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Therapeutic Effects of S-Petasin on Disease Models of Asthma and Peritonitis

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Abstract

To explore the anti-allergic and anti-inflammatory effects of extracts of *Petasites* genus, we studied the effects of s-petasin, a major sesquiterpene from *Petasites formosanus* (a butterbur species) on asthma and peritonitis models. In an ovalbumin-induced mouse asthma model, s-petasin significantly inhibited the accumulations of eosinophils, macrophages, and lymphocytes in bronchoalveolar fluids. S-petasin inhibited the antigen-induced degranulation of β-hexosamidase but did not inhibit intracellular Ca^{2+} increase in RBL-2H3 mast cells. S-petasin inhibited the LPS induction of iNOS at the RNA and protein levels in mouse peritoneal macrophages. Furthermore, s-petasin inhibited the production of NO (the product of iNOS) in a concentration-dependent manner in the macrophages. Furthermore, in an LPS-induced mouse model of peritonitis, s-petasin significantly inhibited the accumulation of polymorpho nuclear and mononuclear leukocytes in peritoneal cavity. This study shows that s-petasin in *Petasites* genus has therapeutic effects on allergic and inflammatory diseases, such as, asthma and peritonitis through degranulation inhibition in mast cells, suppression of iNOS induction and production of NO in macrophages, and suppression of inflammatory cell accumulation.

Key Words: S-petasin, Anti-allergy, Anti-inflammation, COX-2, Degranulation, Mast Cell, Macrophage

INTRODUCTION

Extracts from *Petasites* genus have been shown to have anti-allergic and anti-inflammatory effects (Fiebich *et al*., 2005; Lee *et al*., 2011; Zhang *et al*., 2011). Furthermore, lipophilic extracts of rhizomes of *Petasites hybridus* L., Asteraceae (the butterbur) have been used to treat asthma (Danesch, 2004; Brattstrom *et al*., 2010). Pharmacological studies on its active ingredients and action mechanism have suggested that petasin, a sesquiterpene, inhibits L-type Ca^{2+} channels, decreasing intracellular Ca^{2+} concentration, and thus, inhibits the synthesis inhibition of leukotriene B_4 and cysteinyl leukotrienes in eosinophils and neutrophils (Bickel *et al*., 1994; Thomet *et al*., 2001; Resnati *et al*., 2002; Fiebich *et al*., 2005; Wang *et al*., 2010). Although extracted fractions of *Petasites hybridus* have been reported to inhibit COX-2 activity and prostaglandin E_2 synthesis *in vitro* via p42/44 MAPKs in rat primary microglia, petasin and isopetasin did not exhibit direct COX-2 inhibition (Fiebich *et al*., 2005). Therefore, we hypothesized that there are other active ingredients for anti-allergic and anti-inflammatory effects of *Petasites* genus. We examined whether s-petasin, a methylthio derivative of petasin (Fig. 1)(Aebi *et al*., 1958) and a major sesquiterpene from *Petasites formosanus* (a butterbur species), has such activities (Shih *et al*., 2011). In the present study, we investigated therapeutic effects of s-petasin on disease models of asthma and peritonitis.

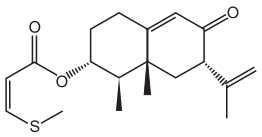

Fig. 1. Structure of s-petasin.

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MATERIALS AND METHODS

Materials

Fura 2-AM was obtained from Calbiochem (Darmstadt, Germany), while s-petasin and other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Rat RBL-2H3 mast cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), cultured at 37°C in a 5% CO_2 humidified incubator, and maintained in high glucose DMEM containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate (Song *et al*., 2012).

Induction of asthma in BALB/c mice and drug administration

Six-week-old male BALB/c mice were obtained from Daehan Biolink (DBL; Seoul, Korea) and adapted for a week beforehand in the Laboratory Animal facility at Pusan National University. The mice were divided into three groups (n=7/group), that is, into a PBS-injected control group, an ovalbumin-injected asthma group, or an s-petasin-treated asthma group (1 mg/kg). Asthma was induced by injecting ovalbumin and alum i.p. on the 1st and 14th days. From the 28th day, mice were exposed to nebulized ovalbumin for 10 min for three consecutive days (Aoki *et al*., 2010). S-petasin (1 mg/kg) dissolved in PBS was administrated i.p. 1 h before ovalbumin nebulization on days 28, 29, and 30. Two days after treatment with nebulized ovalbumin, BALF (bronchoalveolar lavage fluid) samples were collected from lungs and cell densities in BALF were determined by staining and counting (Lee *et al*., 2013c).

