Saucerneol F, a New Lignan Isolated from *Saururus chinensis*, Attenuates Degranulation via Phospholipase Cγ1 Inhibition and Eicosanoid Generation by Suppressing MAP Kinases in Mast Cells

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Abstract

During our on-going studies to identify bioactive compounds in medicinal herbs, we found that saucerneol F (SF), a naturally occurring sesquilignan isolated from *Saururus chinensis* (*S. chinensis*), showed in vitro anti-inflammatory activity. In this study, we examined the effects of SF on the generation of 5-lipoxygenase (5-LO) dependent leukotriene C4 (LTc4), cyclooxygenase-2 (COX-2) dependent prostaglandin D2 (PGD2), and on phospholipase Cγ1 (PLCγ1)-mediated degranulation in SCF-induced mouse bone marrow-derived mast cells (BMMCs). SF inhibited eicosanoid (PGD2 and LTC4) generation and degranulation dose-dependently.

To identify the molecular mechanisms underlying the inhibition of eicosanoid generation and degranulation by SF, we examined the effects of SF on the phosphorylation of PLCγ1, intracellular Ca2+ influx, the translocation of cytosolic phospholipase A2 (cPLA2) and 5-LO, and on the phosphorylation of MAP kinases (MAPKs). SF was found to reduce intracellular Ca2+ influx by inhibiting PLCγ1 phosphorylation and suppressing the nuclear translocations of cPLA2 and 5-LO via the phosphorylations of MAPKs, including extracellular signal-regulated protein kinase-1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38. Taken together, these results suggest that SF may be useful for regulating mast cell-mediated inflammatory responses by inhibiting degranulation and eicosanoid generation.

Key Words: Saucerneol F, Mast cells, Prostaglandin D2, Leukotriene C4, Degranulation, Mitogen-activated protein kinase

INTRODUCTION

During recent years, it has been increasingly realized that mast cells play a central role in the induction of allergic and inflammatory reactions and that they express multiple receptors on their surfaces (Iwaki *et al.*, 2008). Of these high affinity receptors for IgE (FceRI) is the most well known and is stimulated during IgE/antigen-induced mast cells activation (Gillfillan and Tkaczyk., 2006). In addition, mast cells also express Kit (CD117), a receptor for stem cell factor (SCF); also known as Kit ligand, KL), which plays important roles during mast cell differentiation, survival, and activation (Roskoski, 2005; Simon *et al.*, 2008; Orinska *et al.*, 2010). IgE and SCF bind to their receptors on the mast cell membrane, which activates the cells and results in the release of preformed inflammatory mediators, such as, histamine, proteases, and proteoglycans, and of newly synthesized mediators, such as, proinflammatory cytokines and eicosanoids (PGD2 and LTC4), the latter of which are formed by the metabolism of arachidonic acid (AA) (Stevens and Austen, 1989; Murakami *et al.*, 1995; Moon *et al.*, 1998; Yamaguchi *et al.*, 1999).

Kit is a transmembrane protein with an external ligand-binding domain for SCF and intrinsic tyrosine kinase activity in its cytoplasmic domain. When SCF binds to Kit, it causes dimerization, autophosphorylation, and activation of the receptor. When mast cells are activated by SCF, they trigger the activations of several signal molecules, such as, tyrosine kinases (Blume-Jensen *et al.*, 1994), tyrosine phosphatases SHP1 and SHP2 (Yi and Ihle, 1993), PLCγ (Reith *et al.*, 1991), PI3K (Marone *et al.*, 2008), and mitogen-activated protein kinases (MAPKs), including Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) (Lu *et al.*, 2012a; Lu *et al.*, 2012b). Previously we reported that COX-2 dependent PGD2 and 5-LO dependent LTC4 generation are regulated by MAPK/NF-κB pathway (Lu *et al.*, 2012a; Lu *et al.*, 2012b). In mouse bone marrow-derived mast cells (BMMCs), the initial step in arachidonic acid (AA) metabolism is initiated by the liberation of AA from membrane phospholipids via the activations...
of 85-kDa cPLA₂ and 14-kDa type II secretory PLA₂ (Murakami et al., 1994). Furthermore, many reports have demonstrated that cPLA₂ activity is regulated via phosphorylation by MAPKs families in IgE/Ag or SCF plus IL-10 and LPS-simulated mast cells (Lu et al., 2012b).

