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Oregonin inhibits inflammation and protects against barrier disruption in intestinal epithelial cells

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ABSTRACT

Background and aims: Oregonin, a major diarylheptanoid derivative isolated from *Alnus japonica*, exerts anti-inflammatory effects; however, little is known about the effect of oregonin in intestinal inflammation. The current study investigated the potential of oregonin for clinical applications in the treatment of inflammatory bowel disease (IBD) and elucidated its underlying molecular mechanisms.

Methods: The anti-inflammatory effect of oregonin in tumor necrosis factor-α (TNF-α)-stimulated human intestinal epithelial HT-29 cells was investigated. In addition, the protective effect of oregonin was determined against disruption of the intestinal barrier in *tert*-butyl hydroperoxide (*t*-BH)-stimulated human intestinal epithelial Caco-2 cells.

Results: Oregonin suppressed the expression of cyclooxygenase-2 (COX-2), intercellular adhesion molecule-1 (ICAM-1), IL-8, and IL-1β, and inhibited activation of nuclear factor κB (NF-κB) in HT-29 cells stimulated with TNF-α. Oregonin increased heme oxygenase-1 (HO-1) expression through the ERK1/2 and JNK-dependent signaling pathway, which contributed to the oregonin-mediated suppression of COX-2 expression in the HT-29 cells stimulated with TNF-α. Moreover, oregonin induced AMP-activated protein kinase (AMPK) activation. Knockdown of AMPK abolished the induction of HO-1 protein by oregonin and suppression of oregonin-mediated ICAM-1 and COX-2 expression in the HT-29 cells stimulated with TNF-α. Oregonin prevented the *t*-BH-induced increase in monolayer permeability through inhibition of the reduction in expression of zonula occludens-1 and occludin in Caco-2 cells. Targeting HO-1 by siRNA transfection attenuated the oregonin-mediated prevention of loss of tight junction proteins and increase in permeability.

Conclusion: The findings of this study suggest that oregonin is a potential candidate for treatment of IBD by preventing mucosal inflammation and barrier disruption.

1. Introduction

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, has been associated with chronic and relapsing inflammation of the intestinal tract, which is characterized by an abnormal immune response leading to a persistent inflammatory state [1]. Thus, although the etiology of IBD is largely unknown, most therapies have targeted the suppression of inflammation in IBD [2].

The intestinal epithelial barrier of healthy subjects plays an important part in maintaining intestinal permeability for limiting access of microbes to host tissues and mediating the antigenic traffic from the lumen to the lamina propria. It is now evident that defects in the epithelial barrier function play a major role in several intestinal disorders including IBD [3,4]. Thus, protecting the integrity of the intestinal barrier is an important goal for the development of therapeutic agents for IBD [5]. The formation of a tight protective barrier depends on the activity of junctional complexes, such as tight junctions (TJs) [6]. TJs are located at the most apical end of the lateral membrane and involve protein complexes composed of different proteins, including occludin, tricellulin, claudin, and zonula occludens (ZO) [7]. Recently, several studies have shown that suppression of increases in epithelial permeability along with suppression of decreases in TJ protein expression contributes to protection of the intestinal epithelial barrier [8,9].

Alnus japonica has been used as a health food to help strengthen immunity against infection. Oregonin is a major diarylheptanoid derivative isolated from *A. japonica* [10]. Various biological activities of oregonin have been reported, including antioxidative, hepatoprotective, and anti-inflammatory effects [11,12]. Recently, it has been determined that oregonin inhibits lipopolysaccharide-induced inflammation in mouse macrophages and primary human macrophage [13].

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However, little is known about the role of oregonin in intestinal inflammation.

To investigate the potential of oregonin for clinical applications in the treatment of IBD, in this study, we determined the effects of oregonin on inflammation in intestinal epithelial cells by using human intestinal epithelial HT-29 cells. In addition, we also determined whether oregonin has protective activity against *tert*-butyl hydroperoxide (*t*-BH)-induced dysfunction of the intestinal epithelial barrier by using human intestinal epithelial Caco-2 cells.

2. Materials and methods

2.1. Reagents and cell culture

Oregonin was purchased from Sigma–Aldrich (St. Louis, MO, USA). Extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor (U0126), JNK inhibitor (SP600125), and p38 kinase inhibitor (SB203580) were all obtained from Calbiochem (San Diego, CA, USA). Unless otherwise indicated, reagents were all purchased from Sigma–Aldrich. HT-29 cells and Caco-2 cells were obtained from American Type Cell Culture (Manassas, VA, USA) and maintained under standard cell culture conditions at 37 °C under 5% CO_2 in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) that was supplemented with antibiotics (100 IU/mL of penicillin G and 100 μg/mL of streptomycin) and 10% heat-inactivated fetal bovine serum (Life Technologies Co., Grand Island, NY, USA). For induction of cell growth and monolayer formation on filters, Caco-2 cells were plated on polycarbonate membranes in Transwell inserts (6.5 mm; Costar, Cambridge, MA, USA) and cultured for two weeks before the measurement of transepithelial electrical resistance (TER).

