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Abstract: Astilbin, which is one of polyphenolic compounds isolated from the leaves of Engelhardtia chrysolepis HANCE (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.

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Editor-in-Chief:
European Journal of Pharmacology
Dear Prof. F.A. Redegeld,

Thank you for your e-mail of March 15, 2017 regarding our manuscript entitled "Astilbin from *Engelhardtia chrysolepis* Enhances Intestinal Barrier Functions in Caco-2 Cell Monolayers" (Manuscript#: EJP-45625R1). We attach here our revised manuscript as well as point-by-point responses to the Editor's comments.

We consider that the revised manuscript includes the suitable response to Editor's comments, and moreover has been improved over the initial submission. We trust that it is suitable for publication in *European Journal of Pharmacology*.

Thank you in advance for your kind consideration of this manuscript.

Sincerely yours,

Shin Nishiumi

Dear Editor,

We are much grateful for evaluating our manuscript. According the Editor's comments, we have revised our manuscript, and the revision parts have been indicated with red-highlighted letters in our revised manuscript (The yellow- and green-highlighted letters show the parts that were revised previously). We are looking forward to a publication of our manuscript (**Manuscript #: EJP-45625R1**).

Our responses to the Editor's comments are as follows:

Response for Editor
- Use ml (not mL); use h (not hr).
Response: We changed 'mL' to 'ml', and 'hr' to 'h'.

- Use S.D. (not SD) for standard deviaiton.
Response: We changed 'SD' to 'S.D.'.

- Heading Conclusion should be numbered as 5.
Response: The Conclusion section was numbered as '5'.

- Figure legends should be given on a separate page and not be attached to the figures.
Response: Figure legends were transferred to a separate page, but were not attached to Figures.

- Retype tables according EJP style: use a,b,c for significance as symbols are for figures only.
Response: In Table 2, the marks to show the significant differences were changed from '*' to 'a'.

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.

Key Words Astilbin ・ Caco-2 cell ・ Tight junction ・ Claudin-1 ・ Occludin ・ ZO-2

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 4,000 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., 1998), *Hypericum perforatum* (Tatsis et al., 2007) and other

plants. Astilbin and crude extracts from *E. chrysolepis* including astilbin could exert the antioxidant effects (Landrault et al., 2002; Igarashi K 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., 2011). In this study, we investigated whether astilbin can enhance the TJ's functions.

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.

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 800-1,000 $\Omega \cdot cm^2$. 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 $\Omega \cdot cm^2$. 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 sec 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 cell monolayers, and then the TER value and protein expressions of TJ-related molecules were evaluated.

2.9. Statistical analysis

All data were expressed as the mean ± S.D. of at least three independent determinations for each experiment. The technical replicates for each experiments were 1 (for real-time PCR), 2 (for immunoblot analysis) or 3 (for TER measurement) times. Statistical analyses were performed by the Student's t-test and Tukey-Kramer Multiple Comparisons Test to determine difference between groups. All analyses were performed using SPSS for Windows (version 21;IBM Japan Inc., Tokyo, Japan) In each statistical analysis, a level of probability of 0.05 was used as criteria for significance.

3. Results

3.1. Isolation of astilbin

The 50% aqueous EtOH extract from the leaves of *E. chrysolepis* was subjected to Diaion HP-20 colum and silica gel column chromatography and finally HPLC separation to astilbin. The content of astilbin was 95%.

3.2. Effect of astilbin on intestinal barrier functions with TER

The intestinal barrier functions were evaluated by measuring the TER value in Caco-2 cell monolayers. In this experiment, 10 μM quercetin, which is known to enhance the intestinal barrier functions and was used as the positive control, increased the TER value in Caco-2 cell monolayers (Table 2). The TER value across Caco-2 cell monolayers was also significantly increased after addition of 12.5 and 50 μM astilbin. In Caco-2 cell monolayers treated with 12.5 and 50 μM astilbin, the TER value was rapidly increased to 119% and 114%, respectively, during the first 1 h. After the 3 h treatment, The TER value was 119% and 116%, respectively. The significant increase could be confirmed at 24 h after the treatment with astilbin compared with the negative control. In the negative control Caco-2 cell monolayers, the TER value was kept between 96% and 101% against the initial value (100%) through the incubation period. When the TER value was measured at 15, 30 and 60 min after 50 μM astilbin treatment,

the 111.5, 113.5 and 125.2% increases, respectively, were observed compared with the non-treatment (100%).