S. chinensis (Saururaceae) is a perennial herb found in East Asia, and is used in traditional medicine to treat various conditions, such as, edema, jaundice, gonorrhea, fever, and inflammation (Hwang et al., 2003; Lee et al., 2009). Previously, we reported on the isolation and on the cytotoxicities of compounds from the roots of S. chinensis, namely, on four new lignans saucerneol F (SF) (Fig. 1), saucerneol G, saucerneol H, and saucerneol I (Seo et al., 2008; Lee et al., 2009). SF, a lignan constituent of S. chinensis, is used in traditional medicine to treat inflammation, carbuncles, and skin disorders (Sung et al., 2001). More recently, we reported that SF inhibits LPS-induced iNOS expression by inactivating MAPKs, NF-κB, and AP-1 in LPS-induced RAW264.7 cells (Lu et al., 2012c). In this study, we investigated for the first time the effects of SF on cytokine-stimulated eicosanoid generation in and on the degranulation of BMMCs.

MATERIALS AND METHODS

Measurement of cell viability

Cell viability was assessed by MTT assay. Briefly, BMMCs cells were seeded onto 96-well culture plates at 2×10⁴ cells/200 μl/well. After incubation with the indicated concentrations of SF for 4 h, 20 μl of MTT (5 mg/ml) was added to each well. After 4 h incubation, 150 μl of culture medium was removed, and cells were dissolved in 0.4 N HCl/isopropyl alcohol. The optical densities (OD) at 570 nm and 630 nm were measured using a microplate reader (Sunrise, Tecan, Switzerland).

Cell culture and activation

Mouse BMMCs were isolated from male Balb/cJ mice (Sam Taco, INC, Seoul, Korea) and cultured at 37°C in RPMI 1640 media (Thermo Scientific, Utah, USA) containing 10% FBS, 100 U/ml of penicillin (Thermo Scientific), 10 mM HEPES buffer (Sigma-Aldrich, St. Louis, MO, USA), 100 μM MEM non-essential amino acid solution (Invitrogen, Carlsbad, CA, USA), and 20% PWM-SCM (pokeweed mitogen-spleen cell conditioned medium; as a source of IL-3). After 4 weeks, >98% of the cells was found to be BMMCs as determined by a previously described procedure (Murakami et al., 1994). BMMCs were then washed with PBS, and media were replaced with IL-3 free media. The cells were then pretreated with SF or other inhibitors and stimulated with SCF (30 ng/ml) or SCF/LPS for the indicated times. Reactions were terminated by centrifuging cells at 120 g for 4 min at 4°C. Supernatants were retained to assay mediator release and cell pellets were Western blotted.

Degranulation assays

After stimulating with SCF for 15 min with pre-treating with SF for 30 min, degranulation was determined by measuring release of β-hexosaminidase (β-Hex), a marker of mast cell degranulation, was quantified by spectrophotometric method, as described previously (Son et al., 2005).

Measurement of LTC₄ and PGD₂ generation

BMMCs suspended in enriched medium at a cell density of 1×10⁶ cells/ml were pretreated with SF for 30 min and stimulated with SCF (30 ng/ml) for 15 min. Supernatants were isolated for further analysis by enzyme immunoassay (EIA) kit. The concentration of LTC₄ was determined using an EIA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer’s instruction. To assess COX-2-dependent PGD₂ synthesis, BMMCs were preincubated with 1 μg/ml aspirin for 2 h to irreversibly inactivate preexisting COX-1. After washing, BMMCs were activated with SCF (30 ng/ml) plus IL-10 (100 U/ml) and LPS (100 ng/ml) at 37°C for 7 h with or without SF. PGD₂ levels in supernatants were quantified using the PGD₂ EIA kit (Cayman Chemical) and cells were Western blotted.

Measurement of intracellular Ca²⁺ level

Intracellular Ca²⁺ levels were determined using the FluoForté Calcium Assay Kit (Enzo Life Sciences, Ann Arbor, MI, USA). BMMCs were preincubated with FluoForté Dye-Loading Solution for 1 h at room temperature. After washing the dye from cell surfaces with HBSS, cells (5×10⁶) were seeded into 96-well microplates and pretreated with indicated concentration of SF for 1 h before adding 30 ng/ml SCF. Fluorescent was measured using a fluorometric imaging plate reader at an excitation and emission wavelengths of 485 and 520 nm, respectively (BMG Labtechnologies FLUOSTAR OPTIMA plateread, Offenburg, Germany). All experiments were repeated at least three times.

