The Effect of Prim-O-Glucosylcimifugin on Tryptase-Induced Intestinal Barrier Dysfunction in Caco-2 Cells

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The intestinal barrier dysfunction is a critical pathological change in irritable bowel syndrome (IBS). The objective of this study was to evaluate the effect of Prim-O-glucosylcimifugin (POG) on intestinal barrier dysfunction and reveal possible molecular mechanisms. Human colon adenocarcinoma cell line (Caco-2) cell monolayers induced by tryptase (TRYP) were used to establish an intestinal barrier dysfunction model. Caco-2 cell monolayers from both functional and dysfunctional samples were treated with POG (30, 60 and 120 µg/mL) for 2, 8, 24, 36, 48 and 72h. The Caco-2 cell monolayers were assessed by measurement of trans-epithelial electrical resistance (TEER) and percentage of fluorescein permeation (PFP). The expression of Protease Activated Receptor 2 (PAR-2) and myosin light chain kinase (MLCK) mRNA was analyzed by RT-PCR and the level of Zonula Occludens-1 (ZO-1) protein expression was determined by Western blot. In addition, the impact of POG on the distribution of the tight junction protein of Occludin was performed by immunofluorescence. Our results showed that POG elevated the TEER and decreased the PFP of the functional Caco-2 cell monolayers, as well as the dysfunctional Caco-2 cell monolayers. Furthermore, POG inhibited the expression of PAR-2 mRNA and MLCK mRNA and increased the level of ZO-1 protein expression in dysfunctional Caco-2 cells. The distribution of the Occludin proteins was ameliorated simultaneously. This study demonstrates that POG can enhance the intestinal barrier function of Caco-2 cell monolayers by inhibiting the expression of PAR-2 and MLCK and up-regulating the expression of ZO-1 protein, and ameliorated the distribution of Occludin protein.

Key words Prim-O-glucosylcimifugin (POG); intestinal barrier; irritable bowel syndrome; barrier dysfunction; human colon adenocarcinoma cell line (Caco-2) cell
purpose, human colon adenocarcinoma cell line (Caco-2) cell monolayers of both functional and dysfunctional were treated with POG, at doses of 30, 60 and 120 \( \mu \)g/mL. The trans-epithelial electrical resistance (TEER) and percentage of fluorescein permeation (PFP) were measured at 2, 8, 24, 36, 48, 72h after treatment POG. Additionally, the PAR-2 and MLCK mRNA expression levels in dysfunction Caco-2 cell monolayers were analyzed by RT-PCR, and the level of ZO-1 protein expression was measured by Western blot. Simultaneously, the expression and distribution of the Occludin protein were examined using immunofluorescence.

**MATERIALS AND METHODS**

**Materials** Dulbecco’s modified Eagle medium (DMEM) and phosphate buffered saline (PBS) were obtained from Gibco (U.S.A.). Fetal bovine serum (FBS) and TRIzol Reagent were purchased from Invitrogen (U.S.A.). PAR-2 and MLCK PCR primers were synthesized by Sangon Biotech Co., Ltd. ZO-1, Occludin and \( \beta \)-actin antibodies were obtained from Abcam Inc. (U.K.). Tryptase, FSSRY-NH \(_2\) and fluorescein were purchased from Sigma (U.S.A.). PrimerScript 1st-Strand cDNA Synthesis Kit and Primer Taq were purchased from TaKaRa (Japan). The total protein assay kit was obtained from Nanjing Jiancheng Bioengineering Institute (China). MEM Non-Essential Amino Acids (NEAA) Solution was purchased from Thermo Scientific (U.S.A.). Prim-O-glucosylcimifugin (98% pure) were supplied by Sichuan Weikeqi Biological Technology Co., Ltd. (China).

