JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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pubs.acs.org/JAFC

Differential Effects of Flavonoids on Barrier Integrity in Human Intestinal Caco-2 Cells

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ABSTRACT: Flavonoids, present in fruits, vegetables, and teas, provide beneficial effects for our health. We investigated the effect of a number of flavonoids on tight junction (TJ) barrier integrity in human intestinal Caco-2 cells. Transepithelial electrical resistance (TER; a TJ integrity marker) across cell monolayers was measured in cells incubated with flavonoids for 24 h. Chrysin decreased the TER, indicating a decrease in TJ integrity. Daidzein, hesperetin, naringenin, and morin increased the TER, indicating increased TJ integrity. Luteolin and genistein increased or normalized the TER after a transient decrease. Immunoblot analysis revealed that these changes in TER were caused by modification of the cytoskeletal association and expression of TJ proteins, zonula occludens (ZO)-1, ZO-2, occludin, junctional adhesion molecule-1, and/or claudins. Our results suggest that various flavonoids participate in the regulation of intestinal TJ barrier integrity and that this regulation may partially contribute to the flavonoid-mediated biological effects on our health.

KEYWORDS: *flavonoids, tight junction, intestinal barrier, Caco-2 cells*

1. INTRODUCTION

Flavonoids, polyphenolic compounds containing diphenylpropans, are secondary metabolites ubiquitously distributed throughout the plant kingdom. They are classified into a number of subgroups (chalcones, flavanes, flavones, flavonols, flavanols, flavanones, flavanonols, isoflavones, and anthocyanidis), and >4000 different molecules have been identified. Numerous studies have demonstrated that supplemental feeding with flavonoids provides various beneficial effects, including antioxidant, anti-inflammatory, and anticarcinogenic effects, on our health. However, the effects of flavonoids on intestinal barrier function are poorly understood.

Intestinal barrier function is closely related to intestinal health and disease, and it is regulated by the interaction of a number of barrier components, such as the mucous layer, antibacterial peptides, and tight junctions (TJs).[1] The TJs are arranged in strands that locate at the apical ends of the lateral membranes of epithelial cells. They regulate the paracellular passages of ions and water and also limit the diffusion of undesired foreign molecules, such as bacteria and dietary antigens, into the submucosa. Defects in the TJ barrier can result in chronic intestinal inflammation, resulting in various intestinal diseases, such as inflammatory bowel diseases.[1] For example, increased intestinal permeability is present in Crohn's disease patients [2] and is also associated with disease relapse in these patients during clinical remission.[3] Therefore, promotion and protection of intestinal TJ integrity using food factors including flavonoids could provide a preventive or therapeutic tool for diseases associated with barrier impairment.

The TJ is a multiple protein complex composed of transmembrane and intracellular plaque proteins. To date, four transmembrane proteins, occludin, claudins, junctional adhesion molecule (JAM), and tricellulin, have been identified.[4−7] The intracellular domains of these transmembrane proteins interact with intracellular proteins, such as zonula occludens (ZO) and cinglin, which in turn anchor the transmembrane proteins to the perijunctional actomyosin ring. These interactions are known to have a pivotal role in TJ structure and barrier integrity.[8] Therefore, it is important to examine not only the expression but also the cytoskeletal association of TJ proteins in order to understand the molecular mechanisms underlying intestinal TJ regulation.

Recently, we reported that 3 flavonoids, quercetin, myricetin, and kaempferol, belonging to the flavonol subgroup, exhibit promotive effects on intestinal barrier function.[9,10] Quercetin enhances the assembly of ZO-2, occludin, and claudin-1 and expression of claudin-4 via PKCδ inhibition. Kaempferol enhances the assembly of ZO-1, ZO-2, occludin, claudin-1, claudin-3, and claudin-4 and expression of ZO-2 and claudin-4. The underlying mechanism for the myricetin-mediated decrease in the TJ permeability is totally unknown. This evidence suggests that several flavonoids distributed throughout the plant kingdom may participate in the intestinal barrier regulation; however, their effects on TJ barrier integrity and structure are unknown. The present study examined the effects of some common flavonoids in our foods on TJ barrier integrity in human intestinal Caco-2 cells. Seven flavonoids belonging to four subgroups, flavone (chrysin and luteolin), isoflavone (daidzein and genistein), flavanone (hesperetin and naringenin), and flavonol (morin), were used.

