Anti-inflammatory effects of *Scutellaria baicalensis* extract via suppression of immune modulators and MAP kinase signaling molecules

Eun Hye Kim, Bumsang Shim, Seunghee Kang, Gajin Jeong, Jong-soo Lee, Young-Beob Yu, Mison Chun

**Aim of the Study:** A herbal preparation using *Scutellaria baicalensis* (S. baicalensis) Georgi (Huang Qin, SB) was formulated to effectively protect cancer patients from inflammatory reactions. Although SB, is one of the most widely used herbs in oriental medicine for anti-inflammation, anti-cancer, anti-viral, antibacterial and tonifying the immune response, the underlying mechanism(s) by which these effects are induced remains unclear.

**Results:** Here, we report that SB displays anti-inflammatory effects in a zymosan-induced mouse air-pouch model by reducing the expression of nitric oxide (NO), inducible NOS (iNOS), Cyclooxygenase2 (COX-2), Prostaglandin E2 (PGE2), Nuclear Factor-kappaB (NF-κB) and IkBα as well as inflammatory cytokines, such as IL-1β, IL-2, IL-6, IL-12 and TNF-α. In a similar manner, SB also reduced the production of nitric oxide, PGE2, IL-1β, IL-2, IL-6, IL-12 and TNF-α, by decreasing the expression of iNOS, COX-2, IkB kinase αβ (IKKαβ) phosphorylation, IkBα and IkBβ phosphorylation in LPS-treated Raw 264.7 cells. Additionally, SB interfered with the nuclear translocation of NF-κB p65 and p50, resulting in NF-κB-dependent transcriptional repression. We further demonstrate that SB attenuated the activity of c-Raf1/MEK1/2, Erk1/2, p38 and JNK phosphorylation in LPS-treated Raw 264.7 cells.

**Conclusions:** Taken together, these results confirm the strong anti-inflammatory properties of SB by inhibition of iNOS, COX-2, PGE2, IL-1β, IL-2, IL-6, IL-12 and TNF-α expression. This was achieved through the down-regulation of IKKαβ, IkBα, NF-κB activation via suppression of c-Raf1/MEK1/2 (Mitogen-activated protein kinase/ERK kinase) and MAP kinase phosphorylation in the zymosan-induced mice air-pouch and Raw 264.7 cells. These results support the use of SB herbs for its potent anti-inflammatory activity.

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**1. Introduction**

The search for naturally occurring anti-cancer and anti-inflammatory agents effective for the treatment of many human diseases is currently a worldwide pursuit. Pharmacological options for the treatment of inflammatory diseases that are often chronic are associated with severe side effects. Thus the search for less toxic yet equally efficacious compounds is an area of intense research (McKellar et al., 2007). A myriad of studies have already been conducted to screen the potent mediators of anti-inflammatory responses, as well as many compounds, despite some unfavorable effects, from clinical practice (Sonis, 2002, 2004; Molla and Panes, 2007; Rödel et al., 2007). Particular attention was recently focused on ways of decreasing anti-cancer treatment related to toxicity, which is mainly linked to inflammatory reactions. *Scutellaria baicalensis* (S. baicalensis; SB) Georgi, whose Chinese name is Huang-qin, is one of the most widely used traditional Chinese herbal medicines. Traditionally, its roots have been used in the treatment of inflammation, cancer, bacterial and viral infections of the respiratory and gastrointestinal tract. It has also been used in reducing the temperature, moistening aridity, purging fire, detoxifying toxicity and reducing total cholesterol and blood pressure levels. This herb also possesses cholagogic, diuretic, and cathartic properties (Li et al., 2004; Takashi et al., 2007). Many herbal preparations containing SB as the major ingredient in their prescriptions are traditionally used as herbal medicines in China, Japan and Korea. Studies have reported that SB can inhibit the growth of breast, hepatocellular, pancreatic, prostatic, urothelial and colon cancer cells in vitro (Motoo and Sawabu, 1994; So et al., 1997; Ikemoto et al., 2000; Ye et al., 2002). SB can also inhibit lipid peroxidation in rat liver...
(Kimura et al., 1981, 1982) and attenuate oxidative stress in cardiomyocytes (Shao et al., 1999). Moreover, studies have suggested that the anti-cancer effects of SB may be attributable to the inhibition of PGE2 production by suppressing COX-2 activity in some cancer cells and inflammation (Xu et al., 1989; Zang et al., 2003).

The activation of c-Raf-1 is regulated by phosphorylation of the Ser 259 and Ser 338 sites (Morrison and Cutler, 1997) and induces phosphorylation and activation of MEK1/2, followed by activation of ERK1/2 (Kolch, 2000). A major role of MAP kinase is the transmission of extracellular signals to the nucleus, where the transcriptions of specific genes are induced by phosphorylation and activation of transcription factors. The MAP kinase signaling cascade plays a critical role in innate immune responses (Dong et al., 2002). In response to inflammatory signals including microbial or viral infection, UV-irradiation, and burning, the MAP kinase cascade is activated through phosphorylation of p38, Erk1/2, and JNK, together with activation of NF-κB (Sumbayev and Yasinska, 2006). These are known to regulate the production of anti-inflammatory cytokines following stimulation with LPS, peptidoglycan, or dexamethasone (Hammer et al., 2005; Chi et al., 2006). This reports suggest that c-Raf-1, MEK1/2 and MAP kinase may play a role in the regulation of inflammation and is a promising possible target for anti-inflammatory drugs.

