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The Mycotoxin Patulin Decreases Expression of Density-Enhanced Phosphatase-1 by Down-Regulating PPARγ in Human Colon Cancer Cells

Akihiro Katsuyama,[1] Tomomi Konno,[1] Shuji Shimoyama[2] and Hideaki Kikuchi[1,2]

[1]Department of Biochemistry and Molecular Biology, Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki, Aomori, Japan
[2]Science of Bioresources, The United Graduate School of Agricultural Sciences, Iwate University, Morioka, Iwate, Japan

Patulin is a mycotoxin that is found mainly in apple products and causes symptoms such as bleeding from the digestive tract and diarrhea. Efforts to elucidate the mechanism of its toxicity have focused on protein tyrosine phosphatases (PTPs), which regulate the function of tight junctions (TJs) in colon epithelial cells. Patulin reacts with the conserved cysteine residues in the catalytic domains of PTP isoforms. Treatment of Caco-2 human colon cancer cells, used as a colon epithelial model, with 50 μM patulin decreased the level of density-enhanced phosphatase-1 (DEP-1) protein to 30% of the control level after 6 h. The level of *DEP-1* mRNA was also decreased during 24 h after treatment with patulin. Moreover, knockdown of DEP-1 increased the level of phosphorylated claudin-4. Destruction of TJs by patulin treatment was observed by immunostaining with an antibody against zonula occludens (ZO)-1. To better understand the mechanistic basis of the decrease in *DEP-1* mRNA levels, we searched for a *cis*-element upstream of the *DEP-1* gene and found an element responsive to the peroxisome proliferator-activated receptor gamma (PPARγ) protein. Using a PPARγ-specific antibody, we showed a decrease in PPARγ abundance to 42% of the control level within 6 h after treatment with patulin. PPARγ has four cysteine residues that are involved in zinc finger formation. Our data suggest that DEP-1 affects TJ function and that PPARγ might control *DEP-1* expression. Therefore, the toxicity of patulin to cellular functions might be attributable to its ability to down-regulate the expression of DEP-1 and PPARγ.

Keywords: claudin-4; density-enhanced phosphatase-1; patulin; peroxisome proliferator-activated receptor gamma; tight junction
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Introduction

Patulin is a secondary metabolite produced by several fungi that belong to the genera *Penicillium*, *Aspergillus*, *Paecilomyces*, and *Byssochlamys* (Gonzalez-Mariscal et al. 2000). Contamination of food with patulin causes gastrointestinal inflammation, ulcers, and bleeding (McKinley et al. 1982). The mutagenicity (Pfeiffer et al. 1998; de Melo et al. 2012), embryotoxicity (Roll et al. 1990; Wu et al. 2005), and carcinogenicity (Dickens and Jones 1961) of patulin have also been reported. In addition, it is highly reactive towards the thiol groups of proteins and glutathione (Puel et al. 2010). Symptoms of acute toxicity caused by patulin are bleeding from the digestive tract, diarrhea, and brain edema. The acute toxicity of patulin is thought to result from the destruction of tight junctions (TJs) in gastrointestinal tissue (Mahfoud et al. 2002). Epithelial cells form barriers such as TJs to block the entry of toxic compounds or bacteria from outside of digestive tract tissues.

TJs are cell-cell contacts that form in the epithelial cells of the intestine, pulmonary alveolus, and testes. They regulate the paracellular pathway and limit the transport of small hydrophilic molecules and ions via this route (Van Itallie and Anderson 2006). TJs also act as a barrier in epithelial cells; specifically, they inhibit the transfer of cell membrane protein between the apical and the basal sides of cells to produce cell polarity (Cereijido et al. 2008). TJs comprise intracellular scaffold proteins that belong to the zonula occludens (ZO) family and transmembrane proteins that are occludin, JAM and the claudins (Balda and Matter 2008).