Measurement of degranulation

Degranulation was estimated by measuring β-hexosaminidase release, as previously described by Dearman *et al*. (Dearman *et al*., 2005). RBL-2H3 cells (2×10^5 cells/well in 24-well plates) were sensitized with 0.5 μg/ml monoclonal anti-dinitrophenyl specific mouse IgE (DNP-IgE, D8406, Sigma, St. Louis, MO, USA) overnight at 37°C in a 5% CO_2 incubator. Cells were washed twice with PIPES buffer (pH 7.2), containing 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 0.1% BSA, and 1 mM $CaCl_2$ to remove DNP-IgE before stimulation, and then incubated in PIPES buffer in 500 μl. Different concentrations of s-petasin were then added to each well, incubated at 37°C for 30 min, 10 μg/ml of human dinitrophenyl albumin (DNP-hAb, A6661 Sigma, St. Louis, MO, USA) was added, and cells were incubated for a further 30 min at 37°C to induce degranulation. Aliquots (50 μl) of medium were then transferred to a 96-well microplate and incubated for 60 min with 50 μl of 1 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide (N9376, Sigma, St. Louis, MO, USA) in 0.1 M citrate buffer (pH 4.5). Cells were lysed with an equal volume of 0.5% Triton X-100 at 37°C for 1 h. Total β-hexosamidase activity in RBL-2H3 mast cells was measured at the same rate. The reaction was terminated by adding 250 μl of 0.05 M sodium carbonate buffer (pH 10.0; 0.05 M Na_2CO_3/0.05 M $NaHCO_3$). Absorbances (OD) at 410 nm were measured using a microplate reader. Degranulation (%) was calculated by the ratio of released β-hexosamidase in stimulated cells to total β-hexosamidase activity (Lu *et al*., 2012).

Measurement of intracellular Ca^{2+} concentrations

Cells were trypsin-digested, allowed to sediment, incubated in complete medium containing monoclonal anti-dinitrophenyl specific mouse IgE (0.5 μg/ml, DNP-IgE, D8406, Sigma, St. Louis, MO, USA) at 37°C for 2 h, resuspended in Hepes-buffered medium (HBM), consisting of 20 mM Hepes (pH 7.4), 103 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 15 mM glucose, 2.5 mM probenecid and 0.1% bovine serum albumin (fatty acid free), and then incubated for 40 min with 5 μM of fura 2-AM. $[Ca^{2+}]_i$ levels were estimated by measuring changes in fura 2 fluorescence at an emission wavelength of 510 nm and two excitation wavelengths (340 nm and 380 nm) every 0.1 sec using a F4500 fluorescence spectrophotometer (Hitachi, Japan). The ratios of fluorescence intensities ($\lambda_{340}/\lambda_{380}$) at these two wavelengths were used as a surrogate of $[Ca^{2+}]_i$, as previously described (Chang *et al*., 2006; Ahn *et al*., 2012).

Isolation and culture of mouse peritoneal macrophages

Mouse peritoneal macrophages were isolated from the peritoneal cavity of a 3% thioglycol-treated C57BL/6 mouse and cultured at 37°C in a 5% CO_2 humidified incubator, as previously described (Michaud *et al*., 2010). Isolated macrophages were maintained in RPMI1640 containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/mL penicillin, 50 μg/ml streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate for 24 h and then incubated in 0.5% FBS-containing media for 18 h. Macrophages were pretreated with s-petasin and one hour later LPS (100 ng/ml) was added. Total proteins were sampled after 24 h of LPS treatment (Michaud *et al*., 2010).

Reverse transcriptase-PCR

After treatment with LPS and s-petasin for 5 h, first strand cDNA was synthesized using total RNA isolated using Trizol reagent (Invitrogen, USA). Synthesized cDNA products and specific primers were used for PCR with Promega Go-Taq DNA polymerase (Madison, WI, USA). Specific primers for iNOS (sense 5'- ACC TAC CAC ACC CGA GAT GGC CAG-3', antisense 5'- AGG ATG TCC TGA ACA TAG ACC TTG GG-3'), COX-2 (sense 5'- CCG TGG GGA ATG TAT GAG CA- 3', antisense 5'- CCA GGT CCT CGC TTA TGA TCT G -3'), IL-6 (sense 5'- TGG GAA ATC GTG GAA ATG AG-3', antisense 5'- GAA GGA CTC TGG CTT TGT CT-3'), and GAPDH (sense 5'- TTC ACC ACC ATG GAG AAG GC-3', antisense 5'- GGC ATG GAC TGT GGT CAT GA-3') were used and annealing was undertaken at 60°C. For IL-1β (sense 5'- GGA GAA GCT GTG GCA GCT A-3', antisense 5'- GCT GAT GTA CCA GTT GGG GA-3'), and TNF-α (sense 5'- GAC CCT CAC ACT CAG ATC AT-3', antisense 5'-TTG AAG AGA ACC TGG GAG TA -3'), annealing was undertaken at 57°C. Ten μl of aliquots were electrophoresed in 1.2% agarose gels and stained with ethidium bromide.

