

Homoisoflavanone prevents mast cell activation and allergic responses by inhibition of Syk signaling pathway

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Abstract

Background: Mast cells play important roles in allergic inflammatory responses because they produce leukotrienes (LTs), prostaglandins (PGs), and a variety of inflammatory cytokines. Thus, pharmacological interventions for allergies have focused on inhibiting mast cell activation. Homoisoflavanone (HIF), isolated from *Cremastra appendiculata* Makino, has anti-angiogenic activities; however, its effects on allergic reactions have not been determined. The aim of this study was to assess the inhibitory effects of HIF on mast cell activation, which is critical for anti-allergic reaction and the underlying mechanisms.

Methods: Enzyme-linked immunosorbent assays, quantitative real-time PCR, western blot analyses, and degranulation assay were performed to measure pro-inflammatory and allergic mediators in PMA/A23187- or IgE/antigen-stimulated mouse bone marrow-derived mast cells (BMMCs), HMC-1, RBL-1, or human PBMC-derived mast cells treated with or without HIF. The anti-allergic effects of HIF were determined in mouse models using dinitrophenol-immunoglobulin E-induced passive cutaneous anaphylaxis (PCA) and compound 48/80-induced ear swelling.

Results: Homoisoflavanone down-regulated PGD₂, LTB₄, and LTC₄ production and inhibited the production of pro-inflammatory cytokines, such as interleukin-6 and tumor necrosis factor- α in PMA/A23187- or IgE/antigen-stimulated mast cells. The molecular mechanisms by which HIF caused these inhibitory effects were determined to be the inactivation of spleen tyrosine kinase (Syk) signaling and the concurrent suppression of cPLA₂. HIF inhibited IgE-mediated PCA and compound 48/80-induced ear swelling in mouse.

Conclusions: Homoisoflavanone inhibited mast cell activation through the suppression of Syk pathway together with the inhibition of cPLA₂. Thus, it might be a good candidate molecule for allergic diseases.

Mast cells are derived from hematopoietic progenitor cells and mature in local tissues, such as connective tissues, mucosal surfaces, vascularized tissues, and skin (1). Mature mast cells do

not circulate in the blood, but reside in the tissue where they are matured. Mast cells are implicated in many diseases including allergic, neuroinflammatory, and autoimmune diseases. Among them, the role of mature mast cells in allergic disease is most well studied. Mature mast cells express high levels of c-kit, the receptor for stem cell factor (SCF), and Fc ϵ RI, the high-affinity IgE receptor, and allergic response is initiated by IgE-dependent activation of mast cells. Briefly, for the activation of mast cells, IgE binds to its high-affinity receptor Fc ϵ RI in mast cells and cross-linking of the bound immunoglobulin E (IgE) by antigen induces IgE-mediated allergic diseases such as asthma, rhinitis, and atopic dermatitis (2–4).

Abbreviations

5-LOX, 5-lipoxygenase; AA, arachidonic acid; COX-2, cyclooxygenase-2; cPLA₂, cytosolic phospholipase A₂; DNP-IgE, dinitrophenyl immunoglobulin E; ELISA, enzyme-linked immunosorbent assay; HIF, homoisoflavanone; IL, interleukin; LTB₄, leukotriene B₄; LTC₄, leukotriene C₄; PCA, passive cutaneous anaphylaxis; PGD₂, prostaglandin D₂; PMA, phorbol myristate acetate; Syk, spleen tyrosine kinase.

FcεRI is a heterotetrameric receptor composed of an IgE-binding α-subunit, a membrane-spanning β-subunit, and two identical disulfide-linked γ-subunits. The γ-subunits are responsible for initiating the signaling pathway as each γ-subunit contains an immune receptor tyrosine-based activation motif (ITAM) (5, 6). Cross-linking of IgE bound to FcεRI induces signaling through FcεRI. It activates Lyn that then phosphorylates ITAM and activates spleen tyrosine kinase (Syk). Activated Syk triggers the activation of linker of activated T cells (LAT) and the direct or indirect binding of adaptor molecules, including Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa (SLP-76), growth factor receptor-bound protein 2 (Grb2), Grb2-related adaptor downstream of Shc (GADS), son of sevenless (SOS), and phospholipase C (PLC)-γ, to LAT. Subsequently, the phosphorylation of PLC-γ induces the influx of Ca²⁺ and mast cell degranulation (6, 7). In addition to Lyn activation, the cross-linking of IgE bound to FcεRI activates Fyn. Fyn phosphorylates Grb-associated binder 2 (Gab2) to activate phosphatidylinositol 3-kinase (PI3K), which is involved with the phosphorylation of Akt and regulates gene expression (8). The importance of Syk2 in mast cell activation was demonstrated by the lack of receptor-induced degranulation, cytokine synthesis, and intracellular signaling pathways in the presence of defective Syk2 (9). Inhibition of Syk2 prevents mast cell activation, airway hyper-responsiveness, and ovalbumin-induced degranulation of passively IgE-sensitized bone marrow-derived mast cells (BMMCs) (10). Furthermore, inhibition of Syk2 expression prevents allergic inflammation (11, 12).