To gain insight into the anti-inflammatory effect of SB and its mechanisms of action, we investigated whether, and how, SB was linked to the regulation of anti-inflammation in zymosan air-pouch mice and Raw 264.7 cells. We also evaluated the kinetics of leukocyte recruitment, level of cytokines, prostaglandin E2 (PGE2), iNOS, COX-2, c-Raf-1 (Ser 259 and Ser 338), MEK1/2, MAP kinase, IKKα, IkBα and NF-κB. This study highlights the potential role of SB in anti-inflammation and provides a molecular mechanism for the pharmacological action of SB.

2. Material and methods

2.1. Preparation of the S. baicalensis Georgi extracts

The S. baicalensis Georgi sample was purchased from the Korean Association of Crude Medicinal Herbs. S. baicalensis Georgi specimen had been taxonomically identified with respect to its morphology by Professor Hocheol Kim, a herbalist at Kyunghee University. The voucher specimen (S. baicalensis Georgi, No. 071010) has been deposited at the Herbarium of College of Oriental Medicine, Kyunghee University, Korea.

A dried sample of SB (500 g) was cut in pieces and refluxed with 5 l of distilled deionized water twice for 12 h each at room temperature. The aqueous solution was filtered and evaporated under vacuum to yield a dried powder extract. The aqueous extract was partitioned in succession with 100 ml of hexane, chloroform, ethyl acetate and butanol to obtain a dried hexane-soluble fraction, chloroform-soluble fraction, ethyl acetate-soluble fraction and butanol-soluble fraction. Finally, the water-soluble fraction was discarded. The butanol fraction was concentrated at 60 °C under vacuum using an evaporative system (Eyela, Japan). Yield of SB powder extract was obtained 26.64%. This SB powder extract was dissolved in PBS (phosphate buffered saline).

2.2. Quantitative analysis of active compounds of S. Baicalensis by HPLC

The qualitative and quantitative analysis of unique compounds in the SB butanol extract was accomplished under the analytical conditions described below. Baicalin, baicalein, wogonoside and wogonin marker substances of SB were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). To prepare a standard marker substance solution, baicalin, baicalein, wogonoside and wogonin were accurately weighed and dissolved in 50% acetonitrile in 0.1 M phosphoric acid to give various concentrations within the range of 12.5–285 μg/ml. The lyophilized powder (100 mg) of SB butanol extract was dissolved in 50% acetonitrile in 0.1 M phosphoric acid (5 ml) and was sonicated for 10 min. After filtration through a 0.45 μm PVDF filter (Whatman, Philadelphia, PA), 10 μl aliquots were injected for analysis. The HPLC system consisted of a dual-solvent delivery pump (Shimadzu SIL-10AF, Japan), a diode-array UV/VIS multi-wavelength detector (Shimadzu SPD M10Avp), and a system controller (Shimadzu SCL-10Avp). The signals from the detector were collected and analyzed using a computer equipped with the software of LCsolution (ver.1.21 sp1). Separations were carried out on Gemini C18 110A column (particle size 5 μm, 4.6 × 250 mm i.d., Phenomenex, Torrance CA). The mobile phase was composed of 35% acetonitrile in 0.1 M phosphoric acid with isocratic elution at a flow rate of 1 ml/min. The solvents were filtered through a 0.45 μm Millipore filter (Bedford, MA) and degassed prior to use. The operating temperature was maintained at room temperature.

2.3. Animals

All experiments were carried out according to the Guidelines for the care and use of laboratory animals, approved by United States National Institutes of Health. Female Balb/c mice weighing 19–20 g (12–13 weeks old) were obtained from the Core Breeding Center, and maintained thereafter at the Animal Resource Facility of the Department of Biological Sciences, Seoul National University. Mice were provided with a nutritionally balanced rodent diet (Joong-ang Laboratory Animal Center, Seoul, Korea) and water ad libitum; the animals were maintained under standard conditions. The minimum sample size per group, in all animal experiments, was 10.

2.4. Zymosan-induced mouse air-pouch model

We randomly divided 40 female Balb/c mice into 4 groups: 1 negative control group, 1 control group and 2 experimental groups. S. baicalensis Georgi (SB) extract (experimental groups, at 500 or 750 mg/kg/day) and PBS (control group) samples were administered orally for 10 d before induction of the air pouch. The air pouch was induced by subcutaneous injection of 5 ml of filtered air (0.45 Mm PVDF syringe filter and a 24-gauge syringe) on the back of the mice on the first day. On the fourth day, an additional 5 ml of filtered air was injected again into the pouch. On day 7, the 1-ml aliquots of 1% solution of sterile zymosan (Sigma Chemical Co., St. Louis, MO, USA) were injected into the pouch. After 6 d, the animals were anesthetized with ether, and the pouch was flushed out with 2 ml of heparinized saline (Sigma Chemical Co., St. Louis, MO, USA) saline (Konno and Tsurufuji, 1983; Yoshino, 1995). Whole leukocytes were recovered from each pouch and counted using a hemocytometer.

2.5. Cell culture and treatment

The murine macrophage cell line, Raw 264.7 cells, were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco-Invitrogen, Grand Island, NY) supplemented with 10% FBS (Hyclone, Logan, UT). The cells were grown in six-well plates at a density of ~1 × 10⁴ cells per well and allowed to grow in DMEM containing 10% FBS for 24 h. Cells were incubated with the SB 300 μg/ml or 100 μM curcumin (Sigma Chemical Co., St. Louis, MO, USA) and stimulated...
with 5 μg/ml LPS (Escherichia coli 011:B4, Sigma Chemical Co., St. Louis, MO, USA) for 12 h, 24 h and 48 h.

