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REVIEWS: CURRENT TOPICS

Role of flavonoids in intestinal tight junction regulation

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Abstract

The gastrointestinal tract provides a physical barrier to the diffusion of foreign materials from the lumen into the circulatory system. Impairment of the intercellular tight junction (TJ) shield, which is the major determinant of intestinal barrier function, is associated with various diseases. Dietary flavonoids demonstrate various beneficial effects on our health; however, the information regarding their effects on TJ function is quite limited. To date, four flavonoids — epigallocatechin gallate (EGCG), genistein, myricetin and quercetin — have been reported to exhibit promotive and protective effects on intestinal TJ barrier functions. Genistein, a major soybean isoflavone, protects TJ barrier function against oxidative stress, acetaldehyde, enteric bacteria and inflammatory cytokines. Genistein blocks the tyrosine phosphorylation of the TJ proteins induced by oxidative stress and acetaldehyde, which results in the disassembly of the proteins from the junctional complex. Quercetin, a flavonol, enhances intestinal TJ barrier function through the assembly and expression of TJ proteins. The change in phosphorylation status is responsible for the quercetin-mediated assembly of TJ proteins. TJ protein induction has an additional role in this effect. This review presents the recent advances in our understanding of the flavonoid-mediated promotive and protective effects on intestinal TJ barrier function with a particular focus on intracellular molecular mechanisms.

Keywords: Flavonoid; Tight junction; Genistein; Quercetin; Myricetin; Epigallocatechin gallate

1. Introduction

One of the most important functions of gastrointestinal epithelium is to provide a physical barrier to the diffusion of pathogens, toxins and antigens from the luminal environment into the circulatory system. The intestinal barrier is determined by interactions among several barrier components including the adhesive mucous gel layer, the mucosal immune system and the tight junctions (TJs) [1]. Among these components, the intercellular TJs constitute the major determinant of the intestinal physical barrier (Fig. 1). TJs are multiple-protein complexes located around the apical end of the lateral membrane of the epithelial cells and regulate the paracellular movement of ions, solutes and water through the intestinal epithelium. Four integral transmembrane proteins, occludin [2], claudins [3], junctional adhesion molecule (JAM) [4] and tricellulin [5], have been identified, with the claudin family consisting of at least 24 members [6]. These transmembrane proteins interact with intracellular plaque proteins, such as zonula occludens (ZO) and cingluin, which in turn anchor the transmembrane proteins to the perijunctional actin cytoskeleton. The interaction of TJ proteins with the actin cytoskeleton is vital for maintaining TJ structure and function [6]. Numerous studies have reported that TJ protein expression and association with the actin cytoskeleton, which determines TJ permeability, were dynamically regulated by various intracellular signaling molecules, such as protein kinases [7,8], protein phosphatases [9,10] and phosphatidylinositol 3-kinase [11].

A significant body of evidence has demonstrated that intestinal barrier defects are involved in several intestinal and metabolic diseases, such as inflammatory bowel disease [12], food allergies [13], obesity [14] and alcoholic liver disease [15]. In these diseases, inflammatory cytokines [16,17], reactive oxygen species [11,18] and pathogenic bacteria [19,20] have been found to impair intestinal TJ function. These mediators influence not only the expression but also the cytoskeletal association of TJ proteins through the activation/inactivation of intracellular signaling. However, the causal relationships between these diseases and barrier defects, and also the precise mechanisms underlying the barrier defects remain to be fully established.

A large number of studies have reported that various food components provided beneficial anti-inflammatory and anti-mutagenic effects in the intestines. Although the information regarding these effects on intestinal TJ barrier function is quite limited, some results for glutamine [21,22], fatty acids [23–25] and flavonoids are available. The role of glutamine, which is the primary metabolic fuel

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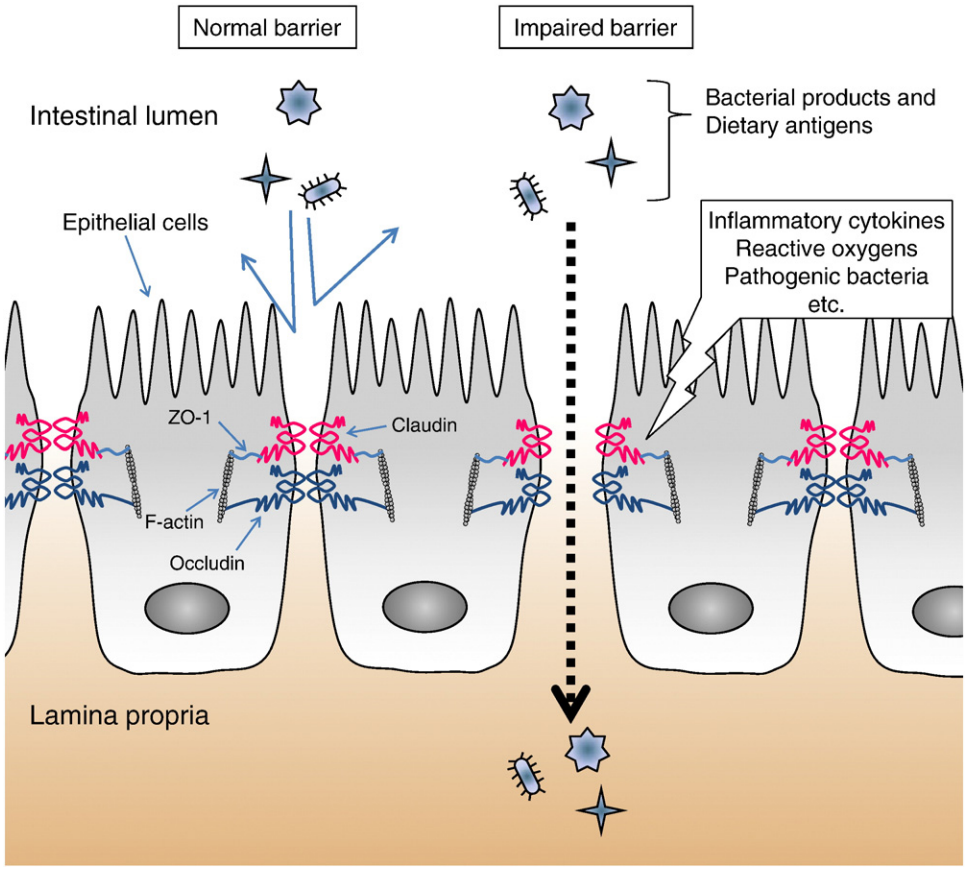

