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Astilbin from *Engelhardtia chrysolepis* enhances intestinal barrier functions in Caco-2 cell monolayers

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ABSTRACT

Astilbin, which is one of polyphenolic compounds isolated from the leaves of *Engelhardtia chrysolepis* H$_{ANCE}$ (Chinese name, huang-qui), is available as the effective component in food and cosmetics because of its anti-oxidant and anti-inflammatory effects. The tight junction (TJ) proteins, which protect the body from foreign substances, are related to adhesion between a cell and a cell. Previously, the enhancement of TJ's functions induced by aglycones of flavonoids has been demonstrated, but the effects of the glycosides such as astilbin have not been observed yet. In this study, we investigated the effects of astilbin on the TJ's functions, and human colon carcinoma Caco-2 cell monolayers were used to evaluate the effects of astilbin on transepithelial electrical resistance (TER) value and the mRNA and proteins expressions of TJ-related molecules. Astilbin increased the TER value, mRNA expression levels of claudin-1 and ZO-2, and protein expression levels of occludin and ZO-2 in Caco-2 cells. Astilbin also increased the TER value in Caco-2 cells co-stimulated with TNF-α plus IFN-γ, and moreover upregulated the protein expression of TJ-related molecules in Caco-2 cells co-treated with TNF-α plus IFN-γ. These results suggest that astilbin can enhance the expressions of TJ-related molecules, leading to upregulation of the barrier functions in the intestinal cells.

1. Introduction

Flavonoids, which are one of secondary metabolites that are generally present in plants, are classified into chalcones, flavanes, flavones, flavonols, flavanols, flavanones, flavanonols, isoflavones and anthocyanidins, and over 4000 different molecules have been identified. Astilbin, which is one of dihydroflavanonol glycosides, is (-)-taxifolin (dihydroquercetin) substituted by α-L-rhamnosyl moiety at position 3 via the glycosidic linkage. It has been reported that astilbin was isolated from *Engelhardtia chrysolepis* (Kasai et al., 1988), *Hypericum perforatum* (Tatsis et al., 2007) and other plants. Astilbin and crude extracts from *E. chrysolepis* including astilbin could exert the anti-oxidant effects (Landrault et al., 2002; Igarashi et al., 1996; Haraguchi et al., 1996), lipoprotein lipase activity (Motoyashiki et al., 1998), amelioration of bladder dysfunction (Levin et al., 2002) and anti-inflammatory activity against the TNF-α, IL-6, IL-1β and IL-10-related biological alterations (Huang et al., 2011).

Gastrointestinal epithelial cells have the important functions as the physical barrier to the invasion of pathogens, toxins and allergens from the external environment into the body. Tight junction (TJ)-related molecules create apical cell-to-cell adhesions to form a barrier, to transport substances, and to help maintain epithelial cell polarity. TJ is comprised of integral proteins, such as claudin and occludin, as well as tight junction-associated proteins, such as zonula occludens (ZO) (Turner, 2009). The characteristics of TJ differ amongst the epithelia of various organs, and these differences are linked to variations of TJ protein expression ratios observed in these organs (Singh and Harris, 2004). When the TJ's functions are declined, foreign substances can easily invade the body from the intestinal tract, and then inflammation and allergy are caused because of immune regulations. Therefore, the enhancement of TJ's functions must lead to alleviation of intestinal diseases. Regarding flavonoids, their aglycones could upregulate the TJ's functions, but there is no reports about the effects of dihydroflavonol glycosides, of which astilbin belongs to the group, on epithelial barrier functions including TJ's functions (Suzuki et al., 2011a, 2011b). In this study, we investigated whether astilbin can enhance the TJ's functions.

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2. Materials and methods

2.1. Materials

Rabbit anti-claudin-1, occludin, ZO-2 and mouse anti-ZO-1 antibodies were purchased from Zymed Laboratories (South San Francisco, CA, USA). Horseradish peroxidase (HRP)-conjugated goat-rabbit IgG antibody was obtained from Jackson Immuno Research (West Grove, PA, USA). Rabbit anti-GAPDH antibody was purchased from MERCK (Kenilworth, NJ, USA). HRP-conjugated Goat anti-mouse IgG antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human TNF-α and human IFN-γ were purchased from R & D Laboratories (Minneapolis, MN, USA).

2.2. Plant materials

The leaves of Kohki (*Engelhardtia chrysolepis*) were collected from South China botanical garden, Chinese Academy of Sciences (Guangzhou, China) in 2000, and then identified by Prof. Chen B of South China Botanical Garden, Chinese Academy of Sciences.

2.3. Cell culture

Human colon carcinoma Caco-2 cells (RCB0988; Riken BRC Cell Bank) were propagated. The grown medium consisted of Dulbecco's modified Eagle's medium (Nissui Pharmaceuticals) with 10% heat-inactivated fetal bovine serum (Hyclone). The cells (passages 46–50) were grown on polyester membranes in Transwell inserts (Pore size: 0.4 μm; 12-well; Costar, Corning, NY, USA) to measure the value of transepithelial electrical resistance (TER), and the experiments were conducted on days 13–14 after seeding.

