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Suppression of Cyclooxygenase-2 Expression in Colonic Epithelial Cells by Ilekudinol B Isolated from *Weigela subsessilis*

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Abstract – Ilekudinol B is one of the flavonoids isolated from *Weigela subsessilis* (Caprifoliaceae). In the present study, the suppression effect of ilekudinol B on tumor necrosis factor (TNF)-α-induced cyclooxygenase-2 (COX-2) expression was investigated in human colon epithelial cell line HT-29. Interleukin-8 (IL-8) production and prostaglandin E_2 (PGE_2) secretion was measured by enzyme-linked immunosorbent assay (ELISA). COX-2 and nuclear factor (NF)-κB expression were determined by Western blot analysis. Ilekudinol B significantly inhibited TNF-α-induced secretion of IL-8 and prostaglandin E_2 (PGE_2) from the human colon epithelial cell line HT-29 in a concentration-dependent manner. In addition, ilekudinol B remarkably diminished TNF-α-induced COX-2 expression and NF-κB p65 subunit translocation to the nucleus. In conclusion, our results indicate that ilekudinol B may have anti-inflammatory activity on TNF-α-dependent colonic inflammation.
Keywords – *Weigela subsessilis,* ilekudinol B, Caprifoliaceae, cyclooxygenase-2, human colon epithelial cells

Introduction

The genus *Weigela*, a member of the family Caprifoliaceae, is comprised of about twelve species (Chang, 1997). All of these plants are widespread and cultivated specifically in Korea, Japan, and Northern China. Among them, four species *W. hortensis, W. praecox, W. florida,* and *W. subsessilis* have been found in Korea (Chang, 1997). *W. subsessilis* is a Korean endemic deciduous shrub, 2-4 m tall. The plant has opposite leaves and yellowish green flowers that bloom on axils in spring, then change to red. *W. subsessilis* grows on sunny mountainous districts. Although this plant is widespread all over Korea, it has been rarely reported for use in folk medicine. Recent phytochemical studies of the leaves of this plant have resulted in the isolation of flavonoids and coumarins (Won *et al.*, 2004; Thoung *et al.*, 2005). The flavonoids from this plant were reported as kaempferol-*O*-3-α-L-(3-*O*-acetyl) rhamnopyranosyl-7-*O*-α-L-rhamnopyranoside sutchuenoside A, kaempferitrin, astragalin, kaempferol 7-*O*-rhamnoside, and kaempferol-3-*O*-α-L-rhamnosyl-7-*O*-β-D-glucoside (Won *et al*, 2004). Four coumarins, scopoletin, cleomiscosin A, scopolin, and fraxin, were also isolated from these leaves. We previously reported that scopoletin and cleomiscosin A, isolated from *Weigela subsessilis*, inhibited low-density lipoprotein (LDL) oxidation involved in the pathogenesis of atherosclerosis (Thuong *et al.*, 2005a).

Inflammatory bowel diseases (IBDs), ulcerative colitis (UC) and Crohn's disease (CD), are chronic recurrent inflammatory disorders of the gastrointestinal tract (Fiocchi, 1998; Podolsky, 1991). They are characterized by a pronounced infiltration of neutrophils into colonic lesion accompanied by epithelial cell necrosis and ulceration. Although the exact pathogenesis of IBDs is poorly understood, there is evidence that it involves interaction between the immune system, genetic susceptibility, and the environment, most notably the bacterial flora. During gut inflammation, intestinal epithelial cells (IECs) receive their activating signals from interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), bacteria, and bacterial products (Nemeth *et al.*, 2002). TNF-α is a potent activator of IECs to produce chemokine IL-8, which is an important mediator of inflammation of the CXC chemokine family that recruits neutrophils into the inflamed tissue (Mitsuyama *et al.*, 1994). An enhanced synthesis of IL-8 has been shown in the intestinal mucosa from patients with inflammatory bowel disease (IBD)

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(Mahida *et al.*, 1992). The current approaches for the treatment of this disease are based on nonspecific suppression of the immune system. NF-κB inhibitor and MAPK inhibitor were used in patients with UC (Waetzig *et al.*, 2002). However, not all patients respond to these drugs. On this line, a number of researchers are in search of the new, potent and less toxic approaches to treat UC.