The ZO family comprises three members (ZO-1, -2, and -3) that belong to the membrane-associated guanylate kinase (GUK) superfamily. ZO-1 contains different types

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Correspondence: Hideaki Kikuchi, Department of Biochemistry and Molecular Biology, Faculty of Agriculture and Life Science, Hirosaki University, 3 Bunkyo-cho, Hirosaki, Aomori 036-8561, Japan.
e-mail: hkikuchi@cc.hirosaki-u.ac.jp

of protein-protein interaction domains such as PDZ, GUK and C-terminal domains. It interacts with occludin through its GUK domain, with claudins through its PDZ domain, and with actin through its C-terminal domain (Cereijido et al. 2008). Moreover, ZO-1 has context-dependent interactions with TJs and binds directly to F-actin and other cytoskeletal proteins; this suggests that ZO-1 regulates cytoskeletal activity at cell junctions in polarized epithelial cells (Fanning et al. 2012). We previously revealed that patulin treatment increased tyrosine phosphorylation of ZO-1 and reduced its abundance in Caco-2 human colon cancer cells (Kawauchiya et al. 2011).

The claudin family comprises 24 members, and has four transmembrane domains with both the C- and N-termini located in the cytoplasm. The phosphorylation of a tyrosine residue (Tyr-208) at the C-terminal end of claudin-4 attenuates its interaction with ZO-1, and Tyr-208 of claudin-4 is phosphorylated by the protein tyrosine kinase EphA2 (Tanaka et al. 2005). The tyrosine phosphorylation of claudin-4 attenuates the association of claudin-4 with ZO-1, decreasing the integration of claudin-4 into sites of cell-cell contact and enhancing paracellular permeability (Tanaka et al. 2005). Destruction of the barrier function of TJs by the inhibition of phosphatase increases the paracellular permeability of small molecules or ions. The function of TJs is usually determined by measuring the electrical resistance between the apical side and the basal side of the epithelial cell layer. Indeed, treatment with patulin decreases the level of transepithelial electrical resistance (TER) in Caco-2 human colon cancer cells (Kawauchiya et al. 2011).

Density-enhanced phosphatase-1 (DEP-1) is a kind of protein tyrosine phosphatase (PTP) that is also known as CD148 and PTP-RJ. DEP-1 has diverse roles in signal transduction; it regulates migration, proliferation, and differentiation as well as cell adhesion (Balavenkatraman et al. 2006; Petermann et al. 2011). DEP-1 dephosphorylates tyrosine residues of ZO-1 and occludin in MCF10A human mammary epithelial cells, and its knockdown increases cell permeability at the TJs (Sallee and Burridge 2009).

Given the reactivity of patulin with the thiol groups of the aforementioned proteins, and the presence of cysteine residues in the active sites of PTPs (Bohmer et al. 2013), we investigated the effects of patulin and the knockdown of DEP-1 on TJs in Caco-2 human colon cancer cells. Our analysis revealed the PTPs targeted by patulin and the molecular mechanisms behind the patulin-induced disruption of TJs. We demonstrated the involvement of peroxisome proliferator-activated receptor gamma (PPARγ) in controlling *DEP-1* expression.

Materials and Methods

Chemicals and cell culture

Patulin was purchased from Enzo Life Sciences International (Plymouth Meeting, PA). Caco-2 human colon cancer cells, purchased from the American Type Culture Collection (ATTC; Manassas, VA), were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 0.1 U/l penicillin, 0.1 g/l streptomycin, and 5% fetal bovine serum, in a 5% CO_2/95% air incubator at 37°C and 100% humidity. The cells were passaged with 0.25% trypsin and 0.02% EDTA in phosphate buffered saline (PBS) after reaching confluence, or the medium was changed twice weekly in instances where growth was not confluent after three days. Patulin was used at doses in the nontoxic range (50 μM) in terms of cell viability. The effect of patulin on TJs has been shown in a previous report (Kawauchiya et al. 2011) using 50 μM patulin; therefore, we used this concentration throughout this study. Troglitazone was purchased from Cayman Chemical (Michigan, USA) and used at a concentration of 50 μM.

