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Quercetin Enhances Intestinal Barrier Function through the Assembly of Zonnula Occludens-2, Occludin, and Claudin-1 and the Expression of Claudin-4 in Caco-2 Cells[1]

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Abstract

Dietary flavonoids provide various beneficial effects for our health. We investigated the promotive effects of quercetin and myricetin on the intestinal barrier function in human intestinal Caco-2 cell monolayers. Transepithelial electrical resistance (TER) across the monolayers increased rapidly during incubation with quercetin, peaking at 6 h. Lucifer yellow flux, a paracellular marker, was dose-dependently lower after quercetin and myricetin treatments, although quercetin exhibited a more potent effect. Immunoblot analysis of tight junction (TJ) proteins revealed that zonnula occludens (ZO)-2, occludin, and claudin-1 were distributed to the actin cytoskeleton fraction by quercetin without increasing their respective whole-cell levels and this distribution was correlated with the increases in TER. The claudin-4 level was elevated by quercetin in both the cytoskeleton fraction and whole cells after 12 h. Confocal microscopy showed the assembly of claudin-1 and -4 at the TJ by quercetin. An inhibitor of protein kinase Cδ (PKCδ), rottlerin, enhanced the barrier function with changes in the distribution and expression of TJ proteins in a manner very similar to that of quercetin. Phosphorylation of PKCδ indicating the enzymatic activity in the cells was decreased by quercetin after 1 h. In the kinase assay, quercetin exhibits direct inhibition of the PKCδ isoform. This study demonstrates that quercetin enhances the intestinal barrier function through the assembly of ZO-2, occludin, and claudin-1 by inhibiting PKCδ and the increase in claudin-4 expression has an additional role after 12 h. J. Nutr. 139: 965–974, 2009.

Introduction

Flavonoids, polyphenolic compounds containing diphenylpropans ($C_6C_3C_6$), are secondary metabolites ubiquitously distributed throughout the plant kingdom. They are classified into 6 major categories; isoflavones, anthocyanidins, flavones, flavonols, flavan-3-ols, and flavonones, and >4000 different molecules have been identified. Quercetin is one of the most abundant flavonoids in the human diet and is a potent antioxidant (1). Antioxidant efficacy of flavonoids is closely related to the number and arrangement of the hydroxyl group on the B-ring (2), although the other characteristics of flavonoids, such as permeability to the cells, also may influence the biological activities in vivo. Meanwhile, recent studies found that quercetin exhibits various biological effects, such as antiinflammatory (3) and anticancer (4) activities, through the activation/inactivation of various enzymes, including intracellular signaling molecules. Quercetin has been reported to inhibit isoform-mixed protein kinase C (PKC)[2] and phosphatidylinositol 3-kinase (PI3K), which were purified from bovine brains and human platelets, in the cell-free assays (5). Other dietary flavonoids, such as myricetin, having an additional hydroxyl group on the B-ring also have functions similar to those of quercetin (6,7). However, the mechanism(s) underlying these flavonoid-mediated biological effects have not yet been fully clarified.

One of the most important functions of gastrointestinal epithelial cells is to provide a physical barrier to the diffusion of pathogens, toxins, and allergens from the external environment into the tissues. Defects in the barrier function play a crucial role in the pathogenesis of various diseases, such as inflammatory bowel disease (8) and alcoholic liver disease (9). The endotoxemia and endotoxin-mediated hepatocellular damage that resulted from elevated intestinal permeability play a role in the

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[2] Abbreviations used: HRP, horseradish peroxidase; JAM, junctional adhesion molecule; LY, lucifer yellow; PI3K, phosphatidylinositol 3-kinse; PKC, protein kinase C; TER, transepithelial electrical resistance; TJ, tight junction; ZO, zonnula occludens.

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pathogenesis of alcoholic liver disease. The major determinant of intestinal barrier function is the intercellular tight junctions (TJ), which are positioned around the apical end of the lateral cell membrane. The TJ are organized by specific interactions between a wide spectrum of proteins (10) and 3 integral transmembrane proteins, occludin (11), claudins (12), and junctional adhesion molecule (JAM) (13), have been identified. These interact with other intracellular plaque proteins such as zonnula occludens (ZO)-1, ZO-2, ZO-3, cingulin, and 7H6, which in turn anchor the transmembrane proteins to the actin cytoskeleton (10). The association of TJ proteins with the perijunctional actin cytoskeleton ring is vital for maintaining the TJ structure and function (10).

A considerable body of evidence indicates that the activities of various intracellular signaling molecules regulate the integrity of TJ (14). The PKC family, consisting of at least 11 isoenzymes, has been studied intensely in regard to the gastrointestinal epithelium and has been shown to be involved in the barrier function in an isoform-specific manner (14). Atypical PKCς and -λ are essential for the maintenance of TJ (15), whereas a novel PKCδ is activated by hydrogen peroxide and causes TJ disruption (16). PI3K also negatively regulates the intestinal barrier function and activation of PI3K by oxidative stress dissociates occludin and ZO-1 from the actin cytoskeleton and disrupts barrier function in epithelial cells (17).

Amasheh et al. (18) recently reported that a 48-h exposure of quercetin enhances the intestinal barrier function through increasing claudin-4 expression in human intestinal Caco-2 monolayers; however, the mechanism is poorly understood. Stimuli, such as nutrients and cytokines, have been reported to influence not only the expression of TJ proteins but also their association with the actin cytoskeleton via activation/inactivation of intracellular signaling molecules and modulate paracellular permeability (19,20). In this study, we investigated the promotive effect of 2 major flavonoids, quercetin and myricetin, on the intestinal barrier function, including the association of TJ proteins with actin cytoskeleton, as well as their expression, and intracellular signaling molecules involved in the flavonoids-mediated effect.

Materials and Methods

Chemicals. Cell culture reagents and supplies were purchased from Invitrogen. Rabbit anti-claudin-1, claudin-3, and JAM-1, mouse anti-ZO-1, ZO-2, and claudin-4, and horseradish peroxidase (HRP)-conjugated anti-occludin were purchased from Zymed Laboratories. Mouse anti-β-actin and HRP-conjugated anti-mouse and -rabbit IgG were purchased from Sigma. Rabbit anti-phospho-PKCδ (Ser643) was purchased from Cell Signaling Technology. Ro-31–8425 (an inhibitor of PKCα/βI/βII/γ/ε), rottlerin (an inhibitor of PKCδ), and LY294002 (an inhibitor of PI3K) were purchased from Calbiochem. All other chemicals were obtained from Wako Pure Chemical Industries.

Cell culture. Caco-2 cells (HTB-37; American Type Culture Collection) were propagated and maintained under standard cell culture conditions as described previously (21). The cells were seeded into permeable polyester membrane filter supports (Transwell, 12-mm diameter, 0.4-μm pore size; Corning Costar) at a density of 0.25×10^6 cells/cm^2. All experiments were conducted on d 13–14 postseeding. Cultures were used between passage 35 and 60 and the medium was refreshed every 3 d.

