



Saucerneol F inhibits tumor necrosis factor- α and IL-6 production by suppressing Fyn-mediated pathways in Fc ϵ RI-mediated mast cells

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ABSTRACT

The aim of this study was to investigate the effect of saucerneol F (SF) on the productions of the pro-inflammatory cytokines, TNF- α and IL-6, in IgE/Ag-induced mouse bone marrow-derived mast cells (BMMCs). SF dose-dependently suppressed the transcriptions of these pro-inflammatory cytokines. To identify the molecular mechanisms responsible for these suppressions, we examined the effect of SF on three important transcription factors; activator protein-1 (AP-1), nuclear factor- κ B (NF- κ B), and STAT5. It was found that SF inhibited the nuclear translocation of the p65 subunit of NF- κ B to the nucleus and its DNA-binding ability. SF also attenuated mitogen-activated protein kinase (MAPK)-mediated AP-1 activation and STAT5 activation. Biochemical analysis of Fc ϵ RI-mediated signaling pathways demonstrated that SF inhibited the phosphorylation of Fyn and multiple downstream signaling processes, including Syk, Gab2, and the Akt/IKK/I κ B and MAPK pathways. Taken together, our results suggest that SF inhibits the production of pro-inflammatory cytokines by suppressing Fyn kinase-dependent signaling events.

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1. Introduction

Mast cell stimulated by crosslinking of high-affinity IgE receptor (Fc ϵ RI) and IgE antigens rapidly degranulate and release granule-stored mediators, such as, tryptase, chymase, histamine, and proteoglycans, and thereby, induce the early phase of allergic responses (Galli et al., 2008). During the late phases of allergic reactions, mast cells produce inflammatory eicosanoids, pro-inflammatory cytokines, and chemokines (Prussin and Metcalfe, 2006). On the other hand, TNF- α and IL-6 are multifunctional cytokines that mediate a variety of inflammatory responses (Gurish et al., 1991; Ishizuka et al., 1997). These two cytokines potentiate inflammatory immune responses via the inductions of other inflammatory mediators. Therefore, the targeting of the production of TNF- α and IL-6 by mast cells provides a potential approach to the treatment of allergic inflammatory diseases.

Crosslinking of Fc ϵ RI activates a variety of Src family kinases, such as, Lyn and Fyn (Eiseman and Bolen, 1992; Gilfillan and

Rivera, 2009). Upon Fc ϵ RI aggregation, immunoreceptor tyrosine-based activation motifs (ITAMs) of the γ and β subunits of Fc ϵ RI are phosphorylated by Lyn, and these phosphorylated subunits then recruit Syk (a non-receptor tyrosine kinase). Syk phosphorylates linker of activated T cells (LAT), which in turn discharges its duty as a SH2-based scaffold, eventually simulating the mitogen-activated protein kinase (MAPK) pathways including that of extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinase (JNK), and p38 MAP kinase, which in turn, activate the de novo transcriptions of various target genes (Lorentz et al., 2003). Furthermore, the concomitant activation of Fyn kinase initiates signaling through a complementary but distinct pathway leading to PI3K activation mediated by Gab2 (Parravicini et al., 2002). The synergic activation of these two pathways orchestrates antigen triggered mast cell responses.

Several transcription factors are known to be involved in allergic inflammation, including NF- κ B, AP-1, and STAT family members (Barnes and Adcock, 1998; Pullen et al., 2012). NF- κ B is now known to regulate the expressions of many inflammatory and immune genes, including IL-1 β , IL-6, TNF- α , iNOS, and COX-2 (Peng et al., 2005). NF- κ B often functions in cooperation with other transcription factors, such as, AP-1 and C/EBP, which are also involved

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in the regulations of inflammatory and immune genes (Xiao et al., 2004). The STAT family of transcription factors is also a critical component of cytokine and growth factor signals (Schindler et al., 2007). In mast cells, one such process is the induction of STAT5 phosphorylation by c-Kit, a receptor for stem cell factor (SCF), or IL-3 receptor via JAK2 activation (Barnstein et al., 2006; Brizzi et al., 1999; Ryan et al., 1997). A recent study demonstrated that STAT5 tyrosine phosphorylation is induced by the crosslinking of Fc ϵ RI. In addition, it was also shown that STAT5 and Fyn co-immunoprecipitated in resting mast cells (Pullen et al., 2012).

In our previous studies, 18 lignans including four new ones were isolated (Seo et al., 2008a,b). Among these compounds, saucerneol D (SD) and saucerneol F (SF), which were a similar derivatives and SD has 3, 4 dimethoxyl groups instead of a 3, 4 methylenedioxy group on the right side benzene ring of SF, were investigated for their inhibitory activity on both LPS-induced iNOS expression in LPS-induced RAW264.7 cells and cytokine-stimulated eicosanoid generation and degranulation in BMMCs (Lu et al., 2012a,b). Both compounds inhibited degranulation, PGD₂ and LTC₄ generation in SCF-LPS/IL-10-induced BMMCs, and SD showed about 2 fold stronger inhibitory activities than that of SF (data not shown).