2.6. Measurement of nitric oxide (NO)

Nitric oxide production was monitored by the assessment of nitrite concentrations accumulated in the exudates. Briefly, the exudates obtained were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylenediamine dihydrochloride, and 2% phosphoric acid) in a 96-well ELISA plate. The optical density at 540 nm was estimated using an ELISA plate reader (ThermoMax; Molecular Devices, Sunnyvale, CA, USA). The total amount of NO production was determined from the pooled exudates of 10 mice from each group by comparing the optical density with the standard curve obtained with NaNO₃.

2.7. RNA isolation and RT-PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer’s instructions. Total RNA (3 μg) was reverse-transcribed into cDNA by using commercially available cDNA synthesis kits (PrimeScript™ One Step RT-PCR Kit Ver. 2; Takara Bio, Japan). The tubes were incubated at 37 °C for 30 min and then at 42 °C for 30 min followed by heating at 95 °C for 2 min. The samples were then stored at −20 °C until further use. RT-PCR was carried out using 1 μg of cDNA. The primer sequences for β-actin, iNOS, COX-2, IL-1β, IL-2, IL-6, IL-12, TNF-α, NF-κB and IκBα are as follows: β-actin, sense 5′-TGG ATT CCT GTG GCA TTC ATG AAA C-3′, and antisense 5′-TAA AAC GCA GCT CAG TTA CAC TGG G-3′; iNOS, sense 5′-CTG AGG GCT CTG TTT AGG TC-3′, and antisense 5′-CCT TGT TCA GCT ACG CCT TC-3′; COX-2, sense 5′-GGA GAG ACT ATC AAG ATA GTG ATC-3′, and antisense 5′-ATG GTC AGT AGA ATA GTA ATC-3′; NF-κB, sense 5′-GGA AGT ACT AAG AGT GGC TAA G-3′, and antisense 5′-TGA GAG AGT GCC CAG ACC-3′; IκBα, sense 5′-AAG CTC TAC AGG CCA AGC-3′, and antisense 5′-ATC CTT GGG AGT TTC AGG-3′; IL-6, sense 5′-TGG AGT CAT AGA AGG AGT GCC TAA G-3′, and antisense 5′-TCT GAC CAC CAT GAG CAA TGG CCA C-3′; IL-12, sense 5′-GTC AGG ACG CGG GTG GTG AG-3′, and antisense 5′-ACC TCA GTT TGG CCA CGG GC-3′; TNF-α, sense 5′-GCC AGG ACC TGG ACC AAG-3′, and antisense 5′-TCC ATG GTG TGG GCC AGG ACC-3′; NF-κB, sense 5′-ACT CTT ACT CGC CTC CCT CT-3′, and antisense 5′-GTC TCT TTC TAC CTC GTG GC-3′; and IκBα, sense 5′-AGG TCA TGG GTG AGG AGT AGG-3′, and antisense 5′-TTG TAG GTT ACC GTG TTC AC-3′. The samples were placed in the thermal cycler (MJ Mini, Bio-Rad, Richmond, CA, USA) and initially denatured at 94 °C for 3 min. These were then denatured at 94 °C for 30 s; annealed at 54 °C (β-actin primer), 64 °C (iNOS primer), 54 °C (IL-1β and TNF-α primers), 56 °C (IL-2 primer), 53 °C (IL-12 primer), or 55 °C (IL-6, NF-κB, and COX-2 primers) for 30 s and extended at 72 °C for 30 s in each cycle. The final extension time was 6 min at 72 °C. We optimized all PCR condition for each primer set. Twenty microliters of the PCR reactants were analyzed using 1% agarose gel electrophoresis. Amplified cDNA bands were detected by ethidium bromide staining, and the amount of mRNA was evaluated by densitometry. The amplification was repeated for 30 cycles for all immune modifiers.

2.8. ELISA

The IL-1 β, IL-2, IL-6, IL-12 and TNF-α concentrations in the air pouch exudates and cell culture supernatant were determined by using IL-1 β, IL-2, IL-6, IL-12 and TNF-α ELISA kits (R&D Systems Europe Ltd., UK), according to the manufacturer’s instructions. All samples were assayed in duplicate, and equivocal results were repeated. The cytokine concentration was calculated from a standard curve of the corresponding recombinant mouse cytokine.

2.9. Prostaglandin E2 (PGE2) assay

Prostaglandin E2 (PGE2) concentrations in the air pouch exudates and cell culture supernatants were determined using the PGE2 assay kit (R&D system Europe Ltd., UK), according to the manufacturer’s instructions. All samples were assayed in duplicate, and equivocal results were repeated.

