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Falcarindiol from *Angelica koreana* Down-regulated IL-8 and Up-regulated IL-10 in Colon Epithelial Cells

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Abstract – *Angelica koreana* is an important medicinal plant for some locals in East Asia including Korea. A few reports have shown the efficacy of its phytochemical constituents. We have isolated and purified one compound falcarindiol (FAL) from the methanolic extract of *A. koreana* roots. At concentrations from to 1 μM to 25 μM, the FAL isolated from the roots of *A. koreana* exerted no significant cytotoxicity and down-regulated LPS-stimulated pro-inflammatory cytokine IL-8 in colon epithelial cells, while up-regulating anti-inflammatory cytokine IL-10. In addition, the FAL decreased the expression of LPS-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 protein by Western blot analysis. Colon epithelial cells play pivotal roles in regulating the colon immune system and thus FAL is expected to be candidate agent as therapeutic potential for the treatment of inflammatory bowel disease (IBD) by modulating LPS-induced inflammation in colon epithelial cells.
Keywords – *Angelica koreana*, Falcarindiol, Inflammatory bowel disease, Colon epithelial cells

Introduction

Inflammation is a defensive physiological reaction of the innate immune system against a variety of stimuli including infections and tissue damage.[1] However, chronic inflammatory response may induce various diseases such as colon carcinogenesis.[2] Intestinal epithelial cells not only present a physical barrier to bacteria but also actively take part in immune and inflammatory reactions. Macrophages are also involved in intestinal homeostasis and the pathology of inflammatory bowel disease (IBD).[2] Tainted colon epithelial cells secrete pro-inflammatory cytokines such as interleukin (IL)-8.[3] On the other hand, IL-10 is important anti-inflammatory cytokine and plays a central role in preventing certain inflammatory diseases by down-regulating inflammatory cascades.[4] Cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) are central enzymes that mediate inflammatory processes.[5] These inducible enzymes are essential components of the inflammatory response, ultimate repair of injury, and carcinogenesis. Moreover, they have critical roles in the response of tissues to injury or infectious agents. Thus, the regulating of these inflammatory mediators is a key strategy for treating chronic inflammatory illnesses including IBD.

The roots of *Angelica koreana* Nakai (Umbelliferae) have been used in oriental herbal medicine for treatment of the common cold, especially for patients that present with symptoms such as chills, fever, headaches, body aches, and pains.[6] *A. koreana* has been studied for anti-oxidant and anti-fungal activity anti-inflammatory effect, melanogenesis inhibition, and anti-cancer activity.[7-9] Regarding the chemical composition, a series of coumarins, monoterpenes, sesquiterpenes, and polyacetylenes have been isolated from this plant.[10] Among them, there have some reports indicating their anti-inflammatory activity of polyacetylenes. Falcarindiol (FAL) isolated from *A. koreana* is a natural polyacetylene and is also contained in a variety of vegetables such as carrot. FAL isolated from *A. furcijuga* significantly reduced nitric oxide (NO) production in lipopolysaccharide (LPS)-activated mouse peritoneal macrophages.[11] Reed et al. found that polyacetylenes containing FAL of *Daucus carota* decreased LPS-induced expression of inflammatory proteins in macrophage and endothelial cells.[12] These previous studies suggest the possible presence of anti-inflammatory polyacetylene, FAL. However, anti-inflammatory activities of FAL isolated

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from *A. koreana* for treating chronic inflammatory illnesses containing IBD have not been investigated yet. HT-29 colon epithelial cells are known as an *in vitro* model for inflammatory based researches.[5] Therefore, in this study, the anti-inflammatory response was conducted using HT-29 human colon carcinoma cells.

Here, to specifically determine whether an anti-inflammatory agent could be developed from FAL of *A. koreana*, its inhibitory activity against the common pathogenic substance was investigated in LPS stimulated colon epithelial cells. In addition, we assessed the anti-inflammatory effects of FAL from *A. koreana* roots by measuring response of inflammatory mediators such as IL-8, Il-10, iNOS, and COX-2 in HT-29 colon epithelial cells.

Experimental

Plant material – The dried roots of *A. koreana* were purchased from Kyung-dong market, Seoul, Korea, in May 2008. A voucher specimen (CBNU 2008017) was deposited at the Herbarium of College of Pharmacy, Chungbuk National University.