Measurement of intestinal barrier function. Intestinal barrier function was evaluated by measurement of transepithelial electrical resistance (TER) and unidirectional flux of lucifer yellow (LY) in Caco-2 cell monolayers in Transwell filter supports (21,22). The cell monolayers show the TER of 1000–1200 Ω·cm^2 and high alkaline phosphatase and sucrase activities (data not shown). Quercetin and myricetin were administrated into the apical wells (0, 10, 30, and 100 μmol/L) and the cells were incubated for 48 h with refreshing media every 24 h. TER was measured before and at 24 and 48 h after administration of the flavonoids using a Millicell-ERS system (Millipore). LY (100 μmol/L), a paracellular

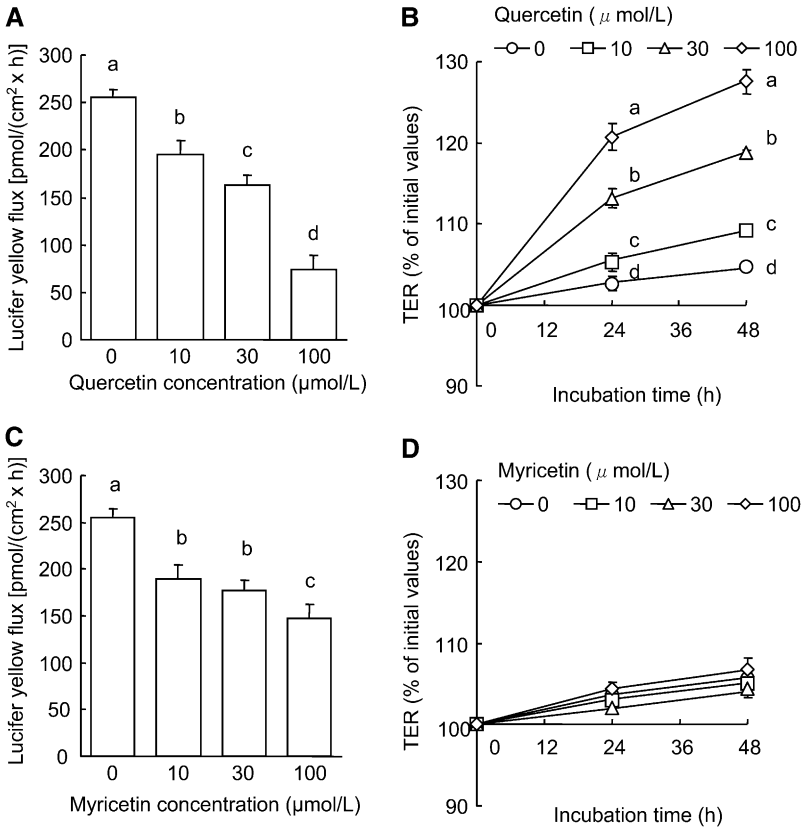

FIGURE 1 LY flux and TER in Caco-2 cells incubated with or without quercetin and myricetin. Caco-2 cell monolayers were incubated with or without 10, 30, or 100 μmol/L quercetin (*A,B*) or myricetin (*C,D*) for 48 h. LY flux (*A,C*), a paracellular marker, was measured for the last 3 h of incubation. TER (*B,D*) was measured before and at 24 and 48 h after the administration of quercetin or myricetin. Values are means ± SEM, $n = 4$. Labeled means at a time without a common letter differ, $P < 0.05$.

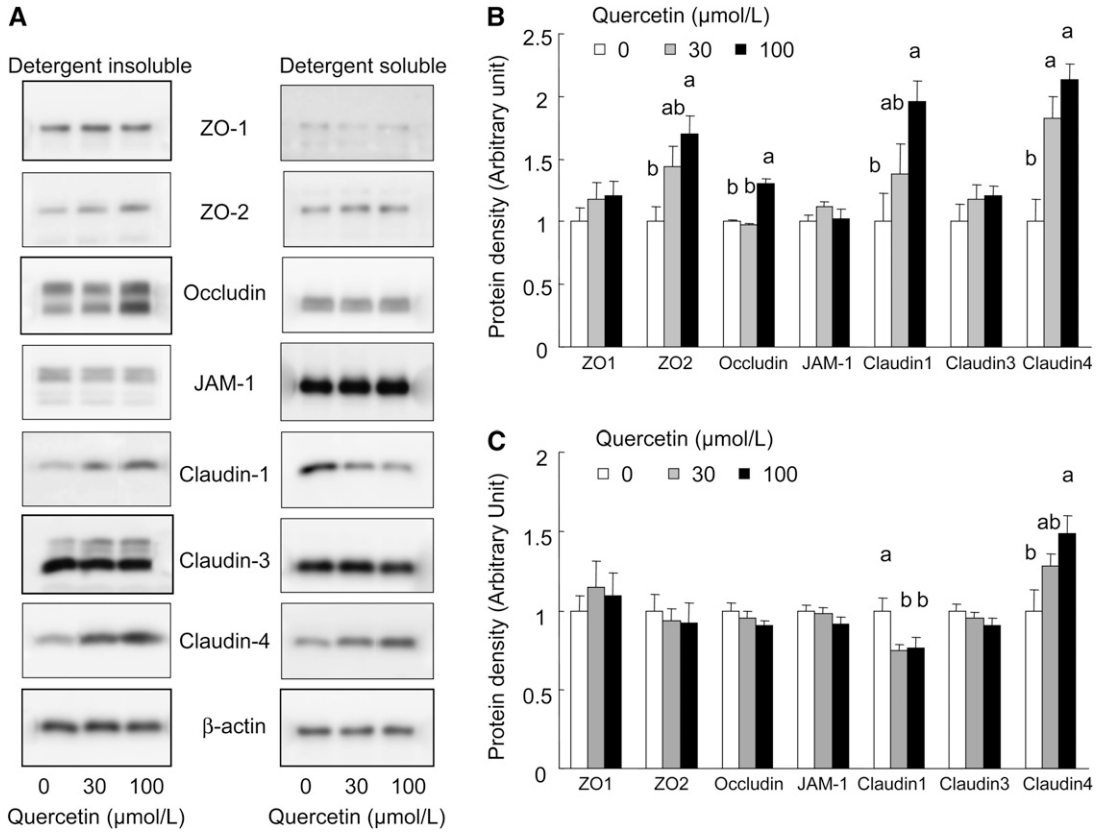

FIGURE 2 Immunoblot analysis of TJ proteins in the detergent-insoluble and -soluble fractions of cells incubated with or without quercetin. Caco-2 cell monolayers were incubated with or without 30 or 100 μmol/L quercetin for 48 h. Detergent-insoluble and -soluble fractions were prepared and immunoblotted for ZO-1, ZO-2, occludin, JAM-1, claudin-1, claudin-3, claudin-4, and β-actin. The immunoblot was representative of 4 monolayers (*A*). Each specific band of proteins was quantitated by densitometric analysis (*B*, detergent insoluble; *C*, detergent soluble). The density values were normalized to the value for the corresponding control monolayer incubated without quercetin. Values are means ± SEM, $n = 4$. Labeled means without a common letter differ, $P < 0.05$.

marker, was injected into the basolateral wells at 45 h and the flux into the apical wells was assessed for 3 h. The paracellular selectivity and conductance are the same in the mucosal and serosal directions (23). The concentration of LY in the apical solution was determined by measuring fluorescence (FP-550; JASCO International). Whole cell extracts and detergent-insoluble and -soluble fractions were prepared for immunoblot analysis of TJ proteins after incubation for 48 h as described below.