**Cell Line and Culture Conditions** Caco-2 was obtained from the American Type Culture Collection (HTB037). Caco-2 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% NEAA at 37°C in an atmosphere with 95% air and 5% CO\(_2\) and utilized for experimentation at approximately 70–80% confluence. And the Caco-2 cell monolayers were formed after 20–22d-cultures, until the TEER value was more than 300 \( \Omega \) cm\(^2\).

**Cell Viability** The POG toxicity was determined using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) (Sigma) assay. Caco-2 cells were seeded into 96-well plates at a density of 10000/cell, with each well containing 200 \( \mu \)L. After adherence, the cells were treated with various concentrations of POG (10, 50, 100, 250, 500, 1000, 5000 and 10000\( \mu \)g/mL) or dimethyl sulfoxide (DMSO, Sigma; control cells) for 24h before 10 \( \mu \)L MTT in medium was added to each well. The cells were incubated at 37°C for 4h. Then the absorbance was detected at 490nm using a microtiter plate reader (Bioteck Instruments).

**Culture of Caco-2 Cell Monolayers and Determination of Barrier Function** The Caco-2 cell monolayers were cultured, and the determination of barrier function was performed as previously described.\(^{25}\) Briefly, Caco-2 cells were transplanted on the apical compartment of collagen-coated polycarbonate membrane Transwell supports (transparent polyethylene terephthalate (PET) membrane: 1.0-cm\(^2\) growth surface area, 0.4-\( \mu \)m pore size) in 24-well plates at 1\( \times \)10\(^5\) cells/well. The apical and basolateral compartment respectively are 100 and 600\( \mu \)M medium and incubated with appropriate culture conditions. The Caco-2 cell monolayers were grown on the Transwell, and the medium was replaced every 48h. Recordings of TEER values were obtained every 24h until they stabilized on 20–22d using an Epithelial Voltometer (World Precision Instruments) in each insert and multiplied by the membrane surface area, corrected by subtracting background resistance of the blank membrane (no cells). A 20–22-d model was used to study barrier function. It has been shown that after day 20–22 the cell monolayers were differentiated. The TEER values of the model Caco-2 cell monolayers was measured at 2, 8, 24, 36, 48 and 72h after treatment with POG (30, 60 and 120\( \mu \)g/mL). The same to the measurement of PFP values, 40\( \mu \)g/mL fluorescein (dioxfluor- oran, C\(_{20}\)H\(_{12}\)O\(_{5}\)) was added to the apical compartment, and the Transwells were incubated for 1h. Then, 100\( \mu \)L of medium from the apical and basal compartments was collected into 96-well plates, and the fluorescein was measured using a microplate reader at excitation and emission wavelengths of 485 and 540nm, respectively. The monolayer permeability of fluorescein was quantified as the PFP from the apical to the basal compartment.

The dysfunctional Caco-2 cell monolayers from the intestinal barrier were induced by TRYP (Sigma) as previously described.\(^{26}\) Briefly, the model Caco-2 cell monolayers were incubated with TRYP (1.3 \( \mu \)mol/L) from the basal side for 72h. To assess the effect of POG on intestinal barrier function, dysfunctional Caco-2 cell monolayers were incubated with POG (30, 60 and 120\( \mu \)g/mL). The TEER and the PFP of the cell monolayers were determined as previously described.