3.3. Effect of astilbin on intestinal barrier functions with mRNA expression

Next, mRNA expressions were evaluated in Caco-2 cells treated with 12.5 and 50 μM astilbin for 30 min (Fig. 2). As a result, mRNA expression of claudin-1 was enhanced by astilbin in a dose-dependent manner, and 50 μM astilbin significantly upregulated its mRNA expression. The mRNA expression levels of occludin and ZO-2 was also increased by 50 μM astilbin treatment, although occludin did not show the significant difference. The claudin-4 mRNA expression was unchanged at 30 min after the treatment with astilbin.

3.4. Effect of astilbin on intestinal barrier functions with protein expression

Then, protein expressions were confirmed at 3, 6 and 24 h after addition of 12.5 and 50 μM astilbin to Caco-2 cells (Fig. 3). In Caco-2 cells treated with astilbin, the protein level of ZO-2 was rapidly enhanced, and 50 μM astilbin significantly increased its protein level during the incubation period. Occludin also had the tendency to be upregulated by the astilbin treatment, and its significant increase could be

confirmed after the 24 h treatment with 50 μM astilbin. Regarding claudin-1, 50 μM astilbin tended to upregulate its protein expression. The ZO-1 protein expression after the treatment with astilbin was unchanged through the incubation period.

3.5. Influence of astilbin on TJ in TNF-α plus IFN-γ treated Caco-2 cells

Finally, it was investigated whether astilbin can influence the TJ's functions in the TNF-α plus IFN-γ treated Caco-2 cells. As shown in Fig. 4, The TER value after TNF-α plus IFN-γ co-treatment in Caco-2 cell monolayers was significantly 66% lower than the negative control treatment, and 50 μM astilbin induced 34% upregulation of the TER value compared with the vehicle control. The co-treatment with 50 μM astilbin and TNF-α plus IFN- γ had the 17% higher value of TER compared with the treatment with TNF-α plus IFN- γ. Then, we examined the effect of astilbin on the expression levels of TJ proteins in Caco-2 monolayers treated with or without TNF-α plus IFN- γ. The expression level of Occludin was decreased by TNF-α plus IFN- γ, and the expression level of Occludin was significantly altered by the treatment with TNF-α plus IFN- γ in the absent or presence of astilbin (Fig. 5).

4. Discussion

Astilbin is one of flavonoids found in various plants of the *Engelhardtia* family,

and its physiological functions, such as antioxidant activity, lipoprotein lipase activity, amelioration of bladder dysfunction and anti-inflammatory activity, have been reported by some researchers. Recently, it was found that flavonoid aglycones can enhance the barrier functions in Caco-2 cell monolayers, which are widely used as a model of the intestinal epithelium (Noda et al., 2012). Astilbin is the glycoside of taxifolin but not the flavonoid aglycone, so we investigated whether astilbin can strengthen the intestinal barrier functions.

Astilbin could increase the TER value in Caco-2 cell monolayers, which is an indicator of intestinal barrier functions. Moreover significantly upregulated the protein expressions of occludin and ZO-2, and the protein expression level of claudin-1 tended to be increased by the astilbin treatment. TJ is regulated via the specific interaction of various transmembrane and cytosolic proteins, and TJ's functions are determined on the basis of the expression, distribution, and association of TJ proteins. These TJ-related events are known to be controlled via the cellular signaling pathways, such as PKCs, MAPKs and PI3K (Gonzalez-Mariscal et al., 2008; Farhadi et al., 2006; Basuroy et al., 2006; Sheth et al., 2003). In Caco-2 cell monolayers treated with astilbin, the significant increase in the TER value was observed through the incubation period from 1 h to 24 h, so astilbin might increase the expression levels of TJ's proteins via the transcriptional

regulations of occludin and ZO-2, potentially claudin-1, resulting in the enhancement of intestinal barrier functions. Inhibition of MEK1/2 caused not only the reduced ERK1/2 phosphorylation but also the decreased ZO-2 expression in in the mouse proximal epididymis, although the ZO-3 expression was upregulated (Kim et al., 2016). Activation of PKC by 12-O-tetradecanoylophorbol-13-acetate (TPA) upregulated the transcription of occludin and ZO-1 in colonic adenocarcinoma T84 cells (Weiler et al., 2005). It was also suggested that the PKC signaling induced by TPA can enhance the barrier functions in human nasal epithelial cells via the transcriptional up-regulations of tight junction proteins, and the involvement of GATA-3, which is one of transcription factors, was shown (Koizumi et al., 2008). Therefore, the phosphorylation signaling seems to regulate the transcription of tight junction molecules, although the mode of actions is different among the types of tissues and cells.