Western blotting

Macrophages were harvested and resuspended in lysis buffer. Concentrations of proteins were determined using the BCA protein assay (Thermo Scientific, Rockford, IL, USA). Equal amounts of proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes, which were blocked in

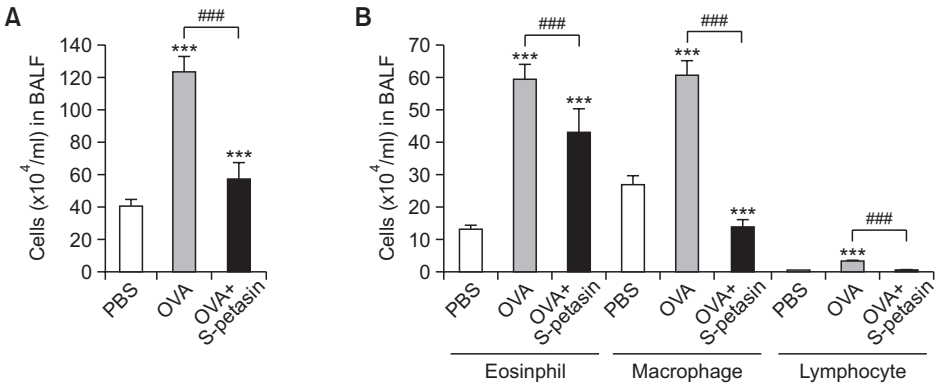

Fig. 2. Effect of s-petasin on the accumulations of eosinophils, macrophages, and lymphocytes in the BALF samples of ovalbumin-induced asthmatic mice. Mice were immunized with ovalbumin twice by i.p. injection (on days 1 and 14) and then sensitized to nebulized ovalbumin on days 28, 29, and 30. S-petasin (1 mg/kg) was administrated i.p. 1 h before ovalbumin nebulization on days 28, 29, and 30. Cells in BALF samples were stained using the May-Grünweld method and counted. (A) Total cell counts in the BALF samples of control mice (PBS), ovalbumin-induced asthmatic mice (OVA), and s-petasin (1 mg/kg)-treated asthmatic mice (OVA + s-petasin). (B) Cell counts of eosinophils, macrophages, and lymphocytes in the BALF samples of the PBS, OVA, and OVA+s-petasin (1 mg/kg) treated groups. Three-day exposure to ovalbumin through airways induced the accumulations of eosinophils, macrophages, and lymphocytes in BALF. However, in OVA+s-petasin treated mice, these accumulations were significantly reduced. Statistical significance: $^{***}p<0.001$ vs. PBS-treated mice and $^{###}p<0.001$ vs. OVA-treated mice.

Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% skim milk, and then incubated with specific rabbit antibodies recognizing COX-2 and iNOS (Cell Signaling Technology, Danvers, MA, USA). Anti-rabbit horseradish-linked IgG was used as the secondary antibody. Signals were developed using an enhanced chemiluminescence system (Advanst, USA) (Kang *et al*., 2013).

Nitrite measurement

The production of NO was estimated by measuring the amount of nitrite, a stable metabolite of NO, using Griess reagent as previously described (Lee *et al*., 2013b). Cells were pretreated with different concentrations of s-petasine for 1 h and then stimulated with LPS (100 ng/ml) for 24 h. Nitrite concentrations in medium were determined using the Griess Reagent System (Promega, Madison, WI, USA).

PGE_2 production

Peritoneal macrophages were incubated with s-petasin (1, 3, 5, 10 μM) for 1 h and subsequently stimulated with LPS (100 ng/ml) for 24 h. Macrophage culture supernatants were harvested and immediately assayed using a PGE_2 EIA kit (Cayman Chemical, Ann Arbor, MI, USA) (Kang *et al*., 2012).

