Journal of Ethnopharmacology xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Ethnopharmacological communication

Modulation of acute and chronic inflammatory processes by a traditional medicine preparation GCSB-5 both *in vitro* and *in vivo* animal models

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ARTICLE INFO

Article history:
Received 26 November 2009
Received in revised form 12 April 2010
Accepted 7 May 2010
Available online xxx

Keywords:
GCSB-5
Acute and chronic inflammation
iNOS
NF-κB
Akt

ABSTRACT

Aim of this study: GCSB-5 is a traditional medicine preparation composed with six oriental herbs which have been widely used for the inflammatory diseases in Asia. In the present study, we have demonstrated the anti-inflammatory effects of GCSB-5 *in vivo* and *in vitro* along with its underlying mechanism of action.
Methods: The acute and chronic inflammation models in animals were applied to investigate the anti-inflammatory effects of GCSB-5. To further investigate the mechanism of the anti-inflammatory activity, lipopolysaccharide (LPS)-induced murine macrophage RAW264.7 cells were also employed.
Results: *In vivo* animal model, oral administration of GCSB-5 significantly inhibited TPA- and carrageenan-induced acute edema and adjuvant-induced arthritis. The vascular permeability, leukocyte migration, and granuloma formation were also inhibited by GCSB-5. In accordance, GCSB-5 suppressed the LPS-induced nitric oxide (NO) production by the downregulation of mRNA and protein expressions of inducible nitric oxide synthase (iNOS). GCSB-5 also suppressed the expressions of cyclooxygenase-2 (COX-2) and inflammatory cytokines such as interleukin-1β and interferon-β. The activation of NF-κB by LPS was also alleviated by GCSB-5, which correlated with its inhibitory effect on IkB degradation. The signaling pathway with the activation of Akt was also attenuated by the treatment by GCSB-5.
Conclusions: Taken together, our results demonstrate that GCSB-5 reduces the development of acute and chronic inflammation and its anti-inflammatory property might in part be a function of the inhibition of iNOS and COX-2 expression via down-regulation of the Akt signal pathway and inhibition of NF-κB activation. These findings suggest that GCSB-5 might be an applicable therapeutic traditional medicine in the regulation of the inflammatory response.

1. Introduction

Inflammation is a complex biological response of the body to cell damage and vascularized tissues (Ferrero-Miliani et al., 2007). Inflammation can be classified as either acute or chronic status depending on onset time. Acute inflammation is the primary response of the body to injurious stimuli and is involved the local vascular and immune response. On the other hand, chronic inflammation is a pathological condition characterized by progressive destruction and recovering of injured tissue from the inflammatory response (Nathan, 2002; Sharon and Elizabeth, 2003).

Macrophages play a central role in inflammation and host defense mechanism. The inflammatory mediators such as nitric oxide (NO) and pro-inflammatory cytokines are involved in host defense mechanisms (Fujihara et al., 2003). The inflammatory response of macrophages initially requires an interaction of signaling proceedings mediated by various enzymes such as phosphoinositide 3-kinases (PI3K), mitogen activated protein kinases (MAPKs) as well as transcription factors (NF-κB) (Sekine et al., 2006). The nuclear factor-κB (NF-κB) family is an essential player in controlling inflammatory mediators such as iNOS and COX-2 (Bresnihan, 1996; Burmester et al., 1997). NF-κB is activated by phosphorylation of IκB via activation of the MAPKs and it is a transcription factor which regulates pro-inflammatory cytokine production in the stimulated macrophages (Christman et al., 1998; Kwon et al., 2002). Because inflammatory mediators can cause severe damage such as sepsis and inflammatory diseases (Stuhlmüller et al., 2000; Hirose et al., 2001), the effective blockade of these

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doi:10.1016/j.jep.2010.05.020

Table 1
Composition of GCSB-5.

GCSB-5	Ratio (g)
Ledebouriellae radix	3.0
Achyranthis radix	3.0
Acanthopanacis cortex	3.0
Cibotii rhizoma	2.0
Glycine semen	2.0
Eucommiae cortex	1.0

inflammatory responses is regarded as an essential therapeutic target.

GCSB-5 (namely, Chungpa-Juhn) consists of a mixture of six crude drugs (Table 1) and each of crude drug has been widely used in traditional medicine to treat various bone disorders such as arthritis, disc, and osteoporosisin. It is reported that Ledebouriellae Radix had the anti-inflammatory effect on the Freund's adjuvant-induced arthritis in rats (Kim et al., 2002). Achyranthis Radix (Ida et al., 1998) has been reported anti-inflammatory properties. Eucommiae Cortex (Hong et al., 1988) was earlier investigated in various inflammatory pain models. Cibotii Rhizoma, which is contained in GCSB-5, had therapeutic effect for Masugi's nephritis models and the growth hormone secretion of adolescent rats (Wang et al., 1989). Furthermore, shinbaromecin of Cibotii Rhizoma showed anti-inflammatory effect (data not shown), which might account for the strong inhibitory effect of GCSB-5. Therefore, these reported data suggest that GCSB-5 might have a potential to alleviate the inflammatory diseases.

In this study, we investigated the effects of GCSB-5 *in vitro* and *in vivo* inflammatory models as well as examined its underlying molecular mechanism. Our results demonstrate that GCSB-5 reduces the development of acute and chronic inflammation and its anti-inflammatory property might be a function of the inhibition of iNOS and COX-2 expression via down-regulation of the ERK and Akt signal pathways and inhibition of NF-κB activation in RAW 264.7 macrophages.

2. Materials and methods

2.1. Animals

Male ICR mice (18–20 g, 5 weeks old) and male Sprague Dawley (SD) rats (150–170 g, 5 weeks old) were purchased from Central Laboratory Animal Inc. (Seoul, Korea). Animals were housed under standard laboratory conditions with free access to food and water. The temperature was thermostatically regulated to $22 \pm 2\,^{\circ}C$, and a 12-h light/dark schedule was maintained. Prior to their use, they were allowed 1 week for acclimatization within the work area environment. All animal experiments were carried out in accordance with Institutional Animal Care and Use Committee Guidelines of Ewha Womans University.

2.2. Preparation of test samples

The mixture of six crude drugs (Ledebouriellae Radix (300 g), Achyranthis Radix (300 g), Acanthopanacis Cortex (300 g), Cibotii Rhizoma (200 g). Glycine Semen (200 g), and Eucommiae Cortex (100 g)) was boiled in tap water (5 L) for 4 h and then the extract was free-dried to obtain the extract of GCSB-5 (239 g, 17.1%). GCSB-5 was administered orally at a dose of 50, 150, or 450 mg/kg in distilled water (0.3 mL), and the same volume of distilled water was used as a vehicle control group. The crude drugs were purchased from herbal market, Seoul, Korea, and authenticated by Dr. S.H. Lee, Jaseng Hospital of Oriental Medicine, Seoul, Korea. The voucher specimens of the plants used in this study were deposited in the herbarium of Jaseng Hospital of Oriental Medicine.