2. MATERIALS AND METHODS

2.1. Chemicals. The following antibodies were used in this study: rabbit polyclonal anticlaudin-1, anticlaudin-3, anti-JAM-1, anti-ZO-1, and anti-ZO-2, mouse anticlaudin-4, HRP-conjugated antioccludin

Received: January 29, 2012
Revised: April 5, 2012
Accepted: April 16, 2012
Published: April 16, 2012

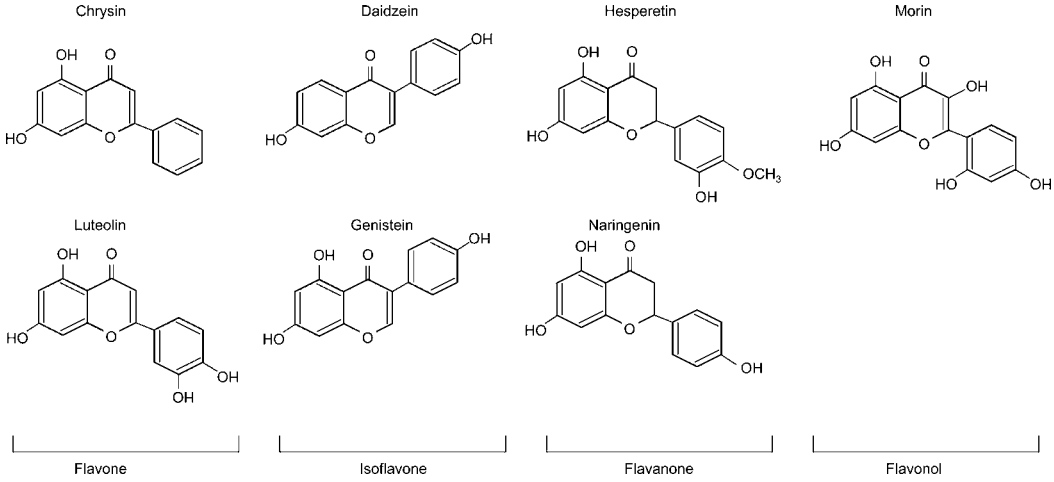

Figure 1. Structures of the flavonoids used in the study.

(Life technologies, San Francisco, CA, USA), mouse anti-β-actin, and HRP-conjugated antimouse and rabbit IgG (Sigma, St. Luis, MO, USA). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Cell Culture. Caco-2 BBe (CRL-2102) intestinal epithelial cells purchased from American Type Cell Culture (Rockville, MD, USA) were grown under standard cell culture conditions as described previously.[9,10] Cells were seeded into permeable polyester membranes in Transwell inserts (12 mm diameter, 0.4 μm pore size; Corning, Cambridge, MA, USA) at a density of 0.25×10^6 cells/cm^2. All experiments were conducted on day 14 postseeding. Cultures were used between passage 55 and 65, and the medium was refreshed every 3 days.

2.3. Measurement of Sucrase and Alkaline Phosphatase Activities of Caco-2 Cells. Caco-2 cell monolayers were homogenated with 200 μL of ice-cold PBS after washing with PBS. To determine sucrase activity, the cell lysates were mixed with the same volume of 112 mmol/L sucrose solution and incubated for 1 h at 37 °C.[11] Reaction was stopped by boiling for 3 min, and the released glucose into the supernatant was measured using a commercially available kit (Glucose CII Test Wako, Wako Pure Chemical Industries). The alkaline phosphatase activity of the cell lysates was determined using a commercially available kit (Alkaline phospha K Test Wako, Wako Pure Chemical Industries).