We previously have reported that ilekudinol B isolated from *Weigela subsessilis* exhibits the inhibitory effect on the IL-8 production in the HT29 cells stimulated by TNF-α (Thuong *et al.*, 2005b). It shows the possibility of ilekudinol B as a candidate of anti-inflammatory drug. In the present study, we evaluated the inhibitory functions of ilekudinol B on the TNF-α-induced COX-2 and PGE_2 production.

Materials and Methods

Plant material and isolation of ilekudinol B – Ilekudinol B was isolated as described previously (Thuong *et al*, 2005b). Briefly, The methanol extracts were concentrated *in vacuo*, resuspended in water, and partitioned successively with hexane and EtOAc. The EtOAc fractions were subjected to column chromatography on a silica gel eluted with hexane-acetone (10:1 to 0:1) to get 8 fractions ($F_1 \sim F_8$). Ilekudinol B was obtained from F_3 fraction by the C-18 column chromatography eluted with MeOH-H_2O (20:1). The amorphous powder of ilekudinol B revealed mp 193 - 195 °C.

Cell culture – HT29 human colonic epithelial cells were purchased from Korean Cell Line Bank (Seoul National University, Seoul, Korea) and grown in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin sulfate. Cells were incubated in humidified 5% CO_2 atmosphere at 37 °C. Ilekudinol B was dissolved in dimethyl sulfoxide (DMSO) and the final concentration of DMSO used for ilekudinol B treatment was adjusted to 0.01%. DMSO (0.01%) did not affect HT29 cells. Recombinant human TNF-α (R&D Systems, Minneapolis, MN) was used as a stimulator.

Enzyme-linked immunosorbent assay (ELISA) for IL-8 – HT29 cells were cultured at 3×10^5 cells into 24-well tissue culture plates (500 μl/well). The cells were pretreated with various concentrations of ilekudinol B for 30 min before TNF-α stimulation. Six hours after TNF-α stimulation, IL-8 levels in the supernatant were measured by ELISA according to the commercial instruction (BD Pharmingen, San Diego, CA). The 96-well plates were coated overnight at 4 °C with antihuman IL-8 monoclonal antibody diluted in coating buffer (0.1 M carbonate, pH 9.5) and washed twice with phosphate-buffered saline (PBS) containing 0.05% Tween-20. All subsequent steps took place at room temperature. Nonspecific protein binding was blocked with assay diluent (PBS containing 10% FBS, pH 7.0) for at least 1 hr. Each samples diluted in assay diluent were applied to wells and incubated for 2 hrs. All standards and samples were assayed in triplicate. The working detector (biotinylated anti-human IL-8 monoclonal antibody and streptavidin-horseradish peroxidase reagent) was applied and incubated for 1 hr. The substrate solution containing tetramethylbenzidine (TMB) and hydrogen peroxidase was added to wells and incubated for 30 min in the dark. The reaction was stopped by the addition of 2N-H_2SO_4, and the absorbance of samples was read at 450 nm within 30 min.

Enzyme immunoassay (EIA) for prostaglandin E_2 (PGE_2) – HT29 cells were cultured at 3×10^5 cells into 24-well tissue culture plates (500 μl/well). The cells were pretreated with various concentrations of ilekudinol B for 30 min before TNF-α stimulation. After 24 hrs of incubation, the supernatants were collected. Supernatants of the cells were used for PGE_2 determination using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). The immunoassay was performed according to manufacturer's protocol. In short, medium from cells added to 96-well plates coated with an anti-mouse antibody, mixed with a PG/acetylcholinesterase tracer and a monoclonal antibody against prostaglandin and incubated at 4 overnight. Unbound PG/acetylcholinesterase was removed and washed extensively and bound acetylcholinesterase was detected by Ellman's reagent and measured at 450 nm.