**RT-PCR Analysis of PAR-2 and MLCK mRNA Expression** The total RNA from the Caco-2 cells was extracted using the TRIzol reagent dissolved in DEPC-treated water according to the manufacturer’s instructions. RNA was reverse-transcribed to cDNA using oligo (dT) primers by PrimerScript 1st-Strand cDNA Synthesis Kit in a final volume of 20\( \mu \)L under the conditions recommended by the supplier (TaKaRa). For PCR amplification of PAR-2 and \( \beta \)-actin, 1\( \mu \)L of the cDNA template and the following specific primers were used: PAR-2F, 5’-CCC TTT GTA TGT C GTG AAA GCA GAC-3’; R, 5’-TTC CGG TGT TTT TTT GAG GTG-3’ (452bp); \( \beta \)-actin F, 5’-TTG TAA CCA ACT GGG ACG ATA TGG-3’; R, 5’-GAT CTT GAT CTT CAT GGT GCT AGG-3’ (754bp). The reaction cycle conditions were the following: 5min at 95°C and 35 amplification cycles consisting of denaturation at 95°C for 40s, annealing at 60°C for 40s, and extension at 72°C for 60s. And for MLCK and \( \beta \)-actin, 1\( \mu \)L of cDNA template and the following specific primers were used: MLCK F, 5’-TCT GAA CTC ACA ACG GTA GG-3’; R, 5’-GCT CAA AGT CCT GGTCAA-3’ (376bp); \( \beta \)-actin F, 5’-TCA CCA CTG TGC CCA TCT ACG A-3’; R, 5’-CAG CCG AAC CGC TCA TTG CCA ATG G-3’ (300bp). The reaction cycle condi-
tions: 5 min at 94°C and 30 amplification cycles consisting of denaturation at 94°C for 30 s, annealing at 54°C for 45 s, and extension at 72°C for 60 s.

The PCR products were resolved via 2% agarose gel electrophoresis and visualized through ethidium bromide staining. The PCR products of predicted size were stained using ethidium bromide and visualized by an UV transilluminator. Quantification of each band was performed using a Scion Image densitometry analysis software package.

**Western Blot Analysis of Expression of ZO-1** Briefly, the cells were washed with pre-cooled PBS and subsequently lysed with RIPA lysis buffer. The protein concentration was determined through the bicinchoninic acid (BCA) assay reagent with the Bradford method. For Western blotting, protein samples (20 µg) of Caco-2 cells were separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were then transferred onto polyvinylidene difluoride (PVDF) membrane and incubated with primary antibody (anti-ZO-1 with dilution: 1:500 or anti-β-actin with dilution: 1:1200) overnight at 4°C and then with the secondary antibody (horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG)) for 2h. The intensity of the immunoblot signal was determined by the ECL Western blotting detection reagent according to the manufacturer’s instructions and the expression of ZO-1 was analyzed quantitatively using the GeneTools software from Syngene.

**Immunofluorescence Analysis of the Distribution of Occludin Proteins** The distribution occludin protein was analyzed with immunofluorescence microscopy. Cells (3×10⁶ cells/well) in 6-well plates were fixed with 4% paraformaldehyde at room temperature for 30 min after POG treatment for 36 h. Then cells were permeabilized with PBS containing 0.1% Triton and then blocked with blocking buffer containing 0.3% bovine serum albumin (BSA) for 30 min at room temperature. Cells were incubated with Occludin antibody (1:1000) overnight at 4 degree and then washed with PBS three times for 15 min and incubated with fluorescein isothiocyanate (FITC) marker Goat anti rabbit IgG (1:100) at room temperature for another 1 h. Observe and record the result as soon as possible with fluorescence microscopy at the condition of excitation wavelength 492 nm and emission wavelength 520 nm.

**Statistical Analysis** All experimental data has been presented as the mean±standard deviation (S.D.), and the analyses of the significance of the differences were performed via one-way ANOVA followed by Student–Newman–Keuls test (S-N-K) using SPSS 17.0. Differences were considered significant if p<0.05.

**RESULTS**

The POG toxicity was illustrated in Fig. 2. The viability of Caco-2 cell incubated with POG (250, 500, 1000, 5000 and 10000 µg/mL) for 24h was decreased compared with the control cells (p<0.05). In the range of POG concentration 10–250 µg/mL, the viability of Caco-2 cell was above 80%.