Induction of peritonitis and neutrophil counting

Peritonitis was induced by injecting 1 mg/kg LPS (Sigma) intraperitoneally (i.p.). Briefly, C57BL/6 mice were pretreated with PBS or 1 mg/kg of s-petasin for 1 h prior to LPS treatment. Peritoneal washing was performed 24 h after LPS treatment using 4 ml of ice-cold RPMI1640 medium. Total cell numbers in peritoneal washings were calculated by counting in trypan blue staining. Cells were washed with 0.1 M phosphate buffer (pH 6.8). The 0.1 M phosphate buffer was made by mixing 153 ml of 0.2 M NaH_2PO_4 (Amresco, Solon, OH, USA) and 147 ml of 0.2 M NaH_2PO_4 (Amresco, Solon, OH, USA) and adding distilled water to make final volume 900 ml. The cells were attached to slides using a Cellspin (Hanil, Anyang, Korea) at 500 rpm for 5 min. The slide was dried at room temperature for 30 min and fixed in methanol for 30 sec. Then, May-Grünwald solution and Giemsa solution were used to stain cells to identify individual cell types (Lee *et al*., 2013a).

Statistics

Results are expressed as the means ± SEs of the indicated numbers of determinations. The statistical significances of differences were determined by analysis of variance (ANOVA) with turkey's post hoc, and statistical significance was accepted for p values <0.05. Analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

S-petasin inhibited the accumulations of eosinophils, macrophages, and lymphocytes in the BALF of mice with ovalbumin-induced asthma

In order to test if s-petasin has anti-allergic effect, ovalbumin-induced asthma model was applied. Anti-asthma effect was measured by measuring accumulations of eosinophils, macrophages, and lymphocytes in bronchioalveolar lavage fluid (BALF). BALF samples from ovalbumin-induced asthmatic mice showed three-fold increase of total cell number (Fig. 2A). Treatment of 1 mg/kg s-petasin blunted significantly the increase of total cell number in BALF about 80% (Fig. 2A). The cells accumulated in BALF were eosinophils, macrophages, and lymphocytes. Mainly eosinophils increased about 6 times by ovalbumin treatments and macrophages about 2 times (Fig. 2B). Lymphocytes were accumulated but their numbers were minor (Fig. 2B). S-petasin treatment significantly inhibited accumulations of three cell types (Fig. 2B). Accumulation of macrophages and lymphocytes were almost completely inhibited to PBS-treated basal levels by s-petasin, but eosinophil accumulation was inhibited mildly about 36% (Fig. 2B).

S-petasin inhibited antigen-induced degranulation in RBL-2H3 mast cells

Next, to confirm the in vivo anti-asthma effect of s-petasin and find underlining mechanisms for the effect, we utilized rat RBL-2H3 mast cells. Because degranulation of histamine, leukotrienes, and prostaglandins from antigen-exposed mast cells is a key step in allergic response, we investigated whether s-petasin inhibits antigen-induced degranulation in rat RBL-2H3 cells. Degranulation was measured by assessing β-hexosamidase activity in media after antigen exposure, as previously described (Dearman *et al*., 2005; Lu *et al*., 2012). Antigen-induced β-hexosamidase release was found to be inhibited concentration-dependently by s-petasin (Fig. 3). IC_{50} value was about 1 nM.

S-petasin did not inhibit antigen-induced Ca^{2+} increase in RBL-2H3 mast cells

Degranulation is evoked by the elevation of $[Ca^{2+}]_i$ after antigen-exposure in mast cells. Therefore, we measured $[Ca^{2+}]_i$ increase caused by antigen exposure and then examined whether s-petasin inhibited the increase (Lee *et al*., 2005). However, s-petasin did not affect antigen-induced $[Ca^{2+}]_i$ increase, indicating that degranulation inhibition by s-petasin occurs in a different mechanism to petasin, which has been reported to block Ca^{2+} channels (Fig. 4) (Lu *et al*., 2012).

S-petasin inhibited inductions of iNOS in peritoneal macrophages

Inflammatory responses are tightly involved in allergy development and asthma responses. Furthermore, s-petasin completely suppressed accumulation of macrophages in the ovalbumin-induced asthma model (Fig. 2). Therefore, we hypothesized that s-petasin negatively acts on macrophage activation, resulting in less macrophage accumulation in BALF and anti-asthmatic responses. Accordingly, we measured effect of s-petasin on expressions of inflammatory genes, that is iNOS, cyclooxygenase 2 (COX-2), IL-1β, IL-6, and TNF-α in peritoneal macrophages. LPS induced the expressions of those genes significantly at the mRNA level in macrophages (Fig. 5). Among the tested genes, s-petasin concentration-dependently inhibited induction of iNOS mRNA but not for others (Fig 5A, B). There were decreasing tendency of COX-2 gene induction but not significant (Fig. 5A, C). The inhibitory effects of s-petasin on iNOS and COX-2 were further confirmed at the protein level in the macrophages. As like the results of mRNAs, s-petasin significantly inhibited protein expression of iNOS in a concentration-dependent manner (Fig. 6A, B). Although COX-2 protein expression was also tend to be decreased by s-petasin, but it was not statistically significant (Fig. 6A, C).