2.10. Preparation of extract and immunoblotting

Raw 264.7 cells were grown in six-well plates at a density of ∼2 × 10⁶ cells per well and incubated with 300 μg/ml SB or 100 μM curcumin (Sigma Chemical Co., St. Louis, MO, USA) and then stimulated with 5 μg/ml LPS (Escherichia coli 011:B4, Sigma Chemical Co., St. Louis, MO, USA) for 60 min. The cells were collected by centrifugation and washed once with PBS. The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM Na fluoride and 0.5 mM Na orthovanadate) containing 5 μg/ml each of leupeptin and aprotinin and incubated for 40 min at 4 °C. Cell debris was removed by micro centrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using a Bio-Rad protein assay reagent according to the manufacturer’s instructions. Nuclear extracts were prepared as described previously (Like et al., 2004) to detect NF-κB p65 and p50, iNOS, COX-2, c-Raf-1 (Ser 259 and Ser 338), MEK1/2, MEK1/2 phosphorylation, IKKαβ, IKKαβ phosphorolayion, IκBα, IκBβ phosphorylation, Erk1/2, p38 and JNK, Erk1/2, p38 and JNK phosphorylation, NF-κB p65 and p50 was detected by immunoblotting with anti-iNOS, anti-COX-2, anti-c-Raf-1 (Ser 259 and Ser 338), anti-phospho-c-Raf-1 (Ser 259 and Ser 338), anti-phospho-MEK-1, anti-phospho-MEK, anti-phospho-IKKαβ, anti-phospho-IKKαβ, anti-phospho-IκBα, anti-phospho-IκBα, anti-phospho-IκBβ, anti-phospho-IκBβ, anti-phospho-IκBβ, anti-phospho-IκBβ, anti-phospho-IκBβ, anti-phospho-IκBβ, anti-phospho-IκBβ, anti-phospho-IκBβ, anti-phospho-IκBβ, anti-phospho-IκBβ, and anti-phospho-IκBβ p50 antibodies (Cell Signaling Technology, Beverly, MA). The immunoreactive band was normalized to that of the control anti-β-tubulin, anti-lamin A/C antibodies (Cell Signaling Technology, Beverly, MA). All blots were developed using ECL reagents (Amer sham, Little Chalfont, UK).

2.11. Statistical analysis

All data are expressed as the mean ± S.E.M. The data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan’s test for multiple comparisons. Statistical significance was set at “p < 0.05,” “p < 0.01.”

3. Results

3.1. Quantitative analysis of active compounds in SB

Representative chromatogram (275 nm) and UV spectrum (Fig. 1) of baicalin, baicalein, wogonoside and wogonin in SB butanol extract were acquired using the HPLC-DAD system. We created a library of known spectra from existing standards and identified peaks by comparing the spectra from unknown peaks to the spectra from standards. Based on HPLC retention time characteristics and the UV-vis spectra of standard compounds in a library, the components of the SB butanol extracts were identified as baicalin, baicalein, wogonoside and wogonin (Fig. 1A).
The amount of baicalin, baicalein, wogonoside and wogonin active molecules in SB butanol extracts was calculated as 17.240%, 1.857%, 3.25% and 0.256% (Fig. 1A).

3.2. Anti-inflammatory effect of SB on zymosan-induced inflammation

We first tested the inhibitory effects of SB on the inflammatory response induced in the air pouch. Inflammation was induced by the administration of zymosan into the air pouches that had previously been produced on the back of mice administered with either PBS or SB as described under Section 2 (Fig. 2A). The mice were closely observed for changes in their body weight and external features including for characteristics, behavior, ability to exercise, and appetite. It was revealed that pretreatment with SB had significant effect on weight gain in mice (Fig. 2B). There was no change in the external features following pretreatment with SB (data not shown). Injection of zymosan into the air pouch induced fluid accumulation and leukocyte infiltration. After SB administration, a statistically significant reduction in leukocyte infiltration was noted (Fig. 2C), and this reduction was believed to be dose dependent. Administration of SB at 500 mg/kg/day reduced the number of leukocytes accumulated in the pouch by approximately 70%, as compared to the control group. Higher dose administration of SB (750 mg/kg/day) reduced the accumulation of fluid and leukocyte by approximately 85%.

3.3. Suppression of synthesis of inflammatory mediators by administration of SB

The effect of SB administration on NO production was evaluated after induction of inflammation. NO levels in the exudates were measured as an index of inflammatory mediators. NO production in the air pouch was significantly inhibited by approximately 77–86% in a dose-dependent manner upon oral administration of SB (Fig. 3A). Furthermore, based on this result, we tested whether SB affected the expression level of inducible nitric oxide synthase (iNOS). RT-PCR analysis showed that administration of SB markedly down-regulated iNOS expression in the zymosan-induced air pouch exudates (Fig. 3C). Next, we examined whether administration of SB exerted an effect on the expression of COX-2, which plays a role in the production of prostaglandins. The results indicate that SB inhibited COX-2 expression that was induced by zymosan-induced inflammation and that this suppression was dose...
dependent (Fig. 3C). The reduction in expression of COX-2 was apparent in the air pouch exudates from the group pretreated with a higher dose of SB (Fig. 3C).

Prostaglandin E2 (PGE2), the most important inflammatory product of cyclooxygenase-2 (COX-2) was quantified in exudates of zymosan-induced inflammation. PGE2 production in the air pouch was significantly and dose-dependently inhibited by approximately 60–80% upon oral administration of SB (Fig. 3B). It is likely that SB reduced the production of NO and prostaglandins, at least in part, by reducing the expression of the key enzymes (i.e., iNOS, COX-2 and PGE2) responsible for their synthesis.

3.4. Down-regulation of pro-inflammatory cytokines by administration of SB

To examine whether pretreatment with SB exerted an effect on the expression of pro-inflammatory cytokines, the total concentrations of IL-1β, IL-6 and TNF-α were measured by ELISA (Fig. 4A–C) and their transcription levels were analyzed using RT-PCR (Fig. 4F). We demonstrate that SB clearly reduced the expression level of the IL-1β protein by 77% at a 500-mg/kg/day dose and by 87% at a dose of 750 mg/kg/day (Fig. 4A). In similar manner, 500 mg/kg/day of SB attenuated the levels of IL-6 protein in the zymosan-induced inflammation air-pouch model by 53%, and by 73% at a dose of 750 mg/kg/day (Fig. 4B). Additionally, 500 mg/kg/day of SB reduced the level of TNF-α protein by 70% in the air-pouch and by 81% at a dose of 750 mg/kg/day (Fig. 4C).