Fig. 1. Intestinal epithelial TJs as a physical barrier. The intestinal TJs tightly regulate intestinal paracellular permeability. The barrier impairment induced by extracellular stimuli, such as inflammatory cytokines and reactive oxygens, allows the lumina bacterial products and dietary antigens to cross the epithelium and enter circulation. This can induce inflammation and immunological reactions in tissues including the intestines, resulting in both intestinal and nonintestinal diseases.

of the small intestinal cells, in intestinal TJ barrier function has been extensively investigated. *In vivo* studies showed that glutamine helps maintain intestinal mucosal integrity, especially during stress, such as radiation therapy [26], chemotherapy [27] and total parenteral nutrition [28]. Furthermore, Li et al. [21] reported that glutamine stabilized TJ integrity by the maintenance of TJ protein expression through the suppression of the phosphatidylinositol 3-kinase/Akt pathway in human intestinal Caco-2 cells. Seth et al. [22] reported that glutamine ameliorated the acetaldehyde-induced TJ dysfunction through the activation of epidermal growth factor-receptor tyrosine kinase in the Caco-2 cells. These results suggest that glutamine and the other food components mentioned above play a role in the regulation of intestinal barrier function and some may have potential applications to the prevention and treatment of diseases associated with intestinal barrier impairment.

This review focuses on flavonoids from the above-mentioned food components and summarizes the protective and promotive effects of flavonoids, especially genistein and quercetin, on intestinal TJ barrier function, with particular emphasis on the intracellular signaling pathways and behavior of TJ proteins involved in the flavonoid-mediated effects on intestinal TJ function.

2. Intestinal absorption and metabolism of dietary flavonoids

Flavonoids, polyphenolic compounds containing diphenylpropans ($C_6C_3C_6$), are secondary metabolites ubiquitously distributed throughout the plant kingdom. They are classified into six major categories: isoflavones, anthocyanidins, flavones, flavonols, flavan-3-ols and flavonones, and more than 4000 different molecules have been identified to date. The chemical structures of the flavonoids presented in this review [genistein, quercetin, myricetin and epigallocatechin gallate (EGCG)] are shown in Fig. 2. The major sources of flavonoids in the human diet are fruits, vegetables and beverages, such as tea and coffee, and the majority of flavonoids are present as glycosides in which one or more sugar groups are bound to the phenolic groups through glycosidic linkage, although a small proportion is present as aglycones. In order to understand the physiological effects of flavonoids, it is important to take into account their intestinal absorption and metabolism.

Flavonoid absorption from the intestine occurs by several different pathways. Flavonoid aglycones can be easily absorbed into the intestinal cells as their lipophilicity facilitates the passage across the mucosal phospholipid bilayer of cells. Lactase phlorizin hydrolase (LPH), which is expressed on the brush border membrane of the small intestinal epithelium, has a crucial role in the absorption of flavonoids bearing β-glycoside linkages. Day et al. [29] demonstrated that LPH was capable of hydrolyzing various flavonoid glycosides, such as quercetin-4′-glucoside, quercetin-3-glucoside, genistein-7-glucoside and daidzein-7-glucoside, to form aglycones.

On the other hand, flavonoid monoglucosides can be transported by sodium glucose transporter-1 (SGLT-1) [30] on the brush border membrane of the intestinal cells. Wolffram et al. [31] demonstrated that SGLT-1 was involved in the uptake of quercetin-3-glucoside in rat small intestinal cells. Most flavonoid glycosides entering the enterocytes are deglycosylated by β-glucosidases, namely, the broad-specificity cytosolic β-glucosidase [32]. The flavonoids appear to be subjected to glucuronidation, sulfation and methylation in the intestinal epithelial cells before entering circulation [33–35],