The TEER value of the normal Caco-2 cell monolayers that were incubated with POG (30, 60 and 120 µg/mL) for 8, 24, 36 and 48 h was significantly elevated compared with the control cells (p<0.05), contrarily to the PFP (Figs. 3A, B). It can be seen in Figs. 4A, B that the TEER and PFP value of the
dyfunctional Caco-2 cell monolayers induced by TRYP was obviously decreased and increased respectively compared with the control cells \((p<0.05)\). And POG \((30, 60 \text{ and } 120 \mu g/mL)\) for 2, 8, 24, 36, and 48 h significantly increased the TEER and decreased PFP compared with the dysfunctional cell monolayers without treatment with POG \((p<0.05)\). Our data showed that the addition of varying doses of POG produced a dose-dependent increase in the TEER and a decrease in the PFP on both functional and dysfunctional cell monolayers, suggesting that POG can promote the barrier function of Caco-2 cell monolayers in a dose-dependent manner.

As shown in Fig. 5, the PAR-2 mRNA expression of the dysfunctional Caco-2 cell monolayers induced by TRYP was much more than the control cells \((p<0.05)\). POG \((30, 60 \text{ and } 120 \mu g/mL)\) reduced the expression of PAR-2 mRNA compared with the dysfunctional cell monolayers \((p<0.05)\). FSSRY-NH\(_2\), which is the PAR-2 antagonist and was used as a positive control drug, distinctly affected the inhibition of the
The Caco-2 cells were incubated with POG (30, 60 and 120 µg/mL) for 36h. The protein expression of ZO-1 was detected via Western blotting as described in the text. The figure represents one of four experiments with similar results. Control: control group; Tryptase: 1.3 µmol/L tryptase; POG-30: 30 µg/mL POG and 1.3 µmol/L tryptase; D: 60 µg/mL POG and 1.3 µmol/L tryptase; E: 120 µg/mL POG and 1.3 µmol/L tryptase. #p<0.05 compared with the control cells; *p<0.05 compared with the cells induced with tryptase.

The MLCK mRNA expression of the dysfunctional Caco-2 cell monolayers induced by TRYP was increased compared with the control cells (p<0.05) in Fig. 6. After treatment with POG (30, 60 and 120 µg/mL), the MLCK mRNA expression was decreased compared with the dysfunctional cell monolayers (p<0.05).

The ZO-1 protein expression in the dysfunctional Caco-2 cell monolayers induced by TRYP was decreased compared with the control cells (p<0.05). After treatment with POG (30, 60 and 120 µg/mL), the ZO-1 protein expression was significantly elevated compared with the dysfunctional cell monolayers (p<0.05). FSSRY-NH2, applied as a positive control drug, significantly increased the expression of ZO-1 protein compared with the dysfunctional cell monolayers (p<0.05) (Fig. 7).

In Fig. 8, there was no significant difference in fluorescence intensity between each group. However, in the dysfunctional Caco-2 cell monolayers induced by TRYP, the occludin protein was loose, apparently serrated, or notched and fissured (Fig. 8B). Apparently, in POG (30, 60 and 120 µg/mL) treatment groups, the green fluorescence distribution showed obvious integrity and clear boundary. And the higher the dose, the more obvious the effect.

DISCUSSION

This study demonstrated that POG can promote intestinal barrier function and ameliorate intestinal barrier dysfunction in Caco-2 cells. These effects are possibly mediated through down-regulating the expression of PAR-2 and MLCK mRNA and enhance the level of ZO-1 protein expression, ameliorate the distribution of Occludin protein.

The intestinal barrier is a crucial component of gut homeostasis that defends against many pathogens, such as bacteria, toxins and food antigens. Caco-2 cells, which exhibit a well-differentiated brush border, tight junctions and intestinal proteins, have been widely used to investigate the drug’s effect on paracellular permeability and as an intestinal barrier model in vitro. In the present study, our results demonstrated that POG can increase the TEER and reduce the PFP in Caco-2 cells, which shows that POG promoted epithelial barrier function in a dose-dependent manner (Fig. 3). In agreement with our present study, Elamin et al. used Caco-2 cells as an intestinal barrier model and found that short-chain fatty acids (SCFAs) can reinforce the barrier function in Caco-2 cells by measuring the TEER and paracellular permeability.