S-petasin inhibited the production of NO in peritoneal macrophages.

To confirm the inhibitory effects of s-petasin on the inductions of iNOS and COX-2, concentrations of their enzyme products, that is, nitric oxide (NO) and prostaglandin E_2 (PGE_2), were measured. As shown in Fig. 7, LPS (100 ng/ml) induced the productions of NO and PGE_2, and s-petasin treatment significantly and concentration-dependently inhibited NO production (Fig. 7A). S-petasin showed concentration-dependent inhibition of PGE_2 production but only statistically significant at 10 μM of s-petasin (Fig. 7B), which is consistent to the effects of s-petasin on mRNA and protein expressions of COX-2 (Fig. 5, 6).

S-petasin inhibited the accumulation of neutrophils in the peritoneal fluid of mice with LPS-induced peritonitis

Based on the in vitro results of s-petasin in peritoneal macrophages, we could presume that s-petasin acts negatively on macrophage activation and this suppression may play a role in the anti-asthma effect of s-petasin. To verify anti-inflammatory effect of s-petasin observed in peritoneal macrophages in vitro, we further examined s-petasin in an acute inflammation animal model of a lipopolysaccharide (LPS)-induced peritonitis (Lee *et al*., 2013a). Peritoneal cavity fluids of mice treated with LPS for 24 h showed accumulations of polymorpho nuclear leukocytes (PMNL: neutrophils, eosinophils, and basophils), mononuclear leukocytes (MNL), and macrophages (Fig. 8).

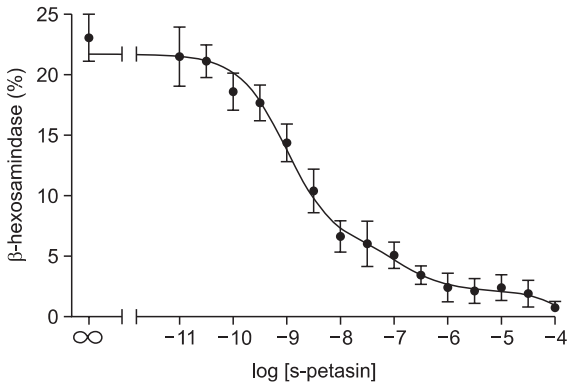

Fig. 3. S-petasin inhibited antigen-induced β-hexosamidase release from RBL-2H3 mast cells in a concentration-dependent manner. RBL-2H3 cells sensitized overnight with anti DNP-IgE were treated with different concentrations of s-petasin, and 30 minutes later challenged with DNP human serum albumin. Antigen-induced degranulation was determined by measuring the amount of released β-hexosamidase activity. S-petasin inhibited antigen-induced degranulation in RBL-2H3 mast cells in a dose-dependent manner. Results are the means ± SEs of three independent experiments.

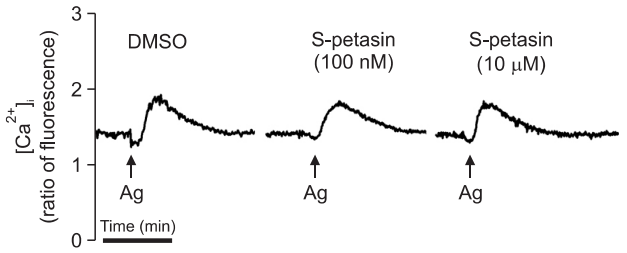

Fig. 4. Effects of s-petasin on antigen-induced $[Ca^{2+}]_i$ increases in RBL-2H3 mast cells. RBL-2H3 cells were sensitized overnight with anti DNP-IgE, loaded with Fura-2 AM for 45 min, and washed twice with PBS. The cells were then preincubated with s-petasin for 10 min and challenged with 10 μg/ml of DNP human serum albumin human. Arrows indicate when the antigen was added. Antigen induced an increase in $[Ca^{2+}]_i$ and this increase was not inhibited by s-petasin at concentrations up to 50 μg/ml. The data shown are representative of three independent experiments. Time scale bar represents 1 minute.