2.4. Isolation of astilbin

The leaves of *E. chrysolepis* (100 g) were cut and then subjected to extraction with 50% aqueous EtOH under reflux. Evaporation of the solvent under reduced pressure provided the 50% aqueous EtOH extract (20.8 g), and the extract (20.0 g) was subjected to Diaion HP-20 column chromatography [200 g, H_2O → MeOH-H_2O (1:1, v/v) → MeOH] to yield the H_2O eluate (6.9 g), the 50% aqueous MeOH eluate (11.3 g), and the MeOH eluate (1.6 g). Reversed-phase silica gel column chromatography [300 g, MeOH-H_2O (30:70, v/v)] of the 50% aqueous MeOH eluate (10.0 g) yielded the dihydroflavonol glycoside mixture (3.5 g). The dihydroflavonol glycoside mixture (3.5 g) was purified by recycling HPLC (MeOH) to give astilbin (2.2 g). Astilbin (Fig. 1) was identified by comparing the physical data ($[\alpha]_D$, IR, and ^{1}H- and ^{13}C-NMR; data not shown) collected from its authentic compound. The following instruments were used to obtain physical data: melting point, Yanaco MP-J3 melting point apparatus (Kyoto, Japan); specific rotation, JASCO P2200 digital polarimeter (l =5 mm) (Halifax, Nova Scotia); IR spectra, JASCO FT/IR-460 spectrometer; MS and high-resolution MS, Waters Xevo G2-Tof MS (Milford, MA, USA); ^{1}H-NMR spectra, JEOL ECS-400 (400 MHz) spectrometer; ^{13}C-NMR spectra, JEOL ECS-400 (100 MHz) spectrometer; preparative HPLC, JAI LC-9201 [column: JAIGEL GS310 (250×20 mm i.d.)]. The following materials were used for chromatography: synthetic absorbent resin column chromatography, Diaion HP-20 (Mitsubishi Chemical Co., Ltd., Tokyo, Japan); reversed-phase silica gel column chromatography, Chromatorex DM1020T (Fuji Silysia, Ltd., Tokyo, Japan); normal-phase HPTLC, silica gel 60 F_{254} (Merck); reversed-phase HPTLC, silica gel RP-18WF_{254s} (Merck). Detection on HPTLC was achieved by spraying with 10% aqueous H_2SO_4 followed by heating.

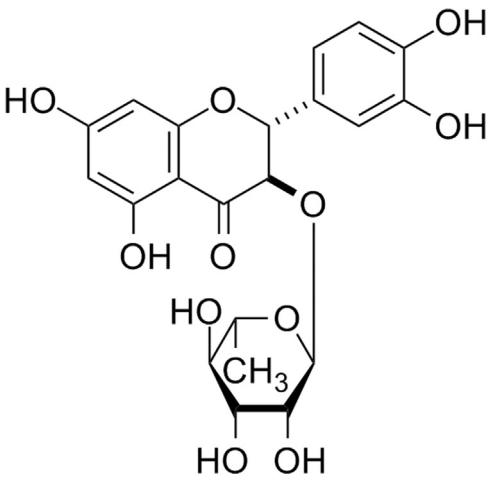

Fig. 1. The chemical structure of astilbin.

2.5. Measurement of TER

Intestinal barrier functions were evaluated by measuring the TER value in Caco-2 cell monolayers grown on polyester membranes in Transwell inserts. Caco-2 cells were seeded in the apical chamber and the changes of TER were measured with the MILLICELL®-ERS voltohmmeter system (Millipore, USA). Before starting each experiment, it was confirmed that Caco-2 cell monolayers had the TER value of 800-1000 Ω cm^2. Astilbin at 12.5 or 50 μM and quercetin at 10 μM were added into the apical wells of the Caco-2 cell monolayers, and dimethyl sulfoxide was used as a vehicle control. To investigate the time-dependency for the effect of astilbin on intestinal barrier functions, the TER value was measured before addition of astilbin to the Caco-2 cell monolayers and at 1, 3, 6, 12 and 24 h after the astilbin treatment. Electrical resistance was measured until similar values were recorded on three consecutive measurements. Values were corrected for background resistance due to the membrane insert and calculated as Ω·cm^2. The technical replicates for TER measurement were 3 times.

2.6. Quantitative real-time PCR

Total RNA from Caco-2 cells was extracted using the ISOGEN II reagent (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's protocol. Reverse transcription (RT) was performed using a cDNA Synthesis Kit (TAKARA, Shiga, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (TAKARA) according to the manufacturer's protocol. The reaction conditions included 40 cycles of two-stage PCR consisting of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min after an initial denaturation step at 95 °C for 10 min. The primers shown in Table 1 were used for PCR reactions. The technical replicates for real-time PCR were 1 time.

2.7. Immunoblot analysis

Proteins were separated by SDS-PAGE (Mini-Protean 4–20%, Bio-Rad, Hercules, CA, USA) and then transferred to polyvinylidene difluoride membranes (Trans Blot Pack, Bio-Rad). The membranes were blotted with specific antibody to claudin-1, occludin, ZO-1, ZO-2 or GAPDH in combination with HRP-conjugated anti-mouse IgG or anti-rabbit IgG antibody. The blots were developed using the enhanced chemiluminescence method (GE Healthcare, Little Chalfont, UK). Quantification was performed by densitometric analysis of specific bands on the immunoblots by using Image J software. The technical replicates for immunoblot analysis were 2 times.

2.8. Treatment with TNF-α plus IFN-γ

Recombinant human TNF-α and IFN-γ were added to the basal chamber, at 10 ng/ml, and/or 50 μM astilbin were applied to Caco-2