Homoisoflavanone-1 cell line is a widely used human mast cell line for *in vitro* experiment, but it does not express FcεRI, a receptor for IgE. Thus, HMC-1 cell cannot be activated by IgE/antigen. Instead, it is activated with PMA and A23187. On the other hand, mouse bone marrow-derived mast cells (BMMCs) express FcεRI; thus, it can be activated by IgE/antigen stimulation, which is more similar to *in vivo* condition. Both PMA/A23187-stimulated HMC-1 cells and IgE/antigen-stimulated BMMCs release prestored mediators, including biogenic amines (histamine, serotonin) and proteases (tryptase and chymase), and newly synthesized mediators, including cytokines (interleukin [IL]-4, IL-5, IL-6, IL-13, and tumor necrosis factor [TNF]-α) and arachidonic acid (AA) metabolites (leukotrienes [LTs] and prostaglandins [PGs]) (13). PGs and LTs are derived from AA by cyclooxygenases (COXs) and 5-lipoxygenase (5-LOX), respectively (14–16). COXs convert AA to thromboxanes, PGs, and prostacyclin. PG production results in inflammation and pain. Conversely, 5-LOX is involved in the synthesis of LTB₄ and LTC₄, which are produced from LTA₄ and play a role in the pathogenesis of asthma and anaphylaxis (17). Therefore, down-regulation of LTA₄ causes decreased production of LTB₄ and LTC₄ and eventually inhibits allergic responses.

5,7-Dihydroxy-3-(3-hydroxy-4-methoxy-benzyl)-chroman-4-one (HIF), isolated from the extract of *Cremastra appendiculata* Makino, exhibits anti-angiogenic activities (18, 19). Here, we found that HIF can also inhibit activation and degranulation of mast cells via the inactivation of Syk2 together with

the inhibition of COX-2 expression and 5-LOX nuclear localization in mast cells. We confirmed the anti-allergic effects of HIF *in vivo* using anti-dinitrophenol (DNP)-IgE-mediated passive cutaneous anaphylaxis (PCA) and compound 48/80-induced ear edema in mice. Our results suggest that simultaneous inhibition of Syk signaling pathway and calcium-dependent cPLA₂ might be a potential therapeutic technique to prevent allergic diseases.

Methods

Mice

Hairless mice, Balb/c mice, and C57BL/6 mice were obtained from the Korean Research Institute of Biotechnology (Daejeon, Korea). All experiments with the mice were approved by the Review Board of Experimental Animals Study Committee at the Catholic University and performed following the guidelines provided by the committee.

Reagents

3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) was obtained from the USB Corporation (Cleveland, OH, USA). RPMI 1640 was purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). Dinitrophenol (DNP)-specific monoclonal IgE, DNP-human serum albumin (HSA), Evans blue, and compound 48/80 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies for COX-1 and COX-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and 5-LOX was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Antibodies for p-PLCγ-1 (pY783) and PLCγ-1 were purchased from Biosource (Camarillo, CA, USA). The antibodies against the phosphorylated forms of Syk, PDK1, Akt (S473), cPLA₂ (s505), JNK, and ERK and the unphosphorylated forms of PDK1, Akt, cPLA₂, and ERK were purchased from Cell Signaling Technology (Beverly, MA, USA).

Preparation of homoisoflavanone

Homoisoflavanone was extracted from the blub of *Cremastra appendiculata* by eluting with ethanol as previously described (18).

Cell culture

HMC-1 and RBL-2H3 cells were grown in IMDM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS at 37°C under humidified atmosphere containing 5% CO₂.

MTT assay

MTT assay was performed as previously described (20). Briefly, HMC-1 cells were cultured in a 96-well plate and treated with varying concentrations of HIF for 24 h. After

removing the supernatant of each well, MTT solution was introduced. After incubation for 4 h, the resultant formazan crystals were dissolved in dimethyl sulfoxide and the absorbance intensity was measured at 490 nm in a microplate reader (Molecular devices, Sunnyvale, CA, USA).

Generation of primary mast cells

Bone marrow-derived mast cells (BMMCs) were generated as previously described with minor modification (21). Briefly, bone marrow cells from C57BL/6 mice were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, nonessential amino acids, and sodium pyruvate in the presence of mIL-3 (10 ng/ml) and mSCF (50 ng/ml) for 8 weeks at 37°C in a humidified atmosphere with 5% CO₂. Human PBMC-derived mast cells were generated as previously described with minor modifications (22). Briefly, we obtained human blood samples from healthy volunteers after IRB approval from the Catholic University of Korea (approved number; KC12TISI0433), and PMBC was isolated from the blood samples. CD34⁺ cells were purified from the PBMC by CD34⁺ cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). PBMC-derived CD34⁺ cells (2×10^5 cells/ml) were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, nonessential amino acids, and sodium pyruvate in the presence of hIL-3 (10 ng/ml) and hSCF (100 ng/ml) for 8 weeks at 37°C in a humidified atmosphere with 5% CO₂. FACS analysis showed that >97% of the cells were CD117 (c-kit) and FcεRI positive.

Enzyme immunoassay

The concentrations of histamines, PGD₂, LTB₄, and LTC₄ from the culture supernatants were determined using enzyme immunoassays according to the manufacturer's instructions (Sapphire Bioscience, Montigny le Bretonneux, France).

Enzyme-linked immunosorbent assay

For the detection of cytokines, BMMCs were sensitized with 1 µg/ml DNP-IgE overnight. After washing the cells with PBS, the cells were treated with HIF for 30 min and then activated by 10 ng/ml DNP-HSA for 24 h. The supernatants were collected and enzyme-linked immunosorbent assay (ELISA) was performed as previously described (20).