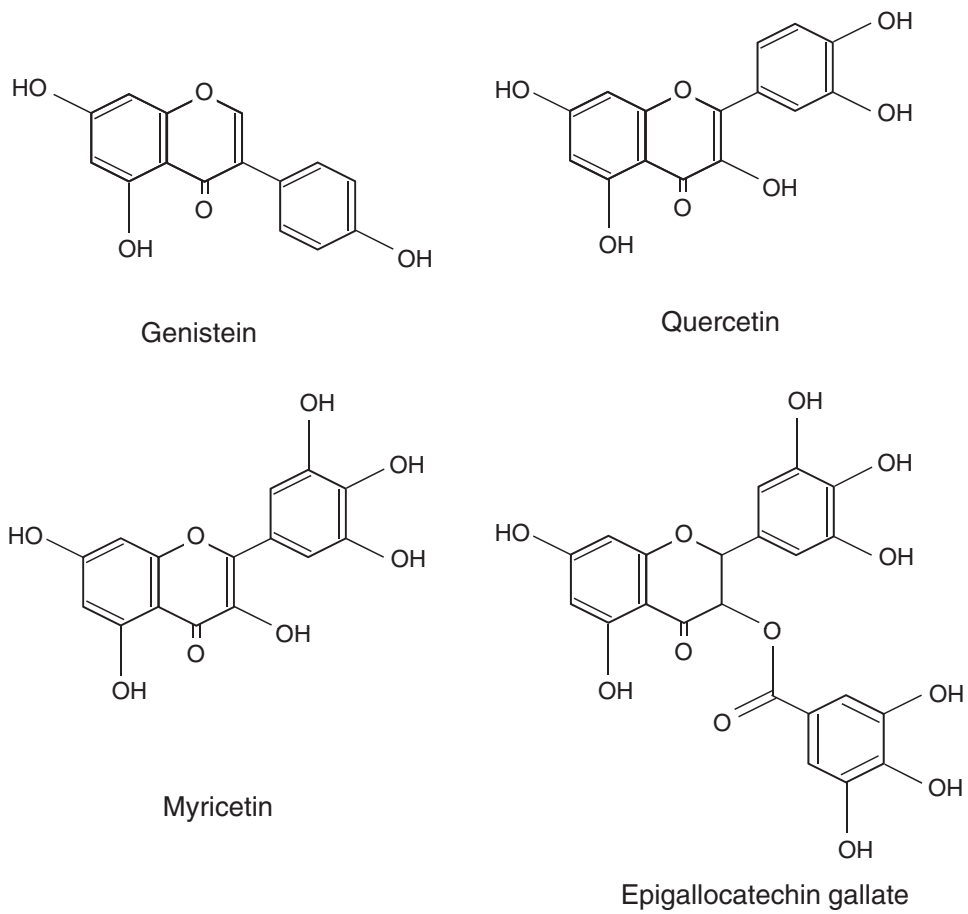

Fig. 2. Chemical structures of flavonoids, genistein, quercetin, myricetin and EGCG.

although the extent of the conversion seems to be lower in isoflavones and catechins than in the other flavonoids [36,37]. The flavonoid conjugates then gain access into hepatocytes where they are further methylated, glucuronidated or sulfated [38,39]. These flavonoid conjugates are excreted into the urine and also into bile fluid, thereby returning to the intestinal lumen [40]. Once there, they may again be reabsorbed by the intestinal cells, mainly in the large intestine. Meanwhile, significant amounts of dietary flavonoids reach the large intestine, because the absorption rate of the flavonoids in the small intestine is typically low. The intestinal microbes have high levels of β-glucosidase and β-glucuronidase activity [41–43], and the glycosylated and/or conjugated flavonoids are quickly hydrolyzed to aglycones or methylated forms by the enzymatic activity in the large intestine. Matsukawa et al. [44] showed that only quercetin and monomethylated quercetin (isorhamnetin and tamarixetin) were detected in the cecal contents and feces of rats fed quercetin-3-glucoside.

Consequently, specific forms of the flavonoids are present in the small and large intestines and plasma, and each of them can produce distinct biological effects on the intestinal epithelial cells.

3. Roles of flavonoids for intestinal barrier function

3.1. Genistein

Much attention has been given to the physiological effects of genistein, one of the major isoflavones found in soybeans. Genistein is naturally present as a glycoside, genistin. Genistein has been often used in studies related to signal transduction as it is a potent inhibitor of protein tyrosine kinases. A large number of studies have demonstrated that the phosphorylation status of TJ proteins was closely related to TJ structure and function [9,45,46]. Occludin is known to undergo tyrosine phosphorylation during the disruption of TJs by various factors [18,47–49], although the tyrosine phosphorylation of TJ proteins is not detectable in the intact intestinal epithelium. Genistein protects TJ barrier function mainly through the above-mentioned inhibition of protein tyrosine kinases.

Genistein is reported to ameliorate intestinal TJ barrier dysfunction induced by oxidative stress. Rao et al. [18] demonstrated that oxidative stress induced by the administration of a mixture of xanthine oxidase and xanthine, which generates superoxide anions in the culture media, decreased the transepithelial electrical resistance (TER) and increased [^{3}H]-mannitol flux, indicators of TJ permeability, in 3 h in human intestinal Caco-2 cells. These changes were prevented by the co-administration of genistein (300 μM). The oxidative stress-induced TJ dysfunction was involved in the tyrosine phosphorylation of TJ proteins, such as occludin and ZO-1, along with that of adherence junction (AJ) proteins, such as E-cadherin, resulting in their disassembly from the junctional complex. The tyrosine phosphorylation is regulated by the balance between phosphorylation by protein tyrosine kinases, such as c-Src kinase, and dephosphorylation by phosphatases, such as protein tyrosine phosphatase (PTP). The oxidative stress induced a rapid activation of c-Src kinase, resulting in the tyrosine phosphorylation of TJ and AJ proteins [50]. Genistein appears to suppress this oxidative stress-induced c-Src kinase activation, thereby protecting TJ barrier function against oxidative stress.