2.3. TPA-induced ear edema

12-*O*-Tetradecanoylphorbol 13-acetate (TPA)-induced ear edema was performed by the method previously reported by Rao et al. (1993). TPA (1.0 μg) dissolved in acetone (20 μL) was applied to the inner and outer surfaces of the right ear of ICR mice. GCSB-5, vehicle, or indomethacin was given orally administration 30 min before the TPA application. The animals were sacrificed by cervical dislocation after 4 h, and ear biopsies were obtained with a punch (a diameter of 5 mm) and weighed. The increase in the weight of the right ear punch over the left indicated the edema (Carlson et al., 1985). The ear sections were homogenized in lysis buffer and centrifuged at 1500 g for 10 min at 4°C. iNOS and COX-2 protein levels of the supernatants were determined by Western blot.

2.4. Carrageenan-induced paw edema

Carrageenan-induced hind paw edema model was used for the assessment of anti-inflammatory activity (Yeşilada and Küpeli, 2002). Thirty minutes after the administration of GCSB-5, vehicle, or indomethacin, paw edema was induced by subplantar injection of 0.1 mL of 1% freshly prepared carrageenan suspension in normal saline into the right hind paw of each rat. The left hind paw was injected with 0.1 mL of normal saline. The paw volume was measured before (0 h) and at intervals of 0.5, 1, 2, 4, and 6 h after carrageenan injection using a plethysmometer (Ugo Basile, Comerio, Italy).

2.5. Acetic acid-induced increase in capillary permeability

Acetic acid-induced increased vascular permeability in mice was determined according to the modified Whittle method as described previously (Yesilada et al., 1988). Briefly, 30 min after the oral administration of GCSB-5, each mouse was injected with 0.1 mL of 4% Evans blue (Sigma, St. Louis, Missouri, USA) in saline solution (i.v.) to the tail vein. Twenty minutes after the i.v. injection of the dye solution, 0.2 mL of 0.6% (v/v) acetic acid was injected intraperitoneally. After 20 min, the mice were sacrificed by cervical dislocation, and the peritoneal exudates were collected after being washed with 5 mL of normal saline, and centrifuged at $1200 \times g$ for 10 min. The dye content in the supernatant was measured at 610 nm by spectrometry.

2.6. Air pouch formation

The formation of air pouch was performed as described previously (Edwards et al., 1981). Briefly, sterile air (8 mL) was injected s.c. on the back of the rats, and 24 h later, 8 mL of sterile air was injected into the same cavity.

2.7. Leukocyte migration

The activity of leukocyte migration *in vivo* was investigated in rats using the method described by Ribeiro et al. (1991). Three days after air pouch formation, 2.5% carrageenan was injected locally in 2 mL of sterile PBS, 30 min after oral administration of GCSB-5. Six hours after injection, rats were euthanized, and pleural fluid was collected by washing the pleural cavity twice with 2 mL of PBS. The cell suspensions were 10-fold diluted in Turk's stain solution (0.01% crystal violet in 3% acetic acid). The number of leukocytes was counted under a light microscope.

2.8. Carrageenan-induced granuloma formation

Three days after the air injection, 2.5% carrageenan in sterile PBS was injected into the air pouch. GCSB-5 was orally adminis-

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tered for 8 days. On day 9 the rats were sacrificed to study the effect of GCSB-5 on granuloma formation. The volume of exudates and weight of granulation tissue were measured from the collected exudates.

2.9. Adjuvant arthritis

Complete Freund's adjuvant, namely 1 mg of *Mycobacterium butyricum* (Sigma) in 10 mL of mineral oil, was injected subplantarly in the right hind paw of rats. Paw volumes were measured at the beginning of the experiment by using a plethysmometer. The change in paw volume between day 20 and day 0 was used as an index of inflammatory edema. GCSB-5, indomethacin, or vehicle was administered orally once daily on days 0 through 20.

2.10. Chemicals

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine, antibiotics–antimycotics solution, and trypsin–EDTA were purchased from Invitrogen Co. (Grand Island, NY, USA). Lipopolysaccharide (LPS, *E. coli* 0111: B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and other chemicals otherwise indicated were from Sigma (St. Louis, MO, USA).

2.11. Cell culture

Mouse macrophage RAW 264.7 cells, obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. Cells was incubated at 37 °C, 5% CO_2 in a humidified atmosphere.

2.12. Nitrite assay

To evaluate the inhibitory activity of the test material on LPS-induced NO production, RAW 264.7 cells in 10% FBS-DMEM without phenol red were plated in 24-well plates (5×10^5 cells/mL), and incubated for 24 h. Cells were washed with PBS, replaced with fresh media, and then incubated with 1 μg/mL LPS in a presence or absence of test compounds. After additional 20 h incubation, the media were collected and analyzed for nitrite accumulation as an indicator of NO production by the Griess reaction. Briefly, 180 μL of Griess reagents (0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in H_2O and 1% sulfanilamide in 5% H_3PO_4) were added to 100 μL of each supernatant from LPS or sample-treated cells in 96-well plates. The absorbance was measured at 540 nm and nitrite concentration was determined by comparison with a sodium nitrite standard curve. % Inhibition was expressed as [1 − (NO level of test samples/NO levels of vehicle-treated control)] × 100. The IC_{50} value, the sample concentration resulting in 50% inhibition of NO production, was determined using non-linear regression analysis (% inhibition versus concentration).

2.13. Cytotoxicity assay (MTT assay)

After Griess reaction, MTT solution (final 500 μg/mL) was added to each well and further incubated for 4 h at 37 °C. Media were discarded, and dimethyl sulfoxide (DMSO) was added each well to dissolve generated formazan. The absorbance was measured at 570 nm and % survival was determined by comparison with control group.

2.14. Preparation of total cell lysates

RAW 264.7 cells (5×10^5 cells/mL in 60 mm dish) were incubated with or without various concentrations of GCSB-5 and LPS (1 μg/mL) for 16 h. To obtain total cell lysates, cells were washed with ice-cold PBS and lysed in boiling 2× sample loading buffer (250 mM Tris–HCl (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromophenol blue, 50 mM sodium fluoride, 5 mM sodium orthovanadate, and 2% β-mercaptoethanol). Cell lysates were boiled for additional 20 min and stored at −20 °C. The protein content of cell lysates was determined by BCA method.

2.15. Western blot analysis

Equal amounts of cell lysates (40–50 μg) were subjected to 8 and 10% SDS-PAGE and electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). Membranes were blocked in PBST (PBS with 0.1% Tween-20) containing 5% non-fat dry milk for 1 h at room temperature. After washing three times with PBST, membranes were incubated with primary antibodies against iNOS, COX-2, or IL1ß (Santa Cruz Biotechnology, Santa Cruz, CA, USA), TNF-α, p-Akt(thr), Akt, p-ERK, ERK (Cell Signaling, Danvers, MA, USA), and β-actin (Sigma) for 3 h at room temperature or overnight at 4 °C. Membranes were washed three times with PBST and incubated with corresponding secondary antibodies (Santa Cruz) for 90 min at room temperature. The blots were washed three times with PBST, and visualized using enhanced chemiluminescence (ECL) Western blotting detection system (Lab Frontier, Suwon, Korea).