Degranulation assay

Degranulation was determined by measuring the release of β-hexosaminidase. BMMCs were sensitized for 16 h with anti-DNP-IgE (1 µg/ml). To measure β-hexosaminidase release, sensitized cells were stimulated with DNP-HSA (100 ng/ml) for 30 min in Tyrode's buffer. The enzymatic activities of β-hexosaminidase in supernatants and cell pellets solubilized with 0.5% Triton X-100 in Tyrode's buffer were measured by adding *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide in 0.1 M sodium citrate (pH 4.5) for 90 min at 37°C.

The reaction was stopped by the addition of 0.2 M glycine (pH 10.7). The release of 4-*p*-nitrophenyl was detected by measuring the absorbance at 405 nm.

Transmission electron microscopy

Transmission electron microscopic examination of HMC-1 cells was performed with the methods as previously described (23).

PCA in mice

An anti-DNP-specific IgE (100 ng/ear) was intradermally injected into a hairless or Balb/C mouse ear, followed 24 h later by topical treatment with HIF (0.5, 1, or 2 µg/ear) or dexamethasone (50 µg/ear) as a control. One hour later, the mice were then challenged with an intravenous injection of antigen (DNP-HSA; 500 µg) in 100 µl PBS containing 4% Evans blue. One hour after the antigen challenge, the mice were killed and the ears were removed to measure the amount of dye extravasated by the antigen. The dye was extracted overnight from the ear in 800 µl formamide at 65°C. The intensity of the absorbance was measured at 620 nm ($n = 6$ mice in each group).

Compound 48/80-induced ear edema in mice

Compound 48/80 (100 µg/ear) was injected intradermally into the hairless mouse ear after topical treatment with HIF (1 or 2 µg/ear), dexamethasone (50 µg/ear) (24), or zileuton (10 µg/ear) (25). The thickness of the ears was measured as the incremental increase in thickness above the baseline control values. The ear swelling response was determined 90 min after the injection of compound 48/80.

Statistical analyses

Statistical comparisons between the groups were made using unpaired two-sided *t*-tests, and differences with $P < 0.05$ (*) and $P < 0.005$ (**) were considered significant. All experiments were performed at least three times.

Results

HIF suppressed the production of pro-inflammatory cytokines in mast cells

To determine whether HIF (Fig. S1) affects cell viability, we performed MTT assays using BMMCs or HMC-1. With 1 or 2 µg/ml (3.16 µM) HIF, cell viability was 103% or 82%, respectively (Fig. 1A). Therefore, we used 1 or 2 µg/ml HIF for the subsequent studies. Allergic inflammatory responses are induced by pro-inflammatory cytokines from activated mast cells. Thus, we examined the effects of HIF on the expression of pro-inflammatory cytokines in activated HMC-1. The mRNA level and release of IL-6 and TNF-α from activated HMC-1 were reduced following the treatment with HIF (Fig. 1B,C). We also determined whether HIF is

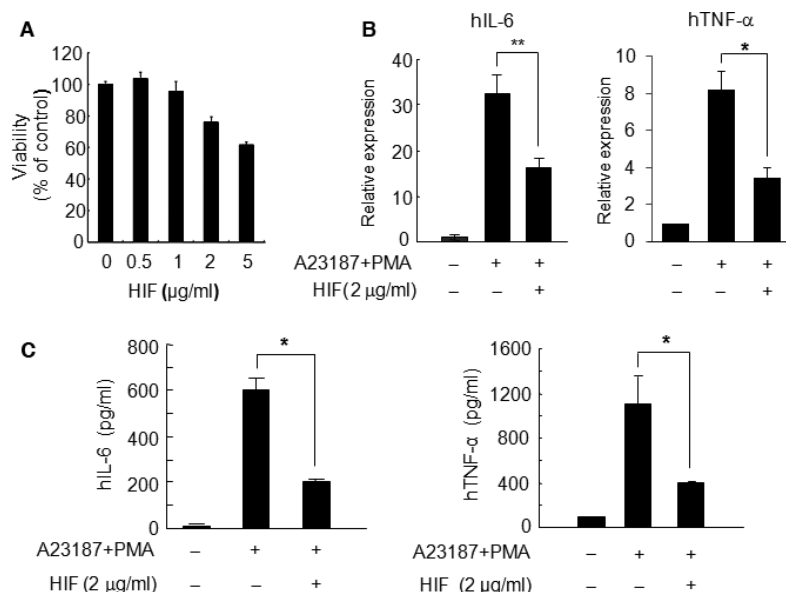


Figure 1 HIF inhibited the release of pro-inflammatory cytokines from activated HMC-1 cell line. (A) HMC-1 was treated with 0–5 µg/ml HIF for 24 h, and cytotoxicity was assessed by MTT assays. (B–C) HMC-1 was treated with HIF for 30 min before stim-

ulation with A23187 and PMA. (B) RNA levels and (C) productions of IL-6 and TNF-α were measured by real-time PCR and ELISA, respectively. Data are representative of three independent experiments. * $P < 0.05$, ** $P < 0.005$.

effective in human and mouse mast cells. Human and mouse mast cells were generated from human PBMC and mouse bone marrow cells, respectively (Fig. 2A). Human PBMC-derived mast cells and mouse bone marrow cell-derived mast cells (BMMCs) were activated by anti-human DNP-IgE and anti-mouse DNP-IgE, respectively, followed by the treatment with DNP-HSA. Figure 2B,C shows that treatment with HIF inhibited expression and production of TNF-α and IL-6 in human and mouse mast cells. HIF especially inhibited the expression of pro-inflammatory cytokine in the activated BMMCs dose dependently. Although 0.5 µg/ml of HIF did not suppress the expression and release of the pro-inflammatory cytokine, 1 and 2 µg/ml of HIF inhibited the expressions and productions of TNF-α and IL-6 (Fig. 2D,E).