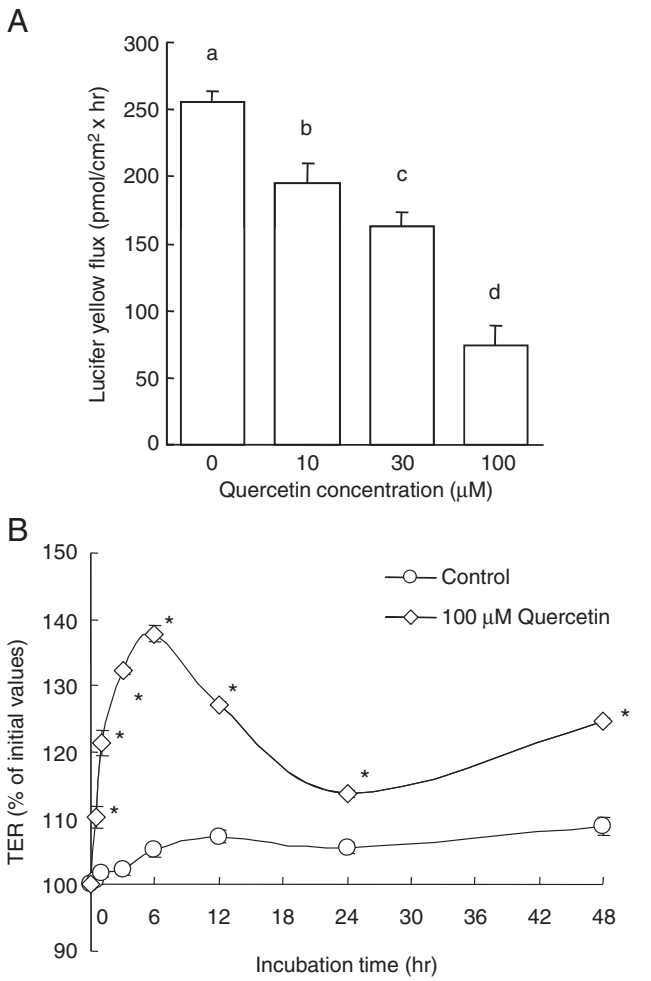

Fig. 3. Promotive effect of quercetin on intestinal TJ barrier function. Human intestinal Caco-2 cells were incubated with or without 10–100 μM quercetin for 48 h in a Transwell culture system. Lucifer yellow flux (A) across the cell monolayers was evaluated for the last 3 h of incubation, and TER (B) was measured before and at 0.5, 1, 3, 6, 12, 24 and 48 h. Values are means±S.E.M., $n=4$. Values not sharing a common letter differ significantly, $P<.05$ (A). Asterisks indicate a significant difference from the values before quercetin administration, $P<.05$ (B).

Genistein has also been reported to protect intestinal TJ barrier function from acetaldehyde-induced insult [51,52]. A significant body of evidence suggests that endotoxemia plays a crucial role in the pathogenesis of alcoholic liver disease [53]. One of the possible mechanisms underlying endotoxemia is the luminal acetaldehyde-induced disruption of intestinal TJ barrier structure [54]. It is reported that luminal acetaldehyde is derived from ethanol oxidation by colonic bacterial fermentation [55], and the intracolonic acetaldehyde level in men consuming alcohol reaches as high as 1 mM [56]. Recent studies showed that acetaldehyde (0.1–0.8 mM) disrupted the intestinal TJ barrier in a tyrosine protein kinase-dependent mechanism [51,52,57]. Acetaldehyde did not increase the overall tyrosine kinase activities, but potently inhibited PTP-1B, -C and -D in the intestinal Caco-2 cells [51]. The inhibition of PTPs by acetaldehyde induced the tyrosine phosphorylation of TJ and AJ proteins and increased paracellular permeability within hours, as indicated by an increase in [^{3}H]-mannitol flux and a decrease in TER. Genistein (30–300 μM) dose-dependently blocked the acetaldehyde-induced intestinal permeability [51]. Confocal immunofluorescence microscopy also showed that the acetaldehyde-induced tyrosine phosphorylation in the junctional region and the dissociation of the TJ proteins, ZO-1 and occludin, were blocked by genistein.

Furthermore, it is reported that genistein ameliorates the impairment of intestinal TJ barrier function by inflammatory cytokines [58] and enteric bacteria [59]. Serosal administration of tumor necrosis factor-α, an inflammatory cytokine, in colonic epithelial HT-29/B6 cells decreased the TER at 8 h to approximately 20% of the initial value, although this decrease was completely blocked by the administration of 185 μM genistein [58]. Genistein also blocked decreases in TER induced by *Salmonella typhimurium* and *Escherichia coli* in Caco-2 cells [59]. However, the precise mechanisms underlying the genistein-mediated protective effects remain unclear.

