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WIN-34B May Have Analgesic and Anti-Inflammatory Effects by Reducing the Production of Pro-Inflammatory Mediators in Cells via Inhibition of IκB Signaling Pathways

Kyoung Soo Kim[1,]*, Hyun Mi Choi[1], Hyung-In Yang[2] and Myung Chul Yoo[1]

[1]East-West Bone & Joint Research Institute, [2]Division of Rheumatology, Department of Internal Medicine, Kyung Hee University at Gangdong, College of Medicine, Kyung Hee University, Seoul 134-727, Republic of Korea

Abstract

WIN-34B showed analgesic and anti-inflammatory effects in various animal models of pain and osteoarthritis. However, the molecular mechanism by which WIN-34B inhibits pain and inflammation *in vivo* remains to be elucidated. We investigated the molecular mechanisms of the actions of WIN-34B using various *in vitro* models using fibroblast-like synoviocytes from patients with rheumatoid arthritis (RA FLSs), RAW264.7 cells and peritoneal macrophages. WIN-34B inhibited the level of IL-6, PGE_2, and MMP-13 in IL-1β-stimulated RA FLSs in a dose-dependent manner. The mRNA levels were also inhibited by WIN-34B. The level of PGE_2, NO, IL-1β, and TNF-α were inhibited by WIN-34B at different concentrations in LPS-stimulated RAW264.7 cells. The production of NO and PGE_2 was inhibited by WIN-34B in a dose-dependent manner in LPS-stimulated peritoneal macrophages. All of these effects were comparable to the positive control, celecoxib or indomethacin. IκB signaling pathways were inhibited by WIN-34B, and the migration of NF-κB into the nucleus was inhibited, which is consistent with the degradation of IκB-α. Taken together, the results suggest that WIN-34B has potential as a therapeutic drug to reduce pain and inflammation by inhibiting the production of pro-inflammatory mediators.

Key Words: Lonicera japonica Thunb, Anemarrhena asphodeloides BUNGE, Analgesic effect, Anti-inflammatory effect, WIN-34B

INTRODUCTION

Among the elderly, osteoarthritis is the most common joint disease and an important cause of physical illness (Ameye and Chee, 2006). Symptoms induce joint pain, stiffness, limited movement, joint deformity, and varying degrees of joint inflammation (Lark *et al*., 1997). Current therapeutic strategies for osteoarthritis focus on alleviating symptoms, particularly pain and inflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids are the mainstream treatments for osteoarthritis (Kidd, 2006). Although NSAIDs are recommended as an initial drug therapy to reduce joint inflammation and pain, their chronic use is limited by gastrointestinal-related toxicities including nausea, dyspepsia, upper gastrointestinal bleeding, and ulcer perforation (Wolfe and Cathey, 1991). To minimize these toxicities, a new generation of NSAIDs, the cyclooxygenase (COX)-2 selective inhibitors (celecoxib, rofecoxib, and valdecoxib), have been developed in an attempt to improve gastrointestinal tolerance. However, reported cardiovascular risks, including myocardial infarction and stroke, are leading to the removal of rofecoxib from the market (Gottlieb, 2001; Hippisley-Cox and Coupland, 2005; Lenzer, 2005). Although other COX-2 inhibitors provide effective symptomatic relief, their substantial toxicities limit long-term use. Additionally, this therapeutic approach is not curative, but relieves clinical signs and symptoms of the disease; thus, a more effective and safe drug must be developed for the curative treatment of osteoarthritis.

Joins™, an herbal drug combining the extracts of *Clematis mandshurica*, *Trichosanthes kirilowii*, and *Prunella vulgaris* is commonly used for the curative treatment of osteoarthritis in Korea. The major bioactive components of their plants are known to be triterpene saponins from *Clematis mandshurica* (Shi *et al*., 2006), a naturally rare isoaurone, 4',6-dihydroxy-4-methoxyisoaurone, cucurbitacin B, 6-(3-hydroxy-4-methoxystyryl)-4-methoxy-2H-pyran-2-one, and blumenol A from *Trichosanthes kirilowii* (Dat *et al*., 2010), and rosmarinic acid from *Prunella vulgaris* (Huang *et al*., 2009). Clinical studies have demonstrated that Joins™ relieves joint pain and improves functionality in osteoarthritis patients. Its efficacy