However, the effect of both SF and SD on the production of pro-inflammatory cytokines in mast cells has not been studied to date. In this study, we investigate the effect of SF on the production of TNF- α and IL-6 and clarified the mechanism responsible for its inhibition of proinflammatory cytokines in IgE/Ag-stimulated BMMCs.

2. Materials and methods

2.1. Plant material

SF was isolated from the ethyl acetate fraction of the roots of *Saururus chinensis* as described previously (Seo et al., 2008a,b). SF used in this study showed a single spot on TLC and the purity of this compound was above 99.5% based on HPLC analysis. This compound was prepared by dissolving in dimethyl sulfoxide (DMSO) and the final concentration of DMSO was adjusted to 0.1% (v/v) in culture media. DMSO alone as a control was run in all cases.

2.2. Reagents

Mouse anti-dinitrophenyl (DNP) IgE and DNP-human serum albumin (HSA) were purchased from Sigma-Aldrich. The following primary antibodies were used: rabbit polyclonal antibody for phospho-I κ B, IKK α / β , ERK1/2, JNK, p38, AKT, Gab2, Stat5, c-Jun, β -actin, and total form for I κ B, ERK1/2, JNK, p38, c-Fos and AKT were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Polyclonal antibodies against NF- κ B p65, Syk, Fyn, Gab2, IKK α β and Lamin B were from Santa Cruz (Santa Cruz, CA, USA). The Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG were also purchased from Santa Cruz. Antibody for phosphotyrosine was from Millipore (Millipore, Billerica, MA, USA). PP2 was purchased from Calbiochem (Millipore, Billerica, MA, USA).

2.3. Preparation of mouse bone marrow-derived mast cells (BMMCs) and culture

Bone marrow derived mast cells (BMMCs) were isolated from male Balb/cj mice (Sam Taco, INC, Seoul) and were cultured for up to 6 weeks in RPMI 1640 medium (Thermo Scientific, Utah, USA) containing 10% fetal bovine serum, 100 U/ml penicillin (Thermo Scientific), 10 mM HEPES buffer (Sigma-Aldrich), 100 μ M MEM non-essential amino acid solution (Invitrogen, NY, USA) and 20% PWM-SCM (pokeweed mitogen-spleen cell conditioned medium) as a source of IL-3 in an atmosphere containing 5% CO₂ at 37 °C. After 6 weeks in culture, cells were used for experiments. BMMCs were washed with PBS, and the medium was replaced with the IL-3 free medium. The cells were sensitized with 500 ng/ml of DNP-specific IgE overnight and then stimulated for various times with 100 ng/ml of DNP-HSA after pretreated with SF or PP2. The reactions were terminated by centrifugation of the cells at 3000 rpm for 5 min at 4 °C.

2.4. Determination of TNF- α and IL-6 concentration

BMMCs were sensitized to DNP-specific IgE (500 ng/ml, overnight), pretreated with SF and PP2 for 1 h, and then stimulated with DNP-HSA (100 ng/ml) for 6 h. Mouse TNF- α and IL-6 ELISA kits were purchased from R&D Systems Inc.

(Minneapolis, MN) and used with culture supernatants according to the manufacturer's instructions. All results are presented as arithmetic means of triplicate determinations.

2.5. Real-time reverse transcriptase-polymerase chain reaction analysis (RT-PCR)

BMMCs were sensitized to DNP-specific IgE (500 ng/ml, overnight), pretreated with SF and PP2 for 1 h, and then stimulated with DNP-HSA (100 ng/ml) for 4 h. Total RNA from BMMCs was extracted using easy-BLUE™ Total RNA Extraction Kit (iNtRON Biotechnology, Inc., Sungnam, Korea). First strand cDNA was generated from 0.5 μ g of total RNA using the one-step RT-PCR kit (Enzynomics, Daejeon, Korea). The primers used for SYBR Green real-time RT-PCR were as follows: for TNF- α , sense primer, 5'-AGCACAGAAAGCATGATCCG-3' and antisense primer, 5'-CTGATGAGAGGGAGGCCATT-3'; for IL-6, sense primer, 5'-GAGGATACCACTCCCAACAGACC-3' and antisense primer, 5'-AAGTGCATCATCGTTTTCATACA-3'; for β -actin, sense primer, 5'-ATCACTATTGGCAACGAGCG-3' and antisense primer, 5'-TCAGCAATGCCTGGGTACAT-3'. A dissociation curve analysis of TNF- α , IL-6, and β -actin showed a single peak for each. PCRs were carried out for 40 cycles using the following conditions: denaturation at 95 °C for 5 s, annealing at 57 °C for 10 s, and elongation at 72 °C for 20 s. The mean Ct of the gene of interest was calculated from triplicate measurements and normalised with the mean Ct of a control gene, β -actin.