Western blot assay – To investigate cyclooxygenase-2 (COX-2) expression, ilekudinol B-pretreated HT29 cells (5×10^5/ml) were stimulated with TNF-α (50 ng/ml) for 8 hrs. Cells were rinsed twice with ice-cold PBS and harvested by scraping in 1 ml of ice-cold PBS. After centrifugation, cell pellets were lysed in 400 μl of lysis buffer (iNtRON Biotech) for 30 min on ice. The lysates were centrifuged at 13,000 × g for 5 min, and supernatants were immediately aliquoted and stored at −70 °C until use. Protein concentration was determined using a bicinchoninic acid protein assay (Sigma), and 20 μg of protein was separated by 12% SDS PAGE and transferred to PVDF membrane (Invitrogen, Carlsbad, CA). After blocking with 5% skim milk, membranes were incubated with anti-human COX-2 antibody (Santa Cruz Biotech, Santa Cruz, CA) for 16 hrs at 4 °C. After washing twice with Tris-buffered saline, membranes were immunoblotted with the HRP-conjugated anti-IgG antibody (DAKO, Bucks, UK)

for 1 hr at room temperature. Epitopes on proteins recognized specifically by antibodies were visualized by using enhanced chemiluminescence kit (Amersham Pharmacia Biotech). The other membrane was immunoblotted with anti-β-actin antibody (Santa Cruz Biotech) as respective loading controls.

To investigate the translocation of NF-κB to the nucleus, ilekudinol B-pretreated HT29 cells were stimulated for 15 min with TNF-α (50 ng/ml). Nuclear extracts were prepared, as described previously (Kim *et al*, 2004). Briefly, cells were washed in 1 ml of ice-cold PBS, resuspended in 400 μl of ice-cold hypotonic buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF], left on ice for 10 min, and centrifuged at 15,000 × g for 30 s. Nuclear pellets were gently resuspended in 50 μl of ice-cold saline buffer [50 mM HEPES (pH 7.9), 50 mM KCl, 1 mM DTT, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, and 0.5 mM PMSF], left on ice for 20 min, and centrifuged at 15,000 × g for 5 min at 4 °C. Nuclear extracts were stored at −70 °C until use. Protein concentration was determined using a bicinchoninic acid protein assay (Sigma), and 20 μg of protein was separated by 12% SDS PAGE and transferred to PVDF membrane (Invitrogen, Carlsbad, CA). After blocking with 5% skim milk, the membrane was immunoblotted with anti-NK-κB antibody (NF-κB p65, Santa Cruz Biotech) for 16 hrs at 4 °C.

Statistical analysis – The results were expressed as mean ± S.E. for the number of experiments. Statistical significances were compared between each treated group and control by the Student's *t*-test. Each experiment was repeated at least three times and yielded comparable results. Values with $p < 0.05$ were considered significant.

Results

Effects of ilekudinol B on TNF-α-induced IL-8 production in HT29 cells – We examined the peak time and adequate concentration of TNF-α for IL-8 production in HT29 cells. After stimulation with TNF-α, cell-cultured mediums were harvested to measure the level of IL-8 production by ELISA. IL-8 production was clearly detected 6 hrs after stimulation with TNF-α and the level of IL-8 production was steadily increased in a time-dependent manner. IL-8 production also was distinctly increased in 10 ng/ml TNF-α and peaked in 50 ng/ml TNF-α (data not shown). Next, we investigated the inhibitory effect of ilekudinol B on the TNF-α-induced IL-8 production in HT29 epithelial cells. HT29 cells were exposed with ilekudinol B in concentrations of 2, 10, and 50 ng/ml for 30 min before stimulation with 50 ng/ml of TNF-α. After TNF-α stimulation, the level of IL-8 production was analyzed by ELISA. Ilekudinol B significantly inhibited TNF-α-induced IL-8 production in a concentration-dependent manner (Fig. 2). This result shows that ilekudinol B inhibit TNF-α-induced IL-8 production in HT29 epithelial cells.

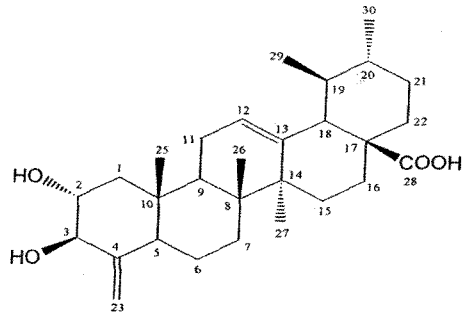

Fig. 1. Chemical structure of ilekudinol B.