Next, we tested whether SB inhibited the synthesis of IL-1β, IL-6 and TNF-α by RT-PCR analysis. Consistent with the ELISA results, the production of IL-1β, IL-6 and TNF-α mRNAs was reduced in the zymosan-induced inflammation of the air-pouch after SB pretreatment (Fig. 4F). In the air-pouch exudates from mice treated with SB at the higher dose (750 mg/kg/day), the expression of IL-1β, IL-6 and TNF-α was significantly inhibited. Together with the ELISA and RT-PCR results (Fig. 4A–C, F), SB appears to mediate an anti-inflammatory response by suppressing the production of pro-inflammatory cytokines.

3.5. Down-regulation of inflammatory cytokines by administration of SB

The influence of pretreatment with SB on the expression of inflammatory cytokines was analyzed. We measured the levels of IL-2 and IL-12 by ELISA (Fig. 4D and E) and RT-PCR (Fig. 4F). IL-2 plays a crucial role in survival signals and cytokine production of effector T cells as well as inhibitory signals for the proper regulation of the inflammatory response. Also, the pleiotropic activity of IL-2 in the context of inflammation is evident in its variety of target cells, participation in pro-inflammatory and regulatory signal pathways as well as in the relationship with other cytokines (Ruth et al., 2008). IL-12 is a heterodimeric cytokine (70-kDa) composed of a p35 and a p40 subunit and is produced primarily by antigen-presenting cells such as macrophages and dendritic cells (Hölscher, 2004). This cytokine presents important physiological regulators in vivo for cellular response to microbial infected inflammation (Hunter, 2005). We demonstrate that SB reduced the level of IL-2 proteins by 55% at 500 mg/kg/day dose and by 72% at a dose of 750 mg/kg/day (Fig. 4D). In a similar manner, 500 mg/kg/day of SB attenuated the level of IL-12 protein in the zymosan-induced inflammation air-pouch model by 48%, and by 63% at a dose of 750 mg/kg/day (Fig. 4E).

Next, we tested whether SB inhibited the synthesis of IL-2 and IL-12 by RT-PCR analysis. Consistent with the ELISA results, production of IL-2 and IL-12 mRNA was reduced by administration of SB in the zymosan-induced inflammation air-pouches. In the exudates from the mice treated with SB at a higher dose (750 mg/kg/day), the mRNA expression levels of IL-2 and IL-12 were significantly inhibited (Fig. 4F). On the basis of these results, we postulate that SB mediates anti-inflammatory effects at the stage of inflammatory cytokine transcription.

3.6. Reduction in expression of NF-κB and I-κBa by administration of SB

We evaluated the mRNA levels of NF-κB and the endogenous NF-κB target genes, in the presence or absence of SB. Pretreatment
with SB potently suppressed the expression of the NF-κB and IkBα genes (Fig. 4F). These observations illustrate that SB can inhibit NF-κB-driven gene expression following inflammation, in addition to the transcription of various pro-inflammatory genes. This implies that pretreatment of the animals with SB inhibited not only NF-κB-driven gene expression that occurred after inflammation but also the transcription of various pro-inflammatory genes.

3.7. SB suppresses synthesis of inflammatory mediators in LPS-stimulated Raw 264.7 cells

We selected an SB concentration of 300 μg/ml as an effective dose for in vitro studies after trials, using various concentrations of SB (data not shown). Another natural product, Curcumin, is known to exert anti-inflammatory effects. For the in vitro study, we used both SB and Curcumin to evaluate the degree of anti-inflammation. Curcumin at 100 μM was used as the positive control. The level of NO in each of the LPS-stimulated Raw 264.7 cells was measured as an index of the inflammatory response. SB (300 μg/ml) was able to significantly inhibit LPS-induced NO production compared to Curcumin (100 μM) in a time-dependent manner (Fig. 5A).

Prostaglandins (PGE2) play an important role in the inflammatory process (Hennebert et al., 2008). To test whether SB inhibited PGE2 production, LPS-stimulated Raw 264.7 cells were treated with 300 μg/ml of SB for 12, 24 and 48 h. SB also inhibited the production of PGE2, in a time-dependent manner (Fig. 5B). COX-2, a key enzyme in the synthesis of prostaglandins, plays an important role in the inflammatory process. RT-PCR and Immunoblotting analysis were performed to determine whether the inhibitory effects of SB on anti-inflammatory mediators, such as NO and PGE2, were modulated by the expression of iNOS and COX2. SB markedly attenuated iNOS and COX-2 expression, in comparison to 100 μM Curcumin in LPS-stimulated Raw 264.7 cells (Fig. 5C and D).

3.8. Down-regulation of pro-inflammatory cytokines and inflammatory cytokines by treatment of SB in LPS-stimulated Raw 264.7 cells

Since SB showed strong inhibition of NO and PGE2 in LPS-stimulated Raw 264.7 cells, we also measured the levels of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α by ELISA at different time points. We have demonstrated that 300 μg/ml of SB significantly reduced the protein expression levels of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α in comparison to 100 μM of Curcumin, in a time-dependent manner (Fig. 6A–C). We further analyzed the effect of SB (300 μg/ml) on the expression of inflammatory cytokines. We measured the levels of IL-2 and IL-12 using ELISA. SB at this concentration significantly reduced the level of IL-2 and IL-12 in comparison to 100 μM of Curcumin, in a time-dependent manner (Fig. 6D and E).