2.16. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RAW 264.7 cells were stimulated with 1 μg/mL LPS in a presence or absence of GCSB-5 for 4 h. Total cellular RNA was extracted with TRI reagent (Sigma) according to the manufacturer's recommended procedure. 1 μg of total RNA was reverse-transcribed using oligo-$(dT)_{15}$ primers and avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA). PCR was performed in a reaction mixture containing the obtained cDNA, 0.2 mM dNTP mixture (Promega), 10 pmol of target gene-specific primers, and 0.25 unit of *Taq* DNA polymerase (Promega) using GeneAmp PCR system 2400 (Applied Biosystems, Foster, CA, USA). Each of PCR steps was performed as follows: initial denaturation step for 4 min at 94 °C; 25–30 cycles of amplification step consisting denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C, and elongation for 30 s at 72 °C; and final extension step for 5 min at 72 °C. PCR products were separated by 2% agarose gel electrophoresis, stained with SYBR-Gold (Molecular Probes, Eugene, OR, USA), and visualized by UV transillumination.

2.17. Statistics

All experiments were repeated at least three times. Data were presented as means ± SD for the indicated number of independently performed experiments. The statistical significances within a parameter were evaluated by one-way and multiple analysis of variation (ANOVA).

3. Results

3.1. Inhibition of TPA-induced ear edema in mice

Ear edema was induced by the applications of TPA in mouse ears. As shown in Fig. 1, the application of TPA for 4 h increased the ear edema and the mean weight of ear in control group was approximately 9.3 mg/mouse. However, the orally administration

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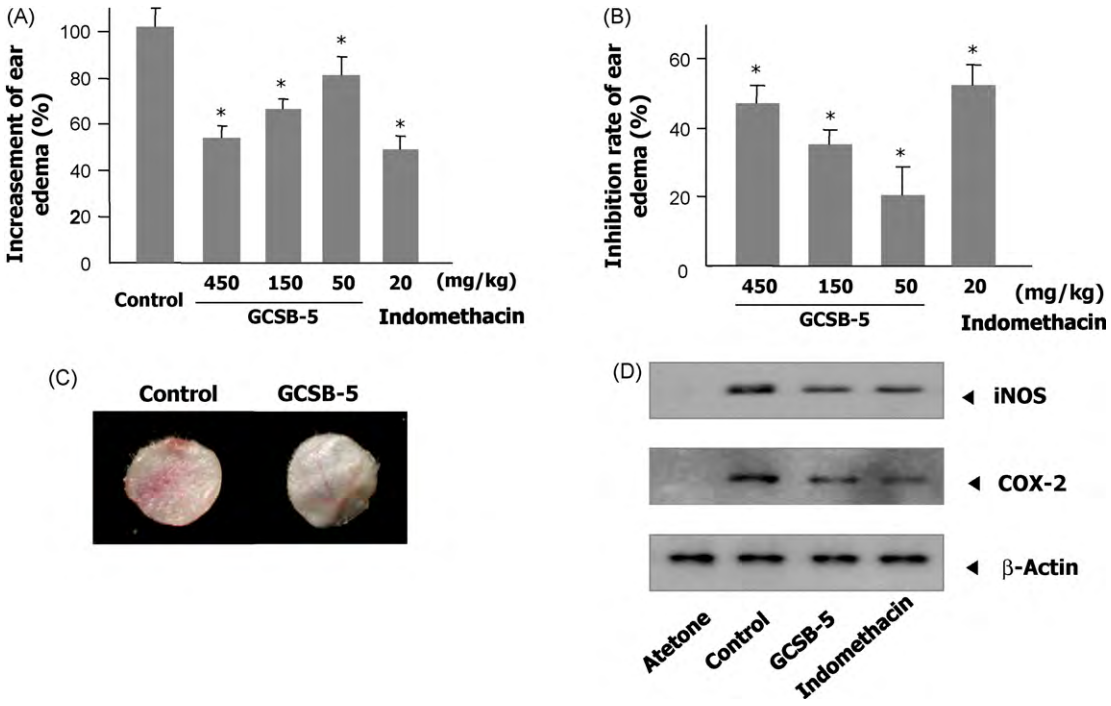

Fig. 1. Inhibitory effect of GCSB-5 on the TPA-induced ear edema. (A, B) GCSB-5 was orally administered 30 min before the TPA (1.0 μg/ear) application in the right ear of ICR mice. The mice were sacrificed 4 h after topical TPA treatment, and ear biopsies were obtained for determination of edema formation by measurement of ear weight and analysis of Western blot. Data represent the mean ± SD ($n = 6$). *$P < 0.01$ indicates statistically significant differences from the TPA treated group. (C) Corresponding image of mouse ear following application of GCSB-5 + TPA (R) or TPA (L). (D) The expressions of iNOS and COX-2 were determined in the prepared homogenate of ear biopsies by Western blot analyses. Data were representative of three separated experiments. β-Actin was used as an internal standard.

of GCSB-5 (50, 150, or 450 mg/kg) significantly ($P < 0.01$) and dose-dependently inhibited the TPA-induced ear edema. Doses of 50, 150, or 450 mg/kg led to 20.6, 35.0, or 47.2% reduction of the edema, respectively. Indomethacin (20 mg/kg), a positive compound, exhibited a similar inhibition (52.3%) with GCSB-5 of the highest dose (450 mg/kg). In addition, the analysis of ear edema showed the suppression of anti-inflammatory biomarkers iNOS and COX-2 protein expressions by GCSB-5 (Fig. 1D).

3.2. Reduction of carrageenan-induced paw edema in rats

To assess the anti-inflammatory effect of GCSB-5, carrageenan-induced paw edema model in rats was performed. Paw edema was induced by the injection of carrageenan (0.1 mL in 1% solution) and monitored the volume of paw edema for 6 h. The paw edema was increased and reached maximally at 4 h after treatment of carrageenan. The treatment of GCSB-5 significantly ($P < 0.01$) reduced the paw edema formation in a dose-dependent manner as shown in Fig. 2. The inhibition rate at 4 h was shown as 37.7, 42.9, and 51.8% with the treatment of GCSB-5 50, 150, and 450 mg/kg, respectively. In the same experimental condition, indomethacin (20 mg/kg) was shown as 56.3% inhibition which was comparable to the treatment of GCSB-5 450 mg/kg.

3.3. Inhibition of acetic acid-induced capillary permeability in mice

To evaluate whether GCSB-5 protects the capillary permeability induced by acetic acid Evans blue dye was injected into tail vein in mice and acetic acid was administered intraperitoneally. The leakage of dye into peritoneal cavity was determined by spectrophotometer. As shown in Fig. 3, GCSB-5 significantly ($P < 0.05$) and dose-dependently reduced the capillary permeability induced by acetic acid.