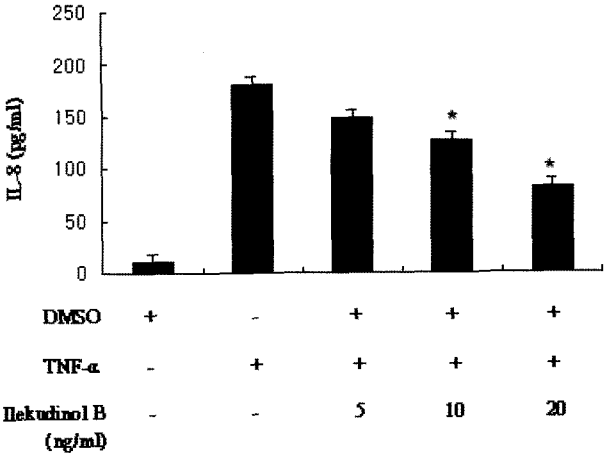

Fig. 2. Effect of ilekudinol B on IL-8 production in TNF-α-stimulated HT29 cells. The cells (3×10^5 cells/ml) were pretreated with ilekudinol B (2, 10, and 50 ng/ml) 30 min before TNF-α (50 ng/ml) stimulation. After TNF-α stimulation for 6 hrs, levels of IL-8 in the supernatants were measured by ELISA. Values are the mean ± S.E. of duplicate determinations from three separate experiments (*$p < 0.05$).

Effect of ilekudinol B on TNF-α-induced PGE_2 production in HT29 cells – We investigated the inhibitory effect of ilekudinol B on the TNF-α-induced PGE_2 secretion in HT29 epithelial cells. HT29 cells were exposed with ilekudinol B in concentration of 2, 10, and 50 ng/ml 30 min before stimulation with 50 ng/ml of TNF-α. After TNF-α stimulation, the level of PGE_2 was determined by ELISA. Ilekudinol B significantly inhibited TNF-α-induced PGE_2 secretion in a concentration-dependent manner (Fig. 3). This result indicates that ilekudinol B inhibit TNF-α-

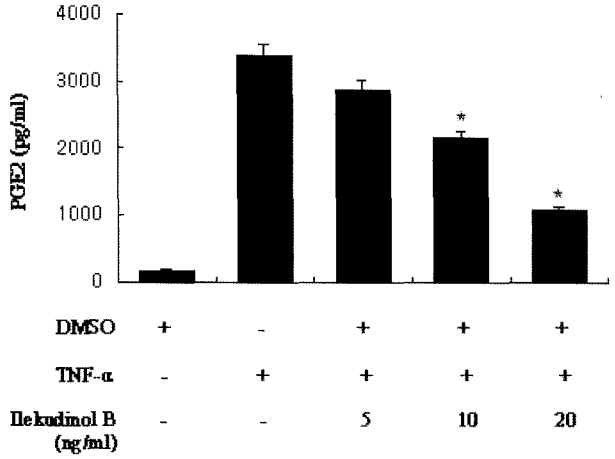

Fig. 3. Effect of ilekudinol B on PGE_2 secretion in TNF-α-stimulated HT29 cells. The cells (1×10^6 cells/ml) were pretreated with ilekudinol B (2, 10, and 50 ng/ml) 30 min before TNF-α (50 ng/ml) stimulation. After 24 hrs, levels of PGE_2 in the supernatants were measured by ELISA. Values are the mean ± S.E. of duplicate determinations from three separate experiments (*$p < 0.05$).

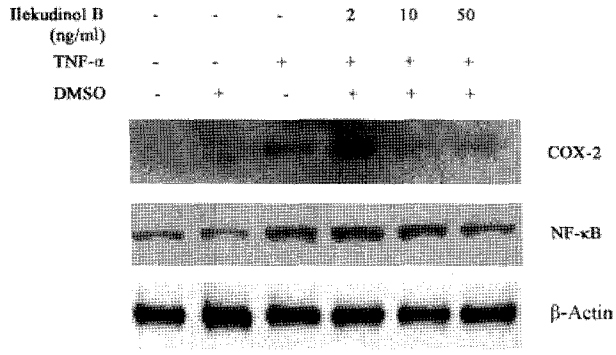

Fig. 4. Effect of ilekudinol B on COX-2 expression and NF-κB translocation in TNF-α-stimulated HT29 cells. The cells (5×10^6 cells/ml) were pretreated with three different concentration of ilekudinol B (2, 10, and 50 ng/ml) 30 min before TNF-α (50 ng/ml) stimulation. After 8 hrs incubation, Cell lysates were obtained, and COX-2 expression were analyzed by Western blot using anti-COX-2 antibody. In addition, after incubation for 15 mins, nuclear extracts were prepared and translocation of NF-κB to the nuclear was analyzed by Western blot using anti-NF-κB antibody (NF-κB p65). β-Actin was used to confirm equivalency of protein. DMSO (0.01%) was used as a vehicle without ilekudinol B. The similar data were obtained in three independent experiments.