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***Corresponding Author**
Email: labrea46@yahoo.co.kr
Tel: +82-2-440-6791, Fax: +82-2-440-6799

may be attributed to cartilage protection and anti-inflammation (Hartog *et al.*, 2008), but its lack of an immediate analgesic effect is a major drawback. Screening of herbs and natural products for a more efficient compound may lead to the development of a superior therapeutic drug, particularly one with stronger immediate analgesic effects. In order to develop a better therapeutic alternative to Joins^{TM}, two herbs, namely, flowers of *Lonicera japonica* Thunb and roots of *Anemarrhena asphodeloides* BUNGE were screened from 200 medicinal herbs used in herbal medicine, which has been widely used for the treatment of inflammatory diseases such as lymphadenitis and arthritis in Far Asia: cartilage protection in human osteoarthritic explant culture, anti-inflammatory effects in RAW264.7 cells *in vitro*, anti-nociceptive in writhing, formalin, paw pressure tests, anti-inflammatory effects in paw edema and ear edema *in vivo*. They demonstrated excellent anti-nociceptive and anti-inflammatory properties in several animal models, as well as cartilage protection and anti-inflammatory properties in *in vitro* assays from our preliminary data. Thereafter, these two herbs were selected and mixed into a 2:1 (w/w) formulation. Then it was extracted with 50% ethanol and finally partitioned with n-butanol, leading to a yield of 7%. The final resultant extract was named as WIN-34B (Kang *et al.*, 2010). The major bioactive constituents of the WIN-34B extract was identified as neomangifrin, chlorogenic acid, 9α-hydroxypinoresinol, mangiferin, sweroside, secologanin, rutin, 3,5-di-O-caffeoylquinic acid, 4,5-di-O-caffeoylquinicacid, quecertin and nyasol in order of retention time by analyses of UPLC and Mass spectrometry.

WIN-34B has an analgesic and anti-inflammatory effect in various pain and osteoarthritis animal models (Kang *et al.*, 2010) and is now in clinical phase II multicenter testing in Korea. However, information about the molecular mechanism by which WIN-34B inhibits pain and inflammation is still lacking. In this study, we tried to elucidate the molecular mechanisms by which WIN-34B exerts anti-osteoarthritic effects in various types of cells. The findings will further support WIN-34B as a candidate for treatment of osteoarthritis.

MATERIALS AND METHODS

Cell isolation and culture

For isolation and culture of fibroblast-like synoviocytes from patients with rheumatoid arthritis, synovial tissues were collected from RA patients after obtaining informed consent. They met the 1987 American College of Rheumatology criteria for the diagnosis of RA and had been treated with nonbiological disease-modifying antirheumatic drugs (DMARDs) and were underwent therapeutic joint surgery. FLSs were isolated and grown in Dulbecco's Modified Essential Medium (DMEM, low glucose) (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco-BRL) and 1× Antibiotic-Antimycotic (Gibco-BRL) as described previously (Kim *et al.*, 2007). After the cells had grown to confluence, they were split at a 1:4 ratio. FLS passages 3-6 from three patients were used for all experiments. Synovial cells (2.5×10^5 cells/60 mm dish/2 ml serum-free media) were treated with recombinant IL-1β (10 ng/ml) (ProSpec, Rehovot, Israel) in the presence or absence of WIN-34B for 24 hr. The culture supernatants were collected and the cultured cells were used for RNA extraction. This study was approved by the Institutional Review Board of Kyung Hee University Hospital at Gangdong.

Murine macrophage RAW 264.7 cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured in a humidified atmosphere and incubated at 37°C in 5% CO_2. Exponentially growing RAW 264.7 cells were plated at a density of 2×10^5 cells/well in 60 mm dish in culture medium and allowed to adhere for 16 h before treatment. WIN-34B was added to the cultures at final concentrations of 100, 200, and 400 μg/ml with 1 μg/ml lipopolysaccharide (LPS) for 24 h.

Resident macrophages were obtained by peritoneal lavage as described in elsewhere (Schiavoni *et al.*, 2004). Briefly, mice were injected intraperitoneally (i.p.) with 1 ml of 3% Brewer thioglycollate media, and macrophages were obtained 3 days later. Exudates from abdomen were centrifuged at 2,000 rpm for 5 min at 4°C. The cell pellets were washed twice with DMEM media containing 10% FBS, 100 u/ml penicillin, and 100 mg/ml streptomycin and washed cells were stimulated with LPS (1 μg/ml) and IFN-γ (1 ng/ml) for 96 h at 2×10^5 cells/well/200 μl of media on 96 well plates for NO assay, or for 24 h at $1x10^6$ cells/well/1 ml of media on 24 well plates for PGE_2 assay.