Immunoblotting

Cells 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 PMSF, 1 M DTT, 200 mM NaF, 200 mM NaVO₄, Protease inhibitor Cocktail). Cell debris was removed by centrifugation at 14,000 × g for 15 min at 4°C and resulting supernatants were western blotted. Protein concentrations were determined using the Qubit Flurometer machine (Invitrogen, Carlsbad, CA, USA). Samples were separated by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA) which were subsequently blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 and incubated with individual antibodies. Primary antibodies were diluted 1:1000-fold (unless otherwise mentioned), and after treatment, membranes were incubated at 4°C overnight. They were then washed three times for 10 minutes with TBS-T buffer, treated with HRP-coupled secondary antibodies (diluted 1:3000-fold)
for 1 h at room temperature, washed three times for 3 min each in TBS-T buffer, and then developed using the enhanced chemiluminescence (ECL) detection kit (Pierce Biotechnology, Rockford, IL, USA).

Statistical analysis
All experiments described were performed three or more times. Average values were expressed as means ± S.E.M. Statistical analyses were performed using SPSS 19.0 (SPSS, Chicago, IL, USA). The Student’s t-test was used for the comparison of two independent groups. For all tests, a p-value < 0.05 was considered statistically significant.

RESULTS
Structure of SF and cytotoxicity to BMMCs
We first measured the cytotoxic effect of SF (Fig. 1A) on BMMCs using the MTT assay. Cell viability was unaffected by SF at 50 μM (Fig. 1B), and therefore, concentrations below 50 μM were used in subsequent experiments.

SF inhibits PLC1-mediated mast cell degranulation and intracellular calcium influx
Since it has been reported that histamine release is closely paralleled β-hexosaminidase (β-Hex; a degranulation marker) (Supajatura et al., 2002). We examined the inhibitory activity of SF on degranulation in SCF-stimulated BMMCs. As shown in Fig. 2A, SF dose-dependently inhibited β-Hex release. In a previous study, increasing intracellular Ca²⁺ concentration stimulated degranulation and the generation of eicosanoid and cytokines in IgE/Ag and in SCF-treated mast cells (Stevens and Austen, 1989; Yamaguchi et al., 1999, Lu et al., 2011; Lu et al., 2012a; Lu et al., 2012b). Thus, we examined the effect of SF on intracellular Ca²⁺ influx in SCF-induced BMMCs, and as shown in Fig. 2B, pretreatment of BMMCs with SF was found to inhibit intracellular Ca²⁺ influx strongly. It has been well established the phosphorylation of PLC1 induces the generations of inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) and that these two second messengers increase Ca²⁺ influx and lead to the activation of PKC, which is a regulator of mast cell degranulation (El-Sibai and Backer, 2007). Therefore, we investigated the role of SF in the phosphorylation of PLC1. Pretreatment of BMMCs with SF was found to suppress the SCF-induced phosphorylation of PLC1 dose-dependently (Fig. 2C), indicating that the inhibitory effect of SF on degranulation was mediated via the inhibition of PLC1-mediated intracellular Ca²⁺ influx.

SF inhibits phosphorylation of cPLA2 and 5-LO dependent LTC4 generation in SCF-induced BMMCs
AA can be converted into LTC4 by the action of 5-LO in BMMCs (Murakami et al., 1995; Lu et al., 2012a). We found that pretreatment of BMMCs with SF or licofelone (positive control) (Bannwarth, 2004) and subsequent stimulation with
SCF for 15 min strongly inhibited LTC4 generation (Fig. 3A). In our recent studies, BMMCs were stimulated with SCF, and maximal times to cPLA2 phosphorylation and the nuclear translocations phosphorylated cPLA2 and 5-LO were 10 to 15 min (Lu et al., 2012a). Thus, we examined the effect of SF on cPLA2 phosphorylation and these nuclear translocations. When BMMCs were pretreated with SF and then stimulated with SCF for 15 min phosphorylated cPLA2 was detected in the cytosol (C-p-cPLA2) and in nuclear (N-p-cPLA2) fractions. As was expected, SF inhibited the phosphorylation of cytosolic PLAr and the nuclear translocation of phosphorylated cPLA2. In resting BMMCs, most 5-LO was localized in the cytosol (C-5-LO) and this was translocated to the nucleus (N-5-LO) after SCF treatment. Furthermore, as shown in Fig. 3B, this reaction was significantly reduced by SF pretreatment (Fig. 3B). These results suggest that SF inhibited LTC4 generation by inhibiting the nuclear translocations of cPLA2 and 5-LO.