2.4. Measurement of Intestinal TJ Barrier Function. Intestinal TJ barrier function was evaluated by measurement of transepithelial electrical resistance (TER) and the unidirectional flux of FITC-conjugated dextran (FD-4; average molecular weight 4000) in Caco-2 cell monolayers in Transwell inserts.[12,13] Cell monolayers showed a TER of 700−800 $\Omega \cdot cm^2$ and high alkaline phosphatase and sucrase activities (data not shown). Each of the seven flavonoids (chrysin, daidzein, genistein, hesperetin, luteolin, morin, and naringenin; 100 μmol/L) was added to the apical wells, and the cells were incubated for 24 h. This experimental condition was chosen according to our previous studies showing that 3 flavonoids, kaempferol, myricetin, and quercetin, at 100 μmol/L reliably exhibited promotive effects on the TJ integrity for 24 h.[9,10] In addition, we expect that a luminal flavonoid concentration of 100 μmol/L can be achievable at an intake level of ~50 mg in humans.[10] The structures of the flavonoids used in this study are shown in Figure 1. TER was measured before and at 1, 3, 6, 12, and 24 h after administration of the flavonoids using a Millicell-ERS system (Millipore, Bedford, MA, USA). FD-4 (100 μmol/L) was injected into the apical wells at 21 h postadministration, and its flux into the basal wells was assessed for 3 h. The concentration of FD-4 in the basal solution was fluorometrically determined at 492 nm for excitation and 535 nm for emission (ARVO x4; Perkin-Elmer, Inc., Waltham, MA, USA). Whole cell extracts and detergent-insoluble fractions of cells incubated with and without 100 μmol/L flavonoids were prepared at 24 h postadministration as described below for subsequent immunoblot analysis.

2.5. Preparation of Detergent-Insoluble Fractions and Whole Cell Extracts. Detergent-insoluble fractions and whole extracts were prepared as described previously.[9,10] The detergent-insoluble fraction corresponds to the actin cytoskeleton-associated protein content. Caco-2 cell monolayers were washed with ice-cold PBS and incubated for 5 min at 4 °C with 200 μL of lysis buffer-CS [1% TritonX-100, 5 mmol/L EGTA in 50 mmol/L 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) containing protease inhibitors (5 μg/L aprotinin, 3 μg/L leupeptin hemisulfate, 5 mmol/L benzamidine hydrochloride, and 1 mmol/L phenylmethylsulfonyl fluoride) and phosphatase inhibitors (25 mmol/L glycerol-2-phosphate, 2 mmol/L sodium orthovanadate, and 10 mmol/L sodium fluoride), pH 7.4]. Cell lysates were centrifuged at 15 600g for 10 min at 4 °C to sediment the high-density actin-rich fraction. The pellet was suspended in 100 μL of lysis buffer F (1% SDS, 1% TritonX-100, 1% sodium deoxycholate, 30 mmol/L Tris, protease, and the phosphatase inhibitors described above, pH 7.4). For preparation of the whole cell extract, 200 μL of lysis buffer F was used after washing cell monolayers with ice-cold PBS. Protein concentrations in the different fractions were measured using the BCA method (Pierce Biotechnology, Inc., Rockford, IL, USA).

2.6. Immunoblot Analysis. Cell extracts were mixed with a one-half volume of Laemmli sample buffer [3× concentrated; 6% (w:v) SDS, 30% (v:v) glycerol, 15% (v:v) 2-β-mercaptoethanol, and 0.02% (w:v) bromophenol blue in 188 mmol/L Tris, pH 6.8] and heated at 100 °C for 5 min. Proteins (20 μg) were separated by SDS-PAGE (8−16% gradient) and transferred to polyvinylidene difluoride membranes. Membranes were blotted for ZO-1, ZO-2, occludin, JAM-1, claudin-1, claudin-3, claudin-4, and β-actin using specific antibodies in combination with HRP-conjugated antimouse IgG or antirabbit IgG antibodies. Blots were developed using the ECL chemiluminescence method (Perkin-Elmer, Inc.). Quantification was performed by densitometric analysis of specific bands on the immunoblots using Image J software.