Extraction and isolation – The dried and powdered roots of *A. koreana* (3 kg) were extracted with methanol (3 × 5 L) at room temperature, and the methanol solution was then evaporated under reduced pressure. The methanol extract (640 g) was suspended in H_2O and partitioned successively with n-hexane, CH_2Cl_2, and ethyl acetate. The CH_2Cl_2-soluble fraction (41 g), which showed the most potent inhibitory activity of NO production was chromatographed on a silica gel column and eluted with n-hexane-acetone gradient system to give nine fractions (M1-M9). M4 (3.8 g) was chromatographed on a RP-18 column (6 × 55 cm) and eluted with methanol-H_2O gradient (from 55:45 to 100:0, 1200 mL for each step) to yield twelve fractions. M410 (300 mg) was chromatographed on a RP-18 column (2 × 30 cm) and eluted with methanol-H_2O (from 55:45 to 0:100, 400 mL for each step) to afford FAL (50 mg).

Falcarindiol Colorless oil; ESIMS *m/z* 283 $[M+Na]^+$; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ_H 5.95 (1H, ddd, J = 17.0, 7.0, 5.5 Hz, H-2), 5.61 (1H, m, H-10), 5.52 (1H, m, H-9), 5.48 (1H, m, H-1), 5.27 (1H, m, H-1), 5.22 (1H, d, J = 8.0 Hz, H-8), 4.95 (1H, d, J = 5.5 Hz, H-3), 2.12 (2H, dq, J = 7.5, 1.5 Hz, H-11), 1.39 (2H, m, H-12), 1.25-1.35 (8H, m, H-13,14,15,16), 0.89 (3H, t, J = 7.0 Hz, H-17); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ_C 135.8 (C-2), 134.5 (C-10), 127.6 (C-9), 117.2 (C-1), 79.8 (C-7), 78.3 (C-4), 70.2 (C-5), 68.6 (C-6), 63.3 (C-3), 58.5 (C-8), 31.8 (C-15), 29.3 (C-12), 29.2 (C-13), 29.1 (C-14), 27.6 (C-11), 22.6 (C-16), 14.1 (C-17)

Cell culture and cell viability – HT-29 human colonic epithelial cells used in this study were purchased from Korean Cell lines bank (Seoul, Korea). The cells were grown in Dulbecco's modified Eagle's medium (Hyclone, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, USA), containing 100 IU/ml penicillin and 100 μg/ml streptomycin. Experiments were performed with HT-29 cells treated with FAL at a final concentration of 0.1 – 100 μM for 1 h, and then treated with LPS for 24 h. The cck-8 assay (Dojindo, Japan) was used to quantify cell viability. We used the bacterial antigen LPS (1 μg/ml; *Escherichia coli* strain B8:0127; Sigma Chemical).

Enzyme-linked immunosorbent assay – Monoclonal anti-IL-8 and IL-10 antibody (R&D Systems) suspended in coating buffer (14.2 mM Na_2CO_3, 34.9 mM $NaHCO_3$, and 3.1 mM NaN_3, pH 9.6) was added to microtiter plates (Costar, Corning, NY, USA) and incubated overnight at 4 ^{o}C. Plates were washed three times with phosphate buffered saline supplemented containing 0.05% Tween-20 (PBST) and blocked with PBST containing 1% bovine serum albumin for 1 h at 37 ^{o}C. One hundred microliters of sample or diluted standard was added to the plates, which were then incubated for 2 h at room temperature (RT). After washing with PBST, 100 μl of biotinylated goat anti-mouse IgG (R&D Systems) was added, and the plate was incubated for 1 h at 37 ^{o}C. Streptavidin-horseradish peroxidase (HRP) conjugate (Amersham, Buckinghamshire, UK) was then added, and color was developed by adding 100 μl of tetramethylbenzidine and incubating for 30 min at RT. The reaction was stopped with 4 M H_2SO_4, and OD values were measured at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader.