To investigate the periodical effect of quercetin on intestinal barrier function, TER was measured before and at 0.5, 1, 3, 6, 12, 24, and 48 h after the administration of quercetin (100 μmol/L) to the cell monolayer. The LY flux was assessed for 3 h to 6 and 48 h. Whole cell extracts and detergent-insoluble fraction were prepared at 0, 1, 3, 6, 12, 24, and 48 h for subsequent immunoblot analysis. The cell viability was examined at 6 and 48 h using a commercially available kit (Cell counting kit-8; Dojindo).

The effect of 3 signaling inhibitors [0–1 μmol/L Ro-31–8425 (an inhibitor of PKCα/βI/βII/γ/ε), 0–10 μmol/L rottlerin (an inhibitor of PKCδ), and 0–10 μmol/L LY294002 (an inhibitor of PI3K)] on intestinal barrier function was also examined to explore the intracellular signaling molecules involved in the flavonoid-mediated effect. The inhibitors were administrated into the apical and basolateral wells and the TER at 0, 24, and 48 h and the LY flux for the last 3 h were measured as described above. Whole cell extracts and detergent-insoluble fractions were prepared after incubation for 48 h.

Preparation of detergent-insoluble and -soluble fractions and whole cell extracts. Detergent-insoluble and -soluble fractions were prepared as described previously (24) and the former corresponds to the proteins associated with the actin cytoskeleton. Caco-2 cell monolayers incubated with flavonoids or signaling inhibitors were washed with ice-cold PBS and incubated for 5 min at 4°C with 200 μL of lysis buffer-CS [1% Triton X-100, 5 mmol/L EGTA in 50 mmol/L Tris containing protease inhibitors (5 mg/L aprotinin, 3 mg/L leupeptin hemisulfate, 5 mmol/L benzamidine hydrochloride, and 1 mmol/L phenylmethylsulfonyl fluoride) and phosphatase inhibitors (2 mmol/L sodium orthovanadate and 10 mmol/L sodium fluoride), pH 7.4]. Cell lysates were centrifuged at 15,600 × *g*; 5 min at 4°C to sediment the high-density actin-rich fraction. The pellet was suspended in 100 μL of RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mmol/L NaCl, 1 mmol/L EGTA, and 1 mmol/L EDTA in 25 mmol/L Tris containing protease and phosphatase inhibitors as described above, pH 7.5). For preparation of the whole cell extract, 200 μL of RIPA buffer was used after washing cell monolayers with ice-cold PBS. Protein concentrations in the different fractions were measured using the BCA method (Pierce Biotechnology). The fractions were mixed with a one-half volume of Laemmli sample buffer [3× concentrated; 6% (wt: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] (25) and heated at 100°C for 5 min.

Immunoblot analysis. Proteins (20 μg) were separated by SDS-PAGE (12%) and transferred to polyvinylidene difluoride membranes. Membranes were blotted for ZO-1, ZO-2, occludin, JAM-1, claudin-1, claudin-3, claudin-4, phospho-PKCδ, PKCδ, and β-actin using specific antibodies in combination with HRP-conjugated anti-mouse IgG or anti-rabbit IgG antibodies. The level of phospho-PKCδ shows the intracellular kinase activities of PKCδ (26). The blots were developed using the enhanced chemiluminescence method (GE Healthcare). Quantification was performed by densitometric analysis of specific bands on the immunoblots using Image J software.

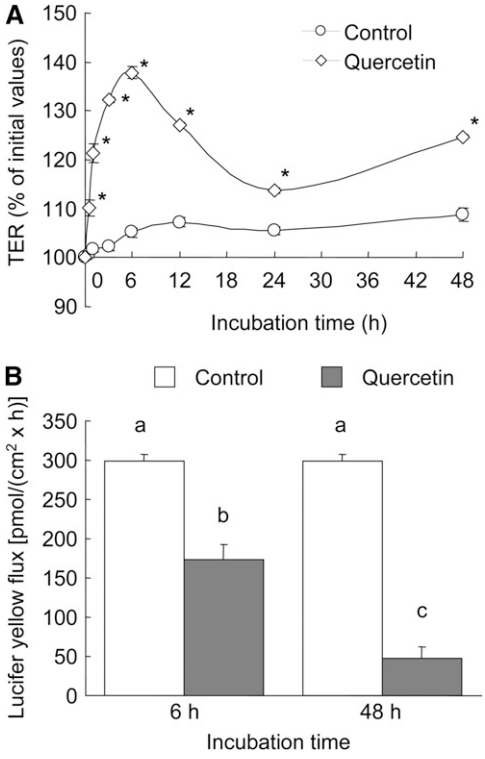

FIGURE 3 TER and LY flux in Caco-2 cells incubated with or without quercetin. Caco-2 cell monolayers were incubated with or without 100 μmol/L quercetin. TER (*A*) was measured at 0, 0.5, 1, 3, 6, 12, 24, and 48 h after the start of incubation (*A*) and LY flux (*B*) was measured for 3 h to 6 and 48 h. Values are means ± SEM, $n = 4$. A: *Different from control at that time, $P < 0.05$. In *B*, means without a common letter differ, $P < 0.05$.

Immunofluorescence microscopy. Cell monolayers incubated with 100 μmol/L quercetin for 6 and 48 h were washed with PBS, fixed in acetone:methanol (1:1) at 0°C for 5 min, and permeabilized with 0.2% Triton-X100 for 5 min. Cell monolayers were blocked in 3% nonfat milk in 20 mmol/L Tris, 150 mmol/L NaCl, 0.5% Tween 20, pH 8.0, and incubated for 1 h with primary antibodies; rabbit polyclonal anti-claudin-1 and mouse monoclonal anti-claudin-4, followed by incubation for 1 h with secondary antibodies, goat AlexaFluor 488-conjugated anti-rabbit IgG and AlexaFluor 546-conjugated anti-mouse IgG antibodies. The fluorescence was visualized using a Zeiss LSM 410 Laser Scanning Confocal microscope (Carl Zeiss) and images from Z-series section (1 μm) were collected.

PKCδ kinase assay. PKCδ kinase assay was performed using a commercially available kit (PKC Kinase Activity Assay kit; Assay Designs) and purified recombinant active PKCδ enzyme (Biomol International) according to the manufacturer's protocol. To examine the inhibitory effect of quercetin on PKCδ isoform activity, 10 ng of purified PKCδ enzyme was preincubated with various concentrations of quercetin (0–100 μmol/L) for 15 min on ice prior to the reactions.

Statistical analysis. All values are expressed as means ± SEM. The TER is expressed as percent of initial values. Unidirectional LY flux is expressed as pmol/cm^2 of surface area. Statistical analyses were performed by a repeated measure 2-way ANOVA or 1-way ANOVA followed by Tukey-Kramer multiple range test. A difference with $P < 0.05$ was considered significant. Statistical analyses were performed using the general linear models procedure of the SAS program (version 6.07; SAS Institute).