**SF inhibits COX-2 dependent PGD2 generation in SCF-induced BMMCs**

Previously, we and other group reported that SCF or IgE/antigen plus IL-10 and LPS induction of BMMCs strongly elicits the delayed phase of PGD2 generation and concomitantly induces COX-2 expression (Murakami et al., 1994; Murakami et al., 1996; Ashraf et al., 1996; Moon et al., 1998). Here, BMMCs were pre-treated with aspirin to abolish preexisting COX-1 activity, and then stimulated with SCF plus IL-10 and LPS with or without SF or licofelone which is used as positive control (Bannwarth, 2004). SCF plus IL-10 and LPS greatly increased PGD2 generation at Fig. 4A and COX-2 protein expression at 7 h (Fig. 4B). PGD2 production was suppressed by SF in a dose-dependent manner and COX-2 protein expression was concomitantly reduced (Fig. 4B). These results demonstrate that like licofelone, SF inhibits the activities of COX-2 and 5-LO (Bannwarth, 2004).

![Fig. 4](image)

**Fig. 4.** Effect of SF on PGD2 generation and COX-2 protein expression. (A) BMMCs were pre-incubated with 1 μg/ml of aspirin for 2 h to abolish pre-existing COX-1 activity, briefly washed, and then stimulated with SCF plus IL-10 and LPS for 7 h. PGD2 release into supernatant was quantified using the PGD2-MOX EIA kit, and cells were immunoblotted for COX-2 protein (B). Values are means ± S.E.M. (n=3). *p<0.05 and **p<0.01 versus SCF plus IL-10 and LPS-stimulated BMMCs.

![Fig. 5](image)

**Fig. 5.** Effect of SF on MAPKs pathways. (A) BMMCs were pre-incubated for 1 h with SF or the MAP kinase inhibitors U0126 (ERK inhibitor), SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), and then stimulated with SCF plus IL-10 and LPS for 15 min. Cytosolic fractions were immunoblotted to evaluate MAPK phosphorylation. The relative ratios of p-ERK/ERK (A), p-JNK/JNK (B) and p-p38/p38 (C) protein levels were determined by measuring immunoblot band intensities by scanning densitometry, respectively (*p<0.05 and **p<0.01). The results from three separate experiments are represented, and the relative ratio (%) is also shown.
SF attenuates phosphorylation of MAPKs in SCF-induced BMMCs

It is well known that enzyme activity of cPLA₂ is markedly increased by MAPK phosphorylation (Lin et al., 1993; Durustin et al., 1994), thus we investigated the effect of SF on MAPK phosphorylation in SCF-induced BMMCs. Recently, we found that when BMMCs are stimulated with SCF or SCF plus IL-10 and LPS, maximal MAPK phosphorylation was reached after 30 min (Lu et al., 2012a; Lu et al., 2012b). As illustrated in Fig. 5, SCF induced-BMMCs showed significantly elevated phosphorylations of ERK1/2, p38, and JNK. Furthermore, pretreatment with SF for 1 h before SCF stimulation for 15 min, markedly and dose-dependently inhibited SCF-induced MAPK phosphorylation without altering the total protein level. Moreover, pretreatment with U0126 (an ERK pathway inhibitor), SP600125 (a JNK inhibitor), and SB203580 (a p38 inhibitor) also inhibited ERK1/2, JNK and p38 phosphorylation, indicating that the activations of MAPK pathways participate in SCF-induced eicosanoid generation, as confirmed by densitometric analysis (Fig. 5).