2.2. Cell viability assay

Cell viability was determined by an MTT assay as described previously [14]. A stock solution of MTT was prepared in phosphate-buffered saline (PBS) and diluted in RPMI-1640 medium. Then, this was added at a concentration of 1 mg/mL to cell-containing wells, after removal of the culture medium. Next, the plates were incubated for 4 h at 37 °C under 5% CO_2. Reduction of MTT to formazan was assessed by using an enzyme-linked immunosorbent assay plate reader.

2.3. Western blot analysis

Nuclear and cytosol lysates were isolated by using a Nuclear/Cytosol Fraction kit (BioVision, Mountain View, CA, USA) in accordance with the manufacturer's instructions. The protein concentration was determined by using the Bio-Rad protein assay dye (Bradford) Reagent (Bio-Rad Laboratories, Hercules, CA, USA). Whole-cell lysates, nuclear extracts, and cytosolic extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Nitrocellulose membranes were incubated with specific antibodies against cyclooxygenase-2 (COX-2), intercellular adhesion molecule 1 (ICAM-1), p65, HO-1, ZO-1, occludin, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Antibodies against ERK1/2, JNK, AMP-activated protein kinase (AMPK), phospho-ERK1/2, phospho-JNK, phospho-I-κBα, and phospho-AMPK were purchased from Cell Signaling Technology (Danvers, MA, USA). TATA binding protein (TBP) was obtained from Abcam (Cambridge, UK) for use as a nuclear protein loading control. Immunoreactive bands were detected by incubating anti-rabbit, anti-goat, or anti-mouse IgG antibodies conjugated with horseradish peroxidase and enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.4. RNA isolation and real-time quantitative polymerase chain reaction (PCR)

The total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). Isolated RNA samples were reverse transcribed using the RETRO script kit (Ambion, TX, USA) and then, gene expression levels were analyzed by real-time PCR using SYBR Green PCR Core Reagents (TAKARA, Warrington, Japan) as previously described [8]. The primer sequences were as follows: IL-8 (NM_000584), sense 5′-GACCACACTG CGCCAACAC-3′ and antisense 5′-CTTCTCCACAACCCTCTGCAC-3′; IL-1β (NM_000576), sense 5′-AACAGGCTGCTCTGGGATTCTCTT-3′ and antisense 5′-ATTTCACTGGCGAGCTCAGGTACT-3′; GAPDH (AF261085), sense 5′-GGTGTGAACCATGAGAAGTATGA-3′ and antisense 5′-GAGTCCTTCCACGATACCAAAG-3′. The amount of target mRNA was determined using the comparative threshold (Ct) method by normalizing the target mRNA Ct values to those for GAPDH (ΔCt). Statistical analysis of real-time PCR data was performed using ΔCt values.

2.5. siRNA transfection

Approximately 1×10^6 cells were transfected with HO-1 siRNA, AMPK siRNA or non-targeting siRNA (Santa Cruz Biotechnology) using Dharmacon's Dharmafect 4 transfection reagent (Dharmacon RNA Technologies, Lafayette, CO, USA) in accordance with the instructions of the manufacturer. Cells for use in subsequent experiments were collected 48 h after transfection.

2.6. Measurement of TER

Filter-grown Caco-2 intestinal monolayers were used for measurement of TER with a Millicell-ERS (electrical resistance system; Millipore, Bedford, MA, USA). TER, expressed in Ω/cm^2, was calculated by dividing measured resistance by the monolayer surface area ($0.33\ cm^2$ for 6.5 mm wells). All readings had the resistance of the polycarbonate membrane in Transwell inserts (ca. $30\ \Omega/cm^2$) subtracted before TER was calculated. TER changes under the experimental conditions are expressed as percentage of the corresponding basal values.

2.7. Unidirectional flux of inulin

Transwells with the cell monolayers were incubated in the different experimental conditions in the presence of FITC-inulin (0.5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) in the basal well. The apical and basal media were individually withdrawn, and the fluorescence was measured using a fluorescence plate reader (excitation: 495 nm, emission: 520 nm; Molecular Devices, Sunnyvale, CA, USA). Flux into the apical well was calculated as a percentage of total fluorescence administrated into the basal well per h per surface area (in cm^2).

2.8. Statistical analysis

GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) was used to perform statistical analysis. One-way analysis of variance and Tukey's multiple-comparison tests were applied when comparing three or more groups. A statistically significant difference was considered as $P < 0.05$.

3. Results

3.1. Oregonin inhibits TNF-α-induced inflammation of intestinal epithelial HT-29 cells

First, we examined the cytotoxicity of oregonin in HT-29 cells by using the MTT assay. Oregonin did not affect the viability of HT-29