Results

Effect of quercetin and myricetin on LY flux and TER. The LY flux was decreased in Caco-2 cells incubated with 10, 30, and 100 μmol/L quercetin in a dose-dependent manner (**Fig. 1***A*). The LY flux in monolayers incubated with 100 μmol/L quercetin was approximately one-fourth of that in control monolayers. TER was dose-dependently higher in the cells incubated with quercetin at 24 and 48 h (Fig. 1*B*). The LY flux was dose-dependently lower in the cell monolayers incubated with myricetin, although the values did not differ between monolayers incubated with 10 and 30 μmol/L myricetin (Fig. 1*C*). TER did not differ among monolayers incubated with myricetin at all time points (Fig. 1*D*). The suppressive effect of quercetin on LY flux was significantly more potent than that of myricetin at a concentration of 100 μmol/L. LY flux was lower in the cells incubated with 100 μmol/L quercetin than in the control cells or those treated with 100 μmol/L myricetin. The reductions in the flux induced by treatment with quercetin and myricetin were 70–80% and 30–40%, respectively, of the values obtained in the absence of those flavonoids.

The absorbance indicating cell viability was higher at 6 h in the cells incubated with 100 μmol/L quercetin (0.36 ± 0.03) than that with control treatment (0.31 ± 0.03) ($P < 0.05$, $n = 3$). The values did not differ at 48 h.

Effect of quercetin and myricetin on TJ protein distributions in detergent-insoluble and -soluble fractions. Quercetin increased the protein levels of ZO-2, occludin, claudin-1, and claudin-4 in the detergent-insoluble fraction of cells 48 h after incubation, indicating an enhancement of their binding to the actin cytoskeleton (**Fig. 2***A*). Densitometric analysis showed that levels of ZO-2, occludin, claudin-1, and claudin-4 in the detergent-insoluble fraction were higher in the cell monolayers incubated with 100 μmol/L quercetin than in the control cells (Fig. 2*B*). Occludin appeared as double bands in the immunoblots with the detergent-insoluble fractions; upper and lower bands correspond to hyper- and non-/less phosphorylated forms (27). The hyper-phosphorylated form in the detergent insoluble fractions was enhanced with 100 μmol/L quercetin treatment. Claudin-4 levels were higher in the detergent-insoluble fraction of cells treated with 30 and 100 μmol/L quercetin than in the control cells and were higher in the soluble fractions of cells treated with 100 μmol/L quercetin (Fig. 2). Claudin-1 was lower in the detergent-soluble fraction of cells treated with 30 and 100 μmol/L quercetin (Fig. 2*C*). ZO-1, ZO-2, occludin, JAM-1, and claudin-3 levels did not differ in the detergent-soluble fraction.

TJ protein levels did not differ in the detergent-insoluble and -soluble fractions on incubation with myricetin for 48 h (data not shown). The amounts of ZO-1 and JAM-1 in the detergent-insoluble fraction were slightly, but not significantly, higher after treatment with 100 μmol/L myricetin.

Effect of quercetin and myricetin on TJ protein expression. Expression of TJ proteins was evaluated by immunoblot analysis using whole cell extracts after incubation with flavonoids for 48 h. Densitometric analysis revealed that claudin-4 expression was enhanced by quercetin in a dose-dependent manner (control: 1.00 ± 0.11; 30 μmol/L quercetin: 1.27 ± 0.02; 100 μmol/L quercetin: 1.58 ± 0.12). The claudin-4 level was higher in the whole cell extract treated with 100 μmol/L quercetin than in that of the control cells. The other TJ proteins (ZO-1, ZO-2, occludin, JAM-1, claudin-1, and claudin-3) did not differ between the cells incubated with or without quercetin (data not shown). Myricetin treatment did not alter the expression of any TJ proteins and protein levels did not differ between the cells incubated with or without myricetin (data not shown).