3.4. Inhibition of leukocyte migration in rats

In the process of inflammation leukocyte migration is generally activated. In order to determine whether GCSB-5 inhibits the migration of leukocytes induced by carrageenan the number of leukocytes in pleural fluids of air pouch was counted. GCSB-5 exhibited the inhibition of leukocyte migration in carrageenan-induced air pouch model in rats in a dose-dependent manner ($P < 0.01$) (Fig. 3C and D).

3.5. Inhibition of granuloma formation in carrageenan-induced air pouch

Carrageenan-induced granuloma formation in air pouch is well-established inflammation model in rats. To investigate whether GCSB-5 affects to the carrageenan-induced granuloma formation carrageenan (2.5%) was injected into the preformed air pouch and the volume and weight of granulation tissues were monitored after GCSB-5 treatment as described in Section 2. As a result, the volume of exudates in the air pouch was significantly ($P < 0.01$) decreased by the treatment of GCSB-5 (Fig. 4A and B) and the weight of granulation tissue was also reduced by the GCSB-5 (Fig. 4C and D).

3.6. Adjuvant arthritis

To investigate the anti-inflammatory effects of GCSB-5 in chronic inflammatory disease, adjuvant-induced arthritis model in rats was performed. As illustrated in Fig. 5, oral administration of GCSB-5 for 20 days after adjuvant injection showed the reduction of paw edema in rats compared to the vehicle-treated control group. At the end of the experiment (on day 20), doses of 50, 150, and 450 mg/kg GCSB-5 led to 28.7, 43.3, and 48.8% inhibition of the paw edema, respectively ($P < 0.01$).

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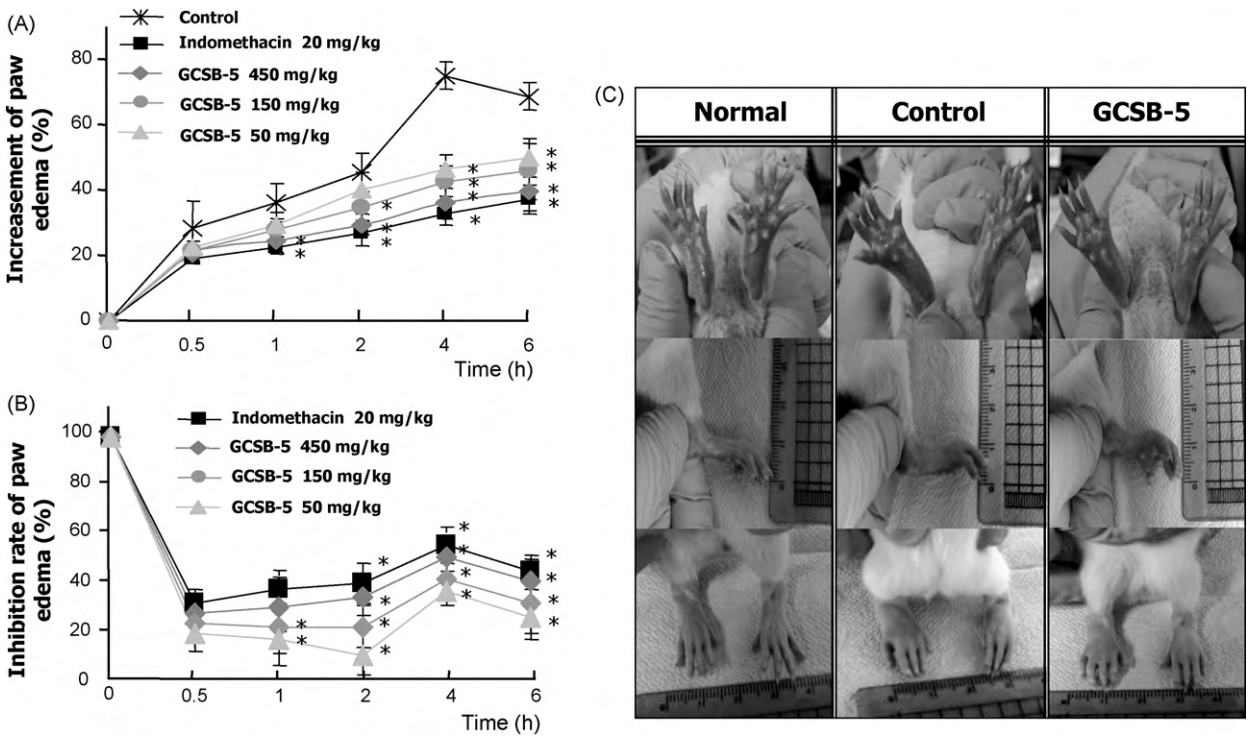

Fig. 2. Inhibitory effect of GCSB-5 on the carrageenan-induced paw edema. (A, B) GCSB-5 was orally administered 30 min before the carrageenan injection in the right paw of SD rats. The paw volume was measured before (0 h) and at intervals of 0.5, 1, 2, 4, and 6 h after carrageenan injection using a plethysmometer. Data represent the mean ± SD ($n = 6$). *$P < 0.01$ indicates statistically significant differences from the control group. (C) Corresponding image of carrageenan-induced paw edema.

3.7. Inhibition of NO production in LPS-stimulated RAW 264.7 cells

In order to evaluate whether GCSB-5 affects to the NO production, the production of nitrite, the stable metabolite of NO and used as an indicator for NO production, was monitored in cultured LPS-stimulated RAW 264.7 macrophage cells. The treatment with LPS (1 μg/mL) markedly increased the production of NO from the basal level of 1.9 ± 0.9 to 25.5 ± 1.5 μM for 20 h incubation. In this assay system, L-NMMA, a positive control of a non-selective inhibitor of NOS, exhibited an IC_{50} of 6.5 μM. When the cells were simultaneously treated with various concentrations of GCSB-5 (0–800 μg/mL) and LPS, NO production was significantly inhibited in a concentration-dependent manner with an IC_{50} value