3.2. Quercetin

Quercetin, a flavonol, is found in high levels in vegetables and fruits such as onions and apples, and is one of the most abundant flavonoids in foods. The intestinal TJ permeability of rats fed the

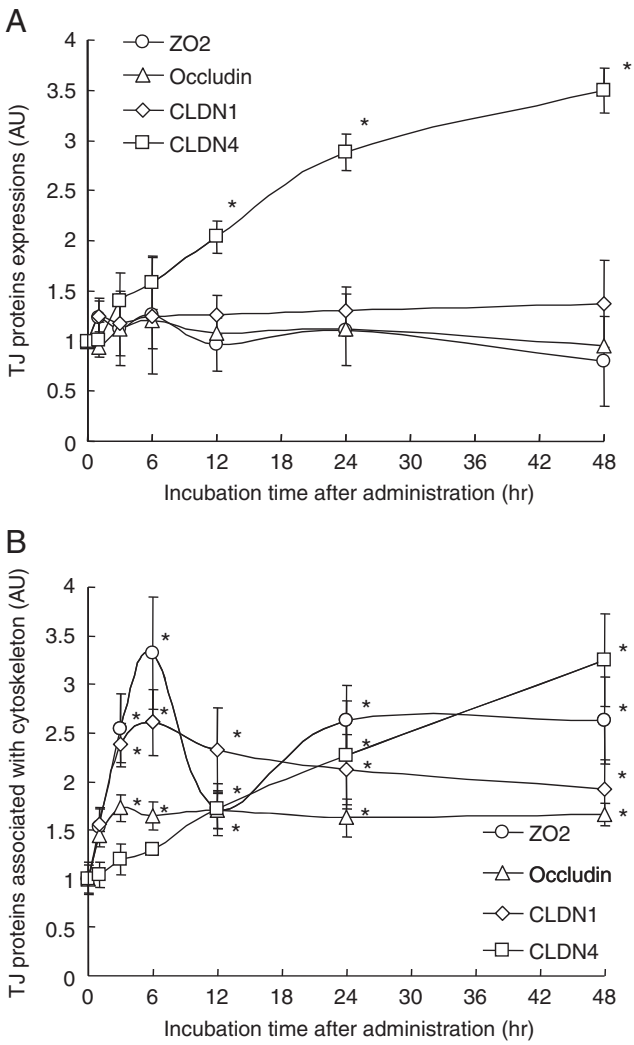

Fig. 4. Effect of quercetin on the expression and cytoskeletal association of TJ proteins. Human intestinal Caco-2 cells were incubated with or without 100 μM quercetin for 48 h in a Transwell culture system. Whole cell extracts (A) and the actin cytoskeleton fraction (detergent-insoluble fraction) (B) were prepared before and at 1, 3, 6, 12, 24 and 48 h, and immunoblotted for TJ proteins, ZO-1, ZO-2, occludin, JAM-1, claudin-1, claudin-3 and claudin-4. Each specific protein band was quantified by densitometric analysis, and the results for ZO-2, occludin, claudin-1 and claudin-4 only are shown, as no differences were found in the other proteins. Values are means±S.E.M., $n=4$. Asterisks indicate a significant difference from the values before quercetin administration, $P<.05$.

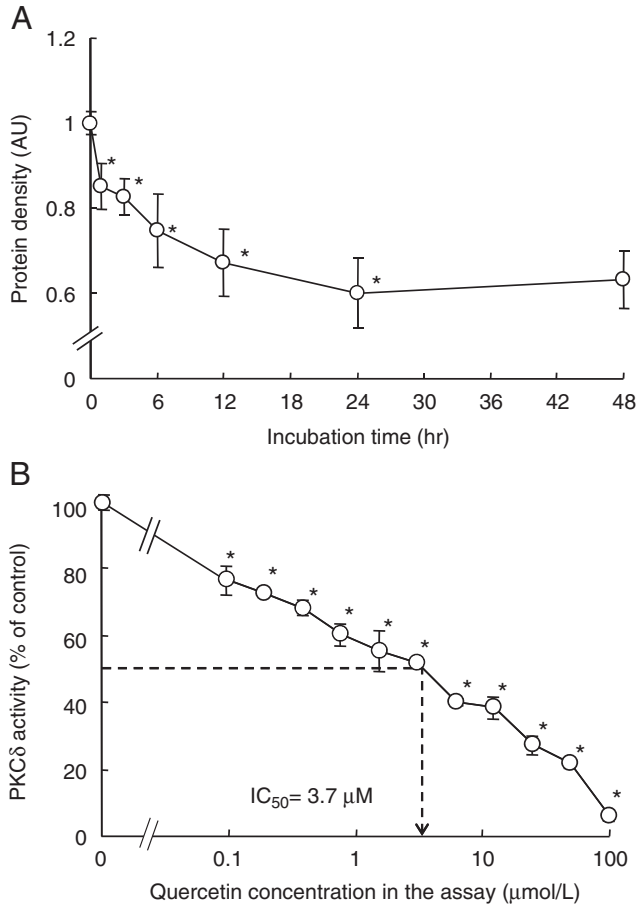

Fig. 5. Inhibitory effect of quercetin on PKCδ activity in Caco-2 cells and in an *in vitro* kinase assay. (A) Human intestinal Caco-2 cells were incubated with or without 100 μM quercetin for 48 h in a Transwell culture system. Whole-cell extracts were prepared before and at 1, 3, 6, 12, 24 and 48 h, and immunoblotted for phospho- and total PKCδ. Each specific phospho- and total PKCδ band was quantified by densitometric analysis, and the ratio of phospho-PKCδ to total PKCδ was calculated. (B) The human recombinant PKCδ enzyme was incubated with the PKC substrate in the absence or presence of quercetin (0–100 μM). The activity values were normalized against the control value obtained from the recombinant PKCδ enzyme incubated with the PKC substrate without quercetin. Values are means±S.E.M., $n=3$. Asterisks indicate a significant difference from the control value, $P<.05$.