HIF inhibited the release of prostaglandin and leukotriene from activated mast cells

Activated mast cells produce LTB_4 , LTC_4 , and PGD_2 . LTB_4 and LTC_4 recruit neutrophils, and PGD_2 recruits Th2 cells, eosinophils, and basophils (26–28). Therefore, these arachidonic acid metabolites contribute to inflammation in allergic diseases, and inhibition of their production is important to prevent or treat allergic diseases. To investigate the effect of HIF on production of LTB_4 , LTC_4 , and PGD_2 from activated mast cells, BMMCs were sensitized with 1 µg/ml DNP-IgE overnight and treated with HIF (2 µg/ml) for 30 min followed by the treatment with DNP-HSA for 24 h. The result showed that treatment of activated BMMCs with HIF suppressed the production of PGD_2 (Fig. 2F). In addition, HIF treatment also inhibited the release of LTB_4 and

LTC_4 in activated BMMCs as compared to untreated activated BMMCs (Fig. 2G). These results indicated that HIF inhibits the release of pro-inflammatory mediators from activated mast cells and imply that it may have an anti-allergic inflammatory effect.

HIF suppressed mast cell degranulation

Degranulation is another hallmark of activated mast cells. Therefore, by measuring the release of β-hexosaminidase or histamine, prestored mediators, we determined whether HIF treatment could inhibit mast cell degranulation. TEM assay showed that activated HMC-1 by A23187 and PMA induced degranulation, but treatment with HIF inhibited degranulation of HMC-1 (Fig. 3A). Inhibited degranulation by HIF was confirmed by reduced secretion of histamine from HIF-treated HMC-1 (Fig. 3B). We also determined the inhibitory effect of HIF on the release of β-hexosaminidase from activated BMMCs (Fig. 3C) and RBL-H2 (Fig. 3D). Consistently, treatment with HIF inhibited the release of β-hexosaminidase from both activated mast cells.

HIF suppressed mast cell activation via inhibition of the FcεRI-induced signaling pathway

Antigen binding to IgE in mast cells induces the cross-linking of FcεRI and activates the Lyn/Syk pathway that leads to calcium influx and MAPK activation. On the other hand, activation of the Syk/Fyn/Gab2/PI3K pathway by the cross-linking of FcεRI is responsible for the activation of AKT and protein kinase C (PKC)δ. Therefore, we examined the effect of HIF