Interestingly, the increases in the TER value and the ZO-2 protein expression level were observed at 1 h after the astilbin treatment. These changes after the 1 h treatment might not be the phenomenon based on the transcriptional regulation, but astilbin might influence TJ's assembly and/or stability of ZO-2 protein, and regulations of the phosphorylation states of TJ-related molecules by the astilbin treatment would be involved in these phenomenon. In the previous study, it was found that the

phosphorylation status of ZO-2, claudins and occludin was involved in TJ's assembly (Sakakibara et al., 1997; Fujibe et al., 2004; Avila-Flores et al., 2001). For example, it seems that the ZO-2 phosphorylation by PKC is involved in TJ's disassembly (Avila-Flores et al., 2001). Thr phosphorylation of occludin was required for TJ's assembly with occludin (Suzuki et al., 2009).

In the article by Suzuki T and Hara H, quercetin, which is one of aglycone flavonoids, could inhibit the PKC activity, resulting in the regulation of epithelial barrier functions (Suzuki and Hara, 2009). In this article, quercetin had the 2 distinct mechanisms: quercetin promoted the assembly of claudin-1, occludin and ZO-2 via inhibition of a novel PKCδ isoform, and additionally upregulated the expression of claudin-4. In addition, kaempferol enhanced TJ's functions through the expressions and actin cytoskeletal association of TJ proteins in Caco-2 cells, and furthermore the membrane lipid microdomains were involved in the kaempferol's effects (Suzuki et al., 2011). There is no report about the relationship between astilbin and PKC, but astilbin could cancel the lipopolysaccharide-induced phosphorylations of JNK, ERK and p38, namely MAPK cascade (Kong et al., 2016). Although the detailed investigations for astilbin's effects are needed in the future, astilbin would enhance the intestinal barrier functions in Caco-2 cells via the 2 distinct mechanisms: regulations of TJ's assembly

and upregulations of TJ proteins accompanying with modulation of the phosphorylation status. Moreover, the moderate increases in the TER value were observed at 15, 30 and 60 min after 50 μM astilbin treatment. These results suggest that astilbin may be also involved in the activity of ion channels in the intestinal cells, because the TER value is moderately increased if ion channels are involved in the intestinal barrier functions (Prashant et al., 2012).

In this study, astilbin could enhance the intestinal barrier functions in Caco-2 cell monolayers stimulated with cytokines. The cytokine-stimulated Caco-2 cell monolayers have been often available as the *in-vitro* model for the intestinal inflammation including inflammatory bowel disease, which is well recognized that it is characterized by overproduction of a broad array of proinflammatory cytokines within the mucosa as well as disruption of epithelial barrier functions, but whether intestinal barrier dysfunctions in inflammatory bowel disease is a primary contributor to mucosal inflammation or a consequence of the action of proinflammatory cytokines has not been understood yet. Plants containing astilbin have been traditionally available for improvement of some diseases, and then astilbin itself has been also reported to exert the beneficial effects. However, the astilbin-caused enhancement effects on TJ's functions in the intestinal cells have been unknown yet, and this study is the first report.

Therefore, astilbin may be also effective for improvement of colitis. In conclusion, we present data demonstrating that a natural compound, astilbin from *E. chrysolepis*, can strengthen the TJ's functions. Taken together, astilbin is one of candidates for the effective compounds for barrier functions in intestinal cells.

5. Conclusion

In this study, we demonstrated that astilbin from *E. chrysolepis* can strengthen the intestinal barrier functions in human intestinal epithelial Caco-2 cells, and furthermore astilbin could exert the same ability under the condition with inflammatory stimuli. We expect that usage of astilbin is beneficial for amelioration of inflammatory and allergic diseases.

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Figure legends

Fig. 1 The chemical structure of astilbin.