2.6. Preparation of nuclear and cytoplasmic extracts

BMMCs were sensitized to DNP-specific IgE (500 ng/ml, overnight) and pretreated with SF or PP2 for 1 h, and then stimulated with DNP-HSA (100 ng/ml) for 30 min. Cultured BMMCs were collected by centrifugation, washed with PBS and lysed in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF and 0.1% NP40 by incubation on ice for 10 min. After centrifugation at 1000 g for 4 min, supernatants were used as a cytosolic fraction. Nuclear pellets were washed and lysed in a buffer containing 20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and the protease inhibitor cocktail. This suspension was incubated for 30 min at 4 °C followed by centrifugation at 10,000 g, and the resultant supernatants were used as a nuclear fraction.

2.7. Immunoprecipitation (IP)

Cell lysates were obtained using modified lysis buffer [0.1% Nonidet P-40, 50 mM HEPES (pH 7.0), 250 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 0.5 mM dithiothreitol]. Total cell lysates (1 mg protein equivalent) were incubated with anti-Fyn or anti-Syk antibodies for 2 h at 4 °C and immunocomplexes were precipitated with 20 μ l of protein A-Sepharose. Immunocomplex precipitates were then extensively washed (3 times) with ice-cold lysis buffer. These precipitates or total cell lysates were subjected to SDS-PAGE and immunoblotted with corresponding antibodies.

2.8. Western blotting

BMMCs were lysed in RIPA lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethanesulfonylfluoride (PMSF), 1 M dithiothreitol (DTT), 200 mM NaF, 200 mM Na₃VO₄, and a protease inhibitor cocktail). Cell debris was removed by centrifugation at 14,000g for 15 min at 4 °C and resulting supernatant were used for western blotting. Protein concentration was measured using the Qubit Fluorometer machine (Invitrogen, USA). Samples were separated by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose transfer membrane (Millipore, Billerica, MA, USA). The membranes were then blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with individual antibodies; primary antibodies were diluted at 1:1000 (unless otherwise stated) and incubated at 4 °C overnight. Membranes were then washed three times for 10 min each with TBS-T buffer, and immunoreactive proteins were incubated with HRP-coupled secondary antibodies diluted at 1:3000 for 1 h at room temperature, washed three times for 10 min with TBS-T buffer, and developed using enhanced chemiluminescence (ECL) detection kits (Pierce Biotechnology, Rockford, IL, USA).

2.9. NF- κ B activation assay

Nuclear fractions were prepared as described previously (Blom et al., 2010). NF- κ B transactivation capacity was measured by using a NF- κ B (p65) transcription factor assay kit (Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturers' instructions. The results from the NF- κ B (p65) transcription factor assay kit are presented as percent activation with the stimulated control set to 100%.

2.10. Image and statistical analysis

Immunoblot films and RT-PCR films were digitized and analyzed using Image J software. Optical density of regions of interest were normalized versus control protein, for example, cytosolic-p-STAT5 was normalized versus β -actin;

nuclear-p-STAT5 was normalized versus LaminB; p-JNK was normalized versus JNK. Normalized values obtained from at least three independent experiments were compared. Statistical analysis was performed using SPSS 18 software ver 18. Results are expressed as means $\pm$ S.D. Analysis was performed using the Student's *t*-test, and statistical significance was accepted for *p* values < 0.05.

3. Results

3.1. SF inhibits production and expression of TNF- α and IL-6 in BMMCs

To explore the cytotoxicity of PP2 on BMMCs, we first performed an MTT assay using various concentrations of PP2 and found that it did not show cytotoxicity up to 100 μ M (data not shown). Further, PP2 inhibited Fyn phosphorylation in a dose-dependent manner, with a complete inhibition at 50 μ M (Supplement Fig. S1). Therefore, 50 μ M of PP2 were used for subsequent experiments. TNF- α and IL-6, which are secreted by activated mast cells, play important roles in mast cell-dependent inflammation. Therefore, we examined whether SF inhibited the expressions and productions of TNF- α and IL-6 in antigen-stimulated BMMCs. SF was found to dose-dependently inhibit the secretions of both TNF- α and IL-6 (Fig. 1). We next investigated whether SF suppressed their expression at the transcription level. Real-time RT-PCR analysis revealed that pretreatment of BMMCs with SF significantly inhibited the inductions of both mRNAs (Fig. 2A and B). Furthermore, PP2 (a general Src-family kinase inhibitor) suppressed the protein and mRNA levels of TNF- α and IL-6, suggesting that certain Src family kinase(s) might be involved in the regulation of the productions of TNF- α and IL-6 in BMMCs.

3.2. Effect of SF on IgE/Ag-induced MAPK pathway and MAPK-dependent AP-1 activation

The MAPK pathway is known to regulate the expressions of TNF- α and IL-6 in many cell types, and several reports have