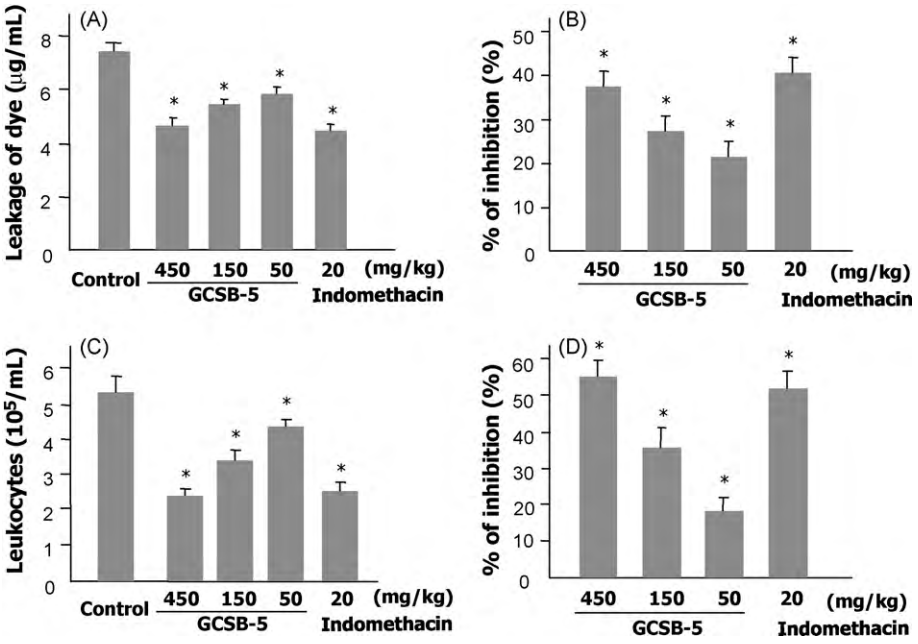

Fig. 3. Inhibitory effect of GCSB-5 on the acetic acid-induced increase in capillary permeability and leukocyte migration. (A, B) Mice recevied oral administrations of GCSB-5 before injection of acetic acid. Data represent the mean ± SD ($n = 6$). *$P < 0.05$ indicates statistically significant differences from the control group. (C, D) GCSB-5 was orally administered 30 min before the carrageenan injection in the air pouch of SD rats. At 6 h after carrageenan injection, the collected leukocyte was counted under a microscope. Data represent the mean ± SD ($n = 6$). *$P < 0.01$ indicates statistically significant differences from the TPA treated group.

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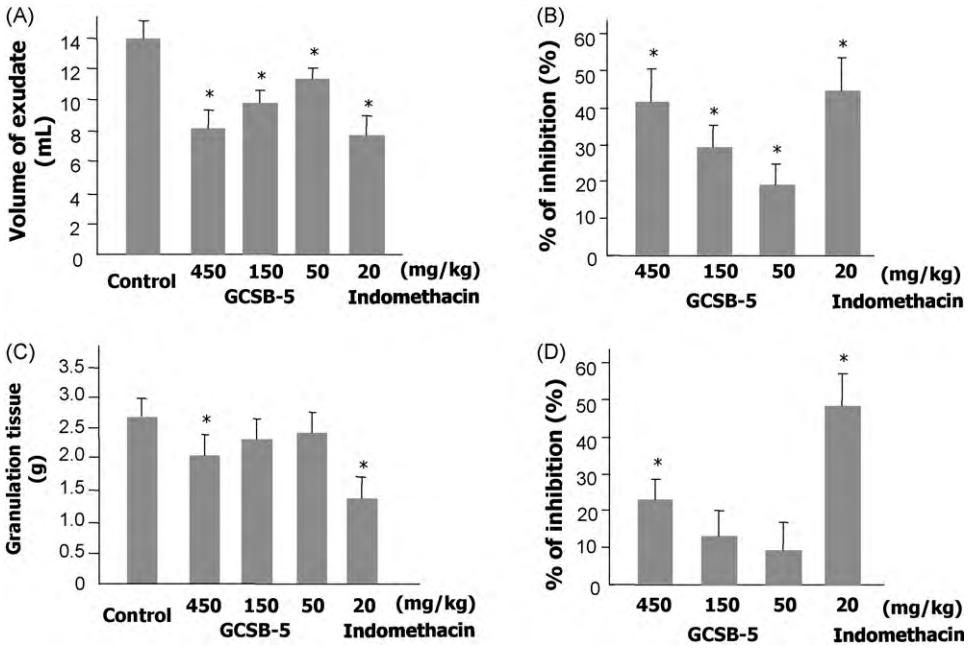

Fig. 4. Inhibitory effect of GCSB-5 on the carrageenan-induced granuloma formation. (A, B) Inhibitory effect of GCSB-5 on the volume of exudates induced by carrageenan. (C, D) Inhibitory effect of GCSB-5 on the granulation tissue induced by carrageenan. GCSB-5 was orally administered 30 min before the carrageenan injection in the air pouch of SD rats. At 8 days after carrageenan injection, the exudates were collected and weighted. Data represent the mean ± SD ($n = 6$). *$P < 0.01$ indicates statistically significant differences from the control group.

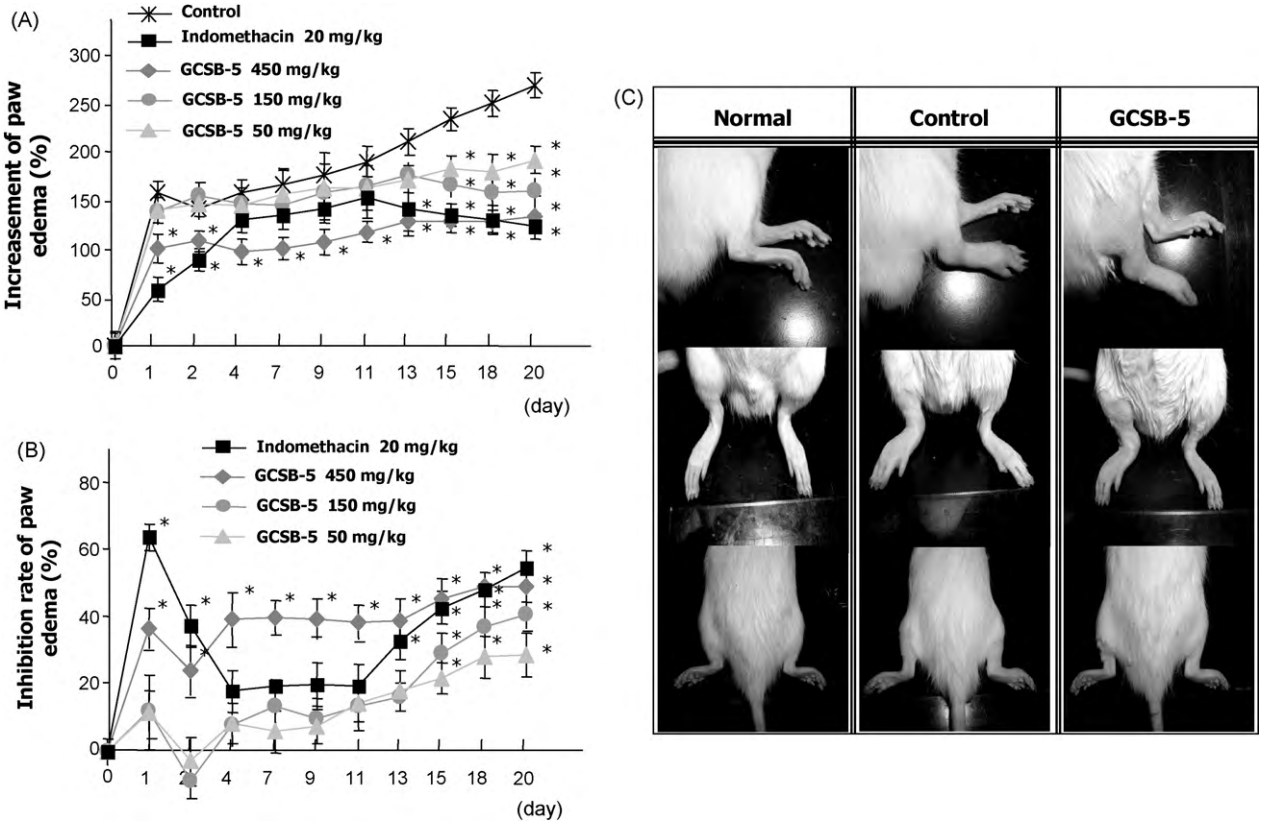

Fig. 5. Inhibitory effect of GCSB-5 on the adjuvant-induced arthritis. (A, B) Arthritis was induced by injecting rats subplantarly with Complete Freund's adjuvant on day 0. GCSB-5 was administered orally once daily on days 0 through the end of the experiments (20 days). Paw volumes were measured at the beginning of the experiment by using a plethysmometer. The change in paw volume between day 20 and day 0 was used as an index of inflammatory edema. Data represent the mean ± SD ($n = 6$). *$P < 0.01$ indicates statistically significant differences from the control group. (C) Representative photographs of joint swelling in the hind paw of GCSB-5-treated and a saline treated rat on day 20.