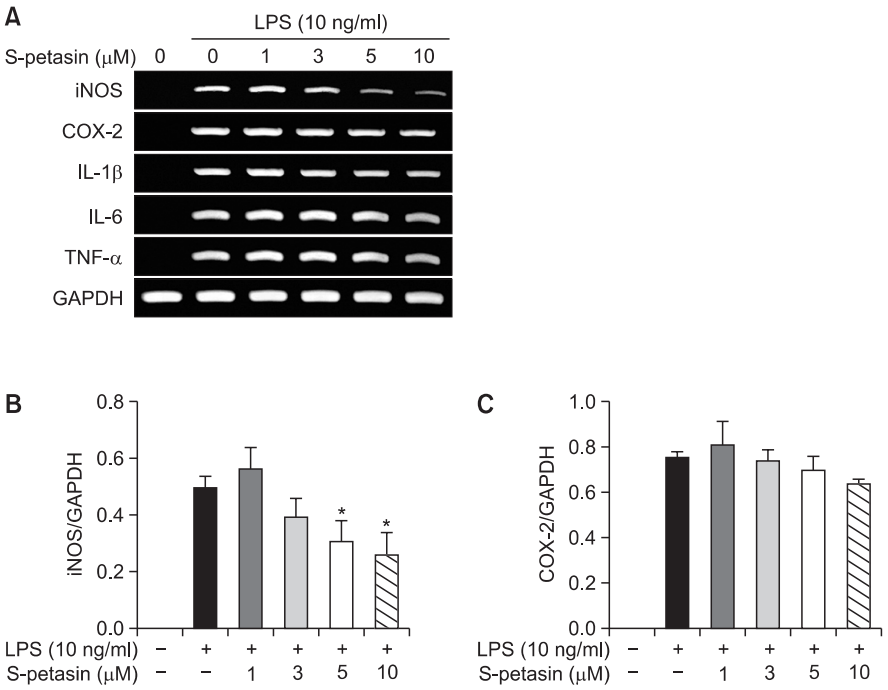

Fig. 5. S-petasin inhibited iNOS expression at mRNA level in mouse peritoneal macrophages. Mouse peritoneal macrophages were treated with s-petasin and 1 h later stimulated with 10 ng/ml LPS for 5 h for RT-PCR analysis. LPS induces the expressions of iNOS, COX-2, IL-1β, IL-6, and TNF-α at the mRNA level (A). S-petasin dose-dependently inhibited iNOS expression at the mRNA level (A, B). Results are representative of three independent experiments that yielded similar results (A). iNOS (B) and COX-2 (C) mRNA levels were quantitated by using ratios to GAPDH mRNA levels. Values are the means ± SEs (n=3). Statistical significance: **p*<0.05 vs. LPS-treated group.

In the LPS-induced peritonitis model, resident macrophages recognize LPS as a bacterial marker, become activated, and give signals to start inflammation. Therefore, suppression of macrophage activation could result in less accumulation of inflammatory cells. The total numbers of peritoneal cells accumulated by LPS stimulation were decreased by s-petasin treatment by 36% (Fig. 8A). Precisely, PMNL accumulation was significantly inhibited about 37% by s-petasin and MNL by 42% but not macrophages (Fig. 8B).

DISCUSSION

The genus *Petasites* has been reported to have anti-allergic and anti-inflammatory effects (Fiebich *et al*., 2005; Lee *et al*., 2011; Zhang *et al*., 2011). However, the active ingredients and their action mechanisms responsible for the effects of the extracts of *Petasites hybridus* have not been fully elucidated. For example, anti-allergic effect of *P. hybridus* extracts was previously suggested to be due to L-type Ca^{2+} channel blockage by petasin and isopetasin (Bickel et al., 1994; Thomet *et al*., 2001; Resnati *et al*., 2002; Fiebich *et al*., 2005; Wang *et al*., 2010). However, neither petasin nor isopetasin inhibited COX-2 enzyme activity *in vitro* (Fiebich *et al*., 2005). Petasin has been shown to inhibit $[Ca^{2+}]_i$ increases and the productions of leukotriene B_4 and cysteinyl leukotrienes in eosinophils and neutrophils (Thomet *et al*., 2001). Furthermore, *P. hybridus* extracts have been reported to inhibit cysteinyl leukotriene biosynthesis in isolated peritoneal macrophages (Bickel *et al*., 1994) and isopetasin and oxopetasan esters, but not petasin, from *P. hybridus* were found to inhibit the biosynthesis of vasoconstrictive cysteinyl leukotrienes (Bickel *et al*., 1994).

We recently identified an anti-allergic component, bakkenolide B in *Petasites japonicus* leaves (Lee *et al*., 2013c). In the present study, we tried to investigate whether s-petasin is responsible for the anti-allergic and anti-inflammatory effects of the genus *Petasites*. We found that s-petasin inhibited antigen-induced degranulation in RBL-2H3 mast cells in vitro (Fig. 3) and inhibited the accumulations of eosinophils, macrophages, and lymphocytes in the BALF samples of ovalbumin-induced asthma mice (Fig. 2). The anti-allergic effect of s-petasin was previously reported in an ovalbumin-induced airway hyperresponsiveness model (Shih *et al*., 2011). However, the effect of s-petasin on mast cell degranulation has not been reported previously (Shih *et al*., 2011). Shih *et al*. failed to observe a decrease in macrophages counts in the BALF samples of s-petasin-treated mice, but in the present study, macrophage, eosinophil, and lymphocyte counts were reduced. We believe that this difference was probably caused by the different animal protocols used, for example, Shin *et al*. collected BALF on Day 74 after immunization, whereas we collected samples on Day 32. Therefore, inhibitory effects of s-petasin on mast cell degranulation and macrophage functions are for the first time investigated in this study.