3.9. SB treatment attenuates phosphorylation of c-Raf, MEK1/2 and MAP kinase activity in LPS-stimulated Raw 264.7 cells

To evaluate the possible mechanisms involved in the anti-inflammatory effects of SB in vivo (Fig. 2), LPS-induced inflammatory responses were studied in cultured Raw 264.7 cells. Activation of c-Raf-1 has been reported to induce modest IκBα degradation by enhancing basal IKKα/β activity as well as regulating the MEK/ERK signaling cascade (Liu et al., 2001; Kyriakis et al., 1992). Also, MEK1/2 has been shown to play a critical role in the activation of the NF-κB signaling pathway (Chakravortty et al., 2001). We thus investigated the effect of SB on the phosphorylation of c-Raf-1 (Ser259 and Ser 338) and MEK1/2 in LPS-stimulated macrophage Raw 264.7 cells following treatment with SB (300 μg/ml). The results indicated that LPS activated c-Raf-1 (Ser259 and Ser 338) and MEK1/2 by including their phosphorylation (Fig. 7A and B). However, SB (300 μg/ml) reduced the LPS-induced c-Raf-1 (Ser259 and Ser 338) and MEK1/2 phosphorylation (Fig. 7A and B). We also show that the total protein levels of c-Raf-1 and MEK1/2 were not changed by LPS or SB alone or in combination (Fig. 7A and B). The assays indicated that SB directly inhibited phosphorylation of c-Raf-1 (Ser259 and Ser 338) and MEK1/2 as the upstream molecules of ERK1/2 in LPS-stimulated Raw 264.7 cells (Fig. 7A and B). SB was also shown to significantly reduce phosphorylation of c-Raf-1 (Ser259 and Ser 338) and MEK1/2 compared to 100 μM of Curcumin in LPS-stimulated Raw 264.7 cells (Fig. 7A and B).

It is known that inflammation is triggered by intracellular signaling pathway events, which involve the activation of MAP
In order to further understand the underlying mechanisms of SB-mediated anti-inflammation, we studied whether SB inhibited LPS-triggered activation of the MAP kinase signaling molecules including p38, Erk1/2, and JNK. The active, phosphorylated levels of p38, Erk1/2, and JNK were analyzed in LPS-stimulated macrophage Raw 264.7 cells following treatment with SB (300 μg/ml). The results showed that LPS activated p38, Erk1/2, and JNK by phosphorylation (Fig. 7C). However, treatment of the cells with SB (300 μg/ml) reduced the LPS-induced phosphorylation of p38, Erk1/2, and JNK (Fig. 7C). Furthermore, the total protein levels of p38, Erk1/2, and JNK were not changed by LPS or SB, alone or in combination (Fig. 7C). These observations indicated that SB might inhibit the activation of the p38, Erk1/2, and JNK kinases compared to 100 μM of Curcumin in LPS-stimulated Raw 264.7 cells (Fig. 7C).

3.10. SB treatment inhibits IKKαβ, I-κBα, phosphorylation of I-κBα and translocation of NF-κB in LPS-stimulated Raw 264.7 cells

It has been reported that activation of MAP kinase phosphorylation transduces signals to activate the transcription of NF-κB-mediated pro-inflammatory cytokines (Hammer et al., 2005; Chi et al., 2006). In unstimulated cells, NF-κB is sequestered in the cytosol by its inhibitor, IκB, which is phosphorylated upon LPS stimulation by its inhibitor IκB kinases. The phosphorylated IκB is then ubiquitinated and rapidly degraded via 26 s proteosome, to release NF-κB (Janssen-Heininger et al., 2000). Here, we investigated whether SB (300 μg/ml) inhibited LPS-stimulated degradation of IκBα in Raw 264.7 cells by an immunoblotting assay with anti-IκBα antibody. LPS-induced IκBα degradation was significantly decreased by treatment of the cells with SB. To determine whether this IκBα degradation was linked to IκBα phosphorylation, we tested the effect of SB on LPS-induced phosphorylation of IκBα. It was found that SB also reduced LPS-induced IκBα phosphorylation (Fig. 8A). Since IKKα and IKKβ are the upstream kinases of IκB in the NF-κB signaling pathway and phosphorylate IκBα and IκBβ (Ghosh and Karin, 2002), we examined the effect of SB (300 μg/ml) on LPS-induced IKKαβ activations by immunoblotting using a phosphorylated IKKαβ antibody. LPS strongly induced IKKαβ phosphorylation, whereas SB markedly inhibited this phosphorylation (Fig. 8A). We evaluated the mRNA levels of NF-κB and endogenous NF-κB target genes in the presence or absence of SB. Treatment of the cells with SB (300 μg/ml) reduced the expression of NF-κB and IκBα genes (Fig. 8B).

In addition, we investigated whether SB prevented the translocations of the p50 and p65 subunit of NF-κB from the cytosol to the nucleus after their release from IκBαs. Treatment of cells with SB attenuated p50 and p65 levels in the nuclear fractions, as shown by the immunoblotting studies (Fig. 8C); β-actin, β-tubulin and Lamin A/C were used as an internal controls. These findings indicate that SB may better inhibit NF-κB p50 and p65 activation by suppression of IKKαβ phosphorylation, IκBα phosphorylation and IκBα compared to Curcumin 100 μM in LPS-induced Raw 264.7 cells (Fig. 8C).