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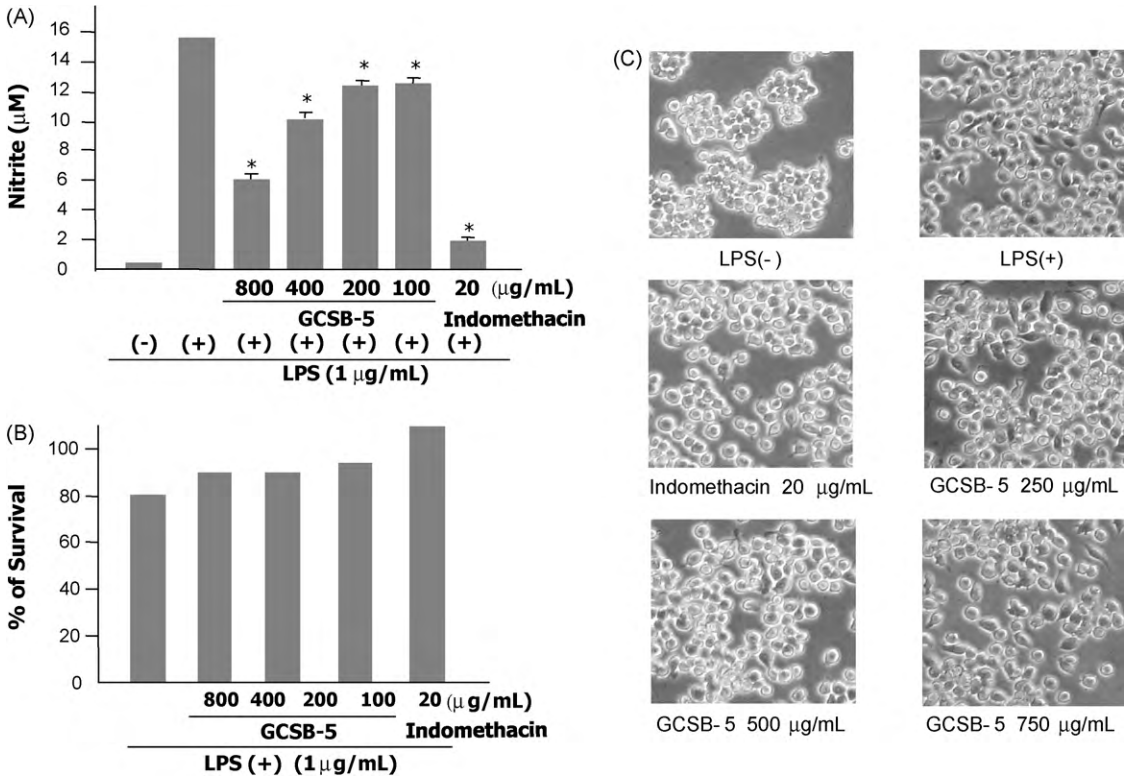

Fig. 6. Inhibitory effects of GCSB-5 on LPS-induced NO production in macrophage cells. (A) RAW 264.7 cells were stimulated with 1 μg/mL LPS in a presence or absence of GCSB-5. After 20 h, cultured media were collected and analyzed nitrite concentration using Griess reaction. The data were expressed as mean ± SD of triplicate tests. $^{*}P < 0.01$ was considered statistically significant. (B) The cell viability was measured by MTT method as described in Section 2. (C) Morphlogical change of RAW 264.7 cells by treatment with GCSB-5 was observed under the inverted phase-contrast microscope and photographed.

of 690 μg/mL (Fig. 6A). Over 63% inhibition of NO production was shown at 800 μg/mL GCSB-5. No significant effect on cell viability was observed at a test concentration up to 800 μg/mL GCSB-5 as determined by MTT assay (>90% cell survival), indicating that the inhibition of NO production by GCSB-5 was not mediated by cytotoxic effect (Fig. 6B).

3.8. Suppression of pro-inflammatory cytokine expression in LPS-stimulated RAW 264.7 cells

To investigate the effect of GCSB-5 on the expression of pro-inflammatory cytokines the steady-state levels of TNF-α, interleukin-1β (IL-1β), and interferone-β (IFN-β) were analyzed by RT-PCR. RAW 264.7 cells expressed very low levels of IL-1β, and TNF-α mRNA in un-stimulated states. As shown in Fig. 7, the treatment with LPS (1 μg/mL) for 4 h markedly increased the expressions of pro-inflammatory cytokines, but co-treatment with GCSB-5 (0–750 μg/mL) and LPS suppressed the expression of IL-1β and IFN-β in a concentration-dependent manner. However, TNF-α mRNA expression was not affected by GCSB-5 treatment. In addition, Western blot analysis showed that GCSB-5 also suppressed the expressions of TNF-α and IL-1β protein in LPS-stimulated RAW 264.7 in a concentration-dependent manner (Fig. 7B)..

3.9. Suppression of iNOS and COX-2 protein and mRNA expression in LPS-stimulated RAW 264.7 cells

To further elucidate the mechanism of action of GCSB-5 on the inhibition of NO production, the effects of GCSB-5 on the protein and mRNA expressions of some pro-inflammatory mediators was determined. As shown in Fig. 8A, the treatment with LPS (1 μg/mL) for 16 h markedly increased the expression of iNOS and COX-2 protein, but this event was suppressed by the co-treatment with GCSB-5 in a concentration-dependent manner. As illustrated in Fig. 7, GCSB-5 significantly decreased iNOS mRNA levels induced by LPS in a concentration-dependent manner, but not COX-2 mRNA, suggesting that the inhibitory activity of NO production by GCSB-5 might be correlated to the suppres-