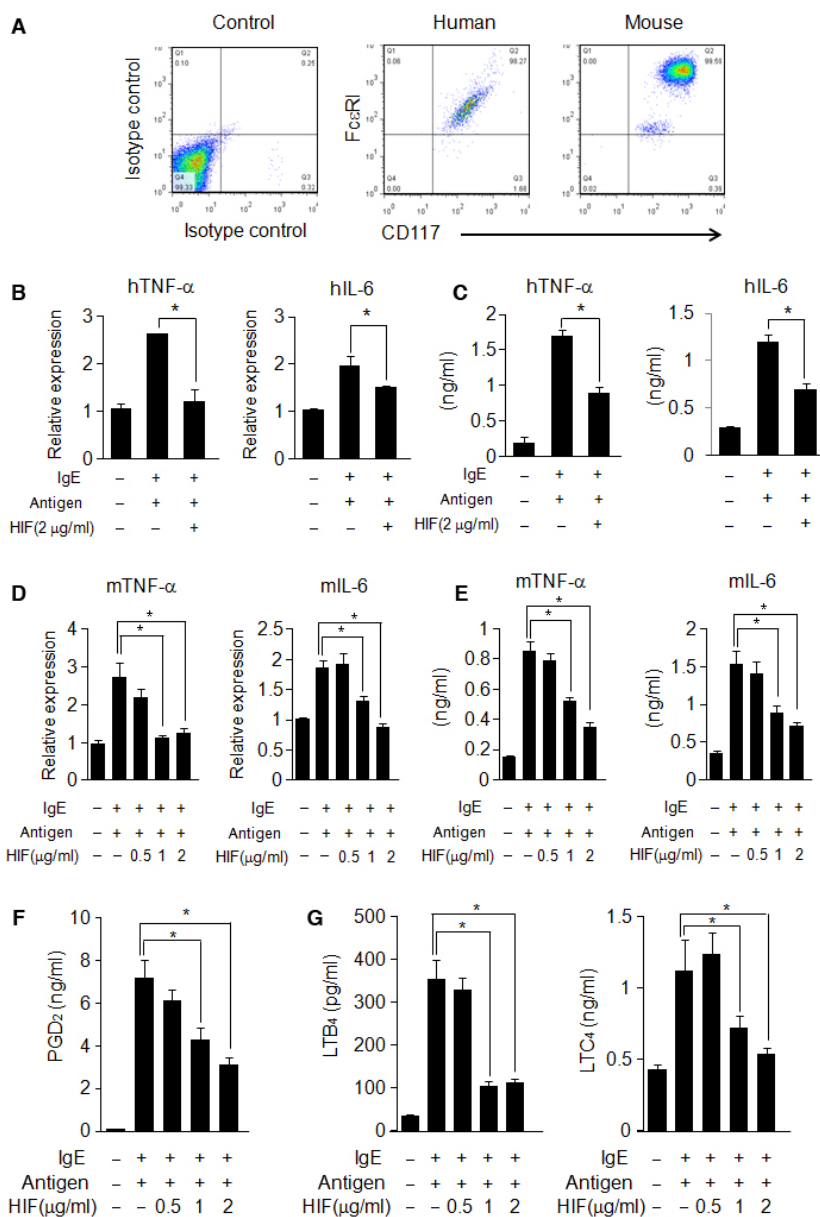


Figure 2 HIF inhibited the expression and secretion of pro-inflammatory cytokines, prostaglandin, and leukotrienes from activated human or mouse mast cells. (A) FACS analysis of human PBMC-derived mast cells and mouse BMMCs. Human PBMC-derived mast cells and mouse BMMCs were generated as described in Methods. CD117⁺ and FcεRI⁺ cells are >99%. (B–E) PBMC-derived mast cells and BMMCs were sensitized with 1 μg/ml anti-DNP-IgE overnight. After sensitization, cells were washed two times and

HIF was treated for 30 min followed by activation with DNP-HSA. (B) RNA levels and (C) secretion of IL-6 and TNF-α from PBMC-derived mast cells decreased by treatment with HIF. HIF also inhibited (D) expressions and (E) productions of IL-6 and TNF-α from BMMCs. Treatment with HIF (1 or 2 μg/ml) inhibited the production of PGD₂ (F), LTB₄, and LTC₄ (G). Data are representative of three independent experiments. *P < 0.05.

on antigen-mediated FcεRI signaling in BMMCs. After overnight sensitization with 1 μg/ml anti-DNP-IgE, cells were treated with HIF for 30 min and were activated by 20 ng/ml DNP-HSA for 5 min. Protein lysates were analyzed by western blot analysis for the phosphorylation of Syk, PLCγ-1, extracellular signal-related kinase (ERK), and cytosolic

phospholipase A2 (cPLA₂). To analyze phosphoinositide-dependent protein kinase (PDK)-1/AKT activation, protein lysates were analyzed 15 min after activation by DNP-HSA. HIF treatment caused down-regulation of antigen-induced phosphorylation of Syk, PLCγ-1, ERK, cPLA₂, PDK-1, AKT, and NF-κB p65 (Fig. 4A). Syk is the most upstream

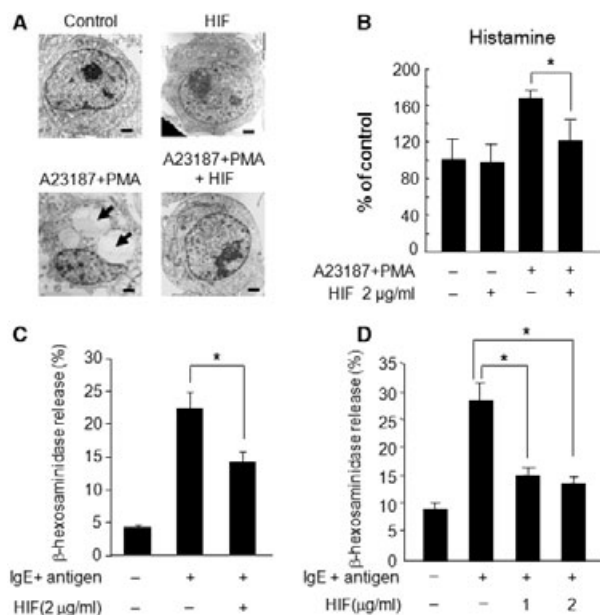


Figure 3 HIF inhibited the degranulation of activated mast cells. (A) Electron microscopy of activated HMC-1 cells. Treatment with HIF (2 µg/ml) inhibited the degranulation of activated HMC-1 cells. (B) Release of histamine molecules from activated HMC-1 cells was suppressed by HIF (2 µg/ml). (C) RBL-2H3 and (D) BMMCs were sensitized with 1 µg/ml DNP-IgE overnight and treated with HIF (1 or 2 µg/ml) for 30 min. Cells were activated for 30 min for the degranulation assay. The enzymatic activities of β-hexosaminidase in supernatants and cell pellets solubilized with 0.5% Triton X-100 in Tyrode's buffer were measured. The percentage of the total enzymatic activity released into the supernatant was calculated using the following formula: Release = (supernatant – blank)/(total – blank) × 100%. Data are representative of three independent experiments. **P* < 0.05.

molecule among the activated signaling molecules during mast cell activation by FcεRI and its antigen. Thus, it seems that inhibition of Syk caused inactivation of downstream molecules. HMC-1 cells do not have FcεRI, so they cannot be activated by IgE/antigen interaction. Instead, they can be activated by A23187 and PMA, which induce calcium influx and phosphorylation of cPLA2. Thus, we determined that treatment with HIF could inhibit the phosphorylation of cPLA2 in HMC-1. The result showed that treatment with HIF suppressed phosphorylation of cPLA2 (Fig. 4B). These results suggest that HIF suppresses mast cell activation by inactivation of Syk and cPLA2 in mast cells.