4. Discussion and conclusions

In the present report, we explored the potential of SB as an anti-inflammatory herb. Use of SB as an oriental remedy for various illnesses is traditional and popular in many Asian countries.
Fig. 5. (A) Inhibitory effects of SB on nitric oxide (NO), (B) prostaglandin E2 (PGE2) production and (C and D) expression of iNOS, COX-2 in LPS-stimulated Raw 264.7. (A) Raw 264.7 cells were treated with SB (300 μg/ml) or LPS (5 μg/ml) for 24 and 48 h. The amount of NO in the culture supernatant was measured by reader absorbance at 540 nm. (B) Raw 264.7 cells were treated with SB (300 μg/ml) or LPS (5 μg/ml) for 12, 24 and 48 h and assayed for PGE2 production as described in Section 2. Statistical significance was set at *p < 0.05, **p < 0.01. (C and D) The effects of SB on LPS-stimulated iNOS, COX-2 protein and mRNA expression in Raw 264.7 cells. (C) Total RNA was prepared for RT-PCR analysis of iNOS and COX-2 gene expression in Raw 264.7 cells treated with SB (300 μg/ml). (D) Cells were treated with SB (300 μg/ml) and LPS. Total cellular proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected with specific antibodies, as described in Section 2. Density ratio of SB treated-group over control group was measured by densitometer.

Fig. 6. SB inhibits LPS-induced production of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α and inflammatory cytokines, IL-2 and IL-12 in LPS-stimulated Raw 264.7 cells. Raw 264.7 cells were treated with SB or LPS for 12 h, 24 h and 24 h. The levels of IL-1β (A), IL-6 (B), TNF-α (C), IL-2 (D) and IL-12 (E) were measured in duplicate using ELISA. Statistical significance was set at *p < 0.05, **p < 0.01.
Although there are various speculations as to its mode of action, the molecular mechanisms by which SB mediates its immunomodulatory effects were poorly understood until now. The mouse air-pouch model of inflammation has previously been used to investigate the immunomodulatory response to a variety of agents, including zymosan and carrageenan (Kono and Tsurufuji, 1983; Martin et al., 1994; Yoshino, 1995; Kon et al., 1999). This model allows a quantitative assessment of the progress of inflammation and measurement of the formation of chemical mediators such as cytokines (Alessandra et al., 2003). The kinetics of leukocyte recruitment, level of cytokines, prostaglandin E2 (PGE2), NF-κB, and IκBα were evaluated. Here, the air-pouch model used to characterize the acute inflammatory response induced by zymosan. Also, it was demonstrated that the leukocyte infiltration and NO production in the zymosan-induced inflammation in the air pouch of mice were significantly suppressed in the SB-treated groups (Figs. 2B, C and 3A). We showed that SB effectively suppressed inflammation by down-regulating the expression of inflammatory mediators and by reducing the production of inflammatory cytokines in the mouse air-pouch model (Figs. 3B, C and 4).

During the inflammatory processes, large amounts of the pro-inflammatory mediators, NO and PGE2 are generated by inducible iNOS and COX-2, respectively (Lee et al., 1992). Many potent anti-inflammatory agents have been screened; these agents act by inhibiting COX-2, whose expression in various types of human cells is often enhanced by inflammatory cytokines such as IL-1β, IL-6 and TNF-α. The expression of COX-2 is up-regulated in cancers, and its overexpression is related to tumorigenesis. The expression of COX-2 is induced by various cytokines, growth factors, lipopolysaccharides (LPS), and radiation (Kuwano et al., 2004). Thus, selective inhibition of COX-2 would prove effective in the prevention and treatment of some cancers (Howe et al., 2001; Kuwano et al., 2004; Nakao et al., 2005). We demonstrated that the gene expression of iNOS and COX-2 was similarly suppressed in SB-treated mice. In addition to the suppression of iNOS and COX-2, it is likely that SB reduced the production of NO and prostaglandins, at least in part, by reducing the expression of the key enzymes responsible for their synthesis (Fig. 3). Since SB showed strong inhibition of NO and PGE2 in the zymosan-induced mice air-pouch, we also measured levels of pro-inflammatory and inflammatory cytokines such as IL-1β, IL-2, IL-6, IL-12 and TNF-α using ELISA (Figs. 3 and 4). The results obtained in the SB-treated zymosan-stimulated acute inflammation mouse model provide evidence that SB exerted anti-inflammatory properties and inhibited the production of all tested pro-inflammatory cytokines and mediators.

Another natural product, Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-hepadiene-3,5-dione), obtained from Curcuma longa L. (Zingiberaceae family) rhizomes, has been widely used in indigenous medicine for centuries and has shown a variety of physiological and pharmacological activities. Interestingly, Curcumin was shown to inhibit the production of various cytokines, including TNF-α, IL-1β and IL-6. In addition, studies involving the systemic administration of Curcumin have shown its beneficial effects on a variety of inflammatory conditions by modulating NF-κB activity (Abe et al., 1999). For the in vitro study, we tested both SB and Curcumin to evaluate the degree of anti-inflammatory exerted by SB. We confirmed that SB exerted strong anti-inflammatory properties that were comparable to that of Curcumin, by inhibition of iNOS, COX-2, PGE2, IL-1β, IL-2, IL-6, IL-12 and TNF-α expression in LPS-stimulated Raw 264.7 cells (Figs. 5 and 6).