Western blotting – The expressions of iNOS and COX-2 were determined by Western blotting. HT-29 cell lysates were prepared using an ice-cold lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, 25 μg/ml leupeptin, and 20 μg/ml pepstatin). Equivalent protein samples were resolved in 10% SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), which were blocked with TBST [10 mM Tris (pH 7.4), 100 mM NaCl, and 0.5% Tween 20] containing 5% non-fat milk for 30 min at RT. For immunodetection, membranes were incubated overnight with anti-iNOS and COX-2 antibody (1:1000, Cell Signaling, MA, USA) in TBST containing 1% milk powder and then incubated with HRP, conjugated anti-mouse-IgG (1:1000, Santa Cruz, CA, USA). Bands were visualized by enhanced chemiluminescence (ECL-kit, Termo, USA). The intensity

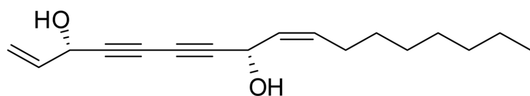

Fig. 1. The chemical structure of falcarindiol (FAL) isolated from *A. koreana*.

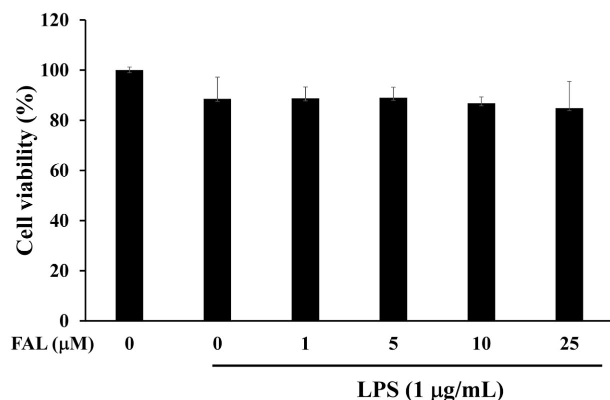

Fig. 2. Effects of FAL on the cell viability of HT-29 colon epithelial cells. The cells were preincubated in the presence of the indicated concentrations of FAL for 1 h and then treated with LPS (1 μg/mL) for 24 h. Cell viability was assessed using the CCK-8 kit. The results are presented as the mean ± SD of three individual experiments.

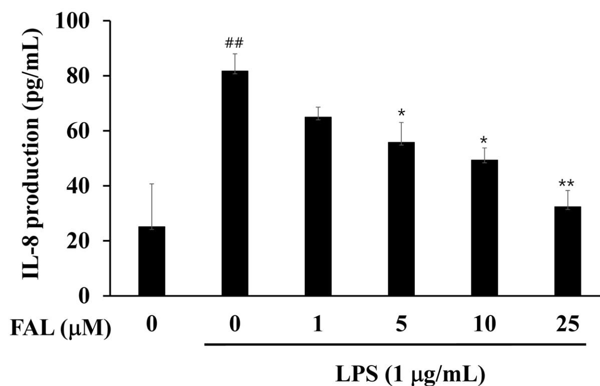

Fig. 3. Effects of FAL on IL-8 production in LPS-stimulated colon epithelial cells. The cells were preincubated for 1h with FAL, then incubated with LPS for 12 h. IL-8 levels were then determined by ELISA (mean ± SD, n = 3). ## $p < 0.01$, compared with untreated control; *$p < 0.05$ and ** $p < 0.01$, compared with LPS-treated control.

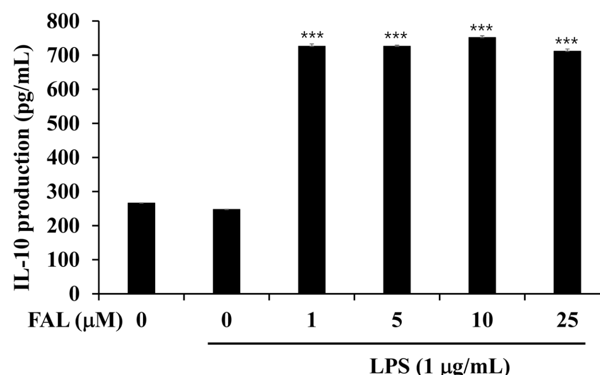

Fig. 4. Effects of FAL on IL-10 production in LPS-stimulated colon epithelial cells. The cells were preincubated for 1 h with FAL, then incubated with LPS for 12 h. IL-10 levels were then determined by ELISA (mean ± SD, n = 3). *** $p < 0.001$, compared with LPS-treated control.

of the bands was measured by densitometry.

Statistical analysis – The evaluation of stastical significance was determined by an "one-way ANOVA" test using computerized statistical package. All data were expressed as means ± standard deviation (SD) of at least three independent experiments, and statistical significance was shown at $p < 0.05$.