HIF down-regulated COX-2 and 5-LOX

Treatment with HIF inhibited the release of prostaglandins and leukotrienes (Fig. 2F,G), which are critical for allergic inflammation and whose synthesis is controlled by COX-2 and 5-LOX, respectively. Thus, we investigated whether HIF inhibited the production of COX-2 and the translocation of 5-LOX through western blot analyses. HIF suppressed

COX-2 expression (Fig. 4C) and the nuclear translocation of 5-LOX (Fig. 4D). Surprisingly, HIF was even more potent at inhibiting the expression of COX-2 than was celecoxib, a specific inhibitor of COX-2 (Fig. 4C). Furthermore, HIF did not inhibit the expression of COX-1. These results indicate that HIF may inhibit the production of prostaglandins and leukotrienes by the dual inhibition of COX-2 and 5-LOX without the side-effects caused by the inhibition of COX-1.

Anti-allergic effects of HIF in mice

To confirm the anti-allergic inflammatory effects of HIF *in vivo*, anti-DNP-IgE-mediated PCA and compound 48/80-induced ear swelling responses were used. For PCA, the ears of mice were passively sensitized with anti-DNP-IgE for 24 h and topically treated with HIF or dexamethasone, a control drug. One hour after the treatments, mice were intravenously injected with DNP-HSA containing 4% Evans blue dye (Fig. 5A). Topical treatment with either HIF or dexamethasone decreased ear thickness and the amount of diffused dye. Treatment with HIF reduced the ear thickness by 53% and the amount of dye by 34%, suggesting the suppression of PCA by HIF (Fig. 5C–E). In addition, treatment with HIF inhibited the ear swelling induced by compound 48/80, which induces mast cell degranulation. HIF, dexamethasone, or zileuton was pretreated one hour before intradermal injection of compound 48/80, and ear thickness was measured 90 min after the injection. Pretreatment with 1 and 2 µg of HIF inhibited the ear thickness by 52% and 56%, respectively. In addition, pretreatment with 1 and 2 µg of HIF decreased the amount of dye by 48.6% and 55%, respectively. Interestingly, HIF was more effective than the control drugs dexamethasone (50 µg) and zileuton (10 µg) that inhibited ear swelling by only 44% and 37%, respectively, on a compound 48/80-induced ear swelling mouse model (Fig. 5B and Table 1). These results imply that HIF has an anti-allergic effect through the inhibition of mast cell activation and might be effective for allergic diseases.

Discussion

Mast cells are key effector cells in allergic diseases (5, 29). In this study, we demonstrated that the anti-allergic effects of HIF were mediated by the inhibition of mast cell activation *in vitro* and *in vivo*. Using BMMCs, we showed that HIF inhibited the release and production of pro-inflammatory mediators, such as β-hexosaminidase, prostaglandins, leukotrienes, and pro-inflammatory cytokines, from activated mast cells (Figs 1B,C and 2B–G).

Mast cell activation is initiated by binding of IgE to FcεRI on mast cells. After antigens bind to IgE, FcεRI aggregate and mast cells are activated by the phosphorylation of Syk (30). Activated Syk triggers downstream pathways, including the phosphorylation of LAT and the recruitment of adaptor molecules such as Grb2, Gads, SLP76, PLC-γ, and SOS, for degranulation, cytokine secretion, and production of eicosanoids or lipid mediators (3, 31). Thus, activated Syk molecule induces the expression of pro-inflammatory cytokines