Inhibitory effect of s-petasin on $[Ca^{2+}]_i$ was previously shown to occur in rat aortic smooth muscle cells via blockage of voltage gate Ca^{2+} channels (Resnati *et al*., 2002; Sheykhzade *et al*., 2008), and subsequently, s-petasin was found to blockage L-type voltage-gated Ca^{2+} channels in rat atria, cardiac myocytes, and in NG108-15 neuronal cells (Wu *et al*., 2003; Wang

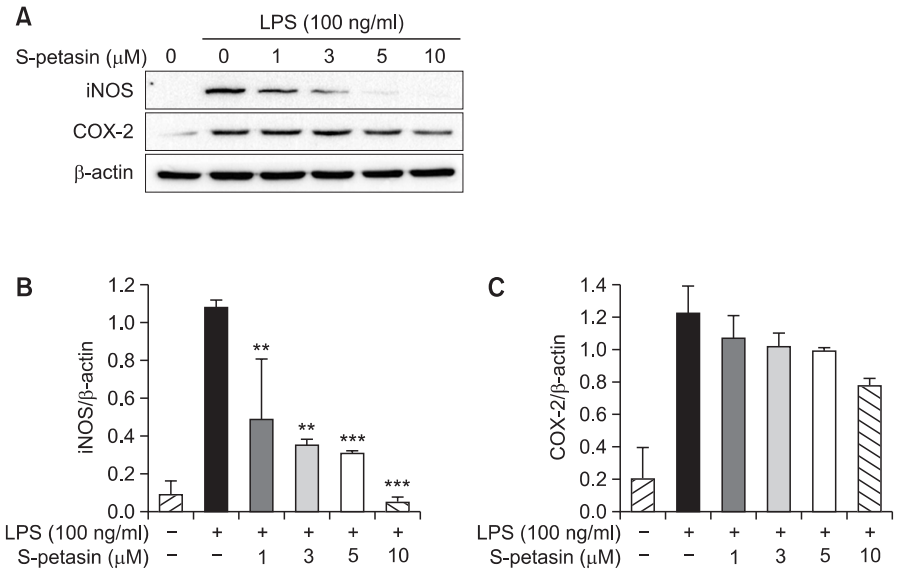

Fig. 6. S-petasin inhibited iNOS expression at protein level in mouse peritoneal macrophages. Mouse peritoneal macrophages were treated with s-petasin and 1 h later stimulated with 100 ng/ml LPS for 24 h for protein analysis. LPS induced the expressions of iNOS and COX-2 at the protein level (A), and s-petasin pretreatment dose-dependently inhibited the induced expression of iNOS at the protein level (A, B). Results are representative of three independent experiments that yielded similar results (A). Protein levels were quantitated by using ratios to β-actin protein levels (B, C). Values are the means ± SEs (n=3). Statistical significance: ***p*<0.01, ****p*<0.001 vs. LPS-treated group.

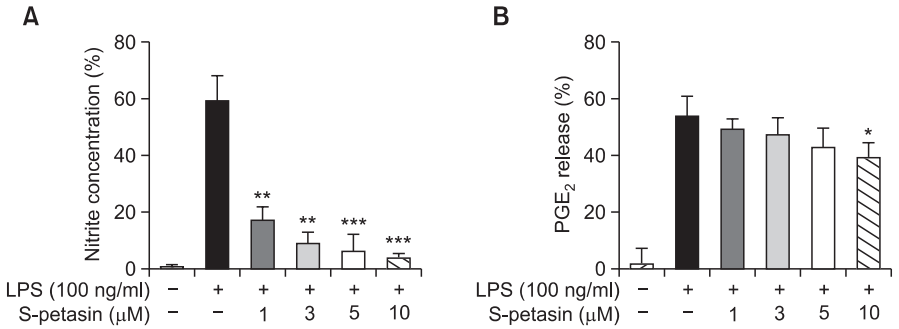

Fig. 7. S-petasin inhibited the productions of NO and prostaglandin E_2 in mouse peritoneal macrophages. Mouse peritoneal macrophages were treated with s-petasin and 1 h later stimulated with 100 ng/ml LPS. LPS-induced productions of nitrite and PGE_2 were measured. S-petasin was found to dose-dependently inhibit nitrite production (A) and PGE_2 production (B). Results are the means ± SEs of three independent experiments in triplicate. Statistical significance: **p*<0.05, ** *p*<0.01, ****p*<0.001 vs. LPS-treated group.