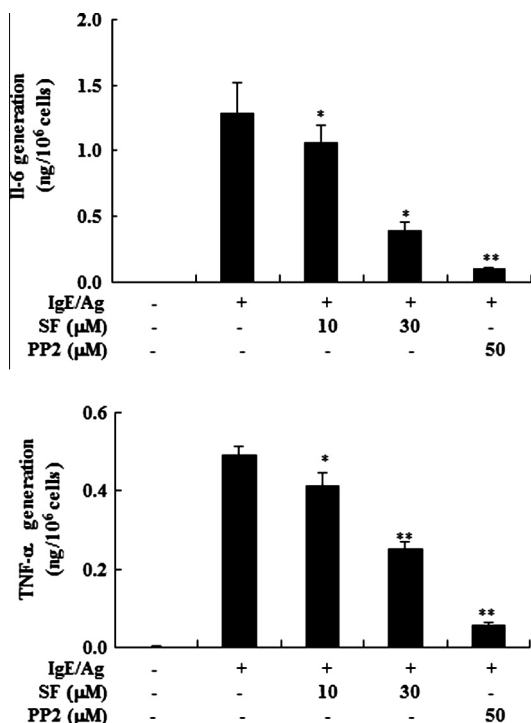


Fig. 1. SF inhibits cytokine production by mast cells. Effects of SF on TNF- α and IL-6 production were measured by ELISA after stimulation of IgE-sensitized BMMCs with DNP-HSA for 6 h. Data are expressed as the means $\pm$ S.D. from three independent experiments. * *P* < 0.05 and ** *P* < 0.01 were compared to IgE/Ag-stimulated BMMCs.

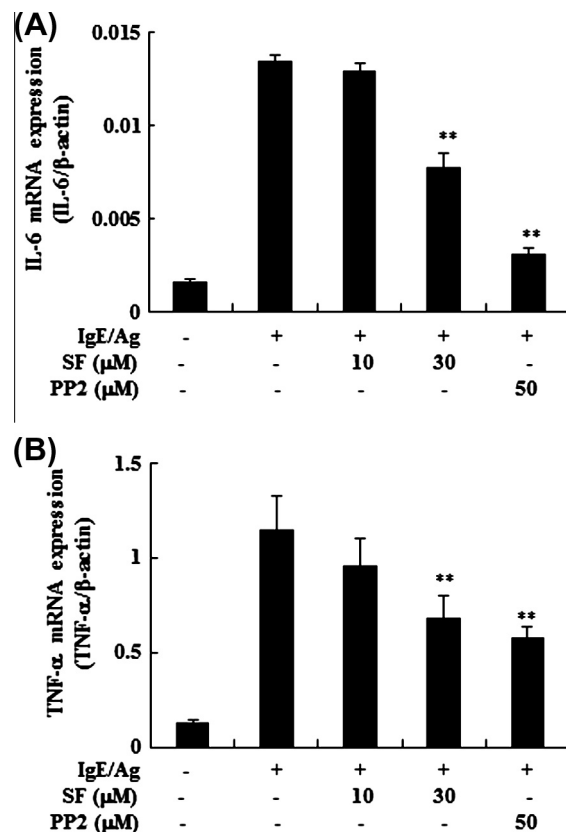


Fig. 2. Inhibitory effect of SF on gene expression of pro-inflammatory cytokines. IgE-sensitized BMMCs were pretreated with the indicated concentrations of SF for 1 h and then stimulated with DNP-HSA for 4 h. The expression levels of IL-6 (A) and TNF- α (B) mRNAs were analysed by real-time RT-PCR. Expression levels were standardized using expression of the housekeeping gene β -actin. Data are expressed as the means $\pm$ S.D. of three independent experiments. ** *P* < 0.01 versus IgE/Ag-stimulated BMMCs.

demonstrated that the activations of several transcription factors are regulated by MAPKs. The activation of ERK contributes to the transcriptional activation of AP-1 via the induction and phosphorylation of c-Fos. Furthermore, p38 MAPK is necessary for the recruitment of Elk-1 to c-Fos promoter, and JNK is required for the inducible phosphorylation of c-Jun (Caelles et al., 1997; Ferreira et al., 2010; Lu et al., 2011; Vaysberg et al., 2008). AP-1 is one of the most important transcription factors with respect to the regulations of the expressions of various cytokines. AP-1 is composed of the Jun and Fos families, and c-Jun is a major component of the AP-1 complex and c-Fos is its best known partner (Ferreiro et al., 2010). To determine whether IgE/Ag-induced cytokine production was mediated by AP-1 in BMMCs, we measured the expression levels of phospho-c-Jun and c-Fos in the presence and in the absence of SF or PP2. As shown in Fig. 3, pretreatment of BMMCs with SF or PP2 suppressed the phosphorylations of all three MAPKs and c-Jun phosphorylation and c-Fos expression in both cytosol and nuclear fractions in a dose-dependent manner. These results indicate that SF suppresses the expression TNF- α and IL-6 by inhibiting MAPK-dependent AP-1 activation.