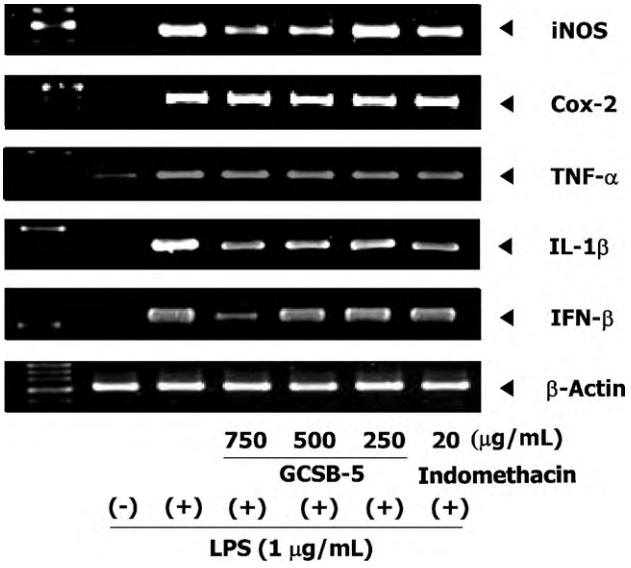

Fig. 7. Effect of GCSB-5 on LPS-induced pro-inflammatory cytokine mRNA expression in macrophage cells. RAW 264.7 cells were treated with LPS (1 μg/mL) and GCSB-5 for 4 h. Total RNA was isolated and further analyzed by RT-PCR as described in Section 2. Data were representative of three separated experiments. β-actin was used as an internal standard.

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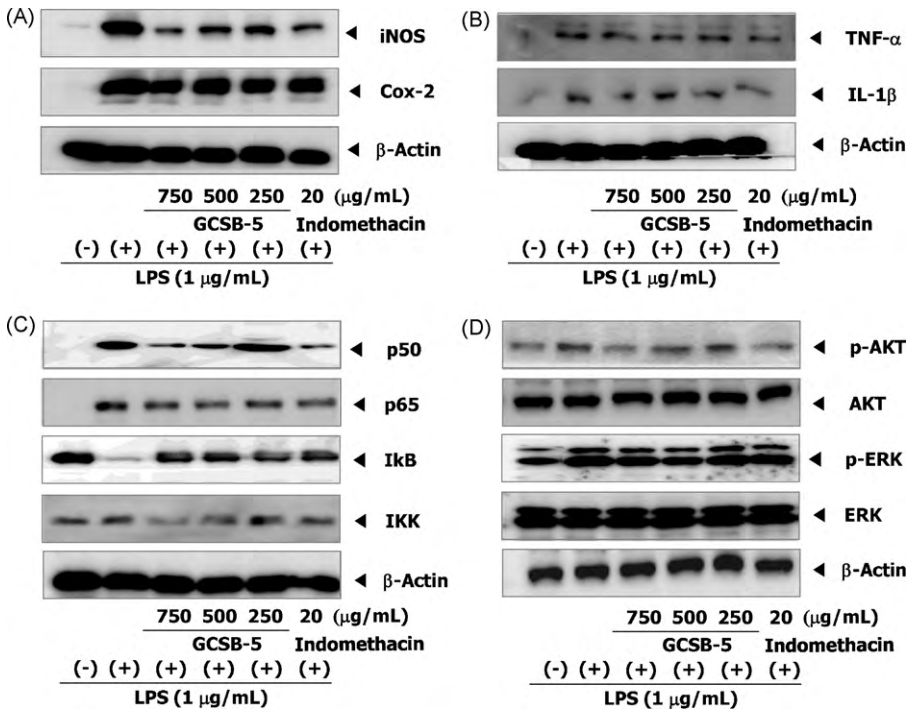

Fig. 8. (A) Effects of GCSB-5 on iNOS and COX-2 protein expressions in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells (5×10^5 cells/mL) were incubated for 24 h, and then treated with LPS (1 μg/mL) and GCSB-5 for additional 4 and 16 h. After incubation, total cell extracts were obtained and subjected to Western blot analysis as described in Section 2. Data were representative of three separated experiments. β-actin was used as an internal standard. (B) Effects of GCSB-5 on TNF-α and IL-1β protein expressions in LPS-stimulated RAW 264.7 cells. (C) Effects of GCSB-5 on the activation of NF-κB and the degradation of IκB-α in LPS-stimulated RAW 264.7 cells. (D) Effects of GCSB-5 on the phosphorylation of Akt and ERK in LPS-stimulated RAW 264.7 cells. RAW264.7 cells were treated with various concentrations of GCSB-5 for 30 min prior to LPS treatment. After stimulation with LPS for an additional 30 min, proteins were extracted and analyzed by Western blot analysis.

sion of iNOS expression at the translational and transcriptional level.

3.10. Suppression of the NF-κB activation and IκBα degradation in LPS-stimulated RAW 264.7 cells

The activation of NF-κB is an essential step in the onset of the inflammatory response. Since p65 and p50 are the major component of NF-κB activated by LPS-stimulated macrophages, we examined the protein levels of p65 and p50 in nuclear extracts. As shown in Fig. 8C, the treatment with LPS (1 μg/mL) for 1 h markedly increased the expressions of p65 and p50 protein, but co-treatment with GCSB-5 (0–750 μg/mL) and LPS suppressed the expression of p65 and p50 in a concentration-dependent manner. Since nuclear translocation is preceded by IκBα degradation, we determined the cytoplasmic levels of IκBα degradation by GCSB-5. The IKK pathway is also involved in the activation of NF-κB, we next examined the effect of GCSB-5 on IKK activation. GCSB-5 showed the effects on the inhibition of IκBα degradation and IKK activation as shown in Fig. 8C. These finding suggest that GCSB-5 may have the inhibitory effect for LPS-stimulated IκBα degradation and IKK activation.

3.11. Suppression of the phosphorylation of Akt and ERK in LPS-stimulated RAW 264.7 cells

To investigate the molecular mechanism of NF-κB inhibition by GCSB-5, we examined the effect of GCSB-5 on LPS-induced phosphorylation of Akt and ERK in RAW 264.7 cells using Western blot analysis. As shown in Fig. 8C, GCSB-5 suppressed LPS-induced activation of Akt in a concentration-dependent manner, whereas that of ERK was not affected by treatment with GCSB-5. These results indicate that GCSB-5 inhibited LPS-induced NF-κB activation through down-regulation of Akt phosphorylation.

4. Discussion

The search for natural products with anti-inflammatory activity has extremely increased in recent years. Herbal extracts, especially crud drugs, are known to possess a diversity of components or secondary metabolites which have various biological activities. However, to identify the active substances and to clarify their unambiguous mechanism of action is a still facing task. GCSB-5 is a complex preparation with six traditional folk medicines which each of these components has been widely used to treat a variety of inflammatory diseases in Asia. GCSB-5 has also been used clinically to treat inflammation-related diseases in Korea. However, the exact pharmacological studies and underlying mechanisms of actions of the preparation were poorly elucidated yet. Therefore, the present study was performed to investigate the anti-inflammatory activity of GCSB-5 in acute and chronic models in animals and to further elucidate the underlying mechanisms of actions in *in vitro* and *in vivo*.