2.7. Statistical Analysis. All values are expressed as means with their SEM. TER is expressed as % of initial values. Statistical analyses were performed by 1-way ANOVA followed by Duncan's multiple range test. A difference with $P < 0.05$ was considered significant. Statistical analyses were performed using the general linear models procedure of the Statistical Analysis Systems program (version 6.07; SAS Institute Inc., Cary, NC, USA).

3. RESULTS

3.1. Effects of Various Flavonoids on Intestinal TJ Permeability. The effects of flavonoids on the TER across the Caco-2 cell monolayers were varied (Figure 2A and 2B).

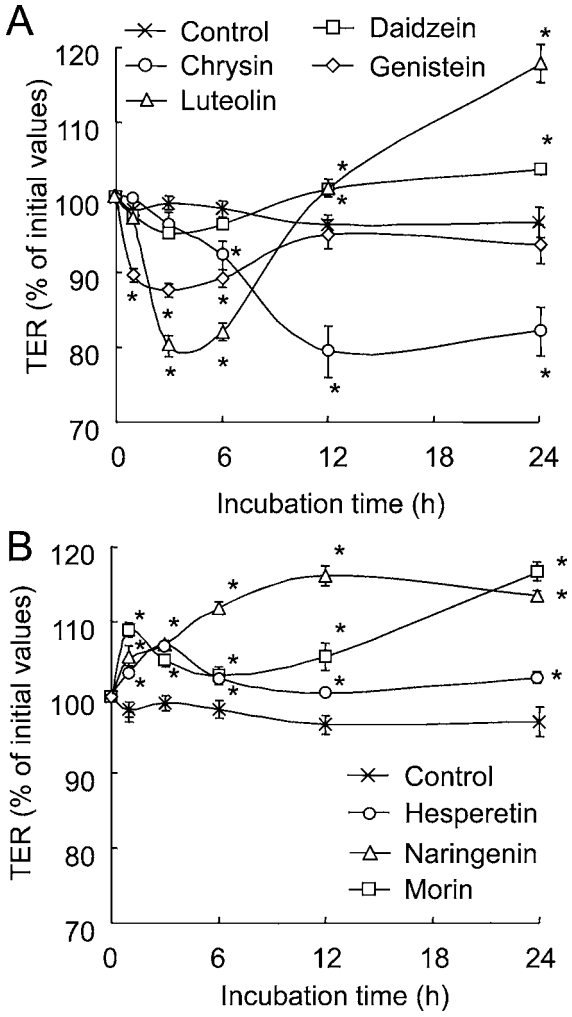

Figure 2. Transepithelial electrical resistance (TER) in Caco-2 cell monolayers. TER was measured across Caco-2 cell monolayers incubated with or without 100 μmol/L flavonoids ((A) chrysin, luteolin, daidzein, and genistein; (B) hesperetin, naringenin, and morin) at various time points. Values are expressed as means ± SEM, $n = 4$. Asterisk (∗) indicates different from the control value at each time point, $P < 0.05$.

Chrysin had a negative effect on the TER, indicating an increase in intestinal permeability, with the TER values in the cells incubated with chrysin at 6, 12, and 24 h lower than the control values at each time point. Daidzein, hesperetin, morin, and naringenin had a positive effect on the TER, indicating the decreases in intestinal permeability. The TER values in the cells incubated with daidzein at 12 and 24 h, hesperetin at and after 1 h, morin at 1, 3, 12, and 24 h, and naringenin at and after 1 h were higher than the control values at each time point. Genistein and luteolin normalized or increased the TER after a transient decrease. The TER values in the cells incubated with genistein at 1, 3, and 6 h and luteolin at 3 and 6 h were lower than the control values at each time point. However, the TER values in cells incubated with luteolin at 12 and 24 h were higher than the control value.

Unidirectional FITC−dextran flux across the cells incubated with chrysin was higher than that in the control cells (Figure 3), whereas the fluxes in cells incubated with hesperetin and naringenin were slightly, but not significantly, lower than the control values.