c-Raf-1 is a serine threonine kinase that phosphorylates and activates a family of Mitogen-activated protein kinase/ERK kinases (MEKs) (Kyriakis et al., 1992). Also, c-Raf-1, phosphorylated by various stimuli, plays an important role in LPS-induced NO production and NF-κB activation (Hambleton et al., 1996). The present study showed that SB inhibited LPS-induced phosphorylation of c-Raf-1 at Ser 259 and Ser 338, which is a critical step in c-Raf-1 activation (Fig. 7A). Moreover, c-Raf-1 activation has been reported to induce modest IκBα degradation by enhancing basal IKK activity as well as regulating the MEK/ERK signaling cascade (Kyriakis et al., 1992; Liu et al., 2001). Mitogen-activated protein kinase/ERK kinase is a tyrosine (Y-) and S/T-dual specificity protein kinase (Alessi et al., 1994). Its activity is positively regulated by c-Raf phosphorylation on S residues in the catalytic domain (James et al., 2007). The present study showed that SB directly inhibited phosphorylation of c-Raf-
1 (Ser259 and Ser338) and MEK1/2 as the upstream molecules of Erk is thought to be involved in LPS-induced macrophage responses, such as, in the increased production of pro-inflammatory cytokines and iNOS (Bhat et al., 1998; Ajizian et al., 1999). Moreover, LPS-stimulation of Raw 264.7 cells rapidly activated the JNK pathway (Hambleton et al., 1996). The identities of the mechanisms by which MAP kinases are activated during LPS-induced signaling are another important issue that requires resolution (Chakravortty et al., 2001). Thus, the activation of p38, Erk1/2 and JNK is used as a hallmark of LPS-induced signal transduction in Raw 264.7 cells. To further confirm the inhibitory mechanisms of NF-κB activation by SB, we investigated the effects of SB on p38, Erk1/2 and JNK phosphorylation in Raw 264.7 cells stimulated with LPS, SB suppressed the phosphorylation of p38, Erk1/2 and JNK (Fig. 7C).

Recent studies showed that the phosphorylation of IκBα was also regulated by α and β isoforms of IκK. These kinases may represent a novel site for pharmacological intervention in a number of inflammatory conditions (Karin and Delhase, 2000), and therefore, we observed the inhibitory effect of SB on IκKαβ activation in Raw LPS-stimulated Raw 264.7 cells. In the present study, IκKαβ, IκBα phosphorylation and IκBα were found to have increased in cells treated with LPS alone, but SB may inhibit IκKαβ, IκBα phosphorylation and IκBα (Fig. 8A). Moreover, SB may better inhibit IκKαβ, IκBα phosphorylation and IκBα compared to Curcumin in LPS-stimulated Raw 264.7 cells (Fig. 8A and B).

NF-κB is an important regulator of the activity of cytokines and other biological defenses. It is a transcription factor that has been implicated in the role of “gatekeeper” and thereby functioning as an “on/off switch” in the pathogenesis of mucositis. NF-κB controls a number of genes including iNOS, COX-2, IL-1β, IL-6 and TNF-α, which are important for immunity and inflammation; furthermore, NF-κB is activated by LPS (Barnes and Karin, 1997). In unstimulated cells, NF-κB is present in the cytosol as a homodimer or heterodimer, and is linked to the inhibitory protein IκB. NF-κB activation results from the phosphorylation, ubiquitination, and proteasome-mediated degradation of inhibitory IκB proteins, and this is followed by the nuclear translocation of NF-κB (Brown et al., 1993; Rodriguez et al., 1999). The present study showed that SB reduced the production of zymosan-stimulated, NF-κB and IκBα in mice air-pouch model (Fig. 4F). SB also reduced the production of zymosan-stimulated, NF-κB and IκBα in mice air-pouch model (Fig. 4F). This study showed that SB inhibited the LPS-stimulated nuclear translocations of p65 and p50 proteins, as well as the phosphorylation of IκBα and IκBα in Raw 264.7 cells (Fig. 8). Moreover, we showed that SB attenuated inflammation by down-regulating the expression of various inflammatory mediators (TNF-α, IL-1β, IL-2, IL-6, and IL-12), and inflammation-related genes (iNOS, COX-2), in LPS-stimulated Raw 264.7 macrophage cells (Figs. 5 and 6). Accordingly, collaboration between immuno-stimulatory and anti-inflammatory effects of SB may contribute to the best possible immune response, which can be the integrated action mode for various medicinal efficacies of SB. Taken together, our study suggested that SB inhibited LPS-induced NF-κB activation by down-regulating the c-Raf1/MEK1/2, phosphorylation of p38, Erk1/2 and JNK.

We identified baicalin (17.240%), baicalein (1.857%), wogonoside (3.25%) and wogonin (0.256%) as the major components of SB responsible for its anti-inflammatory effect. HPLC analysis of SB displayed the presence of several secondary metabolites such as polyphenolic flavonoids including baicalin, baicalein, wogonoside and wogonin (Fig. 1), which have various biological effects on cell signaling, cell growth and gene expression (Lin and Shieh, 1996;
Chen et al., 2001; Shen et al., 2002). These metabolites are also presumed to contribute, at least in part, to the anti-inflammatory effects of SB. Although additional studies are required to determine potential compound(s) and their in vivo mechanisms, it seems that polyphenolic flavonoid(s) from SB may have important implications for clinical applications.

In summary, we have demonstrated that SB displayed significant anti-inflammatory effects not only in vitro (LPS-stimulated Raw 264.7 macrophage cells) but also in vivo (in an acute inflammatory mouse model). These experiments further demonstrate that SB potently inhibited the expression of pro-inflammatory genes and inhibited inflammation both in vivo and in vitro. Additionally, our results show that SB inhibited the activation of MEK/ERK and IκKβ/IκBα-dependent pathways via c-Raf-1 activation, and thereby altered the inflammatory mediator synthesis and release in vitro. Moreover, SB interfered with LPS-induced NF-κB translocation in vivo and in vitro. Taken together, this study extends our understanding of the molecular mechanisms underlying SB’s anti-inflammatory activity observed in oriental medicine. Thus, this work provides scientific support for the use of SB as an oriental prescription for the treatment of various human inflammatory diseases.

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