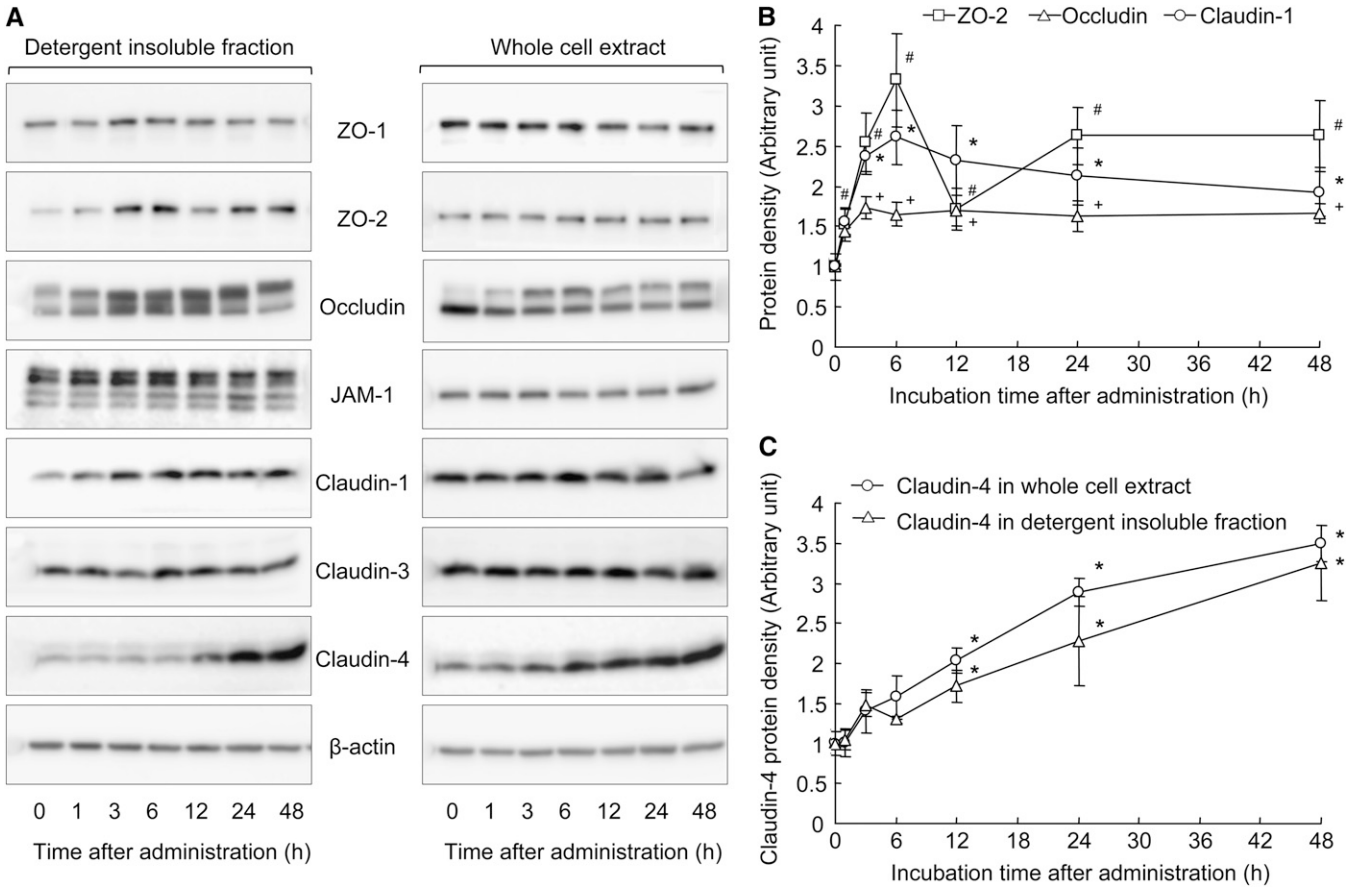

FIGURE 4 Immunoblot analysis of TJ proteins in the detergent-insoluble fraction and whole cell extract of cells incubated with or without quercetin. Caco-2 cell monolayers were incubated with or without 100 μmol/L quercetin. The detergent-insoluble fractions and whole cell extracts were prepared before and at 1, 3, 6, 12, 24, and 48 h after the start of incubation and immunoblotted for ZO-1, ZO-2, occludin, JAM-1, claudin-1, claudin-3, claudin-4, and β-actin. The immunoblot was representative of 4 monolayers (*A*). Specific bands of ZO-2, occludin, and claudin-1 in the detergent-insoluble fractions (*B*) and claudin-4 in the detergent-insoluble fractions and whole cell extracts (*C*) were quantitated by densitometric analysis. The density values were normalized to the value before incubation. Values are means ± SEM, $n = 4$. Symbols indicate different from time 0, $P < 0.05$.

Biphasic effect of quercetin on intestinal barrier function. The TER across the cells had a biphasic increase after the administration of 100 μmol/L quercetin (**Fig. 3***A*). The TER value in the presence of quercetin rapidly increased to 138% of the initial value during first 6 h. The value then gradually decreased to 114% at 24 h before increasing again to 125% at 48 h. In the control monolayers, the TER values remained between 100 and 109% of the initial value through the incubation period. The TER values in the cells incubated with quercetin were higher than the control values at all time points. The suppressive effect of quercetin on LY flux was found in a time-dependent manner (Fig. 3*B*). The LY flux across the cells incubated with quercetin was lower than each control value at 6 and 48 h and the effect was more potent at 48 h than at 6 h.

The protein levels of ZO-2, occludin, and claudin-1 rapidly increased over the first 6 h in the detergent-insoluble fraction and remained elevated throughout the incubation period, except for ZO-2, which transiently decreased at 12 h (**Fig. 4***A*,*B*). The ZO-2 levels at 1, 3, 6, 12, 24, and 48 h and the occludin and claudin-1 levels at 3, 6, 12, 24, and 48 h were higher than the initial value in the detergent-insoluble fraction. The claudin-4 protein levels gradually increased up to 48 h in both the detergent-insoluble fraction and whole cells treated with quercetin and were significantly higher at 12, 24, and 48 h than at 0 h in both fractions (Fig. 4*A*,*C*). No significant increases in other TJ proteins, ZO-1, JAM-1, and claudin-3, were found in the detergent-insoluble fraction and whole cell extracts during the incubation period (data by the densitometric analysis not shown).

The higher immunofluorescence intensity of claudin-1 at 6 h and claudin-4 at 48 h was observed at junctional region of cell monolayers incubated with 100 μmol/L quercetin compared with the control monolayers in the confocal images (**Fig. 5**).

Effect of signaling inhibitors on LY flux and TER. Inhibition of PKCδ and PI3K, but not PKCα/βI/βII/γ/ε, enhanced intestinal barrier function, indicated by reduced LY flux and increased TER (**Fig. 6**). The LY flux was lower in cells incubated with 3 and 10 μmol/L rottlerin, a specific PKCδ inhibitor, than in control cells (Fig. 6*A*). The TER in cells incubated with 3 and 10 μmol/L rottlerin was higher than the control value at 24 and 48 h after the start of incubation; however, the TER value in the cells treated with 3 μmol/L were higher than that in the cells treated with 10 μmol/L (Fig. 6*D*). Treatment of cells with a PI3K inhibitor, LY294002, decreased the LY flux in a dose-dependent manner and the flux was lower in the cells incubated with 3 and 10 μmol/L LY294002 than in the control cells (Fig. 6*B*). The TER in the cells incubated with 3 and 10 μmol/L LY294002 was higher than that in the control at 24 and 48 h (Fig. 6*E*). Increasing the dose of LY294002 resulted in a decrease in TER. LY flux and TER did

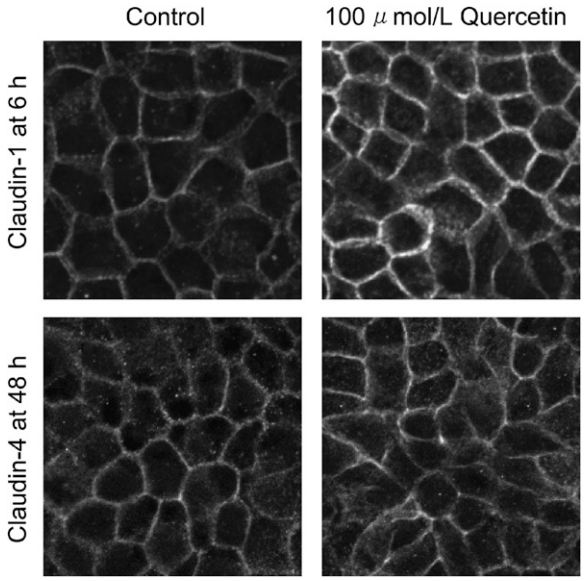

FIGURE 5 Immunolocalization of claudin-1 and 4 in Caco-2 cells incubated with or without quercetin. Caco-2 cell monolayers were incubated with or without 100 μmol/L quercetin, fixed, and stained for claudin-1 at 6 h and claudin-4 at 48 h using an immunofluorescence method. Images were collected by confocal microscopy.

not differ at any time points in the cells treated with or without Ro-31–8425, a PKCα/βI/βII/γ/ε inhibitor (Fig. 6*C*,*F*).

Effect of PKCδ inhibition on TJ protein levels in detergent-insoluble fractions and whole cell extracts. ZO-2, occludin, claudin-1, and claudin-4 levels in the detergent-insoluble fractions increased in the cells incubated with rottlerin, a PKCδ inhibitor (**Fig. 7***A*,*B*). The level of ZO-2 in this fraction increased with increases in rottlerin in a dose-dependent manner and the level in cells incubated with 10 μmol/L rottlerin was higher than in the control cells. Claudin-1 and claudin-4 levels in the detergent-insoluble fraction of cells treated with 3 and 10 μmol/L rottlerin were both higher than the respective control values and the increments induced by lower and higher doses of rottlerin were similar. The occludin level was slightly, but significantly, higher in the detergent-insoluble fraction of cells incubated with 3 and 10 μmol/L rottlerin than in the control cells. The rottlerin treatment increased the intensity of upper bands corresponding to hyperphosphorylated occludin in the detergent-insoluble fraction. Claudin-4 levels in the whole cell extracts increased with increases in rottlerin in a dose-dependent manner and the level in cells incubated with 10 μmol/L rottlerin was higher than that in the control cells (Fig. 7*A*,*C*). No significant increases were observed in the other TJ proteins in the whole cell extracts.