Splenocytes were prepared by disrupting mouse spleens between glass slides in complete RPMI 1640 medium, as described previously (Park *et al.*, 2006). After centrifuging for 10 min at 1500 rpm to separate cells from debris, they were washed in RPMI medium, and erythrocytes were lysed in lysis buffer (0.15 M NH4Cl, 1mM $KHCO_3$, 0.1 mM Na2EDTA) at 4°C for 10 min. The splenocytes obtained were stimulated with LPS (1 μg/ml) in complete RPMI 1640 medium containing 50 μM mercaptoethanol for 3 days in the presence of WIN-34B, Joins, Celecoxib, or indomethacin. The inhibitory effect of WIN-34B on cell proliferation was evaluated using MTS assay kits (Promega, Madison, WI, USA) in accordance with the manufacture's protocol.

Plant material

Dried flowers of *Lonicera japonica* and dried root of *Anemarrhena asphodeloides* were purchased from Song Lim Pharmaceutical Company (Seoul, Korea). They were identified by the Korea Pharmaceutical Trading Association (Seoul, Korea). Voucher specimen of *Lonicera japonica* Thunb (No. OA-LOJ-15) and *Anemarrhena asphodeloides* BUNGE (No. OA-ANA-11) were deposited at Central Research Institute, WhanIn Pharm.,Co., Ltd (Suwon, Korea).

WIN-34B preparation

WIN-34B was prepared as reported previously (Kang *et al.*, 2010). Briefly, 2:1 (w/w) mixture of the two medicinal herbs, 100 g of such chosen dried flowers of *Lonicera japonica* and 50 g of such chosen dried roots of *Anemarrhena asphodeloides* were extracted with 1.35 L of 50% (v/v) ethanol in water for 4 h at 85°C. After the extracted solution was filtered and evaporated in vacuo, the resulting concentrate was dissolved in 225 ml of distilled water and partitioned with 195 ml of n-butanol. The n-butanol layer was evaporated in vacuo and lyophilized for complete removal of the residual solvent to a yield brown powder of 11 g, with a yield of 7%. The final resultant extract was called WIN-34B. Voucher specimen of WIN-34B (No. OA-WIN-34B-15) were deposited at Central

Research Institute, WhanIn Pharm., Co., Ltd (Suwon, Korea).

Enzyme-linked immunosorbent assay (ELISA)

The collected supernatants were analyzed for TNF-α, IL-1β, IL-6, prostaglandin E2 (PGE_2), and MMP-13 using commercial kits according to the manufacturer's instruction (ELISA; R&D Systems, Inc., Minneapolis, MN, USA). For the measurement of transcription factor, NF-κB, in the nucleus, RAW264.7 cells were seeded (1×10^6 cells) into 100 mm dishes and grown to 80% confluence. The cells were serum-starved overnight and stimulated by LPS (1 μg/ml) for 90 min in the presence or absence of WIN-34B as described above. Subsequently, the cells were washed twice in PBS and treated with lysis buffer and the extraction of transcription factors from the nucleus was performed according to the manufacturer's protocol (Active Motif, Seoul, Korea).

Measurements of nitric oxide (NO) content

Total NO production can be determined by assaying for nitrite because NO is rapidly converted to nitrite and nitrate in the presence of H_2O. Thus, 100 μl of culture supernatant was incubated at room temperature for 10 min with 100 μl of Griess reagent (1% sulfanilamide, 0.2% N-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% H_3PO_4). Sample OD val-