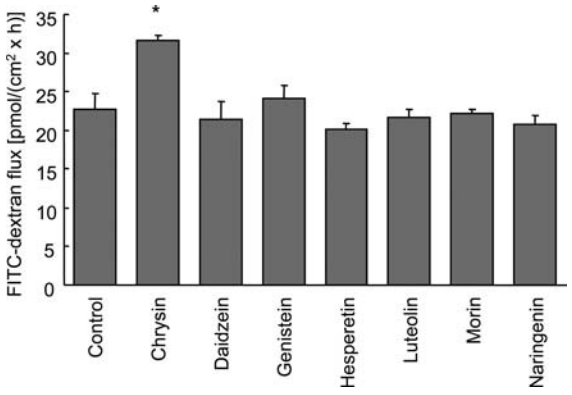

Figure 3. Unidirectional FITC−dextran flux in Caco-2 cell monolayers. FITC−dextran flux was measured across Caco-2 cell monolayers incubated with or without 100 μmol/L flavonoids (chrysin, daidzein, genistein, hesperetin, luteolin, morin, and naringenin) for 24 h. Values are expressed as means ± SEM, $n = 4$. Asterisk (∗) indicates different from the control value, $P < 0.05$.

3.2. Effect of Various Flavonoids on TJ Protein Expression. Chrysin and luteolin were found to decrease the expression of some TJ proteins in the cells. Occludin, JAM-1, and claudin-1, -3, and -4 expression in the cells incubated with chrysin were lower than those in the control cells (Figure 4). Claudin-1, -3, and -4 expression in the cells incubated with luteolin were also lower than those in the control cells, whereas ZO-2 expression in the presence of luteolin was higher than the control value. On the other hand, hesperetin and naringenin increased the expression of some TJ proteins, with occludin and claudin-4 expression in the cells incubated with hesperetin and naringenin higher than those in the control cells. Daidzein, genistein, and morin had no effect on TJ protein expression.

3.3. Effect of Various Flavonoids on TJ Protein Association with the Actin Cytoskeleton. TJ protein association with the actin cytoskeleton was affected more dynamically than was total expression (Figure 5). Chrysin and luteolin decreased the levels of some TJ proteins in the detergent-insoluble fractions, which correspond to the cytoskeleton-associated protein content. The levels of all TJ proteins immunoblotted, except for ZO-2 in the detergent-insoluble fractions of the cells incubated with chrysin, were lower than those in the control cells. JAM-1, claudin-1, and claudin-4 in the fraction of cells incubated with luteolin were lower than those in the control cells, whereas daizdein, genistein, hesperetin, morin, and naringenin all increased the level of one or more TJ proteins in the fraction. The level of claudin-1 in the fractions of cells incubated with daidzein and genistein were higher than that in the control cells. Occludin and claudin-3 were also slightly, but not significantly, higher in the cells incubated with these 2 flavonoids than in the control cells. The levels of occludin and claudin-1, -3, and -4 in the fraction of cells incubated with hesperetin were higher than those in the control cells. In addition, the levels of claudin-3 in the fraction of cells incubated with morin and those of ZO-2, occludin, and claudin-1, -3, and -4 in the fraction of cells incubated with naringenin were all higher than those in the control cells.

4. DISCUSSION

Intestinal barrier function has a crucial role in intestinal health and disease.[1] Defects in intestinal barrier integrity followed by