Results

During searching the natural products for therapeutics of IBD, we found that FAL isolated from *A. koreana* roots potentially exhibited anti-inflammatory activity through previously reported studies and conducted the isolation of FAL for assay the anti-inflammatory effects in colon epithelial cells. The methanolic extract of *A. koreana* roots was successively fractioned into *n*-hexane, CH_2Cl_2, ethyl acetate, and H_2O fractions. Among those fractions, the CH_2Cl_2 fraction yielded a polyacetylene compound using column chromatography. The polyacetylene compound was identified as falcarindiol from spectral data comparison with those reported in the literature (Fig. 1). A panel of colon epithelial cells (HT-29) was treated with vehicle (DMSO, 0.01%) or 1, 5, 10, 25, 50 μM FAL for 1 h, then cultured with 1 μg/mL LPS for 24 h, and followed by the CCK-8 assay. In Fig. 2., all cells showed that FAL exerted no significant cytotoxicity up to 25 μM (50 μM data not shown); subsequent experiments were conducted with up to 25 μM FAL. IL-8 is a multifunctional member of the chemokine family that is elevated in tissues from IBD patients.[13] It is produced by a variety of cell types including monocytes and macrophages as well as epithelial cells.[14] Also, IL-10 is known as an anti-inflammatory cytokine.[4] Therefore, we investigated the effects of FAL on the expression of IL-8 and IL-10 in LPS-stimulated colon epithelial cells (Fig. 3. and 4). LPS induced IL-8 expression by three-fold compared to untreated cells and FAL down-regulated the LPS-increased IL-8 expression. At concentrations of 1-25 μM, control and LPS treated cells showed the IL-10 expression levels of 260 and 240 pg/mL and FAL recovered the IL-10 levels to 720 - 750 pg/mL. The colon epithelial cells produce

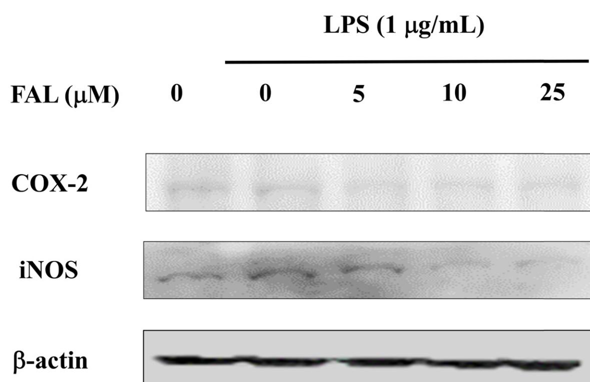

Fig. 5. Effects of FAL on the expression of LPS-induced iNOS and COX-2 protein in HT-29 colon epithelial cells. Cells were pretreated with FAL at the indicated concentrations for 1 h and then treated with LPS (1 μg/mL) for 24 h. Cell lysates were prepared and the iNOS, COX-2 and actin protein levels were determined by western blotting. The relative intensity of iNOS and COX-2 to actin bands was measured by densitometry.

inflammatory mediators, such as interleukins, iNOS and COX-2, when activated by an endotoxin such as LPS.[15] To evaluate whether FAL has an anti-inflammatory effect, we investigated the inhibitory effect of FAL against the overexpression of iNOS and COX-2 in LPS-stimulated colon epithelial cells. As shown in Fig. 5., LPS without FAL treatment induced overexpression of iNOS and COX-2 proteins, while pretreatment of FAL suppressed LPS-mediated overexpression of iNOS and COX-2 proteins. These data indicate that FAL shows anti-inflammatory activity in colon epithelial cells.

Discussion

Chronic inflammation is related to cell proliferation and escape from apoptosis and ultimately contributes to carcinogenesis. Abundant evidence supports a close relationship between inflammation and carcinogenesis, as in chronic inflammatory bowel disease (IBD), such as Crohn's disease and ulcerative disease.[2] COX-2 and iNOS are immediate gene products in the early stage of inflammation and their expression is transiently up-regulated by inflammatory stimuli. Moreover, COX-2 and iNOS expression is enhanced in inflamed tissue and tumors, but is not prominent in the normal colonic epithelium.[16] NO is generated enzymatically by nitric oxide synthases (NOS), and macrophages trigger inflammation via generation of free radicals such as NO in stimulated conditions.[17] In addition, iNOS can be induced by inflammatory cytokines such as IFN-γ, TNF-α, and IL-1.[4,17] These cytokines play a key role in the pathogenesis of IBD, and some may lead to colon carcinogenesis.[2]