Preparation of siRNA

For the RNA interference (RNAi) experiments, siRNA duplexes were synthesized using a Silencer siRNA Construction Kit (Ambion Inc., Austin, TX, USA) in accordance with the manufacturer's protocol. The siRNA against DEP-1 was described previously (Petermann et al. 2011). The siRNA against claudin-4 was designed using siDirect (http://sidirect2.rnai.jp/, antisense: 5′-AAUACUUGGCGGAGUAAGGCU-3′; sense: 5′-CCUUACUCCGCCAAGUAUUCU-3′). As a negative control of knockdown, siRNA against green fluorescence protein (GFP) was used as a transfection control (target RNA sequence: GGCUACGUCCAGGAGCGCACC).

Immunofluorescence microscopy

The Caco-2 cells were seeded on cover glasses at a density of 2 × 10^5 cells/12-well plate and cultured for 2 d at 37°C. The siDEP-1 was then transfected to the cells using Lipofectamine™ RNAiMAX (Invitrogen Corporation, Carlsbad, CA, USA), to give a final concentration of 20 nM. After transfection, the cells were incubated for 72 h. For the patulin treatment, the Caco-2 cells were seeded on cover glasses at a density of 2 × 10^5 cells/12-well plate, and cultured for 4 d at 37°C. Patulin was added to the cells at a final concentration of 50 μM, and the cells were then incubated for 24 h.

For immunostaining with the ZO-1 antibody (kindly provided by Professor S. Tsukita, Osaka University), the cells were fixed with 0.1% formaldehyde/calcium- and magnesium-free phosphate-buffered saline [PBS(−)] for 25 min at room temperature. Then, they were permeabilized with 0.2% TritonX-100/PBS(−) for 40 min. The cover glasses were then washed three times with 0.05% Tween-20/PBS(−) (T-PBS) and the cells were incubated at room temperature for 3 h in the dark with the secondary antibody (Alexa Fluor 488 conjugated to rabbit anti-mouse IgG; Invitrogen Co., Carlsbad, CA) that had been diluted 250-fold with 1% BSA/PBS(−). The cells were washed three times with T-PBS and stained for 30 min in the dark with Hoechst 33258 (1.6 μg/ml). After washing with T-PBS three times, the cells on the cover glasses were embedded with PermaFluor™ Aqueous Mounting Medium (Funakoshi Co., Ltd., Tokyo, Japan).

For immunostaining with claudin-4 antibody (Invitrogen Co., Carlsbad, CA), the cells were fixed and permeabilized with 100% ethanol at room temperature for 20 min. After blocking with 1% BSA/PBS(−) for 30 min at room temperature, the cells were incubated with mouse anti-claudin-4 antibody diluted 100-fold with 1% BSA/PBS(−) at 4°C overnight. The cover glasses were then washed with T-PBS three times and the cells were incubated at room temperature for 3 h in the dark with the secondary antibody (Alexa Fluor 488 conjugated to rabbit anti-mouse IgG; Invitrogen Co., Carlsbad, CA) diluted 250-fold with 1% BSA/PBS(−). The cells were washed three

times with T-PBS and stained for 30 min in the dark with Hoechst 33258 (1.6 μg/ml). After washing with T-PBS three times, the cells on the cover glasses were embedded with PermaFluor™ Aqueous Mounting Medium.

Images were captured using a confocal laser scanning microscope (FV1000D; Olympus, Tokyo, Japan).

Western blot analysis

The siRNAs were transfected to Caco-2 cells (2×10^5 cells/3.5-cm dish, cultured for 2 d) to give a final concentration of 20 nM, and the cells were incubated for 72 h. For the patulin treatment, patulin was added to the Caco-2 cells (2×10^5 cells/3.5-cm dish, cultured for 4 d) to give a final concentration of 50 μM, and incubated for 24 h.

The cells were washed with PBS(−) twice, scraped from the bottom of the dish, and centrifuged for 5 min at 4°C and 800 g. The pellets were sonicated using a Vibra-Cell (Sonics & Materials, Inc., Newtown, CT) instrument, followed by the addition of lysis buffer [1% Nonidet® P-40, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1× Protease Inhibitor Cocktail (Roche Diagnostics GmbH, Mannheim, Germany)]. The lysates were centrifuged for 5 min at 4°C and 14,000 g. The supernatants were diluted using 5× sample buffer [40% glycerol, 10% SDS, 4 M 2-mercaptoethanol, 250 mM Tris-HCl (pH 6.8), 0.02% bromophenol blue], and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and western blot analysis. Mouse anti-DEP-1 antibody (Santa Cruz Biotechnology Inc., California), mouse anti-claudin-4 antibody, mouse anti-ZO-1 antibody, or rabbit anti-PPARγ antibody (Santa Cruz Biotechnology Inc.) was used as the primary antibody. Appropriate secondary antibodies [rabbit polyclonal anti-mouse IgG/horseradish peroxidase (HRP) (Dako, Denmark) or goat polyclonal anti-rabbit IgG/HRP (Dako)] were also used.