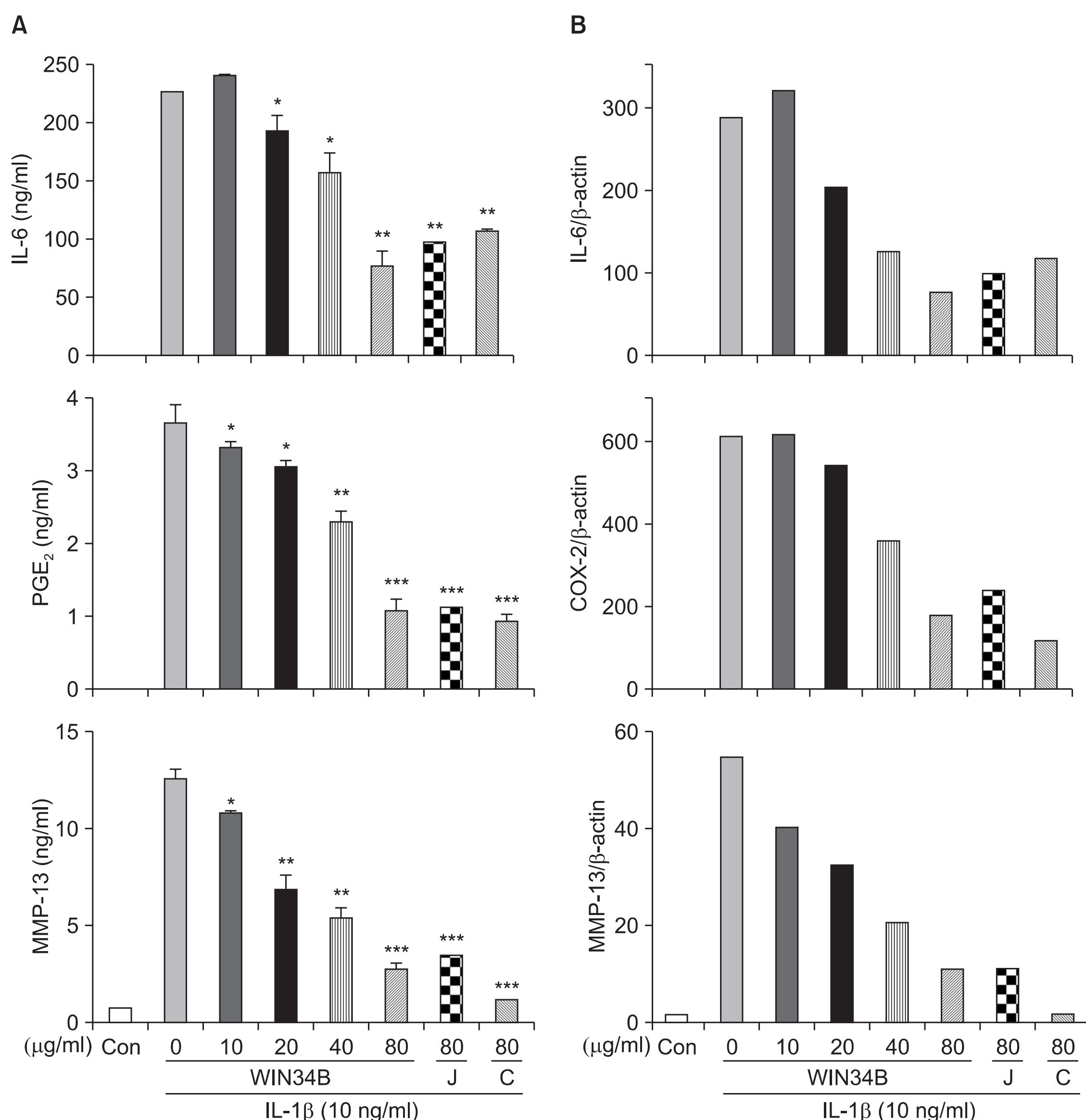

Fig. 1. Effect of WIN34B on the production of IL-6, PGE_2, and MMP-13 in IL-1β-stimulated RA FLSs. 2.5×10^5 cells/60 mm dish in 2 ml serum-free media were treated with WIN-34B dissolved in DMSO at various concentrations (0-80 μg/ml) 30 min before treatment with IL-1β (10 ng/ml) for 24 hr. For the comparison test, Joins™ (J) and celecoxib (C) was used as positive controls. The culture supernatants were collected for ELISA and the cultured cells used for RNA extraction. The expression levels of IL-6, PGE_2, and MMP-13 was measured by (A) ELISA and (B) real time PCR. Three independent experiments were performed in quadruplicate. Similar results were obtained for all three, and the data shown are representative. Values are expressed as mean ± S.E.M. **p*<0.05, ***p*<0.01, ****p*<0.001 versus IL-1β-treated group in the absence of WIN-34B. J: Joins, C: Celecoxib, Con: control.

ues were read at 570 nm, and a standard curve was then used to determine the $NO2^-$ concentration.

Real-time PCR

For real-time quantitative PCR, the reaction was carried out using the LightCycler PCR system (Roche Diagnostics, Meylan, France) using the DNA binding SYBR Green I dye and primers to detect the PCR products as described previously (Choi *et al.*, 2009; Choi *et al.*, 2010). The results are calculated as ratios of gene transcripts to β-actin transcripts, with the quantity of transcripts in each sample expressed as a copy number. The ratio of IL-6, IL-8 and COX-2/β-actin mRNA was assigned a value of 100%, with the corresponding results calculated as relative percentages.

Western analysis

RAW264.7 cells were pretreated with WIN-34B at 100-200 μg/ml 2 hr before stimulation with LPS (1 μg/ml) for 30 min. The cells are prepared for Western blot analysis and the samples were separated using 12% SDS-PAGE, and were then transferred to Hybond-ECL membranes (Amersham, Arlington Heights, IL, USA). The membranes were first blocked with 6% nonfat milk dissolved in TBST buffer (10 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.05% Tween 20). The blots were then probed with various rabbit polyclonal antibodies for IκB-α, and β-actin (Cell Signaling Technology, Beverly, MA, USA) diluted 1:1000 in TBS at 4°C for overnight, and incubated with 1:1000 dilutions of goat anti-rabbit IgG secondary antibody coupled with horseradish peroxidase. The blots were developed using the ECL method (Amersham). For re-probing, the blots were incubated in the stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl [pH 6.7]) at 50°C for 30 min with occasional agitation.