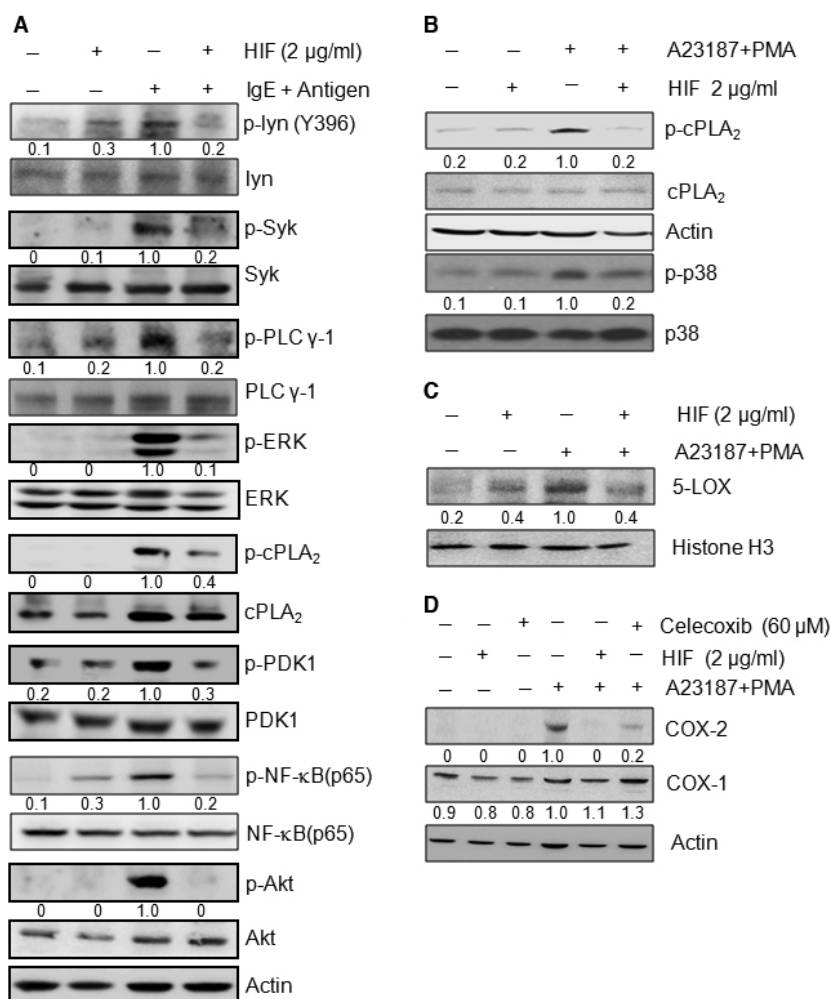


Figure 4 HIF inhibited the antigen-mediated Fc ϵ RI signaling pathway. (A) BMMCs were cultured without IL-3 for 3 h before sensitization. After overnight sensitization with anti-DNP-IgE (1 μ g/ml), cells were washed with phosphate-buffered saline (PBS) three times and treated with HIF (2 μ g/ml) for 30 min prior to the challenge with DNP-HSA (10 ng/ml) for 15 min prior to western blot analysis. HMC-1 cells were treated with HIF (2 μ g/ml) for 1 h before stimulation. (B) Phosphorylation of cPLA₂ was determined 30 min after stimulation. Treatment with HIF inhibited phosphorylation of cPLA₂ in activated HMC-1 cell line. (C) 5-LOX was deter-

mined from nuclear extracts 5 h after stimulation. Nuclear pellets were lysed in cold extraction buffer (50 mM HEPES/KOH, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, protein inhibitor cocktail, and 0.5 mM PMSF, pH 7.9). Nuclear extracts were obtained from supernatants following centrifugation of nuclear pellet lysates at 15 000 $\times g$ for 15 min at 4°C. (D) Expression of COX-1 and COX-2 was determined 21 h after stimulation. The numbers of below band indicate the relative intensity of the band. Data are representative of three independent experiments.

through activated PI3K, AKT, and NF- κ B pathways. Several lines of evidence suggest that the regulation of Syk could be the main target for the pharmacological management of allergic diseases. For example, aerosolized Syk antisense oligonucleotides delivered directly to airways inhibit allergic inflammation (11).

To determine the mechanisms of HIF action, we examined the activation status of signaling molecules. Treatment of activated BMMCs with HIF inhibited the activation of Syk (Fig. 4). Therefore, HIF blocks mast cell activation at the initial stage, which is advantageous over drugs that inhibit a

single downstream event because HIF blocks multiple downstream pathways that are required for the pathogenesis of allergic disorders. HIF inhibited the pathway downstream of Syk activation that includes PI3K pathway molecules, such as PKD and Akt, and MAPK pathway molecules, such as ERK and cPLA₂ (Fig. 4A). Additionally, HIF inhibited the activation of HMC-1 cells, which is due to the lack of Fc ϵ RI and Syk signaling pathway, through the suppression of cPLA₂ (Fig. 4B). These results suggest that HIF inhibits mast cell activation through the inactivation of Syk signal pathway and cPLA₂.