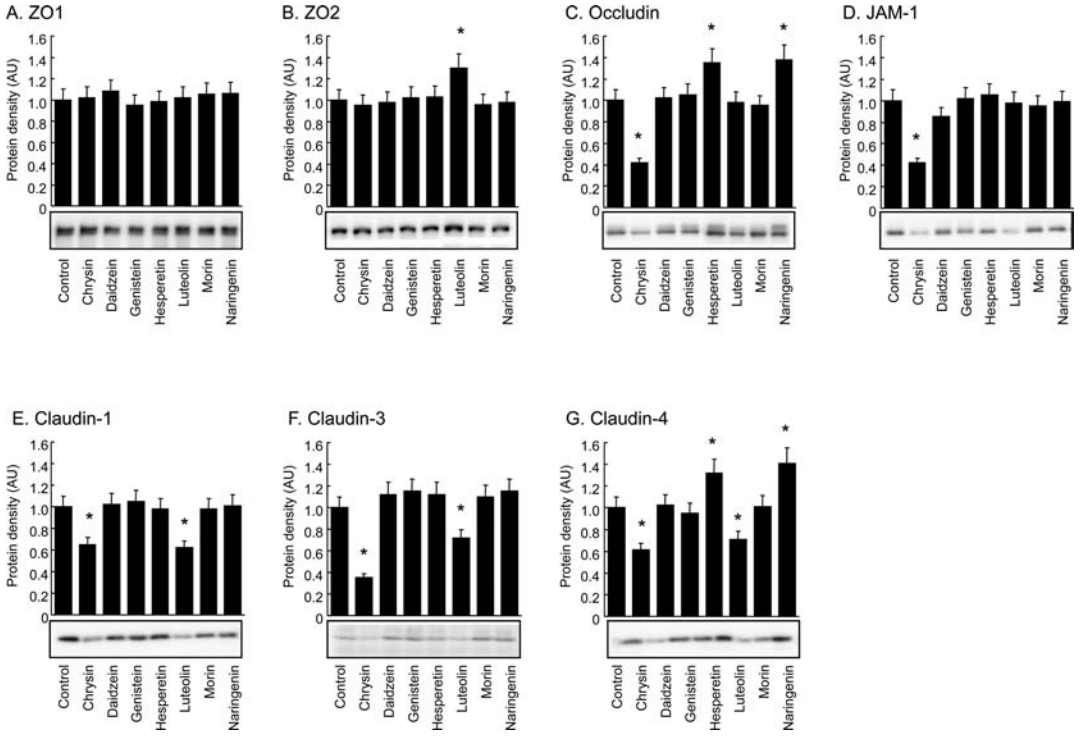

Figure 4. Immunoblot analysis of TJ proteins in the whole extracts of Caco-2 cell monolayers. Whole extracts of Caco-2 cell monolayers incubated with or without 100 μmol/L flavonoids (chrysin, daidzein, genistein, hesperetin, luteolin, morin, and naringenin) for 24 h were immunoblotted for ZO-1 (A), ZO-2 (B), occludin (C), JAM-1 (D), claudin-1 (E), claudin-3 (F), and claudin-4 (G). Each immunoblot was representative of 4 monolayers. Specific bands for each protein were quantitated by densitometric analysis. Density values were normalized to the values before incubation. Values are means ± SEM, n = 4. Asterisk (∗) indicates different from the control value, $P < 0.05$.