RESULTS

Effect of WIN-34B on the production of pro-inflammatory mediators in fibroblast-like synoviocytes, RAW264.7 macrophages, and mouse peritoneal macrophages

In order to understand the molecular mechanism by which WIN-34B inhibits pain and inflammation *in vivo*, its *in vitro* analgesic and anti-inflammatory effects were tested in fibroblast-like synoviocytes (FLSs) from patients with rheumatoid arthritis (RA) and macrophages. First, we determined whether WIN-34B inhibits the increased production of IL-6, PGE_2, and MMP-13, which play important roles in inducing inflammation, pain, and bone degradation, respectively, in IL-1β-stimulated RA FLSs (Fig. 1). WIN-34B at a concentration of 10-80 μg/ml significantly inhibited production of these proteins in a dose-dependent manner. Consistent with the protein levels, the mRNA levels were also inhibited by WIN-34B. The inhibitory effects of WIN-34B were comparable to those of Joins™ and celecoxib. When the cells were cultured for 3 days in the presence of 400 μg/ml WIN-34B, Joins™, and celecoxib, they did not show any cytotoxic effects when examined under the microscope or with MTT assay (data not shown). To demonstrate the anti-inflammatory and analgesic effects of WIN-34B in LPS-stimulated RAW264.7 murine macrophages, RAW264.7 cells were activated with LPS in the presence or absence of WIN-34B. As shown in Fig. 2, WIN-34B inhibited the increased production of PGE_2 in LPS-stimulated RAW264.7 cells in a

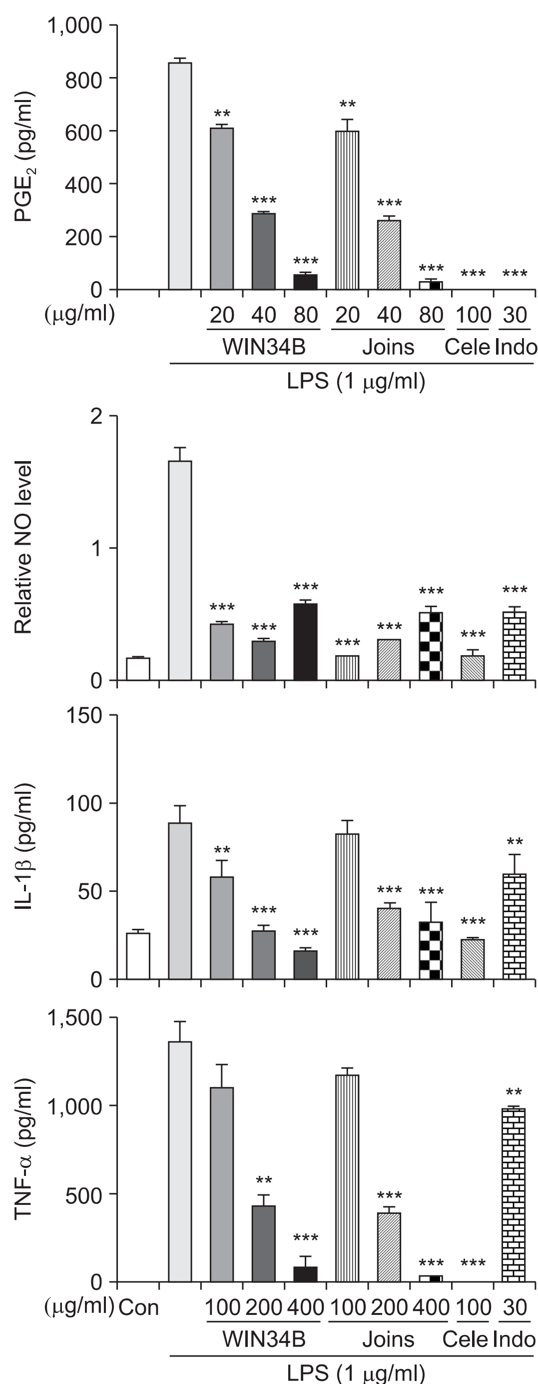

Fig. 2. Effect of WIN-34B on the production of pro-inflammatory mediators in LPS-stimulated RAW264.7 macrophages. The levels of PGE_2, NO, IL-1β, and TNF-α were measured in LPS-stimulated RAW264.7 cells in the presence of WIN-34B as described in the Materials and methods. Three independent experiments were performed in quadruplicate. Similar results were obtained for all three, and the data shown are representative. Values are expressed as mean ± S.E.M. $^{**}p<0.01$, $^{***}p<0.001$ versus LPS-treated group in the absence of WIN-34B. Cele: celecoxib, Indo: indomethacin, Con: control.