et al., 2004) and to block $Ca_v2.1$ channel (Horak *et al*., 2009). Therefore, we studied the $[Ca^{2+}]_i$ in RBL-2H3 mast cells, because increase of $[Ca^{2+}]_i$ is an important step in the process of degranulation. However, s-petasin was not found to inhibit increase in $[Ca^{2+}]_i$ by antigen treatment in these cells (Fig. 4). Antigen-induced Ca^{2+} increase in mast cells was proposed to be mediated through a sphingosine kinase dependent pathway and may be independent to voltage-gated Ca^{2+} channels (Ryu *et al*., 2009). Therefore, we may not be able to observe inhibitory effect of s-petasin on Ca^{2+} rise in mast cells. Although we couldn't find how s-petasin inhibited degranulation in mast cells, a possibility of that s-petasin inhibits Ca^{2+} channel or Ca^{2+} rise in mast cells was excluded.

Anti-inflammatory effects could contribute to anti-asthma responses, because asthma is a common chronic inflammatory disease of the lung (Wills-Karp, 2004). The pathogenesis of asthma is associated with increased infiltration of inflammatory cells and excessive mucus secretion into airways (Wills-Karp, 2004). Especially, macrophage accumulation was significantly inhibited by s-petasin in the ovalbumin-induced asthma model experiment (Fig. 2). Therefore, we focused on effects of s-petasin on macrophage functions. In the present study, we found that s-petasin inhibited LPS-induced iNOS induction completely at the protein level and NO production in mouse peritoneal macrophages (Fig. 6, 7). Although there was a tendency of inhibition on COX-2 by s-petasin, it was not significant except PGE_2 production inhibition by 10 μM of s-petasin (Fig. 7). Therefore, suppression of iNOS expression and NO production by s-petasin in macrophages play a major role in anti-inflammatory effects in not only in vivo peritonitis model but also in vivo asthma model. In the experiment of LPS-induced peritonitis model, among PMNL, neutrophils are the mainly recruited cells at peritoneal cavity between 6-24 h. Macrophages are usually recruited during 48-96 h after LPS

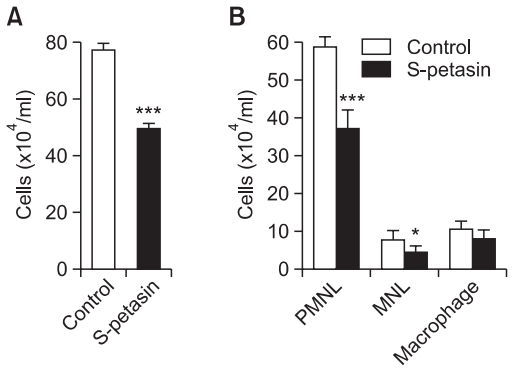

Fig. 8. Effect of s-petasin on LPS-induced peritonitis in mice. C57BL/6 mice were treated with vehicle (n=6) or s-petasin (n=6) at 1 mg/kg by intraperitoneal injection, and 1 h later, LPS (1 mg/kg, i.p.) was administrated. After 24 h, mouse peritoneal cells were collected, stained using the May-Grünwald & Giemsa method, and counted. (A) Total cell counts in the peritoneal fluids of LPS-induced peritonitis mice (control), and s-petasin (1 mg/kg)-treated mice with LPS-induced peritonitis (S-petasin). (B) Cells counts of polymorpho nuclear leukocytes (PMNL), mononuclear lymphocytes (MNL), and macrophages from the peritoneal cavity fluids of LPS or s-petasin (1 mg/ml)+LPS treated mice. Statistical significance: *p<0.05, ***p<0.001 vs. LPS-treated control group.

treatment in order to clean up apoptotic neutrophils and resolve inflammation. Because we counted accumulated cells in peritoneal cavity at 24 h after LPS treatment, no significant change of macrophage numbers was observed (Fig. 8). Although expression of IL-1β, IL-6 and TNF-α was also investigated, but there was no significant suppression by s-petasin (Fig. 5). It is necessary to investigate in the future why iNOS was mainly suppressed by s-petasin.

In conclusion, we found that s-petasin is an active ingredient in *Petasites* genus extracts with known anti-allergic and anti-inflammatory effects. In addition, s-petasin inhibitions of degranulation in mast cells and of the induction of iNOS in macrophages probably contribute to effects, especially regarding the accumulation of immune cells in BALF and peritoneal cavity.

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