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Saucerneol F inhibits tumor necrosis factor-α and IL-6 production by suppressing Fyn-mediated pathways in FcεRI-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-κB (NF-κB), and STAT5. It was found that SF inhibited the nuclear translocation of the p65 subunit of NF-κB 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 FcεRI-mediated signaling pathways demonstrated that SF inhibited the phosphorylation of Fyn and multiple downstream signaling processes, including Syk, Gab2, and the Akt/IKK/IκB and MAPK pathways. Taken together, our results suggest that SF inhibits the production of pro-inflammatory cytokines by suppressing Fyn kinase-dependent signaling events.

1. Introduction

Mast cell stimulated by crosslinking of high-affinity IgE receptor (FcεRI) 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 FcεRI activates a variety of Src family kinases, such as, Lyn and Fyn (Eiseman and Bolen, 1992; Gilfillan and Rivera, 2009). Upon FcεRI aggregation, immunoreceptor tyrosine-based activation motifs (ITAMs) of the γ and β subunits of FcεRI 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-κB, AP-1, and STAT family members (Barnes and Adcock, 1998; Pullen et al., 2012). NF-κB is now known to regulate the expressions of many inflammatory and immune genes, including IL-1β, IL-6, TNF-α, iNOS, and COX-2 (Peng et al., 2005). NF-κB 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 FcεRI. 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, PGD_2 and LTC_4 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-IκB, IKKα/β, ERK1/2, JNK, p38, AKT, Gab2, Stat5, c-Jun, β-actin, and total form for IκB, ERK1/2, JNK, p38, c-Fos and AKT were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Polyclonal antibodies against NF-κB p65, Syk, Fyn, Gab2, IKKα 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 μM MEM non-essential amino acid solution (Invitrogen, NY, USA) and 20% PWM–SCM (pokeweed mitogen–spleen cell conditioned medium) as a souse of IL-3 in an atmosphere containing 5% CO_2 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 μg of total RNA using the one-step RT PCR kit (Enzynomics, Daejon, 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′-AAGTGCATCATCGTTGTTCATACA-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 $MgCl_2$, 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 μl 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 Na_3VO_4, 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-κB activation assay

Nuclear fractions were prepared as described previously (Blom et al., 2010). NF-κB transactivation capacity was measured by using a NF-κB (p65) transcription factor assay kit (Cayman Chemicals, Ann Arbor, MI, USA) according to the manufacturers' instructions. The results from the NF-κB (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;