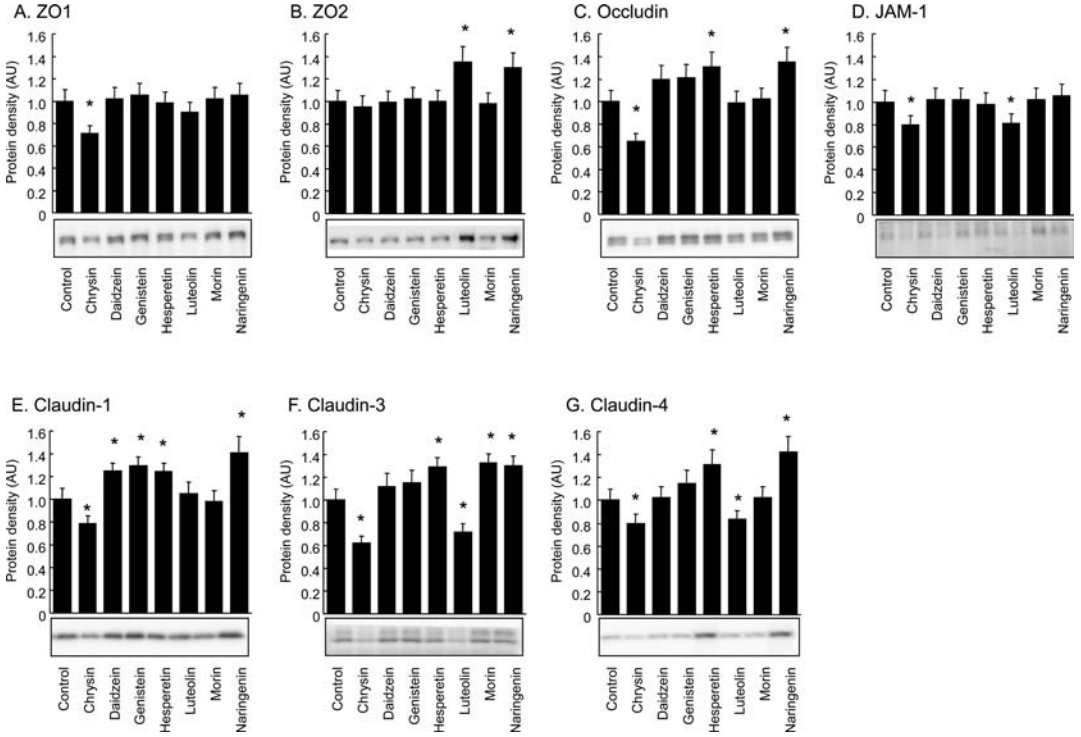

Figure 5. Immunoblot analysis of TJ proteins in the detergent-insoluble fractions of Caco-2 cell monolayers. Detergent-insoluble fractions of Caco-2 cell monolayers incubated with or without 100 μmol/L flavonoids (chrysin, daidzein, genistein, hesperetin, luteolin, morin, and naringenin) for 24 h were immunoblotted for ZO-1 (A), ZO-2 (B), occludin (C), JAM-1 (D), claudin-1 (E), claudin-3 (F), and claudin-4 (G). Each immunoblot was representative of 4 monolayers. Specific bands for each protein were quantitated by densitometric analysis. Density values were normalized to the values before incubation. Values are means ± SEM, n = 4. Asterisk (∗) indicates different from the control value, $P < 0.05$.

dx.doi.org/10.1021/jf300382h | J. Agric. Food Chem. 2012, 60, 4628−4633

permeation of pro-inflammatory molecules from the lumen can induce excessive activation of the mucosal immune system, resulting in sustained inflammation and tissue damage. Indeed, increases in intestinal permeability are found in various inflammatory diseases, including ulcerative colitis, Crohn's disease, celiac disease, and food allergies.[14] Dietary flavonoids have been reported to exhibit ameliorative effects on some of these diseases, although the precise mechanisms behind these effects remain to be clarified.[15,16] The present study demonstrates that some flavonoids participate in the regulation of intestinal barrier function. Our results suggest that the flavonoid-mediated regulation of the intestinal barrier may be partially associated with their biological effects on our health.