3.3. Effect of SF on IgE/Ag-induced NF- κ B pathway

Since NF- κ B has been identified as an important regulator of the expressions of TNF- α and IL-6, we examined whether SF suppresses the NF- κ B pathway, in which IKK-dependent phosphorylation and degradation of inhibitory I κ B result in the nuclear

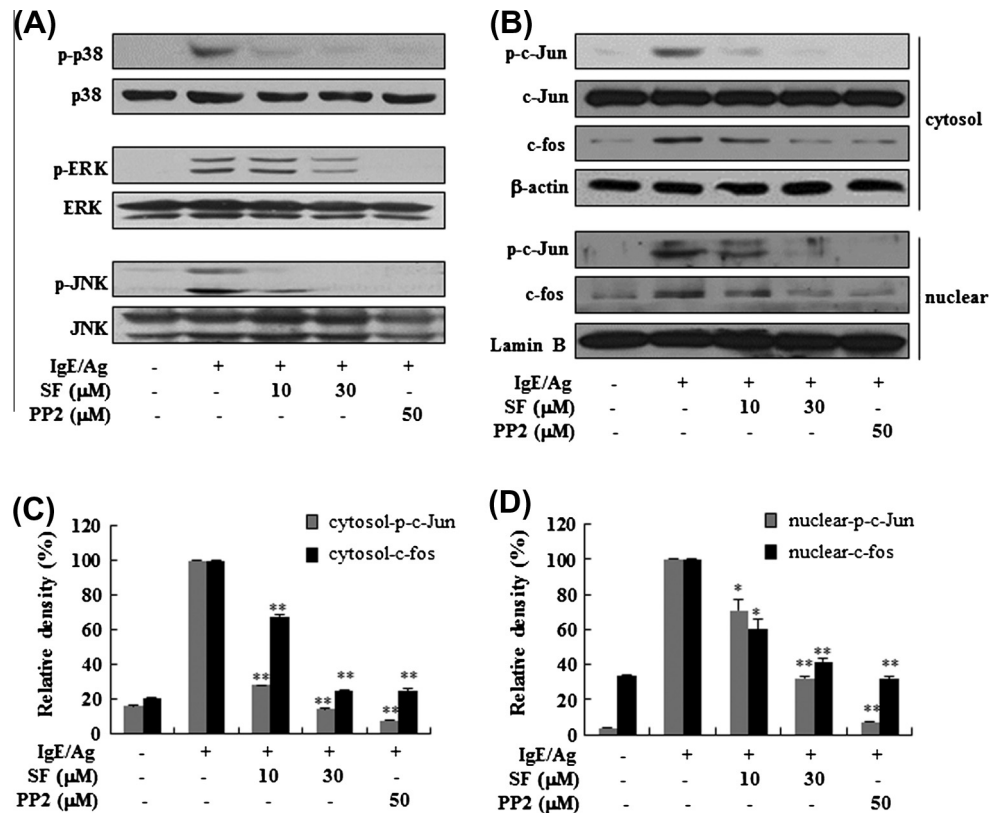


Fig. 3. MAPK and AP-1 activities are suppressed by SF. (A) IgE-sensitized BMMCs were pre-incubated with SF or PP2 for 1 h and stimulated with DNP-HSA for 15 min. Cell lysates were immunoblotted with antibodies for phospho-ERK, phospho-JNK, and phospho-p38, and followed by stripping and reprobing with antibodies against their respective total proteins. (B) Cytosol and nuclear extracts were prepared from BMMCs that had been incubated with IgE in the presence or absence of SF or PP2 for 1 h and then stimulated with DNP-HSA for 30 min. Immunoblots of β-actin and Lamin B were used as protein control for cytosol and nuclear fractions, respectively. The relative ratios of C-p-c-Jun/total c-Jun and C-c-fos/β-actin protein levels (C) and N-p-c-Jun/Lamin B and N-c-fos/Lamin B (D) were determined by measuring immunoblot band intensities by scanning densitometry (* $P < 0.05$ and ** $P < 0.01$). The results from three separate experiments as relative ratios (%) are represented.

translocation of NF-κB (Blackwell and Christman, 1997; Tak and Firestein, 2001). After IgE/Ag activation, the phosphorylations of IKK and IκB were increased in BMMCs, with concomitant nuclear translocation of NF-κB and a decrease in total IκB. SF blocked the phosphorylations of IKK and IκB, the degradation of IκB, and the nuclear translocation of NF-κB (Fig. 4A). PP2 also inhibited these signal events, demonstrating that the IKK-IκBα-NF-κB pathway was under the control of certain Src-family kinase(s). To investigate the activation of NF-κB further, we examined the DNA-binding activity of NF-κB using an NF-κB activation assay. Nuclear extracts were obtained from BMMCs pretreated with SF or PP2 for 1 h were stimulated with DNP-HSA for 30 min. It was found that both SF and PP2 markedly reduced IgE/Ag-induced NF-κB-binding activity (Fig. 4B). These results indicate that SF inhibits the NF-κB pathway by inhibiting the IKK-IκBα-NF-κB signaling pathway.

3.4. SF suppressed Fyn-mediated STAT5 phosphorylation

Recent studies indicate STAT5 expression is required for IgE-mediated mast cell cytokine production, and that the activation of STAT5 is dependent upon Fyn kinase, but independent of Syk, PI3K, Akt, Bruton's tyrosine kinase, and JAK2 (Pullen et al., 2012). We found that SF significantly inhibited STAT5 phosphorylation in both cytosol and nuclear fractions (Fig. 5). In addition, PP2 almost completely inhibited STAT5 phosphorylation, suggesting that the phosphorylation of STAT5 is regulated by Fyn.