Quantitative PCR (qPCR)

For the patulin treatment, Caco-2 cells were seeded at a density of 2×10^5 cells/6-cm dish, and cultured for 4 d. Patulin was added to give a final concentration of 50 μM. For the troglitazone treatment, Caco-2 cells were seeded at a density of 2×10^5 cells/6-cm dish, and cultured for 4 d. Troglitazone, a ligand for PPARγ, was added to give a final concentration of 50 μM to test a possibility that the increase of DEP-1 mRNA is PPARγ dependent.

The cDNA was synthesized using M-MuLV reverse transcriptase (Fermentas, Hanover, MD). The qPCR was performed in a reaction mixture that contained THUNDERBIRD™ SYBR qPCR Mix (Toyobo, Osaka, Japan). The amplification was performed in a LightCycler® nano (Roche Diagnostics GmbH, Mannheim, Germany), where the reaction mixture was heated to 95°C for 10 min, and then underwent 50 cycles of denaturing at 95°C for 5 s, annealing at 55°C for 10 s, and elongation at 72°C for 20 s. The primer set for qPCR of *DEP-1* was as described previously (Balavenkatraman et al. 2006), and the primers used to amplify GAPDH cDNA were as follows: forward: 5′-CATCACCATCTTCCAGGAGC-3′; reverse: 5′-GGATGA TGTTCTGGAGAGCC-3′. GAPDH cDNA was amplified in each sample as an internal control for all the qPCR reactions.

Immunoprecipitation

For the knockdown of DEP-1, the Caco-2 cells (2×10^5 cells/3.5-cm dish, cultured for 2 d) were transfected with siRNA described above to give a final concentration of 20 nM. After the transfection, Caco-2 cells were incubated for 72 h. For the patulin treatment, patulin was added to the Caco-2 cells (2×10^5 cells/3.5-cm dish, cultured for 4 d) to give a final concentration of 50 μM, and the cells were incubated for 24 h.

The cells were washed with PBS(−) twice, then scraped into PBS(−), and centrifuged for 5 min at 4°C and 800 g. The pellets were suspended in the phosphatase inhibitory buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 1 mM Na_3VO_4, 0.1 mM NaF, 1 mM dithiothreitol, 10% glycerol, 0.5% Triton-100, 0.1% Tween®-20, 1× Protease Inhibitor Cocktail], followed by sonication. The lysates were centrifuged for 5 min at 4°C and 14,000 g and used for immunoprecipitation. For the crosslinking between the antibody and Dynabeads® Protein G (Invitrogen), 1 μg of antibody [mouse anti-phosphotyrosine PY20 (BD Biosciences, San Jose, CA, USA) or normal mouse IgG (Santa Cruz Biotechnology Inc.)] was incubated with 3 μl of Dynabeads® Protein G at 4°C overnight, and reacted with 20 mM dimethyl pimelimidate dihydrochloride (DMP) (Sigma-Aldrich)/ethanolamine for 30 min at room temperature, followed by washing with 0.1% T-PBS twice. The whole-cell lysates (1 mg of protein) were then incubated with the antibody-Dynabeads® Protein G complex. After washing with the phosphatase inhibitory buffer three times, adsorbed proteins were eluted using 0.1 M glycine/HCl (pH 2.8). The supernatant was suspended in 5× sample buffer and subjected to SDS-PAGE and western blot analysis. As a primary antibody, mouse anti-claudin-4 antibody was used. As a secondary antibody, rabbit polyclonal anti-mouse IgG/HRP was used.