Clinical data suggest that a high level of TRYP released by mast cells upon degranulation in D-IBS, is associated with alteration in intestinal barrier function. TRYp increases the permeability of colon tissue in diarrhea-predominant IBS patients. A study conducted by Ludidi et al. revealed that TRYp can increase the permeation flux of 4-kDa fluorescein isothiocyanate-labelled dextran (FD4) in Caco-2 cells, indicating an effect of enhancing paracellular permeability and intestinal barrier dysfunction. In our study, we induced Caco-2 cell monolayers with TRYp, and this treatment reduced the TEER and increased the PFP. After treatment with POG, the TEER obviously increased, and the PFP decreased compared
with the values obtained in the TRYP-induced intestinal barrier dysfunction Caco-2 cells (Fig. 4). The current evidence of the effect of POG on the intestinal barrier is limited. Two similar studies conducted showed that the active ingredients of SD affect the intestinal permeability and decrease the level of nitric oxide (NO) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. 33–35.

The intestinal barrier dysfunction in IBS is associated with the disturbance of gut sensory and motor functions. PAR-2 activation is responsible for intestinal barrier dysfunction and the sensitization of sensory neurons in patients with IBS. 34–36 PAR-2 belongs to the PARs family and is distributed throughout the gastrointestinal tract, localized in epithelial cells, myocytes and enteric neurons. 37–38 The activation of PAR-2 on the intestinal epithelial barrier directly affects cytoskeletal contraction by triggering relevant paracellular pathways with subsequent changes in tight junction permeability, PAR-2 is activated by serine-proteases, such as trypsin and tryptase, which in turn brings great impact on epithelial migration, integrity, and barrier function. 39–40 A study conducted by Piche et al. demonstrated that Caco-2 cells exposed to tryptase in the plasma of IBS-D patients elevates the expression of PAR-2 mRNA, leading to an increase in paracellular permeability and a reduction in TEER. 41 In the present study, the results showed that the level of PAR-2 mRNA significantly elevated in dysfunctional Caco-2 cells and POG would down-regulated the expression of PAR-2 mRNA (Fig. 5).

The activation of MLCK is vital to the enhancement of paracellular permeability and is essential for tight junction barrier regulation in response to Na+-nutrient cotransport, inflammatory cytokines, or pathogenic bacteria. 38,39 MLCK can change the junctional actomyosin ring structure and tight junction morphology. 40–41 The integrity of tight junctions depends on many tight junction proteins, which make up the normal intestinal architecture. ZO-1 and occludin proteins are essential for cytoskeletally-mediated barrier regulation, 42–43 Disruption and redistribution of the ZO-1 and occludin proteins could lead to tight junction barrier dysfunction. These changes in ZO-1 and occludin proteins are the result of the activation of MLCK. 44 In the dysfunctional Caco-2 cells, our data suggested that MLCK mRNA expression was significantly increased and ZO-1 protein expression was remarkably reduced. Although the expression level of occludin did not decrease obviously, the distribution of occludin protein changed remarkable. After treatment with POG, the expression of MLCK mRNA were down-regulated and the level of ZO-1 were enhanced. Beyond that, the POG would ameliorate the distribution of Occludin proteins (Figs. 6–8).

In conclusion, our research shows that POG has potential therapeutic efficacy in the treatment of IBS. Down-regulation of PAR-2 and MLCK expression, up-regulation of ZO-1 protein expression and ameliorated the distribution of Occludin proteins may be the possible molecular mechanism. Therefore, the beneficial effects of POG on intestinal barrier dysfunction suggest that POG may be a promising therapeutic drug for some diseases associated with intestinal barrier dysfunction, such as IBS.

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Conflict of Interest The authors declare no conflict of interest.

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