3.5. SF inhibits Fyn-dependent signaling events in IgE/Ag-stimulated BMMCs

To gain insight of how SF suppresses the productions of TNF-α and IL-6, we examined its effects on the FcεRI-mediated activation of Fyn and on signaling events downstream of Fyn. As shown in Fig. 6, the phosphorylations of Fyn, Syk, Gab2, and Akt were inhibited by both SF and PP2. When the cells were treated with Fyn-specific siRNA, the reduced Fyn expression was accompanied by concomitant reductions of c-Jun and STAT5 phosphorylation, c-fos expression, NF-κB translocation, and cytokine production (Supplement Fig. S2), as in the experiments using SF or PP2. This result suggests SF inhibits the IgE/Ag-induced phosphorylation of Fyn, and that this, in turn, regulates the activations of transcription factors leading to TNF-α and IL-6 expression.

4. Discussion

Previously, our group reported that SF inhibits NO production by inhibiting the phosphorylations of MAPKs and the translocation of NF-κB in LPS-induced RAW264.7 cells (Lu et al., 2012b). In addition, we also found that SF inhibits eicosanoid generation by suppressing the phosphorylations of MAPKs in SCF/LPS/IL-10-induced BMMCs (Lu et al., 2012a). In the present study, we investigated the effects of SF on IgE/Ag-induced TNF-α and IL-6 production in BMMCs. To address how SF inhibits TNF-α and IL-6 production, we focused on Fyn, which lies upstream of several signaling pathways in IgE-Ag induced BMMCs.

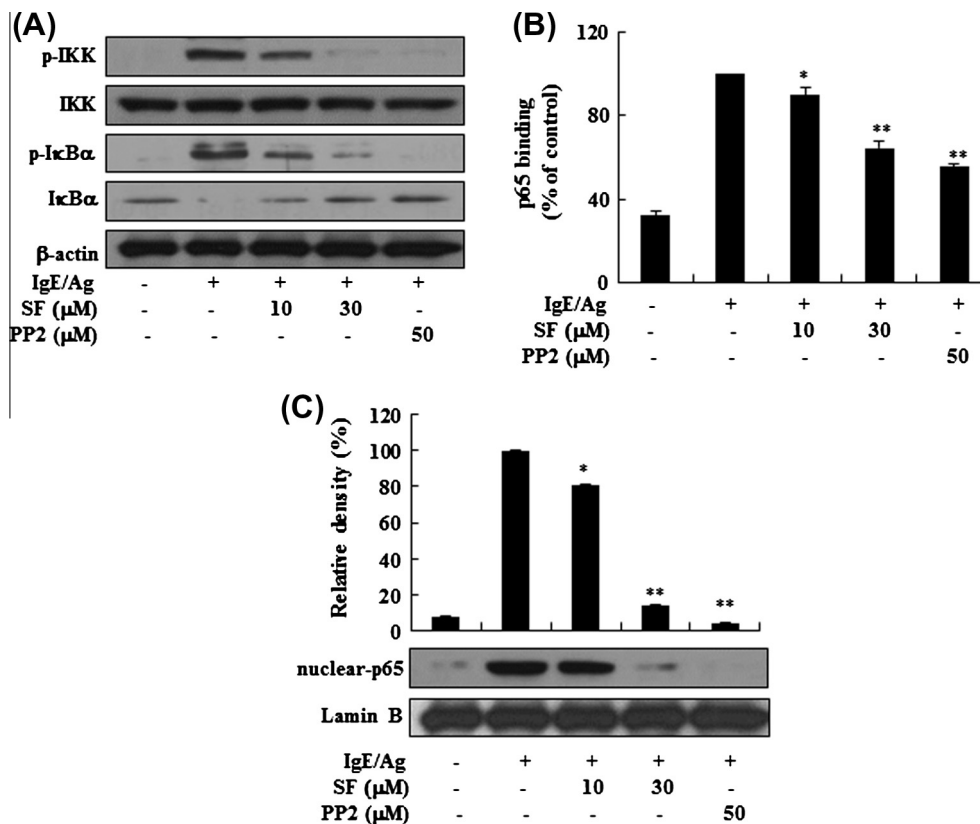


Fig. 4. Pretreatment of BMMCs with SF suppresses the NF-κB pathway. (A) IgE-sensitized BMMCs were pre-incubated with SF or PP2 for 1 h and stimulated with DNP-HSA for 15 min. Cells lysates were then immunoblotted for IKK, IκBα, NF-κB-p65, β-actin, and Lamin B. Blots are representative of three independent experiments that provided similar results. (B) IgE-sensitized BMMCs were preincubated with SF or PP2 for 1 h and stimulated with DNP-HSA for 30 min. Nuclear extracts were used for NF-κB (p65) EMSA. The relative ratios of nuclear-p65/Lamin B level (C) was determined by measuring immunoblot band intensities by scanning densitometry. The results from three separate experiments as relative ratios (%) are represented. Data are the means $\pm$ S.D., * $P < 0.05$ and ** $P < 0.01$ versus IgE/Ag sensitized BMMCs.