Measurement of TER

For the transfection with siRNA, Caco-2 cells were seeded on Transwell polycarbonate cell culture inserts with a mean pore size of 0.4 μm (#665641, Greiner Bio-One GmbH, Frickenhausen, Germany) at 5×10^4 cells/well, and cultured for 4 d. Each siRNA was then introduced to the apical side of the insert, to give a final concentration of 20 nM. After the transfection, the cells were incubated for 24 h, and the medium was changed to DMEM. For patulin treatment, patulin was added to the cells (5×10^4 cells/well, cultured for 5 d) on the apical side to give a final concentration of 50 μM. TER was monitored using a Millicell®-ERS (Electrical Resistance System) instrument (Millipore Co., Bedford, MA). TER was normalized with the area of the monolayer, and the background TER of a blank filter was subtracted from the TER of the cell monolayer.

FITC-dextran permeability assay

For the transfection with siRNA, Caco-2 cells were seeded on Transwell polycarbonate cell culture inserts at 5×10^4 cells/well, and cultured for 4 d. Each siRNA was then introduced to the apical side, to give a final concentration of 20 nM. After the transfection, the cells were incubated for 24 h and the medium was changed to Phenol-red-free DMEM (Sigma-Aldrich). For the patulin treatment, the cells were seeded at 5×10^4 cells/well and cultured for 5 d. On the day before the experiment, the medium was changed to Phenol-red-free DMEM, with incubation for 24 h. Patulin was added to the apical side to give a final concentration of 50 μM. Four-kDa FITC-dextran (Sigma-Aldrich) was also added at a final concentration of 350 μg/ml. At each time point, 50 μl of medium was withdrawn from the basal side of the insert and its fluorescence was measured using Fluoskan Ascent FL (Labsystems, Helsinki, Finland).

Statistical analysis

Numerical values from individual experiments were pooled and

are expressed throughout as the mean ± standard deviation (S.D.) of the mean. Single set comparison was performed by Student's *t*-test. A p value of < 0.05 was considered significant.

Results

Effect of patulin on DEP-1

Patulin reacts with sulfhydryl groups of proteins and PTPs that have a cysteine residue in their active sites (Wang et al. 2003); therefore, we investigated the effect of patulin on DEP-1, a member of the PTP family, by western blot analysis and qPCR. Following treatment with patulin, the abundance of DEP-1 decreased by 67% and 66% after 6 h and 24 h, respectively (Fig. 1A and B). The level of *DEP-1* mRNA relative to that of *GAPDH* mRNA decreased in a time-dependent fashion until 24 h (Fig. 1C). The DEP-1 protein level remained unchanged between 6 and 24 h (Fig. 1B), despite the continuous decrease in its mRNA level (Fig. 1C).

Effects of patulin or knockdown of DEP-1 on the epithelial barrier

To estimate the effects of patulin or DEP-1 knockdown on the epithelial barrier function, we measured both the TER and the permeability to FITC-dextran. The transfection with each siRNA was carried out 24 h before the measurement. Patulin was added at the start point of the measurement (Fig. 2A). The expression level of DEP-1 was decreased by transfection with siDEP-1 (Fig. 2B). Compared with the effect of siGFP (control), the value of TER was decreased by 90% at 36 h after patulin treatment. The knockdown of DEP-1 also decreased the level of TER by only 10% at 36 h after the start of measurement compared with that of siGFP (Fig. 2C). The knockdown effect of claudin-4 on TER was similar to that of DEP-1 knockdown at 36 h (Fig. 2C). Permeability to FITC-dextran increased significantly (5- and 7.4-fold after 24 and 36 h, respectively, $p < 0.05$) after the patulin treatment, compared with that of the siGFP (control). However, no substantial difference in permeability was observed following transfection with either siDEP-1 or siGFP (Fig. 2D).

We applied immunofluorescence microscopy to observe whether patulin or the knockdown of DEP-1 affects the subcellular location of TJ proteins. Patulin treatment, but not transfection with siDEP-1, altered the localization of ZO-1 (Fig. 3A). In contrast, the localization of claudin-4 was not affected by the addition of patulin or transfection with siDEP-1 (Fig. 3B). Protein-protein interactions in TJs are regulated by phosphorylation of their proteins. Therefore, we tried to elucidate the levels of phosphorylation in TJ proteins to clarify the mechanism of destruction of TJs in the next section.