DISCUSSION

Previously, we reported that saucerneol D (SD) and saucerneol F (SF), isolated from S. chinensis, suppressed LPS-stimulated iNOS expression by blocking the activation of MAPKs and NF-κB in LPS-induced RAW264.7 cells (Moon et al., 2008; Lu et al., 2012c). However, the effect of SF on COX-dependent PGD₂ generation, 5-LO dependent LTC₄ generation, and degranulation in mast cells has not been previously examined. As described above, eicosanoid generation and degranulation play important roles in allergic-inflammatory responses. Therefore, we investigated the effects of SF on eicosanoid generation and degranulation in SCF-induced BMMCs. We found that SF inhibited degranulation and LTC₄ generation in SCF-induced BMMCs and inhibited PGD₂ generation in SCF plus IL-10 and LPS-induced BMMCs.

Histamine is one of the most important chemical mediators in various allergic diseases (Yamaguchi et al., 1999). Furthermore, β-Hex release to culture medium has been used as a degranulation marker because β-Hex, like histamine, is stored in the granules of mast cells. In addition, β-Hex is released with histamine when mast cells are activated (Lobe et al., 1983). Therefore, we examined degranulation by measuring β-Hex release in SCF-mediated BMMCs, and we found that SF dose-dependently inhibited β-Hex release. Several studies have shown that the activation of PLC₁ and an increase in intracellular Ca²⁺ concentration are essential for the degranulation of and eicosanoid synthesis in mast cells (El-Sibai and Backer, 2007), and thus, we investigated the effect of SF on the phosphorylation of PLC₁ and on intracellular Ca²⁺ influx in SCF-induced BMMCs. It was found that SF dose-dependently inhibited PLC₁ phosphorylation and Ca²⁺ influx, which suggests that the inhibition of degranulation by SF required the activation of PLC₁-dependent Ca²⁺ influx.

To synthesis PGD₂ and LTC₄ in mast cells, AA must be liberated from membrane phospholipids by the activation of Ca²⁺-dependent cPLA₂ (Chang et al., 2006). The released AA is then converted to LTA₄, which is metabolized to LTB₄ by LTA₄ hydrolase or to LTC₄ by LTC₄ synthase (Wertz and Steinhiber, 2005). Therefore, we first examined the effect of SF on 5-LO dependent LTC₄ generation, and found that SF strongly and dose-dependently inhibited LTC₄ generation. Since increased intracellular Ca²⁺ influx is considered important for the release of AA and the generation of LTC₄ by 5-LO in BMMCs (Lu et al., 2012a), we also examined the effect of SF on intracellular Ca²⁺ influx. As shown in Fig. 2C, intracellular Ca²⁺ concentration was elevated in SCF-induced BMMCs, and this increase was dose-dependently inhibited by SF. It has been established that both cPLA₂ and 5-LO have a C2 domain in their N-terminal, which are required for their translocations to the nuclear membrane (Rouzer and Kargman, 1988; Kramer and Sharp, 1997). In the present study, we found that SF significantly suppressed cPLA₂ phosphorylation and nuclear translocation and consequently suppressed the release of AA used for LTC₄ generation. Furthermore, SF also strongly inhibited the nuclear translocation of 5-LO. Since MAPKs play an essential role in the activation of cPLA₂ during SCF-induced mast cell activation (Lu et al., 2012a), we examined whether SF affects the phosphorylations of the MAPK family members ERK1/2, JNK, and p38. The results obtained showed that the inhibition of LTC₄ generation by SF was mediated by via inhibition of MAPs phosphorylation (Fig. 4). Mounting evidence now demonstrates that PGD₂ synthesis in BMMCs occurs in a biphasic manner, that is, COX-1 dependent PGD₂ generation occurs within 2 h, whereas delayed PGD₂ generation occurs after several hours (2-10 h). This delayed PGD₂ synthesis is associated with the de novo synthesis of induced COX-2 protein after stimulation with IgE/Ant IgE combination with SCF plus IL-10 and LPS (Ashraf et al., 1996; Moon et al., 1998). When BMMCs were activated with SCF plus IL-10 and LPS in the presence or absence of SF, COX-2-dependent PGD₂ generation was inhibited in a dose-dependent manner, with concomitant inhibition of COX-2 protein expression. Taken together, our findings suggest that SF may be a useful biochemical and pharmacological tool for determining the role of COX-2/5-LO dual inhibitors and/or degranulation inhibitory agents during certain allergic-inflammatory responses.

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