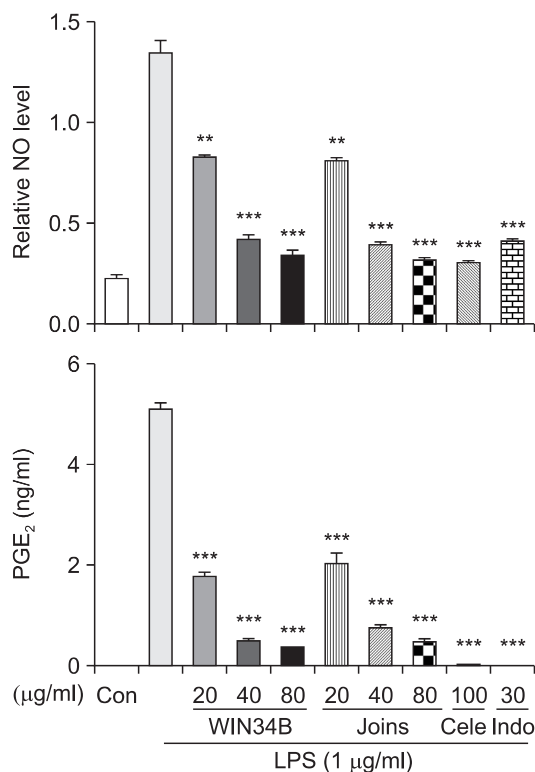

Fig. 3. Effect of WIN-34B on the production of pro-inflammatory mediators in LPS-stimulated peritoneal macrophages. The levels of PGE_2 and NO were measured in LPS-stimulated peritoneal macrophages in the presence of WIN-34B as described in the Materials and methods. Three independent experiments were performed in quadruplicate. Similar results were obtained for all three, and the data shown are representative. Values are expressed as mean ± S.E.M. ***p*<0.01, ****p*<0.001 versus the LPS-treated group in the absence of WIN-34B. Cele: celecoxib, Indo: indomethacin, Con: control.

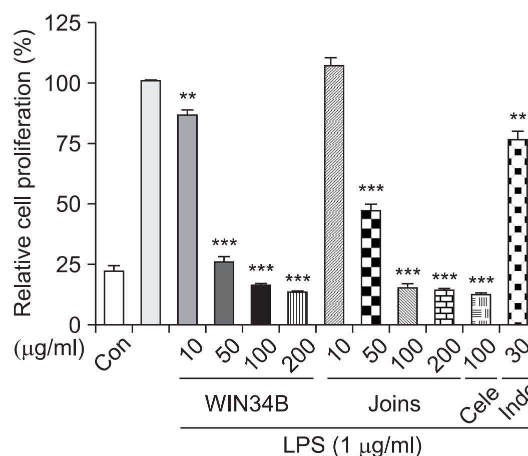

Fig. 4. Effect of WIN-34B on LPS-stimulated lymphocyte proliferation. The lymphocytes were obtained from the spleen and stimulated with LPS (1 μg/ml) in the presence of WIN-34B as described in the Materials and methods. Three independent experiments were performed in quadruplicate. Similar results were obtained for all three, and the data shown are representative. Values are expressed as mean ± S.E.M. ***p*<0.01, ****p*<0.001 versus the LPS-treated group in the absence of WIN-34B. Cele: celecoxib, Indo: indomethacin, Con: control.

dose-dependent manner. Also, the levels of nitric oxide (NO), IL-1β, and TNF-α were significantly inhibited by WIN-34B in a dose-dependent manner. To demonstrate the anti-inflammatory effect in murine peritoneal macrophages, peritoneal macrophages were obtained from mice and stimulated with LPS. The production of NO and PGE_2 in LPS-stimulated peritoneal macrophages was inhibited by WIN-34B in a dose-dependent manner, similar to that of RAW264.7 (Fig. 3). In contrast, the levels of NO were significantly inhibited by WIN-34B at a lower concentration than in RAW264.7 cells. Also, the levels of TNF-α and IL-1β were too low to be detected in culture supernatant from peritoneal macrophages (data not shown).

