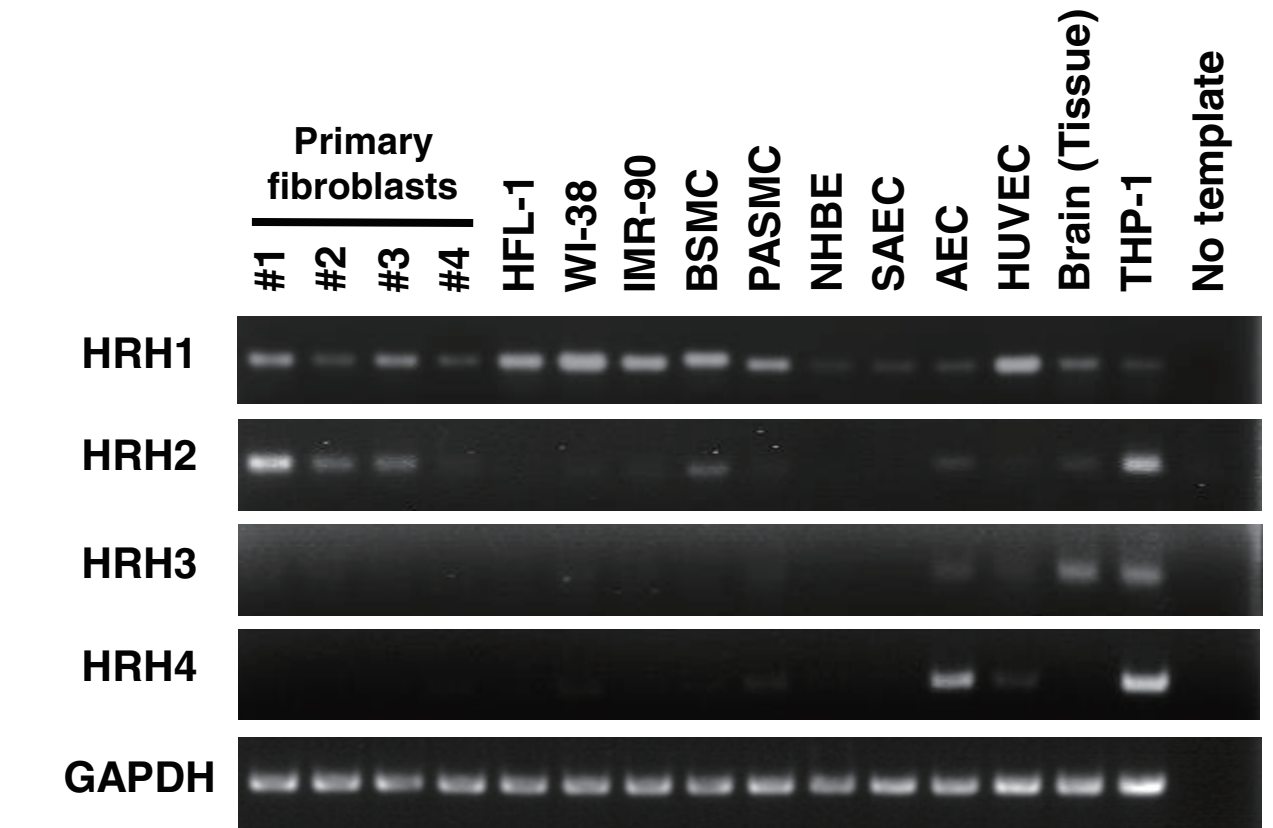




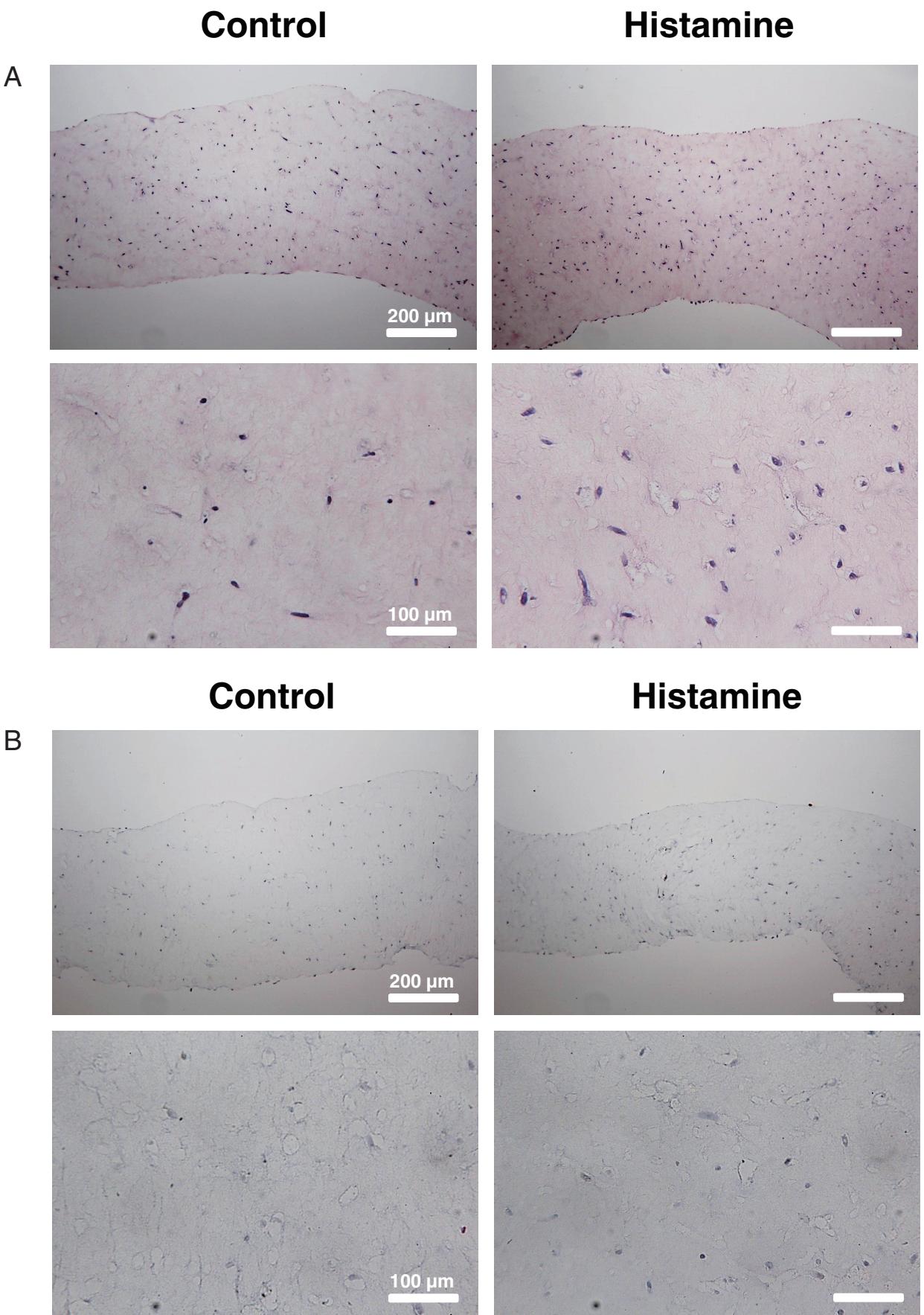




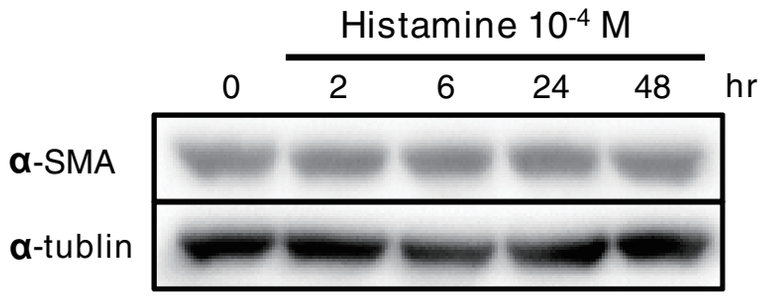
Supplementary Fig. 2. RT-PCR for each histamine receptor. Thirty cycles of RT-PCR was performed for detection of HRH1, HRH2, and HRH3. Thirty-five cycles of RT-PCR was performed for detection of HRH4. Twenty-five cycles of RT-PCR was performed for detection of GAPDH. BSMC = bronchial smooth muscle cells, PASMC = pulmonary artery smooth muscle cells, NHBE = normal human bronchial epithelial cells, SAEC = small airway epithelial cells, AEC = alveolar epithelial cells, HUVEC = human umbilical vein endothelial cells, HRH1 = histamine receptor h1, HRH2 = histamine receptor h2, HRH3 = histamine receptor h3, and HRH4 = histamine receptor h4. HUVEC was used as positive control for HRH1, THP-1 was used as positive control for HRH2 and HRH4, and brain tissue was used as positive control for HRH3.