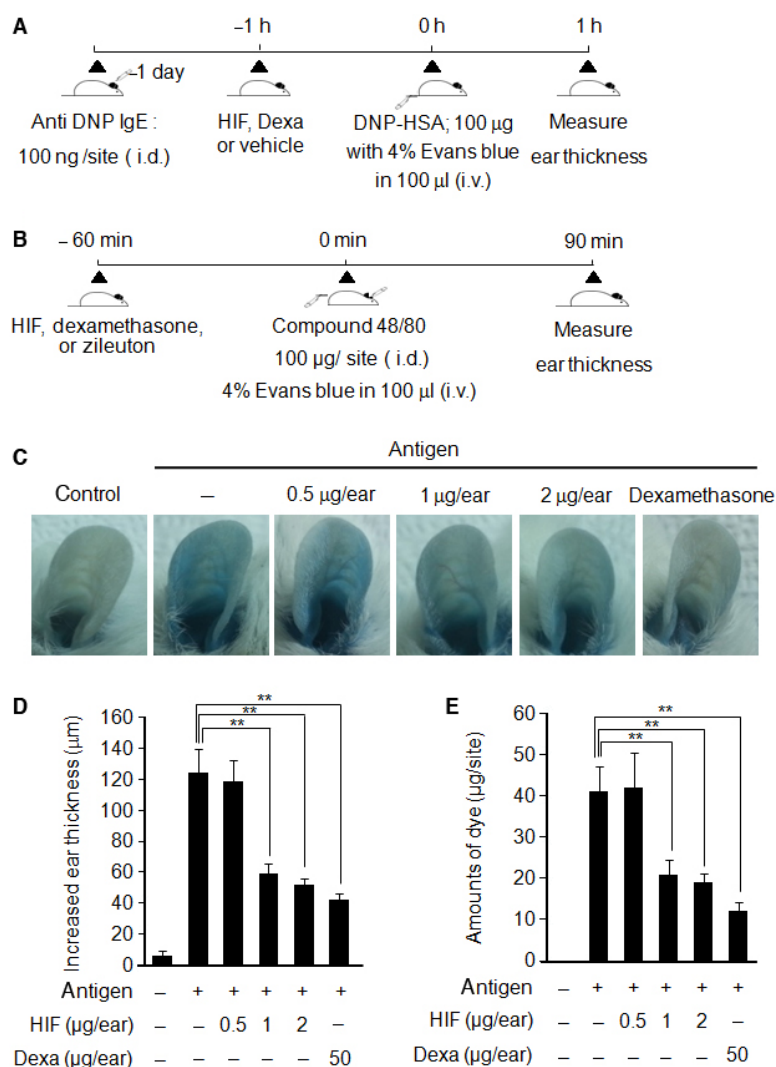


Figure 5 HIF prevented passive cutaneous anaphylaxis. (A) Experimental scheme for passive cutaneous anaphylaxis experiment. Balb/c mice were passively sensitized with 100 ng anti-DNP-IgE. Twenty-four hours after injection, HIF (0.5, 1, or 2 µg/ear) or dexamethasone

(50 µg/ear) was applied for 1 h. Mice received 100 µg DNP-HSA containing Evans blue dye (5%) via intravenous injections. One hour later, ear thickness and the amount of Evans blue dye were measured (C and D). Data represent the mean ± SEM. ** $P < 0.005$.

Table 1 The effects of HIF on compound 48/80-induced ear swelling in mice

Stimulus	Drug	Ear swelling* (10^{-2} mm)	Inhibition (%)
Vehicle	Vehicle	-0.25 ± 0.35	
Compound 48/80	Vehicle	13.75 ± 1.29	
Compound 48/80	HIF 1 µg	6.5 ± 1.18	53
Compound 48/80	HIF 2 µg	7.25 ± 1.97	47
Compound 48/80	Dexamethasone	7.7 ± 0.91	44
Compound 48/80	Zileuton	8.63 ± 1.65	37

*The groups of mice ($n = 6$, 10 week, 20–25 g) were topically pre-treated with 20 µl of PBS, HIF, dexamethasone, or zileuton 1 h before the compound 48/80 (100 µg/ml) intradermal injection. Mean ± SEM.

Mast cell-derived TNF contributes to airway hyper-reactivity in a mouse asthma model (32). Mast cell-derived LTB_4 may also play a critical role in the development of airway hyper-responsiveness via the release of IL-13 from $CD8^+$ T cells following interactions with its receptors (33, 34). HIF treatment reduced the production of pro-inflammatory cytokines, including TNF- α (Figs 1C and 2C,E) and the leukotriene LTB_4 (Fig. 2G).

It was previously reported that homoisoflavanoid molecules isolated from the roots of *Ophiopogon japonicus* has anti-inflammatory effects in bronchial epithelial cell line, BEAS-2B (35). Together with our experimental results showing the inhibitory effect of HIF on mast cell activation, it seems that homoisoflavanoid may be a good candidate

molecule to treat asthma because it acts on both bronchial epithelial cells and mast cells.

Leukotrienes and prostaglandins play important roles in many diseases including inflammatory and allergic diseases. Treatment with HIF inhibited the nuclear translocation of 5-LOX and the production of COX-2, which are key enzymes involved in the synthesis of leukotrienes and prostaglandins, respectively. These results suggest that HIF may work as a dual inhibitor of COX-2 and 5-LOX probably through the inhibition of Syk and cPLA₂. Compared to single inhibitors of the COX or LOX pathways, dual inhibitors contain a wide range of anti-inflammatory activities. Licofelone is a well-known dual inhibitor of COX-2 and 5-LOX and is under clinical trial for the treatment of anti-inflammatory diseases. HIF does not inhibit COX-1, but selectively inhibits COX-2, implying that HIF may not cause gastric toxicity, the most troublesome side-effect of nonselective COX inhibitors. It has been also reported that dual inhibition of 5-LOX and COX-2 induces apoptosis and suppresses cancer formation of colon cancer cells. Thus, it will be also interesting to investigate whether HIF can inhibit the formation and survival of colon cancer cells (36, 37).

The present study showed that HIF inhibited the production of prostaglandins, leukotrienes, and pro-inflammatory cytokines and degranulation from mast cells by the inactivation of FcεRI signaling pathway. In addition, HIF reduced PCA- and compound 48/80-induced ear edema in mice, which are induced by activated mast cells. There has been an increasing demand for therapeutic intervention in allergic diseases in proportion to the growing number of people affected

by allergies or related disorders, including atopic dermatitis, allergic rhinitis, asthma, and anaphylactic shock. Our results imply that dual inhibition of Syk signaling pathway and calcium-dependent cPLA₂ activation may be a useful therapeutic method to treat allergic diseases.

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Author contributions

Tae-Yoon Kim designed the experiments, analyzed the data, and wrote the manuscript. Yun Sang Lee designed and performed the experiments, analyzed the data, and wrote the manuscript. Seulgi Hur performed the experiments.

Conflicts of interest

The authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Chemical structure of HIF.

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Supplementary figure S1