quercetin-containing diet (~1.0%) for 9 days was examined using an Ussing chamber system. Both the small and large intestines of rats fed the quercetin diets showed dose-dependent decreases in lucifer yellow (LY) flux (personal observation). Treatment of intestinal Caco-2 cells with quercetin (~100 μM) for 48 h also decreased the LY flux (Fig. 3A) and increased the TER across the monolayers in a dose-dependent manner [60]. The TER began to increase within 0.5 h of the administration of 100 μM quercetin and markedly increased over the first 6 h (Fig. 3B). Thereafter, the TER decreased until 24 h before again gradually increasing between 24 and 48 h. At the same time, total protein expression of claudin-4, but not the other TJ proteins, continuously increased from 12 h after quercetin treatment (Fig. 4A) [60]. Another research group has reported, on the basis of reporter gene assays in Caco-2 cells, that quercetin stimulates claudin-4 promoter activity, indicating that quercetin enhanced the claudin-4 expression at a transcriptional level [61]. Furthermore, the cytoskeletal association of ZO-2, occludin and claudin-1 was promoted during the first 6 h after quercetin administration, although the association of claudin-4 remained at increased levels at and after 12 h (Fig. 4B) [60]. These results show that the promotion of ZO-2, occludin and claudin-1 assembly is responsible for the quercetin-mediated increase in TER in the early phase in the first 6 h, and the stimulation of claudin-4 expression has an additional role in the increases in TER observed in the later phase.

This raises the question as to what signaling molecules are involved in the quercetin-mediated enhancement of the intestinal TJ barrier function. Recently, the cellular biological effects of flavonoids are believed to be mediated through the interaction with signaling molecules, such as protein kinases, rather than through the antioxidant properties of the flavonoids. Among several signaling inhibitors, a selective protein kinase C δ (PKCδ) inhibitor was found to exhibit a promotive effect on the TJ barrier function in a very similar manner to that of quercetin, i.e., by promoting the cytoskeletal association of ZO-2, occludin and claudin-1, as well as total claudin-4 expression [60]. The PKCδ activity in Caco-2 cells began to decrease within 1 h of quercetin administration and reached 60% of the initial activity at 24 h (Fig. 5A) [60]. Furthermore, an *in vitro* kinase assay showed the direct inhibition of PKCδ by quercetin and revealed that the quercetin concentration required for the 50% inhibition of PKCδ was as low as 3.7 μM (Fig. 5B) [60]. Taken together, these results show that the quercetin-mediated promotion of intestinal TJ barrier function occurs *via* the inhibition of the PKCδ isoform.

Although the precise mechanism underlying the quercetin-mediated promotion of TJ protein assembly and expression remains unclear, it is clear that quercetin induced the phosphorylation of occludin in the Caco-2 cells concomitantly with the assembly of the TJs. Occludin phosphorylation was estimated by migration in the SDS-PAGE to be enhanced within 1 h of quercetin administration, and the enhancement was closely associated with the occludin assembly

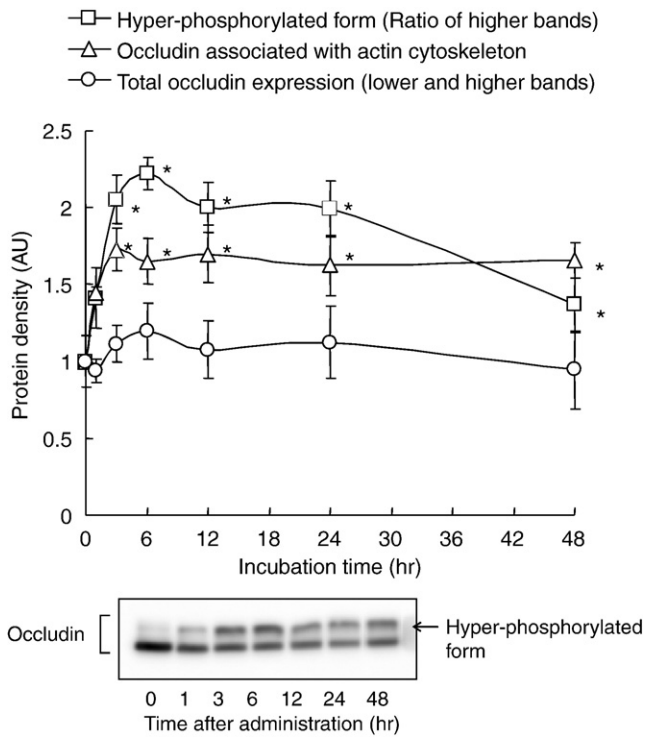

Fig. 6. Possible involvement of occludin phosphorylation in the quercetin-induced assembly. Human intestinal Caco-2 cells were incubated with or without 100 μM quercetin for 48 h in a Transwell culture system. Whole-cell extracts and actin cytoskeleton fractions of cells were prepared before and at 1, 3, 6, 12, 24 and 48 h, and immunoblotted for occludin. Occludin showed double bands in the immunoblots, with the higher band corresponding to the hyper-phosphorylated form of occludin. Each band was quantified by densitometric analysis. The total expression (sum of the lower and higher bands) and the ratio of the hyper-phosphorylated form to total expression were calculated. Values are means±S.E.M., $n=4$. Asterisks indicate a significant difference from the values before quercetin administration, $P<.05$.