Supplementary Fig. 3. Hematoxylin and eosin staining and immunostaining for Ki67 in collagen gels. (A) Collagen gels were fixed in formalin solution and embedded in paraffin, and vertical sections (4 μm) were stained with hematoxylin and eosin. Upper left: control, scale bar, 200 μm. Upper right: the gel treated with 10−6 M histamine for 3 days. Lower left: control, scale bar, 100 μm. Lower right: the gel treated with 10−6 M histamine for 3 days. (B) Immunostaining for Ki67. Upper left: control, scale bar, 200 μm. Upper right: the gel treated with 10−6 M histamine for 3 days. Lower left: control, scale bar, 100 μm. Lower right: the gel treated with 10−6 M histamine for 3 days.



Supplementary Fig. 4. Immunoblotting for α-SMA. HFL-1 cells were treated with histamine at the concentration of 10–4 M for 2, 6, 24, and 48 hour. The cell lysates were collected at the indicated time points, and immunoblotting was performed for α-SMA. α-tubulin was used as loading control.



Supplementary table 1. Histamine induces collagen gel contraction in 4 primary cultured human lung fibroblasts (values in Fig. 1B).

	Control	Histamine 10^{-7}	Histamine 10^{-6}	Histamine 10^{-5}	Histamine 10^{-4}
#1	100	73.5112	79.6348	79.6348	87.0787
	100	83.9045	84.6910	83.8483	82.5843
	100	85.1685	85.8146	79.2135	62.9213
#2	100	90.7510	76.1474	70.0974	71.5577
	100	90.9597	72.3922	71.3491	75.9388
	100	88.8734	74.6871	72.3922	73.6439
#3	100	63.2044	59.0412	53.3642	55.1304
	100	75.0631	58.5366	57.1489	58.6627
	100	74.8108	58.2843	34.8192	55.7611
#4	100	96.1353	76.3285	64.7343	72.7858
	100	85.3462	74.8792	69.5652	79.7101
	100	98.0676	65.3784	67.1498	71.4976

Data are shown as % of control gel size of day3.

Supplementary table 2. Histamine induces collagen gel contraction in 3 human fetal lung fibroblast cell lines (values in Fig. 2A).

HFL-1	day0	day1	day2	day3
Control	100	85.1256	65.9747	63.1653
	100	80.6774	63.0248	59.7472
	100	78.3830	62.0883	58.9980
Histamine	100	66.6771	47.5261	43.2184
	100	63.8208	46.4960	41.5327
	100	61.2923	44.2017	38.9574
WI-38				
Control	100	74.4186	63.3350	51.0143
	100	77.9317	61.6526	54.4780
	100	84.0178	67.4419	55.4676
Histamine	100	73.2806	55.2202	43.6912
	100	74.0722	57.7437	47.3528
	100	80.0099	60.3167	46.3632
IMR-90				
Control	100	63.2184	52.3946	45.0192
	100	63.2663	58.1418	36.2548
	100	72.8448	59.4828	54.4540
Histamine	100	55.0287	22.2222	19.7797
	100	53.9751	25.4789	17.9598
	100	50.3831	23.0843	17.3851

Data are shown as % of the gel size of day 0.

Supplementary table 3. Histamine induces collagen gel contraction in HFL-1 (values in Fig. 2B).

	Control	Histamine 10^{-7}	Histamine 10^{-6}	Histamine 10^{-5}	Histamine 10^{-4}
HFL-1	100	92.4638	83.5072	73.1014	81.9710
	100	92.3402	46.4286	61.6541	45.4887
	100	135.4501	58.9867	58.1423	81.5350
	100	88.5201	58.7339	63.7749	69.3694
	100	112.6353	95.8828	102.0237	52.4483

Data are shown as % of control gel size of day3.