Effect of PI3K inhibition on TJ protein levels in detergent-insoluble fractions and whole cell extracts. A higher dose of LY294002, an inhibitor of PI3K, increased occludin levels in the detergent-insoluble fraction of cells (control: 1.00 $\pm$ 0.07; 10 μmol/L LY294002: 1.45 $\pm$ 0.04; $P < 0.05$; $n = 4$). ZO-1, ZO-2, JAM-1, claudin-1, claudin-3, and claudin-4 did not differ in the detergent-insoluble fraction or in any TJ proteins in the whole cell extracts (data not shown).

Effect of quercetin on phosphorylation of PKCδ in Caco-2 cell monolayers. Phosphorylation of PKCδ on Ser 643, but not total PKCδ level, gradually decreased in the cells incubated with 100 μmol/L quercetin in the first 24 h and remained decreased throughout the incubation period (**Fig. 8***A*). The densitometric analysis showed that the ratio of phosphorylated:total PKCδ was lower at all time points after 1 h than the initial value and the values at 24 and 48 h were ~60% of the initial value (Fig. 8*B*).

Inhibitory effect of quercetin on PKCδ activity in vitro. Quercetin showed a dose-dependent inhibitory effect on PKCδ isoform activity in vitro (**Fig. 9**). PKCδ activity was 76.4, 55.3, 38.4, and 6.0% of the control value in the presence of 0.1, 1.6, 12.5, and 100.0 μmol/L quercetin, respectively. The quercetin concentration required for 50% inhibition of PKCδ was calculated to be 3.7 μmol/L.

Discussion

This study demonstrates that 2 flavonoids, quercetin and myricetin, enhance barrier function in Caco-2 cell monolayers, which are widely used as a model of the intestinal epithelium. The enhancing effect of quercetin, but not that of myricetin, results from the promotion of the assembly of TJ proteins, claudin-1, occludin, and ZO-2, and the expression of claudin-4 through the inhibition of a novel PKCδ isoform. Quercetin and myricetin are well-known antioxidants. However, the promotive effect of these flavonoids on the barrier function do not seem to be involved in their antioxidant activity, because quercetin has a more potent effect than that of myricetin. Myricetin has higher antioxidant activity than quercetin due to another hydroxyl group on the B-ring (2).

In the early part of this study, we examined the effects of quercetin and myricetin on barrier function in Caco-2 cells incubated for 48 h. Treatment of cells with quercetin increased claudin-1, claudin-4, occludin, and ZO-2 levels in the detergent-insoluble fraction, indicating that the association of these proteins with the actin cytoskeleton and their assemblies at the TJ are promoted by quercetin. These results suggest that the assemblies of these 4 proteins at the TJ are responsible for the quercetin-mediated promotion of intestinal barrier function. Levels of TJ proteins bound to the actin cytoskeleton correlate with the permeability of TJ in the epithelium (28). The promotive effect of myricetin on intestinal barrier function was weaker than that of quercetin, as shown by the higher LY flux. Treatment of cells with myricetin did not change the TJ protein levels in the detergent-insoluble/-soluble fractions and whole cell extracts, although myricetin induced a decrease in LY flux without any changes in TER. The mechanism(s) underlying the myricetin-mediated decrease in LY flux should be considered in future studies.

We investigated periodical changes in intestinal barrier function on incubation with quercetin for 48 h. The TER across the cells began to increase within 0.5 h of the quercetin administration. The TER rapidly and markedly increased over the first 6 h and then gradually decreased before increasing again at 24 h, although the suppressive effect of quercetin on LY flux was time dependent. Our results show that the increases in TER for the initial 6 h and after 24 h occur through 2 distinct mechanisms. The protein levels of ZO-2, occludin, and claudin-1 in the detergent-insoluble fractions rapidly increased during the first 6 h after the quercetin administration, although claudin-4 did not increase in the detergent-insoluble fractions or the whole cell extracts before or at 6 h. On the other hand, the claudin-4 protein level in the detergent-insoluble fractions and whole cell