induced PGE_2 secretion in HT29 cells.

Effect of ilekudinol B on TNF-α-induced COX-2 expression and NF-κB translocation in HT29 cells – The possibility that ilekudinol B may reduce the TNF-α-induced COX-2 expression via an inhibition of the transcription factor NF-κB was then addressed. The ilekudinol B-pretreated HT29 cells were stimulated with TNF-α for 8 hrs. COX-2 expression was analyzed by Western blot using anti-COX-2-antibody. We observed that TNF-α treatment led to an increase in COX-2 expression, and ilekudinol B remarkedly diminished TNF-α-induced COX-2 expression at concentration of 10 and 50 ng/ml (Fig. 4).

Next, we examined the effect of ilekudinol B on TNF-α-induced translocation of NF-κB to the nucleus in HT29 cells. The ilekudinol B-pretreated HT29 cells were stimulated with TNF-α for 15 min. Cell lysates were prepared for Western blot analysis using anti-NF-κB antibody. We observed that TNF-α treatment led to an increase in the translocation of NF-κB p65 to the nucleus, and ilekudinol B inhibited in a concentration-dependent manner (Fig. 4). These results indicate that ilekudinol B might suppress TNF-α-induced-COX-2 expression through inhibition of the translocation of NF-κB p65 subunit to the nucleus.

Discussion

The chemokines are induced by TNF-α stimulation and are abundantly expressed and secreted in IBD (Ajuebor and Swain, 2002; Banks *et al*, 2003; Papadakis, 2004). TNF-α also showed potent effect on the inflammatory pathway inducing PGE_2 production via COX-2 expression (Kim *et al.*, 1988).

We previously have reported only effect of twelve compounds containing ilekudinol B isolated from *Weigela subsessilis* on TNF-α-induced IL-8 production in the HT29 cells (Thuong *et al.*, 2005b). In the present study, we demonstrated that ilekudinol B significantly diminished COX-2 expression and PGE_2 production in the human colon epithelial cells. It supports that ilekudinol B may interfere with the recruitment of polymorphonuclear leukocyte and thus may impede progression and aggravation of inflammation (Sugimoto *et al.*, 2002; Choi *et al.*, 2005). Furthermore, we observed that ilekudinol B inhibits PGE_2 production and COX-2 expression. Prostaglandins have been known to be important regulators of gastrointestinal fluid secretion. The food allergies or invasion of microorganisms lead to a synthesis of Ca^{2+}-dependent PGE_2 in the intestinal epithelium and the high level of PGE_2 was observed in IBD patients (August *et al.*, 1999). Exogenous PGE_2 has been reported to up-regulate IL-8 expression in human colonic epithelial cells (Yu and Chadee, 1998). Our results thus indicate that ilekudinol B could diminish IL-8 production through blockage of PGE_2 synthesis and COX-2 expression in HT29 cells.

Recent studies have documented the increased NF-κB activation in mucosa tissue of UC patients (Gan *et al.*, 2002), as well as *in vitro* evidence showing a role of NF-

κB on TNF-α-mediated gene expression in IECs (Jobin *et al.*, 1999; Kim *et al.*, 2004). TNF-α-mediated NF-κB activation also is involved in COX-2 expression in HT-29 cells (Jobin *et al.*, 1998). In this study, we observed that ilekudinol B inhibit the translocation of NF-κB to nuclei in a concentration-dependent manner. In conclusion, ilekudinol B might modulate COX-2 expression, PGE_2 synthesis, and IL-8 secretion through inhibition of the NF-κB activation. Therefore, ilekudinol B could be a useful in intestinal inflammation and its effect remain to be seen in various animal models.

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