Primarily, the anti-inflammatory activity of GCSB-5 was evaluated in acute and chronic animal models. Acute inflammation is a short-term process which is characterized by the typical signs of inflammation, such as swelling, pain, and loss of function due to the infiltration of the tissues by plasma and leukocytes. Among them, edema is one of the fundamental actions of acute inflammation and it is an essential parameter to be considered when evaluating compounds with a potential anti-inflammatory activity (Morris, 2003). Therefore, we first tested the anti-inflammatory activity of GCSB-5 on mouse ear edema model. As shown in Fig. 1A and 1B, GCSB-5 significant inhibited the TPA-induced ear edema, suggesting the anti-inflammatory activity of GCSB-5 in acute animal model system. As one possible mechanism of action the suppression of TPA-induced expression of iNOS and COX-2 by GCSB-5 might be suggested in this acute inflammation mouse ear model (Fig. 1D).

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Subsequently, the carrageenan-induced paw edema model in rats was employed (Vinegar et al., 1987; Otterness and Moore, 1988). In this study, the formation of edema reaches a maximum at 4 h after carrageenan injection and the oral administration of GCSB-5 dose-dependently inhibited the paw edema induced by carrageenan (Fig. 2). These findings demonstrate that GCSB-5 has a potent *in vivo* anti-inflammatory activity in acute inflammation model systems.

It is also known that the increase of vascular permeability is one of the major events of the acute inflammatory process. The vascular permeability induced by acetic acid in mouse model is a fundamental capillary permeability assay (Winter et al., 1962). In this assay system, GCSB-5 significantly reduced the increase of vascular permeability, indicating the suppression of the vascular response in the process of acute inflammation by GCSB-5 (Fig. 3A and B).

The migration of leukocytes at the site of inflammation is an important parameter in the inflammatory response. The migration of leukocyte occurs as a result of different processes including adhesion and cell mobility (Meade et al., 1986). The present study showed that GCSB-5 inhibited the leukocyte migration induced by carrageenan in rats (Fig. 3C and D).

The chronically inflamed tissue in chronic inflammation includes a proliferation of fibroblasts and the infiltration of neutrophils and exudation (Dunne, 1990). The method of granuloma formation is widely used to evaluate the anti-inflammatory action on proliferation phase of the chronic inflammation. As shown in Fig. 4, GCSB-5 showed a significant anti-inflammatory activity on granuloma formation in chronic inflammatory conditions.

Arthritis is a well-known chronic disease which affects joints including the cartilage, synovium, tendons, and muscle. Therefore, adjuvant-induced arthritis in rats provides a useful model for assessment of anti-inflammatory agents against chronic inflammation (Billingham, 1983). In this study, GCSB-5 was shown to suppression the inflammation of adjuvant-induced arthritis without any side effects (Fig. 6). These results suggest that GCSB-5 might have a potential to improve the chronic inflammation as well as acute inflammation responses.

Additionally, the mechanisms underlying the anti-inflammatory activity of GCSB-5 were investigated in a mouse macrophage cell line, RAW 264.7. Stimulation of macrophages by LPS induced in the expression of iNOS (Xie and Nathan, 1994) and COX-2 (Lee et al., 1992). The overproduction of NO plays an important role in the process of macrophage activation and is associated with acute and chronic inflammations. Therefore, the inhibition of NO production by down-regulation of iNOS in inflammatory cells is a very significant therapeutic strategy in the development of anti-inflammatory agents. In this study, GCSB-5 was demonstrated to inhibit LPS-induced NO production in a concentration-dependent manner in RAW 264.7 macrophages (Fig. 7). To further explore the mechanism responsible for the inhibitory effect of GCSB-5 on LPS-induced NO production, the effect on iNOS protein and gene expressions in LPS-stimulated RAW 264.7 macrophages was examined. GCSB-5 inhibited the expressions of iNOS and COX-2 protein and mRNA (Figs. 7A and 8). These results support that GCSB-5 exerts its effect through the suppression of the iNOS mRNA transcription step.

The production or function of pro-inflammatory cytokines, such as TNF-α and IL-1β, play roles in many inflammatory responses (De Nardin, 2001). Thus, the modulation of cytokine production or function is a major mechanism in the control of inflammation. TNF-α, a potent activator of macrophages, is initially produced by monocytes, and has various pro-inflammatory effects on many cell types. IL-1β is also an important cytokine, primarily released by macrophages (Dayerihj, 2003). In the present study, we show that GCSB-5 inhibited the protein expression of the pro-inflammatory cytokines TNF-α and IL-1β in RAW264.7 cells stimulated by LPS. These findings provide evidence that GCSB-5 possesses a useful anti-inflammatory activity (Fig. 7).

NF-κB is known to be a major transcription factor to regulate the expressions of pro-inflammatory enzymes and cytokines, such as iNOS, COX-2, and TNF-α (Kujubu et al., 1991; Karin and Ben-Neriah, 2000). NF-κB subunits (p65 and/or p50) are normally sequestered in the cytosol as an inactive complex by binding to inhibitory factor IκB-α in un-stimulated cells. Upon stimulation of pro-inflammatory signals including LPS, IκB-α is phosphorylated by IκB kinase (IKK) and inactivated through ubiquitin-mediated degradation. The resulting free NF-κB is translocated into the nucleus and acts as a transcription factor. As shown in Fig. 8C, the treatment with GCSB-5 blocks the degradation of IkB, activation of IKK, and activation of NF-κB in RAW 264.7 macrophages by LPS. Therefore, these results suggest that GCSB-5 inhibits the expression of iNOS and COX-2, and thus NO production through inactivation of NF-κB by reducing IκBα degradation and IKK activation.

Various intracellular signaling pathways are involved in the regulation of NF-κB activation and cytokine expression. The activation of Akt and MAPK has been demonstrated to be significant in the modulation of iNOS and COX-2 expression through control of the activation of NF-κB (Suh et al., 2006; Pergola et al., 2006). To further investigate the mechanisms of NF-κB inactivation and NO inhibition by GCSB-5, the effects of GCSB-5 on the LPS-induced activation of Akt were examined. The data demonstrated that GCSB-5 inhibited the phosphorylation of Akt in LPS-stimulated RAW 264.7 macrophages (Fig. 8D). These results suggest that the inhibition of NF-κB activation by GCSB-5 might be due to the inhibition of Akt phosphorylation. Overall, our results indicate that the down-regulation of Akt phosphorylation is involved in the inhibitory effect of GCSB-5 on LPS-induced NO production and iNOS and COX-2 expression via NF-κB inactivation. These *in vitro* findings were well correlated with the *in vivo* anti-inflammatory effects of GCSB-5.

Taken together, our results suggest that GCSB-5 has a potential anti-inflammatory activity in in vitro and in vivo inflammatory model systems. The underlying mechanisms of actions by GCSB-5 are correlated with the inhibition of iNOS and COX-2 expression via down-regulation of the Akt signal pathways and inactivation of NF-κB. The data in the present study support the pharmacological basis of the use of GCSB-5 as a traditional herbal medicine for the treatment of inflammation.

Acknowledgment

This work was supported by a grant from JASENG Hospital of Oriental Medicine (JS-2008-1).

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