A. koreana, a member of the Umbelliferae family, commonly was used herbal traditional medicine for the treatment of headache, rheumatism, neuralgia, and cold. *A. koreana* contains compounds such as coumarin derivatives (bergapten, imperetorin, isoimperetorin, kaholinin, oxypeucedanin, oxypeucedanin-hydrate, prangolarin, and xanthotoxol), monoterpene derivatives (camphene, δ-3-carene, p-cymene, limonene, α-phellandrene, α-pinene, and β-pinene), and sesquiterpene derivatives (angelikoreanone, m-cresol, eudesmol, and osthol), and acetylenic compounds.[10] FAL is a natural polyacetylene isolated from *A. koreana*. It is also contained in a variety of vegetables such as carrot and has been reported to have anti-inflammatory, anti-bacterial, and anti-cancer activities.[18-20] However, anti-inflammatory activities of FAL isolated from *A. koreana* for treating chronic inflammatory illnesses containing IBD have not been investigated yet. Thus, we tried to validate the anti-inflammatory effect of FAL, active constituent of *A. koreana* in colon epithelial cells.

To explore the beneficial effects of FAL in colon inflammation, we treated 1 - 25 μM range of FAL on LPS-stimulated HT-29 colon epithelial cells. HT-29 cell line model can mimic the intestinal epithelial cell condition during the disease and serve as a well-known *in vitro* model for inflammatory based studies during LPS stimulation.[15] Also, HT-29 cells have been known to be the most responsive to LPS stimulation and induce the expression of IL-8.[21] The IL-8, a pro-inflammatory cytokine, is a major chemoattractant and activator of neutrophils in the immune response and the promotion of inflammation.[22] IL-8 stimulates proliferation of colon epithelial cells and the related chemokines.[21] The results showed increasing IL-8 in inflamed versus non-inflamed conditions, like those found in inflamed gastrointestinal mucosa, for example in Crohn's disease and ulcerative colitis.[3] On the other hand, IL-10 is important anti-inflammatory cytokine.[4] In our results, elevated IL-10 levels were associated with a down-regulation of pro-inflammatory cytokines (Fig. 4). However, in our experimental systems, LPS did not significantly decrease the IL-10 compared to non-treated cells, but all doses of FAL surprisingly increased IL-10 production in the presence of LPS as compared to non-treated cells and LPS-stimulated cells. IL-10 deficient mice [IL-10(–/–)] express elevated gene expression of IL-8 in infected mice and this is associated with intestinal barrier dysfunction.[23] FAL significantly inhibited LPS-induced phenotype activation and cytokine secretion in bone marrow-derived dendritic cells.[24] NO is endogenously synthesized from L-arginine

through the catalyzed reaction of NOS enzymes. The down-regulation of iNOS has been regarded as a therapeutics target for treating inflammation while the activation of iNOS leads to various inflammatory diseases.[25] COX-2 is another enzyme that has a pivotal role in mediating inflammation by activation the biosynthesis of prostaglandins.[5] COX-2 and iNOS has been implicated in the pathogenesis of many disease processes, as diverse as chronic inflammation and carcinogenesis.[17] At a concentration of 5 - 25 μM, FAL down-regulated the protein productions of iNOS and COX-2 on LPS-stimulated HT-29 colon epithelial cells by Western blot analysis. These inhibitory effects of FAL were accompanied by the decrease in expression of COX-2 and iNOS protein in a concentration-dependent manner.

In summary, FAL, reported as anti-inflammatory constituent of the roots of *A. koreana,* down-regulated the pro-inflammatory cytokine IL-8 and up-regulated the anti-inflammatory cytokine IL-10 in LPS-stimulated colon epithelial cells. In addition, FAL potently inhibited LPS-induced iNOS and COX-2 production at concentrations from to 1 μM to 25 μM, exerted no significant cytotoxicity. Colon epithelial cells play pivotal roles in regulating the colon immune system and thus FAL is expected to be candidate agent as therapeutic potential for the treatment of IBD by modulating LPS-induced inflammation in colon epithelial cells. Moreover, we expect that anti-inflammatory activity of FAL might be potentiated at in vivo model, dextran sodium sulfate induced IBD mice model, which needs to be elucidated by further investigation.

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