Fig. 2 The effect of astilbin on mRNA expressions of TJ-related molecules in Caco-2 cells. Caco-2 cells were treated with 12.5 or 50 μM astilbin for 30 min. Dimethyl sulfoxide was used as a vehicle control. mRNA expression levels of TJ-related molecules were investigated by real-time PCR, and data are expressed as mean ± S.D. (n = 4). *P<0.05 vs. each control group (0 μM) evaluated by Student's t-test.

Fig. 3 The effect of astilbin on protein expressions of TJ-related molecules in Caco-2 cells. Caco-2 cells were incubated with 12.5 or 50 μM astilbin for 0, 3, 6 and 24 h. Dimethyl sulfoxide was used as a vehicle control. The protein extracts obtained from Caco-2 cells were immunoblotted for claudin-1, occludin, ZO-1, ZO-2 and GAPDH. Lane 1, control 0 h treatment; Lane 2, control 3 h treatment; Lane 3, control 6 h treatment; Lane 4, control 24 h treatment; Lane 5, 12.5 μM astilbin 3 h treatment; Lane 6, 12.5 μM astilbin 6 h treatment; Lane 7, 12.5 μM astilbin 24 h treatment; Lane 8, 50 μM astilbin 3 h treatment; Lane 9, 50 μM astilbin 6 h treatment; Lane 10, 50 μM

astilbin 24 h treatment. Representative photographs of the membrane are shown. Each protein was evaluated by immunoblot analysis, and then the area of each band was measured using an image analyzer. Data are expressed as mean ± S.D. (n = 3). *P < 0.05 vs. each control group (0 μM) evaluated by Student's t-test. **A,** claudin-1; **B,** occludin; **C,** ZO-1; **D,** ZO-2.

Fig 4. The effect of astilbin on TER in Caco-2 cells treated with TNF-α plus IFN-γ. Caco-2 cell monolayers were treated with or without 10 ng/ml TNF-α plus 10 ng/ml IFN- γ in the absence or presence of 50 μM astilbin for 72 h, and then the TER values were measured. Dimethyl sulfoxide was used as the vehicle control for astilbin. The TER values were normalized with the control group. Data are expressed as mean ± S.D. (n = 3), and asterisks (*) indicate the significant differences (P < 0.05) between 2 groups evaluated by Tukey-Kramer Multiple Comparisons Test.

Fig. 5 The effect of astilbin and TNF-α plus IFN-γ on the expression level of TJ proteins. Representative photographs of the membrane were shown. Each protein was evaluated by immunoblot analysis, and then the area of each band was measured using an image analyzer. Data are expressed as mean ± S.D. (n = 3), and asterisks (*) indicate

the significant differences ($P < 0.05$) between 2 groups evaluated by Tukey-Kramer Multiple Comparisons Test.