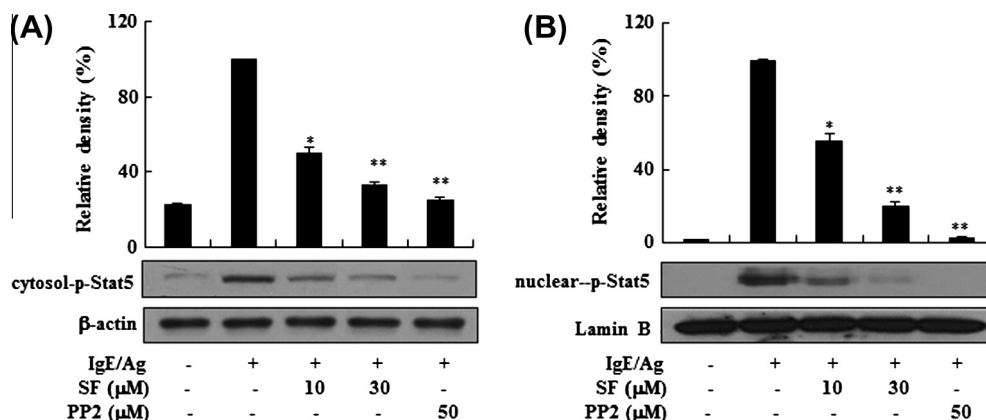


Fig. 5. SF inhibits STAT5 activation. Immunoblotting for phospho-STAT5 was performed using cytosol and nuclear extracts from BMMCs sensitized with IgE in the presence or absence of SF or PP2 for 1 h and then stimulated with DNP-HSA for 15 min. Signal intensities of phospho-STAT5 were normalized versus β-actin (A) or Lamin B (B). A representative blot from three independent experiments is shown. * $P < 0.05$ and ** $P < 0.01$ versus IgE/Ag sensitized BMMCs.

The initiation of an allergic response requires the activation of mast cells and is primarily a consequence of the allergen-induced aggregation of IgE-occupied high-affinity FcεRI. Activated mast cells are able to release potent inflammatory mediators, such as, histamine, proteases, chemotactic factors, arachidonic acid metabolites, and pro-inflammatory cytokines (Borish and Joseph, 1992; Siraganian, 2003). Furthermore, the aggregation of FcεRI triggers the activations of different signal transduction pathways. Activation of Src family tyrosine kinases, such as, Fyn and Lyn, is one of the earliest signaling events induced by FcεRI aggregation on mast

cells. Lyn is required for the activation of Syk, which plays an essential role in the IgE-dependent activation of mast cells. In addition, activation of MAPK downstream of Syk is believed to play an important role in cytokine production by switching on their transcription. Activated JNK phosphorylates a number of substrates, including c-Jun (a component of AP-1 transcription factor), to regulate gene expressions required for stress and immune responses. In particular, activated ERK can directly phosphorylate transcription factors, such as, Elk-1 and Sap-1, which then bind to the serum-responsive element of c-Fos promoter (Hess et al., 2004;

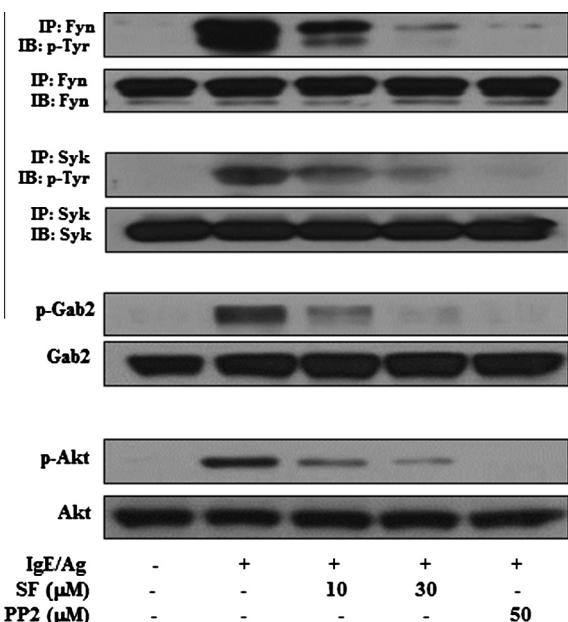


Fig. 6. Effects of SF on the Fyn-mediated phosphorylations of upstream signaling molecules. IgE-sensitized BMMCs were stimulated with or without DNP-HSA for 5 min. Proteins were immunoprecipitated for Fyn or Syk to assess their phosphorylation statuses. The phosphorylations of Gab2 and Akt were determined using total cell lysates. Blots are representative of three independent experiments that provided similar results.

Karin, 1995). Our results show that SF suppresses Syk phosphorylation, and thereby dampens the phosphorylations of all MAPKs and the activations of c-Jun and c-Fos.