Effect of WIN-34B on LPS-stimulated lymphocyte proliferation

To further elucidate the mechanism by which WIN-34B exerts anti-inflammatory effects, we determined whether WIN-34B inhibits the LPS-induced lymphocyte proliferation. As shown in Fig. 4, WIN-34B significantly inhibited cell proliferation in a dose-dependent manner. The inhibitory effect was stronger than that of Joins™ at the same concentration. WIN-34B showed anti-proliferative effects, even at the concentration of 10 μg/ml, though Joins™ did not show an effect at the same concentration.

Effect of WIN-34B on IκB signaling pathways in LPS-stimulated RAW264.7 cells

The IκB signaling pathway is known to play important roles in inflammation and cell proliferation. Thus, we determined whether WIN-34B inhibits the IκB signaling pathway in RAW264.7 macrophages activated by LPS. The level of IκB-α was decreased in response to LPS in RAW264.7 cells. WIN-34B significantly inhibited the LPS-induced degradation of IκB-α at a concentration of 100 μg/ml (Fig. 5A). We also determined whether the decreased degradation of IκB-α by WIN-34B leads to decreases NF-κB migration into the nucleus. The level of p65, which is one component of NF-κB, was significantly inhibited by WIN-34B in a dose-dependent manner, even though there was discrepancy between IκB-α degradation and p65 level in LPS-Indo treated group (Fig. 5B).

DISCUSSION

WIN-34B showed analgesic and anti-inflammatory effects in various animal models of pain and osteoarthritis (Kang *et al.*, 2010). However, the molecular mechanism by which WIN-34B inhibits pain and inflammation *in vivo* remains to be elucidated. In this study, we demonstrated the *in vitro* molecular mechanisms by which WIN-34B exerts analgesic and anti-inflammatory effects. WIN-34B inhibited the production of IL-6, PGE_2, and MMP-13 in IL-1β-stimulated RA FLSs, as well as the production of PGE_2, NO, IL-1β, and TNF-α in LPS-stimulated RAW264.7 macrophages. This effect was also demonstrated in LPS-stimulated peritoneal macrophages.

WIN-34B allows fast significant pain relief and the development of a better therapeutic drug originating from natural herbs, unlike current herbal drugs such as Joins™. The major

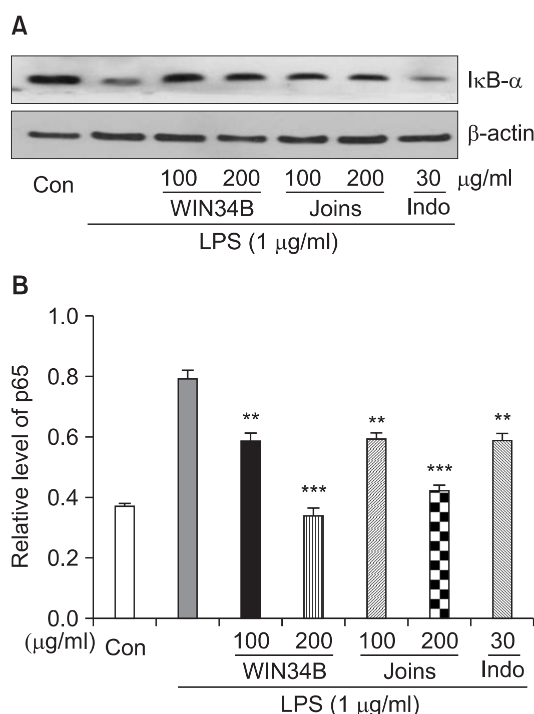

Fig. 5. Effect of WIN-34B on IκB signaling pathways in LPS-stimulated RAW264.7 cells. (A) The cells were activated with LPS for 2 hrs and prepared for Western blot analysis to detect the level of IκB-α as described in the Materials and methods. (B) The level of NF-κB was measured using ELISA for p65, one of the components of NF-κB. Three independent experiments were performed in quadruplicate. Similar results were obtained for all three, and the data shown are representative. Values are expressed as mean ± S.E.M. ***p*<0.01, ****p*<0.001 versus the LPS-treated group in the absence of WIN-34B. Indo: indomethacin, Con: control.

drawback of therapeutic drugs from natural herbs is the lack of an immediate analgesic effect for osteoarthritis. Thus, herbal drugs have been used in clinics with other painkillers, such as COX-2 inhibitors. WIN-34B shows immediate analgesic effects and anti-inflammatory effects in various animal models, such as acetic acid-induced writhing test, formalin-induced pain test, hot plate test, tail-flick test, carrageenan-induced paw pressure test, Hargreaves test, acetic acid-induced vascular permeability test, carrageenan-induced paw edema test, croton oil-induced ear edema test, and MIA-induced osteoarthritis test. WIN-34B is currently in phase II clinical trials. However, the molecular mechanisms by which WIN-34B exerts analgesic and anti-inflammatory effects need to be elucidated in more detail. WIN-34B is composed of two herbs, flowers of *Lonicera japonica* Thunb and roots of *Anemarrhena asphodeloides* BUNGE. The mode of action of WIN-34B on anti-osteoarthritis was suggested in our previous report on the basis of the major constituents of WIN-34B (Kang *et al.*, 2010).