Figures

Fig. 1

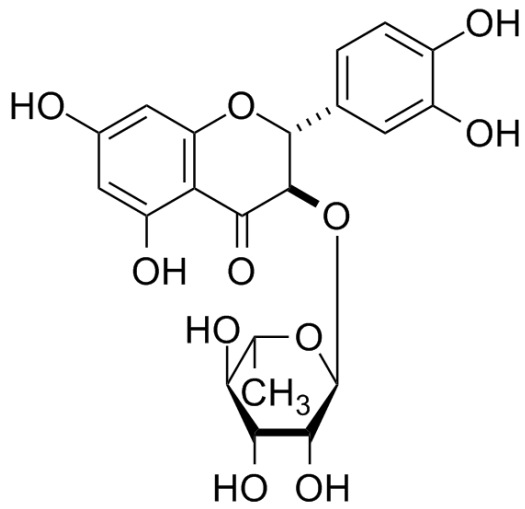

Fig. 2

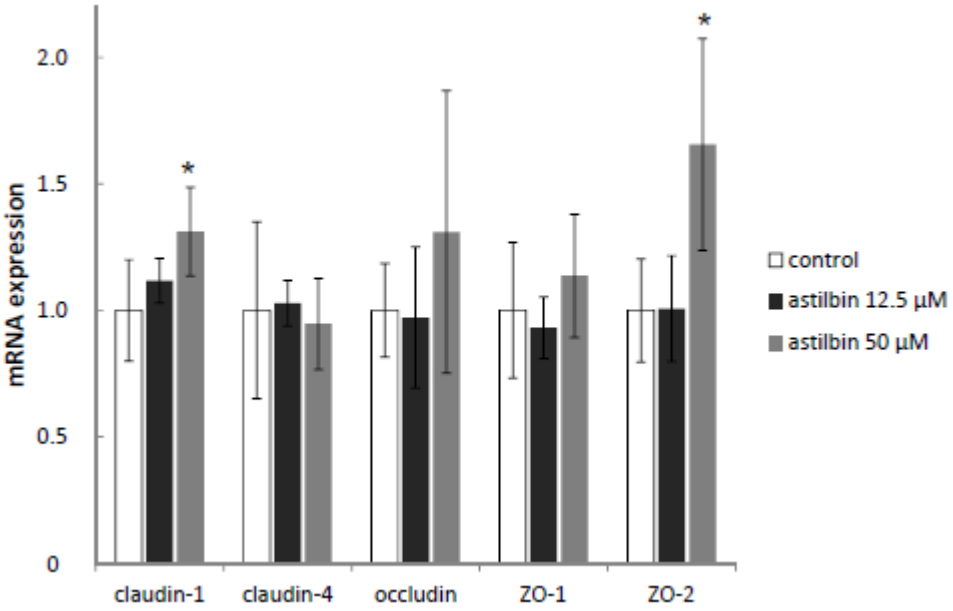
mRNA expression
2.0
1.5
1.0
0.5
0
*
*
claudin-1
claudin-4
occludin
ZO-1
ZO-2
control
astilbin 12.5 µM
astilbin 50 µM

Fig. 3

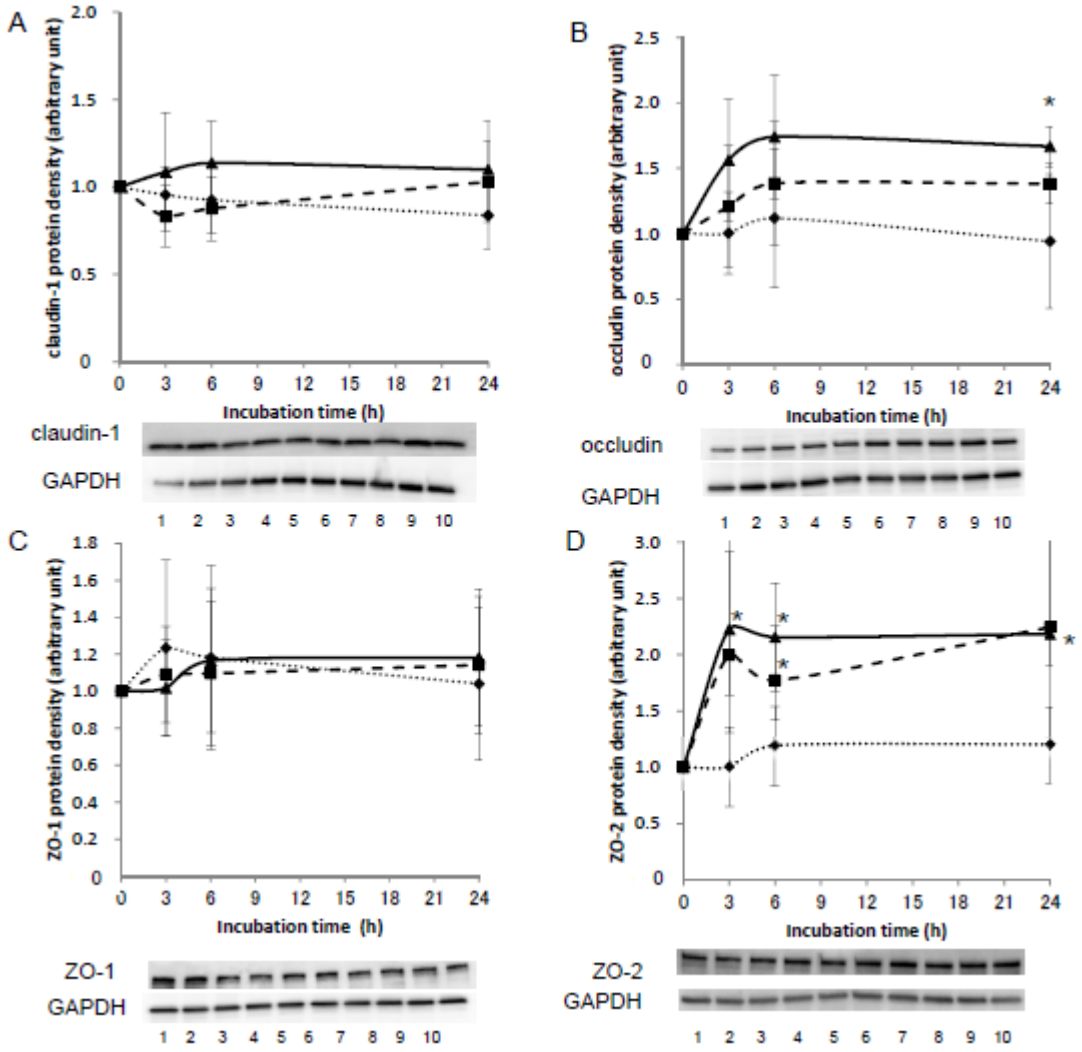
A
claudin-1 protein density (arbitrary unit)
Incubation time (h)
claudin-1
GAPDH
B
occludin protein density (arbitrary unit)
Incubation time (h)
occludin
GAPDH
C
ZO-1 protein density (arbitrary unit)
Incubation time (h)
ZO-1
GAPDH
D
ZO-2 protein density (arbitrary unit)
Incubation time (h)
ZO-2
GAPDH