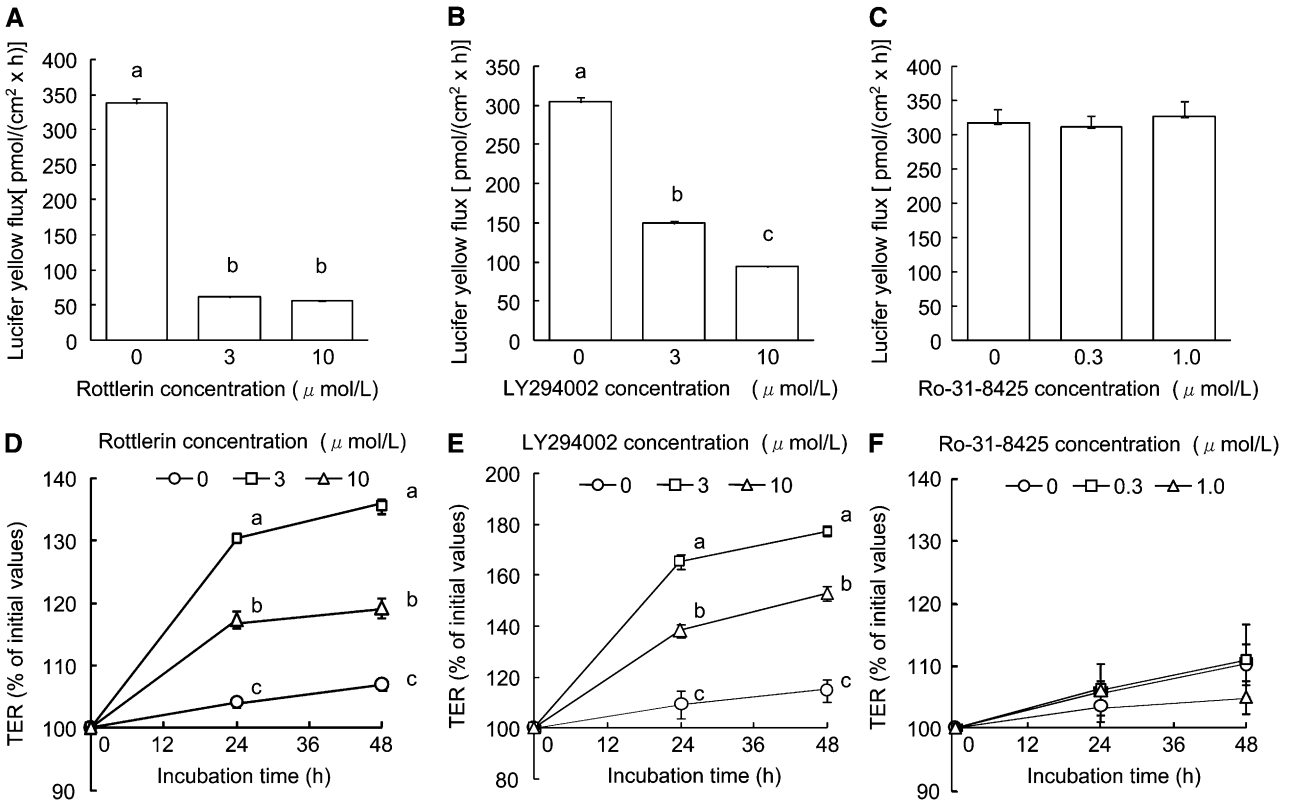

FIGURE 6 LY flux and TER in Caco-2 cells incubated with or without rottlerin (a PKCδ inhibitor), LY294002 (a PI3K inhibitor), or Ro-31–8425 (a PKCα/βI/βII/γ/ε inhibitor). Caco-2 cell monolayers were incubated with or without rottlerin [3 or 10 μmol/L, (*A,D*)], LY294002 [3 or 10 μmol/L, (*B,E*)], or Ro-31–8425 [0.3 or 1 μmol/L, (*C,F*)] for 48 h. LY flux (*A–C*), a paracellular marker, was measured for the last 3 h of incubation. TER (*D–F*) was measured before and at 24 and 48 h after the administration of the inhibitors. Values are means ± SEM, $n = 4$. Labeled means at a time without a common letter differ, $P < 0.05$.

extracts continuously increased after 12 h, although the levels of ZO-2, occludin, and claudin-1 in the detergent-insoluble fractions remained increased from 24 to 48 h. Confocal microscopy demonstrates that quercetin enhances the distributions of claudin-1 at 6 h and claudin-4 at 48 h in the intercellular junctions. These results reveal that the assemblies of ZO-2, occludin, and claudin-1 are responsible for the quercetin-mediated increase in TER and decrease in LY flux in the early phase and the expression of claudin-4 has an additional role in the increased TER and decreased LY flux in the later phase.

The quercetin-mediated assemblies of ZO-2, occludin, and claudin-1 occurred without any concomitant change in their expression levels in the whole cells. Previous reports have shown that the phosphorylation states of occludin (27), claudins (29), and ZO-2 (30) play an important role in their assemblies at the TJ. Occludin has been shown to undergo dephosphorylation on Ser/Thr residues during the disruption of TJ by oxidative stress (24) and calcium depletion (27) and we have reported that the phosphorylation of Thr residues is required for the assembly of occludin at the TJ (31). Quercetin seems to enhance phosphorylation of occludin, which appears as the upper bands in the immunoblot with the detergent-insoluble fraction. Fujibe et al. (29) have reported that claudin-1 in the detergent-insoluble fraction is mainly phosphorylated in mouse F9 embyonal cells. According to these evidences, quercetin is likely to influence the phosphorylation status of ZO-2, occludin, and claudin-1, but not claudin-4, and acts to induce their assemblies.

The level of claudin-4 protein was higher in the whole cell extracts as well as in the detergent-insoluble fraction of cells incubated with quercetin for 48 h, indicating that quercetin promotes claudin-4 expression at a transcription level. Amesheh et al. (18) have shown that quercetin promotes transcriptional expression of claudin-4 using the reporter gene assay, although the mechanism has not been clarified. It has been reported that 2 binding sites of SP1, a transcriptional factor, were identified in the promoter region of claudin-4 and that the sites were required for promoter activity (32). Furthermore, Pan et al. (33) demonstrated that the quercetin-induced expression of plasminogen activator-1 is mediated through SP1 in endothelial cells. It is, therefore, possible that quercetin enhances claudin-4 expression through SP1 activation in Caco-2 cells.

To explore the intracellular signaling mechanism(s) underlying the flavonoid-mediated promotive effect on barrier function, the effects of 2 PKC inhibitors (Ro-31–8425 and rottlerin) and a PI3K inhibitor (LY294002) on intestinal barrier function were examined. Some flavonoids, including quercetin and myricetin, are known to inhibit PKC and PI3K activity in vitro (5), 2 signaling molecules that are well known to be associated with the regulation of epithelial barrier function (15–17,22). Rottlerin, a specific inhibitor of PKCδ isoform, and LY294002 significantly enhance intestinal barrier function, indicated by increases in TER and reductions in LY flux, although it is not clear why their effects on TER were higher with lower doses than with higher doses. The TER values depend on the paracellular ion flux such as sodium and chloride, which may be increased with the high doses of these

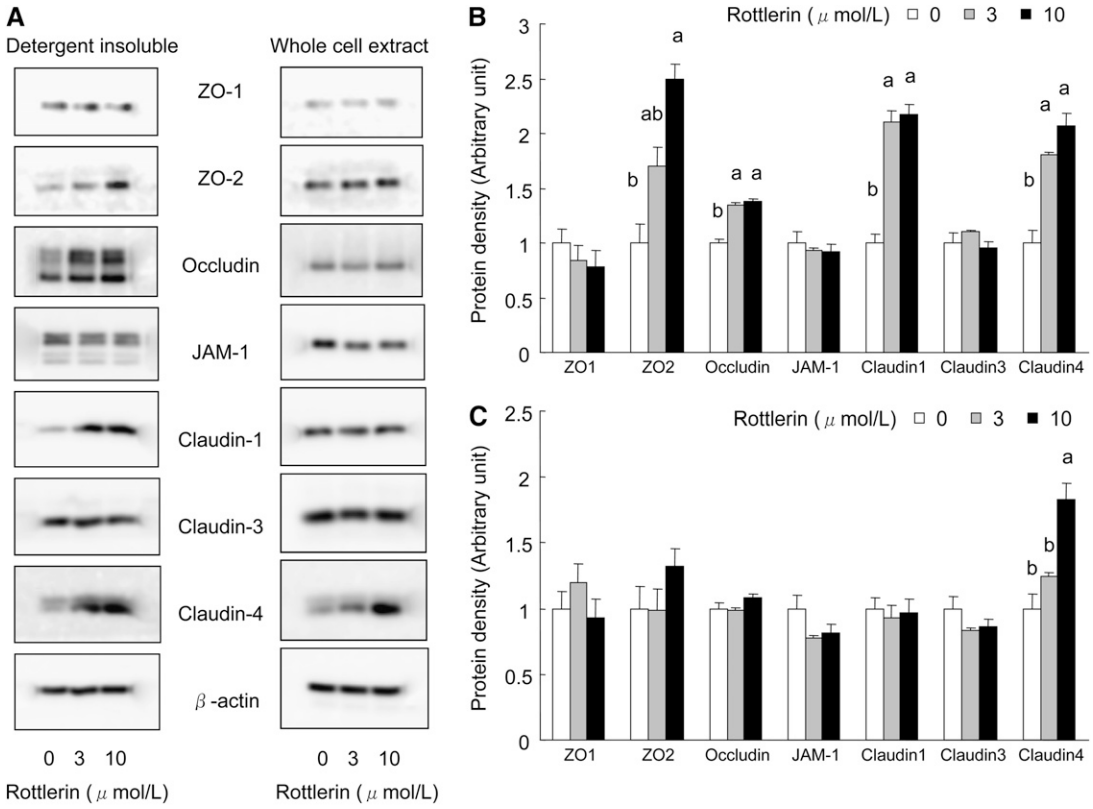

FIGURE 7 Immunoblot analysis of TJ proteins in the detergent-insoluble fraction and whole cell extract of cells incubated with or without rottlerin, a PKCδ inhibitor. Caco-2 cell monolayers were incubated with or without 3 or 10 μmol/L rottlerin for 48 h. Detergent-insoluble fractions and whole cell extracts were prepared and immunoblotted for ZO-1, ZO-2, occludin, JAM-1, claudin-1, claudin-3, claudin-4, or β-actin. The immunoblot was representative of 4 monolayers (*A*). Each specific protein band was quantitated by densitometric analysis (*B*, detergent-insoluble fraction and *C*, whole cell extract). The density values were normalized to the value for the corresponding control monolayer incubated without the inhibitor. Values are means ± SEM, $n = 4$. Labeled means without a common letter differ, $P < 0.05$.