Chlorogenic acid, the primary ingredient in *Lonicera japonica* Thunb, has been shown to have anti-inflammatory effects (Qian *et al.*, 2008). Chlorogenic acid at a concentration of ≥12.5 μg/ml inhibits inflammation by suppressing LPS-induced COX-2 expression in RAW264.7 cells *in vitro*, though it does not inhibit NO production (Shan *et al.*, 2009). Chlorogenic acid also inhibited carrageenan-induced paw edema and formalin-induced pain in rats *in vivo* (dos Santos *et al.*, 2006). Furthermore, chlorogenic acid reduced the release of NO from LPS/IFN-γ-stimulated C6 astrocyte cells (Soliman and Mazzio, 1998). In our study, WIN-34B significantly inhibited the level of PGE_2 and NO in LPS-stimulated RAW264.7 and murine peritoneal macrophages. Considering the concentration of chlorogenic acid contained in WIN-34B, WIN-34B seems to be more effective than chlorogenic acid alone, and other constituents may cooperate to inhibit their production in this system. In addition, mangiferin, which has strong anti-inflammatory properties, is a major active constituent of *Anemarrhena asphodeloieds* BUNGE (Jung *et al.*, 2009). Mangiferin inhibits LPS-induced NO and PGE_2 through NF-κB inactivation in RAW264.7 macrophages and rat microglial cells, which exhibit anti-inflammatory effects *in vitro* (Bhatia *et al.*, 2008). Two components, loiceroside C and anemarsaponin B of *Lonicera japonica* Thunb and *Anemarrhena asphodeloides* BUNGE, have shown anti-inflammatory effects. We expect that WIN-34B also inhibits inflammation through the IκB signaling pathway. Therefore, we first investigated whether WIN-34B has an inhibitory effect on the IκB signaling pathways. WIN-34B significantly inhibited the migration of NF-κB into the nucleus, and it was more active than the positive control indomethacin (30 μg/ml). However, other constituents are also involved in the analgesic and anti-inflammatory effects through other signaling pathways. The amounts of the two components in *Lonicera japonica* Thunb and *Anemarrhena asphodeloides* BUNGE are very small (Kwak *et al.*, 2003; Kim *et al.*, 2009). Thus, WIN-34B may work as an anti-osteoarthritic agent by decreasing inflammation and pain via the inhibition of leukotriene and prostaglandin production by the main components of WIN-34B, such as chlorogenic acid and mangiferin, or by unknown components.

WIN-34B also inhibited LPS-induced lymphocyte proliferation. In particular, WIN-34B was more effective than Joins™, even at the concentration of 10 μg/ml. This effect also seems to contribute to the anti-inflammatory effect of WIN-34B. Chlorogenic acid also suppresses T-cell proliferation stimulated by concanavalin A (Con A, 5 μg/ml) at a concentration of 5-80 μg/ml (Lee *et al.*, 2008), and it shows anti-arthritic effects in mice with septic arthritis caused by *Candida albicans*. Chlorogenic acid also inhibits the proliferation of both LNCap and PC-3 cells by increasing cyclin-dependent kinase inhibitor p27 levels (Reddivari *et al.*, 2007). The inhibitory effect was not tested in mouse peritoneal macrophages because the cells were not easy to obtain. Thus, we investigated the signaling pathways in RAW264.6 cells instead. We think that RAW264.7 macrophages can be used to show the signaling pathways in normal cells.

In conclusion, WIN-34B, which is formulated with 2:1 flowers of *Lonicera japonica* Thunb and roots of *Anemarrhena asphodeloides* BUNGE, exhibits better anti-nociceptive and anti-inflammatory activities than celecoxib or Joins™, even in osteoarthritic animal models. WIN-34B seems to inhibit pain and inflammation through the inhibition of the IκB signaling pathway or unknown mechanisms. WIN-34B also inhibits lymphocyte proliferation, contributing to the inhibition of inflammation. WIN-34B does not appear to exhibit any adverse gastrointestinal or cardiac effects and is successfully undergoing phase II clinical trials. Thus, WIN-34B has strong potential to become a novel therapeutic drug for osteoarthritis with superior efficacy and safety compared to other commonly used

drugs.

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