Fig. 4

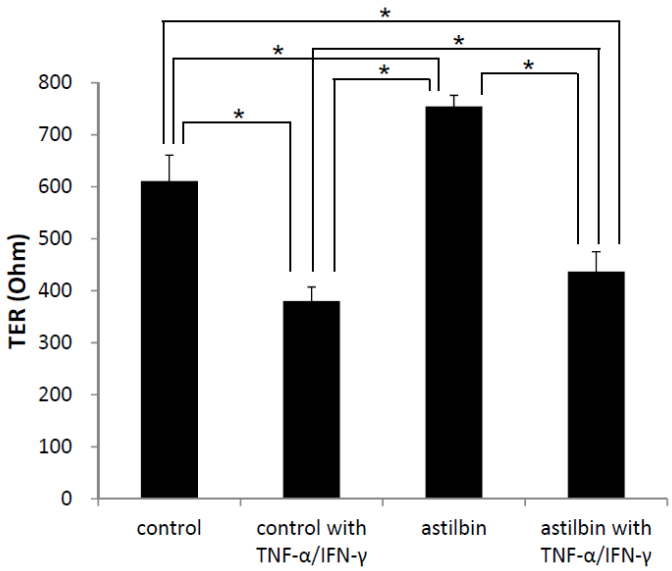
TER (Ohm)
800
700
600
500
400
300
200
100
0
control
control with TNF-α/IFN-γ
astilbin
astilbin with TNF-α/IFN-γ
*
*
*
*
*
*

Fig. 5

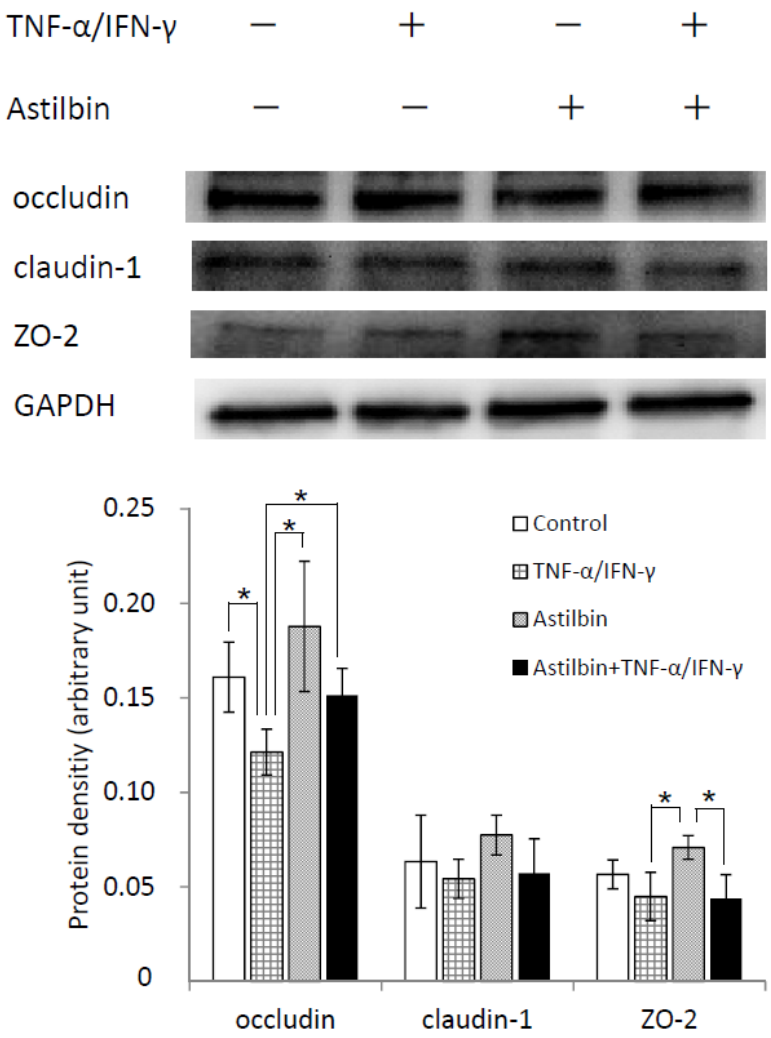
TNF-α/IFN-γ
−
+
−
+
Astilbin
−
−
+
+
occludin
claudin-1
ZO-2
GAPDH
Protein densitiy (arbitrary unit)
0.25
0.20
0.15
0.10
0.05
0
Control
TNF-α/IFN-γ
Astilbin
Astilbin+TNF-α/IFN-γ
*
occludin
claudin-1
ZO-2