The effects of the flavonoids used in our study on TER were varied, although FD-4 flux was only significantly affected by chrysin. It seems that FD-4 flux is low in intact Caco-2 cells and not responsive to the strengthening of TJ integrity by flavonoids, such as naringenin. The TJ integrity and permeability are coordinately regulated by several TJ proteins. The transmembrane TJ proteins such as occludin, JAM-A, claudin-1, claudin-3, and claudin-4 create the paracellular barrier to ions and solutes by homophilic or heterophilic interaction between the adjacent cells.[8] The intercellular domains of the transmembrane proteins interact with the cytosolic plaque proteins such as ZO-1 and -2, which in turn anchor the transmembrane proteins to the perijunctional actin cytoskeleton ring.[8] ZO proteins have roles not only in the stabilization of TJ structures but also in the assembly of TJ proteins at the TJ.[17] Therefore, we evaluated the total expression and cytoskeletal association of TJ proteins by immunoblot analysis to understand the molecular mechanisms underlying the flavonoid-mediated changes in intestinal permeability. Among the flavonoids used in our study, chrysin induced a marked increase in TJ permeability, indicated by the decreased TER and increased FD4 flux. Immunoblot analysis revealed that the chrysin-mediated increases in TJ permeability resulted from the decreased cytoskeletal association of the TJ proteins, ZO-1, occludin, JAM-1, and claudin-1, -3, and -4. These decreases, except for ZO-1, resulted from the impairment of total expression. Four flavonoids, daidzein, hesperetin, morin, and naringenin, all decreased TJ permeability, indicated by the increased TER. However, the decreases in TJ permeability by these flavonoids can be attributed to different changes in expression and/or cytoskeletal association of the TJ proteins. Specifically, naringenin increases the cytoskeletal association of ZO-2, occludin, and claudin-1, -3, and -4, and hesperetin increases the cytoskeletal association of occludin and claudin-1 and-3. Daizdein and morin increase only claudin-1 and claudin-4, respectively. The effects of genistein and luteolin on intestinal TJ integrity are complicated in that the 2 flavonoids increase or normalize the TER after a transient decrease. Immunoblot analysis suggests that the transient TER decrease induced by luteolin is caused by a decrease in the cytoskeletal association of JAM-1 and claudin-3 and -4 and is compensated by increased ZO-2 expression after 6 h, whereas the cause of the transient TER decrease induced by genistein was unclear, because genistein only increases the cytoskeletal association of claudin-1 after incubation. Genistein may have temporally decreased the cytoskeletal association of some TJ proteins along with decreasing the TER at 1, 3, 6 h; however, further studies are needed to uncover the precise mechanism.

The present study examined the effect of flavonoid aglycones on the intestinal TJ integrity using the in vitro model. A large part of flavonoids exists as glycosides in plants and fruits, and they are metabolized to the conjugated and/or methylated forms in our body. Studies with the flavonoid glycosides and metabolites also will provide us useful information to deeply understand the roles of flavonoids on the intestinal TJ regulation. Further, their effects should be investigated in the animal and clinical studies in the future.

The present study used 7 flavonoids belonging to 4 subgroups: flavone (chrysin and luteolin), isoflavone (daidzein and genistein), flavanone (hesperetin and naringenin), and flavonol (morin). Previously, we showed that 2 flavonols, quercetin and kaempferol, decrease TJ permeability.[9,10,18] On the basis of these studies, the 2 flavanones and 3 flavonols can be seen to share a suppressive effect on intestinal TJ permeability but their effects on TJ protein distribution and expression differ. Further, luteolin has 2 additional hydroxy groups on the B ring of chrysin, and genistein has an additional hydroxy group on the A ring of daizdein. Our results showed that these additional hydroxy groups influence the effects on TJ permeability. This evidence indicates that the effect of flavonoids on intestinal barrier function is not simply determined by the type of flavonoid subgroup and that the number and configuration of hydroxy groups also influence the observed effects of flavonoids, even within the same subgroup.

Recent studies have shown that the flavonoid-mediated physiological effects occur via activation/inactivation of various enzymes including intracellular signaling molecules.[19] The cytoskeletal association and expression of TJ proteins are also dynamically regulated by various intracellular signaling pathways.[19] Therefore, modulation of TJ permeability by flavonoids possibly results from activation/inactivation of signaling molecules. We previously reported that a flavonol, quercetin, enhances intestinal TJ integrity through direct inhibition of intracellular protein kinase Cδ.[10] The intracellular signaling mechanism underlying the flavonoid-mediated regulation of intestinal TJ permeability should, therefore, be investigated in future studies.

In conclusion, we demonstrated that various flavonoids participate in the regulation of intestinal TJ integrity. Although the precise mechanisms should be investigated in future studies, this regulation is possibly associated with previously demonstrated flavonoid-mediated biological effects on our health.

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Funding

This study was partially supported by the Japan Society for the Promotion of Science, a Grant-in-Aid for Young Scientists (B) 23780135 and the Uehara Memorial Foundation.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

FD4, FITC-conjugated dextran; JAM, junctional adhesion molecule; TER, transepithelial electrical resistance; TJ, tight junction; ZO, zonula occludens.

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