inhibitors with side effects. On the contrary, Ro-31–8425, which has a broad specificity to PKCα, βI, βII, γ, and ε isoforms, did not affect barrier function. Additionally, rottlerin has very similar effects to quercetin on the distribution and expression of TJ proteins, i.e. inducing increases in ZO-2, occludin, claudin-1, and claudin-4 levels in the detergent-insoluble fractions as well as an increase in the total claudin-4 level. LY294002 exclusively increases occludin in the detergent-insoluble fraction, which is distinct from the effects of the 2 flavonoids. We examined the effect of quercetin on the PKCδ activity in both the cells and cell-free system, because we speculated that the suppression of PKCδ activity is involved in the promotive effect of quercetin on the barrier function. In Caco-2 cells, the phosphorylation of PKCδ on Ser643, an autophosphorylation site, began to decrease within 1 h of the quercetin administration, which indicates that quercetin quickly suppresses PKCδ activity in the cells. The quercetin concentration required for a 50% inhibition of PKCδ was calculated to be 3.7 μmol/L in our kinase assay. We calculated that the concentration is obtainable in the intracellular compartment of cells incubated with 100 μmol/L quercetin on the basis of a previous report (34). This is the first report, to our knowledge, showing the direct inhibition of PKCδ isoform by quercetin, although quercetin reportedly inhibits isoform-mixed PKC enzymes purified from bovine brains (5). In conjunction with the fact that the inhibition of PKCδ by rottlerin promotes the assembly and expression of TJ proteins similarly to quercetin, these data suggest that the quercetin-induced promotive effect on intestinal barrier function is mediated through the inhibition of PKCδ activity in Caco-2 cells. The activity of some PKC isoforms has a role in phosphorylation of TJ proteins, especially occludin, and regulate the assembly (22,31). The inhibition of PKCδ may lead to the activation of other PKC isoforms, followed by the alteration of occludin phosphorylation; however, further investigations are required to identify the downstream signaling molecule(s).

In this study, quercetin aglycone was administrated to the apical side of Caco-2 cells and exhibited a dose-dependent effect in the range of up to 100 μmol/L. Orally ingested quercetin is extensively metabolized to glucuronated, sulfated, and methylated quercetin conjugates in the intestinal cells before its entry into blood (35); however, the unabsorbed majority of quercetin is intact until degradation by large intestinal microbes. Unmetabolized quercetin is predominantly found in the stomach, small intestine, cecum, and colon of rats fed quercetin (35). The daily intake of flavonols, including quercetin and myricetin, has been estimated at 20–35 mg/d (36). A luminal quercetin concentration of >30 μmol/L, which activates the intestinal barrier function, can be expected with an intake of 10 mg quercetin in a meal. Meanwhile, a previous study indicated that a luminal quercetin concentration of up to 100 μmol/L can be achieved by a daily oral intake of 100–250 mg quercetin in humans (37). Therefore, not only the use of a quercetin supplement but also the intake from regular meals might provide us with the protective and ameliorative effects on the gastrointestinal diseases associated with intestinal permeability.

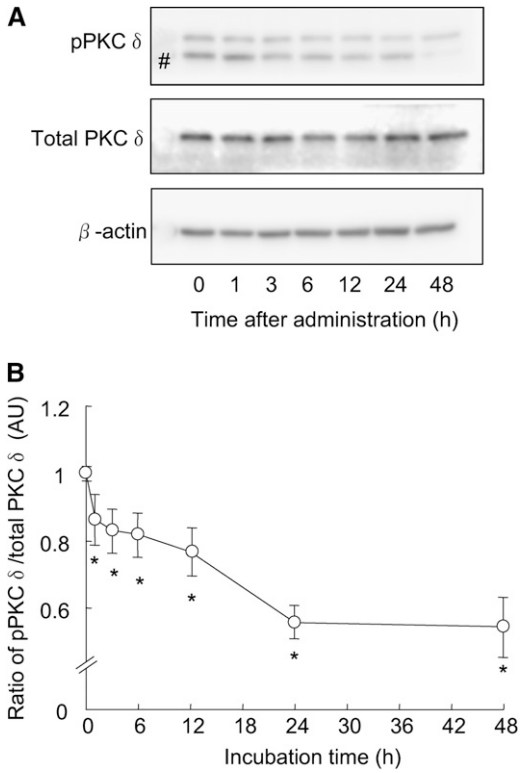

FIGURE 8 Immunoblot analysis of phospho- and total PKCδ in Caco-2 cells incubated with or without quercetin. Caco-2 cell monolayers were incubated with or without 100 μmol/L quercetin. The whole cell extracts were prepared before and at 1, 3, 6, 12, 24, and 48 h after the start of incubation and immunoblotted for phospho-PKCδ (Ser 643), total PKCδ, and β-actin. The immunoblot was representative of 4 monolayers (*A*). Pound signs indicate unknown bands (60 kDa) that appeared below the bands of phospho-PKCδ. Each specific band of phospho- and total PKCδ was quantitated by densitometric analysis and the ratio of phosphorylated:total PKCδ was calculated (*B*). The ratio was normalized to the value before incubation. Values are means ± SEM, $n = 4$. *Different from time 0, $P < 0.05$.

In summary, this study demonstrated that 2 flavonoids, quercetin and myricetin, enhance barrier function in human intestinal Caco-2 cells. Quercetin promotes the assembly of TJ proteins, ZO-2, occludin, and claudin-1 and the expression of claudin-4 by inhibiting the PKCδ isoform.

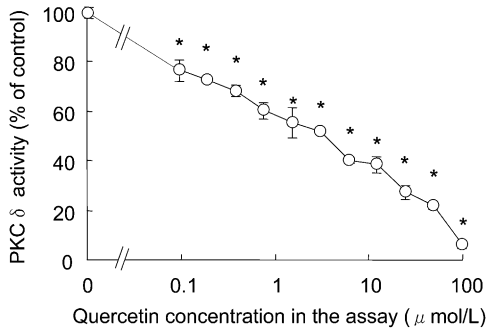

FIGURE 9 The activity of recombinant PKCδ enzyme in the absence or presence of quercetin. The recombinant PKCδ enzyme was incubated with the PKC substrate in the absence or presence of quercetin (0.1–100 μmol/L). The activity values were normalized to the control value obtained from recombinant PKCδ enzyme incubated with the PKC substrate without quercetin. Values are means ± SEM, $n = 3$. *Different from time 0, $P < 0.05$.

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