Fyn is also required for another arm of the critical downstream signaling pathways that involve Gab2 (Yu et al., 2006). One of the key roles of Gab2 in FcεRI-initiated response is to activate the PI3K pathway (Gu et al., 2001), which is required for mast cell degranulation and cytokine production (Huang et al., 2008; Nakanishi et al., 1995). BMMCs from Gab2^{-/-} mice have decreased activation of PI3K, reduced tyrosine phosphorylation of PLCγ1, Akt, and JNK resulting in reduced FcεRI-induced degranulation and cytokine generation (Gomez et al., 2005). In addition, studies using Fyn-deficient BMMCs have suggested that decreased PI3K activation, as reflected by AKT phosphorylation, is accompanied by reductions in the activations of the IKK/IκBα/NF-κB and MAPK/AP-1 pathways in parallel with decreases in degranulation and cytokine production (Gomez et al., 2005; Parravicini et al., 2002). These observations concur with our present findings. Furthermore, PP2 (a Src family kinase inhibitor) was also found to inhibit signaling molecules downstream of Fyn and to block cytokine production.

Members of the STAT family of transcription factors are best known as central players in signal transduction downstream of cytokine or growth factor receptors. In mast cells, the SCF receptor c-Kit/CD117 activates STAT1, 3, 5, and 6 (Chaix et al., 2011; Gotoh et al., 1996; Morales et al., 2010; Ryan et al., 1997) partly via the tyrosine kinase JAK2. Previous studies have shown that FcεRI rapidly induces STAT5 tyrosine phosphorylation and that a STAT5 deficiency greatly reduces early and late mast cell responses (Barnstein et al., 2006; Pullen et al., 2012). Barnstein et al. reported 50% declines in histamine release and LTB₄ secretion and complete loss of cytokine production in STAT5-deficient mast cells, indicating the importance of STAT5 in mast cell activation. Pullen et al. recently reported, using BMMCs from Fyn-deficient mice or those treated with Fyn siRNA, that FcεRI-dependent tyrosine phosphorylation of STAT5 depends on Fyn, but not on Syk, JAK2, BTK, or PI3K-AKT. Because of the important role played by STAT5 in mast cells,

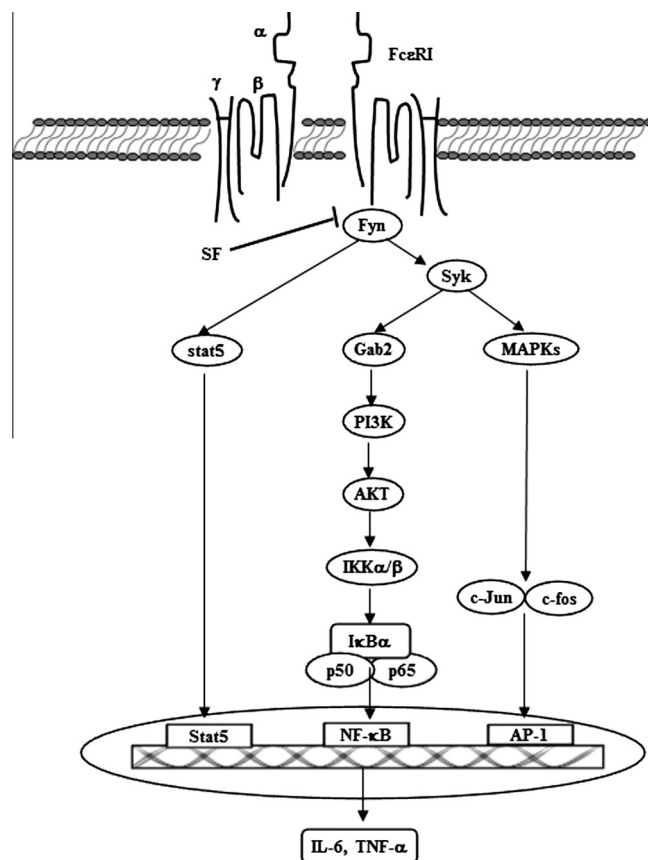


Fig. 7. Possible inhibitory mechanism of FcεRI-dependent cytokine production by SF. Engagement of FcεRI with cognate Ag triggers the activation of Fyn (a major proximal kinase), which in turn activates Syk. Then, activated Syk regulates the Gab2/PI3K/Akt pathway, which plays a key role in PI3K/AKT and NF-κB mediated production, and the MAPK pathway for AP-1 activation. The STAT5 tyrosine phosphorylation is also regulated by Fyn kinase. All these pathways are required for the induction of TNFα and IL-6. SF blocks Fyn and thereby affects these three independent pathways, leading to the suppression of cytokine production.

we examined the effect of SF on STAT5 tyrosine phosphorylation, and found that it dose-dependently inhibited STAT5 tyrosine phosphorylation in cytosol and in nuclear fractions.

In summary, SF suppressed the AKT/IKK/IκBα/NF-κB, MAPK/AP1, and STAT5 pathways, and thus, abrogated the inductions of pro-inflammatory cytokines (Fig. 7). We suggest that the inhibition of Fyn, the most upstream kinase, may underlie the inhibitory effects of SF on all signaling events in FcεRI-activated mast cells. The inhibitory effect of SF on IgE/Ag-induced TNF-α and IL-6 production through inhibiting Fyn-dependent transcription factor pathway.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fct.2013.06.056>.

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