(Fig. 6). Several reports demonstrate that the assembly and disassembly of TJs are involved in the phosphorylation of occludin on Ser, Thr and Tyr residues. In the Caco-2 cells, occludin undergoes dephosphorylation on Thr residues during TJ disassembly under calcium-depleted conditions and is rephosphorylated by calcium replacement [8,10,45]. Several protein kinases, such as PKCζ [9], PKCη [8] and casein kinase I/II [62], and protein phosphatases, such as PP1 and PP2A [9,10], have been identified as taking part in occludin phosphorylation and dephosphorylation. It seems that quercetin affects the activities of these kinases or phosphatases through PKCδ inhibition, resulting in the promotion of occludin phosphorylation and TJ assembly.

3.3. Other flavonoids

Myricetin, one of the flavonols found in grapes and tea, exhibits a promotive effect on intestinal TJ barrier function in Caco-2 cells [60]. Cells incubated with 10–100 μM myricetin for 48 h showed a dose-dependent decrease in LY flux; however, no changes in the cytoskeletal association or expression of TJ proteins were observed. This evidence confirms that the promotive effects of quercetin and myricetin are not associated with their antioxidant activities, because quercetin promotes TJ barrier function more effectively than does myricetin, whereas myricetin has higher antioxidant activity than does quercetin due to an additional hydroxy group on the B-ring.

A green tea flavonoid, EGCG, was found to ameliorate the intestinal TJ barrier dysfunction provoked by interferon (IFN) γ [63]. Human intestinal T84 cells exposed to IFNγ (20 ng/ml) for 48 h showed a decrease in TER and an increase in horseradish peroxidase flux. The mucosal administration of EGCG (100 μM) completely reversed these changes. However, the molecular mechanisms underlying these EGCG-mediated effects remain unclear.

4. Physiological implications and relevance of the flavonoid-mediated effects on intestinal barrier function

Information on the flavonoid-mediated promotive effects on intestinal TJ barrier function in humans is completely lacking. To