Table 1. Primers for real-time PCR.

Gene		Sequence (5’-3’)
Claudin-1	Forward primer	GCGCGATATTTCTTCTTGCAGG
	Reverse primer	TTCGTACCTGGCATTGACTGG
Claudin-4	Forward primer	CGCATCAGGACTGGCTTTATCTC
	Reverse primer	CAGCGCGATGCCCATTA
Occludin	Forward primer	CTCCCATCCGAGTTTCAGGT
	Reverse primer	GCTGTCGCCTAAGGAAAGAG
ZO-1	Forward primer	CGGTCCTCTGAGCCTGTAAG
	Reverse primer	GGATCTACATGCGACGACAA
ZO-2	Forward primer	CCGGAGGCA GAGACA CCC
	Reverse primer	AACTTCTGCCATCAAACTCG
GAPDH	Forward primer	GCACCGTCAAGGCTGAGAAC
	Reverse primer	ATGGTGGTGAAGACGCCAGT

Table

Table 2. The TER value in Caco-2 cell monolayers treated with astilbin or quercetin.

	0 h	1 h	3 h	6 h	12 h	24 h
Negative control	459.5 ± 45.0	451.7 ± 36.3	463.3 ± 24.7	440.7 ± 28.5	444.7 ± 22.7	452.9 ± 24.9
	(100%)	(98.5%)	(100.7%)	(96.4%)	(97.2%)	(98.7%)
12.5 μM Astilbin	434.8 ± 29.1	531.1[a] ± 18.0	531.6[a] ± 12.9	514.6[a] ± 13.2	512.2[a] ± 6.6	508.3[a] ± 7.5
	(100%)	(119.3%)	(119.4%)	(116.0%)	(115.5%)	(114.7%)
50 μM Astilbin	435.5 ± 31.2	504.5[a] ± 24.5	513.5[a] ± 16.9	489.0[a] ± 22.9	482.2[a] ± 19.6	481.7[a] ± 21.9
	(100%)	(113.8%)	(115.6%)	(110.7%)	(109.3%)	(109.2%)
10 μM Quercetin	442.5 ± 28.5	540.5[a] ± 30.1	528.7[a] ± 28.0	505.4[a] ± 19.3	511.7[a] ± 26.5	513.5[a] ± 25.7
	(100%)	(119.3%)	(117.0%)	(112.4%)	(113.7%)	(113.5%)

The Caco-2 cell monolayers were incubated with 12.5 and 50 μM astilbin, or 10 μM quercetin as the positive control, and then TER values were measured. Dimethyl sulfoxide was also used as the negative control. The values in parentheses were the percentage values normalized as 100% before addition of astilbin, quercetin or dimethyl sulfoxide. All data are expressed as mean ± S.D. (n = 3). The significant differences from the corresponding control are shown by '[a]', $P<0.01$.