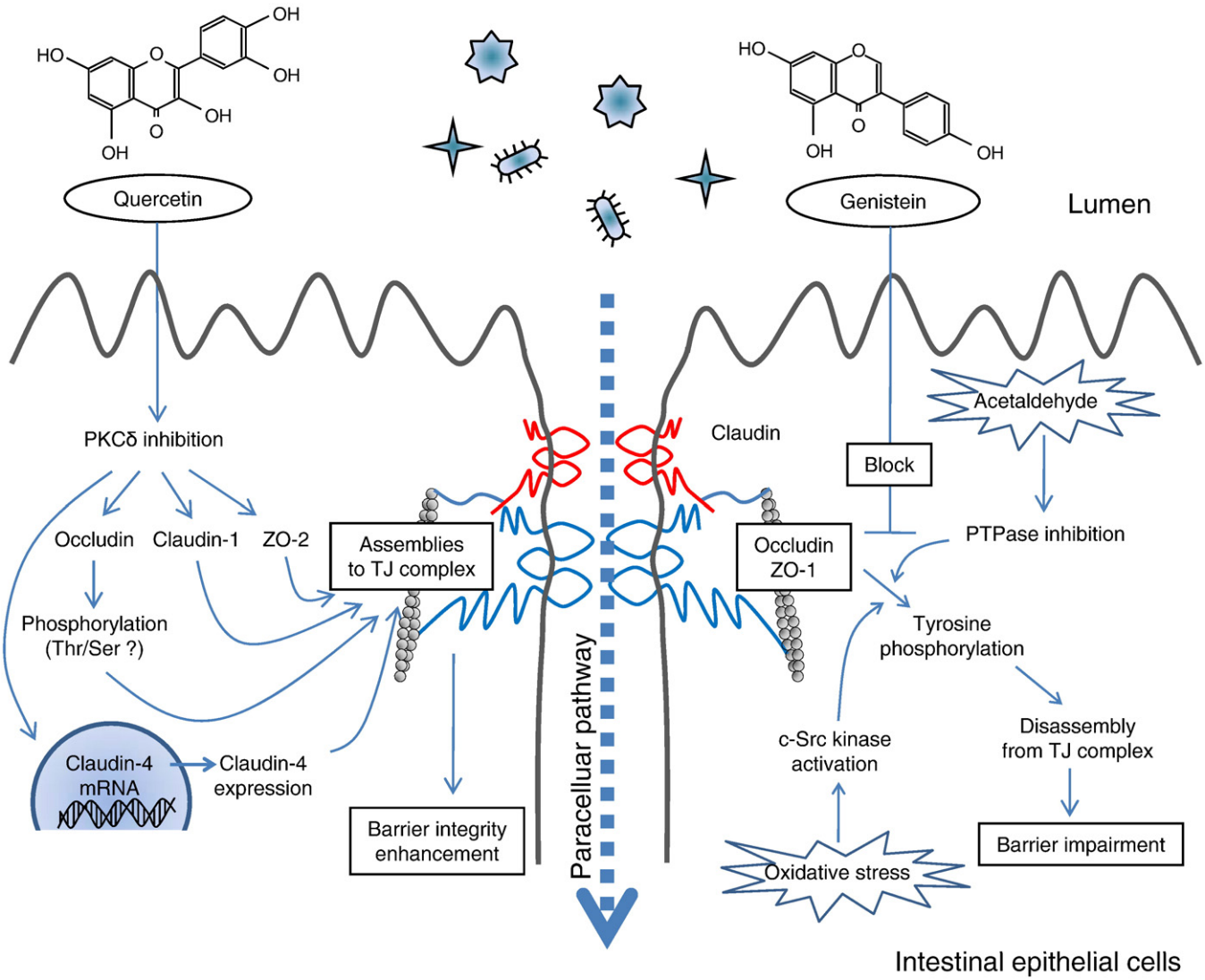

Fig. 7. Diagrams showing the molecular mechanisms underlying the flavonoid-mediated effects on intestinal TJ barrier function. Genistein protects intestinal TJ barrier function against oxidative stress and acetaldehyde. Oxidative stress and acetaldehyde induce tyrosine phosphorylation of TJ proteins through c-Src kinase activation or PTP inhibition leading to barrier impairment. Genistein normalizes the tyrosine phosphorylation of TJ proteins by inhibiting protein tyrosine kinases including c-Src kinase. Quercetin enhances intestinal TJ barrier integrity through PKCδ inhibition. Quercetin promotes total claudin-4 expression and the assembly of ZO-2, occludin and claudin-1 without any changes in their total expression levels. The promotion of claudin-4 expression by quercetin is induced at a transcriptional level leading to an increase in assembly. The quercetin-induced occludin assembly is then possibly involved in the phosphorylation of occludin on Ser or Thr residues.

play a role in humans, effective concentrations of flavonoids are required in the plasma or intestinal lumen. The daily intake of flavonoids in humans is reportedly 20–50 mg for isoflavones, 20–35 mg for flavonols [64–66] and 18–50 mg for catechins [67], and it has been shown that the plasma concentrations of total flavonoid derivatives range from 0 to 4 μM at an intake level of 50 mg aglycone equivalents [68]. These concentrations seem too low to exhibit any promotive or protective effects on intestinal TJ barrier function such as that shown by quercetin at concentrations of >30 μM in the Caco-2 cells [60]. On the other hand, the luminal concentrations of flavonoids are known to be much higher than those in plasma because of the low intestinal absorption rate, although accurate data is not available in humans. Previous studies show that the quercetin derivative concentrations in the ileal and cecal lumens were found to be ~30 and ~4.2 mmol/kg wet content of rats fed 100 mg quercetin aglycone equivalents as diet [44,69]. On the basis of these results, it is calculated that a luminal quercetin concentration of >40 μM can be achievable at an intake level of 1 mg quercetin in rats, and an intake of 1 mg quercetin in rats may correspond to approximately 20 mg in humans, based on daily calorie intakes. Collectively, the promotive and protective effects of flavonoids on intestinal TJ barrier function observed in animals and cell culture models could be translated to humans.

Flavonoid aglycones have been used in most of the experiments presented in this review. Aglycones are definitely present throughout the intestinal lumen even when flavonoid glycosides are ingested [44,70]. However, the effects of glycosylated, conjugated and/or methylated flavonoids on intestinal TJ barrier function should be examined in future studies as they are also distributed in the lumen, show different biological properties and may have different roles from those of aglycones.

The TJs have crucial roles in paracellular nutrient transport as well as in barrier function in the intestines. The paracellular route largely contributes to the transport and absorption of certain minerals, such as calcium [71] and magnesium [72]. The modification of TJ structure and function by flavonoids may influence the paracellular absorption of these nutrients. To confirm their safety for human consumption, the effects of flavonoids on the absorption of these nutrients along with TJ barrier function should be examined in future studies. Meanwhile, these minerals are also known to be absorbed by the active transcellular pathway in the intestine [71,72]. The contribution of each pathway depends on the dietary level of the minerals, and transcellular transport is generally more tightly regulated than is paracellular transport. It is reported that lower calcium intake up-regulates the transient receptor potential vanilloid family 6 (TRPV6) calcium channel and calbindin D9k, which are responsible for transcellular calcium transport, in the intestine [73]. Thus, transcellular transport may compensate for changes in the paracellular transport of these nutrients. Furthermore, there has been no data reported to date showing any signs of physical or physiological problems induced by the prolonged feeding of flavonoids in animal or human studies.

5. Conclusion

To date, only four flavonoids — genistein, quercetin, myricetin and EGCG — have been reported to show protective and promotive effects on intestinal TJ barrier function. Genistein and quercetin interact with intracellular signaling molecules, tyrosine kinases and PKCδ, resulting in the regulation of TJ protein expression and assembly (summarized in Fig. 7). As it is known that TJ barrier defects are involved in several diseases, such as inflammatory bowel disease, dietary supplementation with flavonoids might afford an effective tool for the prevention and treatment of those diseases. Future investigations are required to elucidate the precise mechanisms underlying these flavonoid-mediated protective and promotive effects on intestinal TJ barrier function.

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