Effect of DEP-1 on phosphorylation of TJ proteins

Sallee and Burridge (2009) reported that occludin and ZO-1 were dephosphorylated by DEP-1 in MCF10A human mammary epithelial cells treated with ochratoxin A. An increase of permeability was caused by the removal of claudin-3 and 4 by ochratoxin A treatment in Caco-2 cells (McLaughlin et al. 2004). As such, we tested the effect of patulin and depletion of DEP-1 on the phosphorylation status of claudin-4. Whereas a significant decrease of claudin-4 was not observed at 24 h after siDEP treatment (Fig. 4A), an increase in the level of claudin-4 phosphorylation was detected by an improved immunoprecipitation method

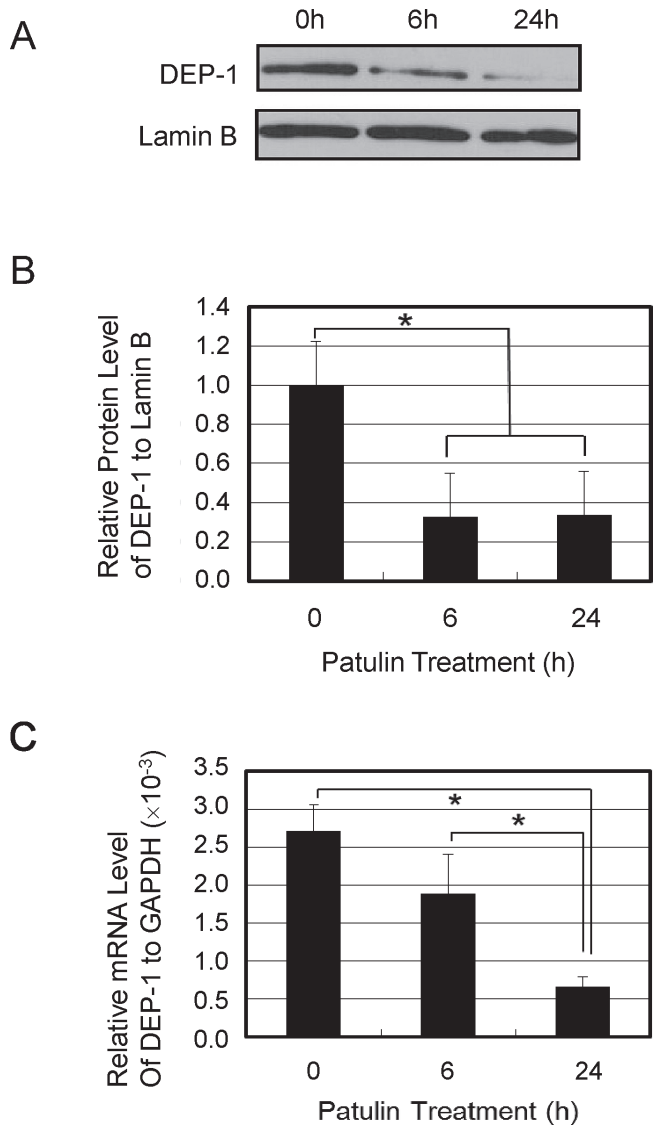

Fig. 1. Effect of patulin on DEP-1 levels.
Caco-2 cells were treated with 50 μM patulin for 0, 6, or 24 h. (A) Western blot analysis. The level of DEP-1 protein was quantified using ImageJ 1.44 (http://rsbweb.nih.gov/ij/) (B). Caco-2 cells were treated with 50 μM patulin for 0, 6, or 24 h. After harvesting of the cells, total RNA was isolated and reverse-transcribed to cDNA. Levels of *DEP-1* and *GAPDH* mRNAs were determined by qPCR using specific primer sets (C). Values are means ± SE ($n = 3$ for each group). An asterisk indicates the mean value of each time point after patulin treatment that is significantly ($p < 0.05$) different from that of the control.

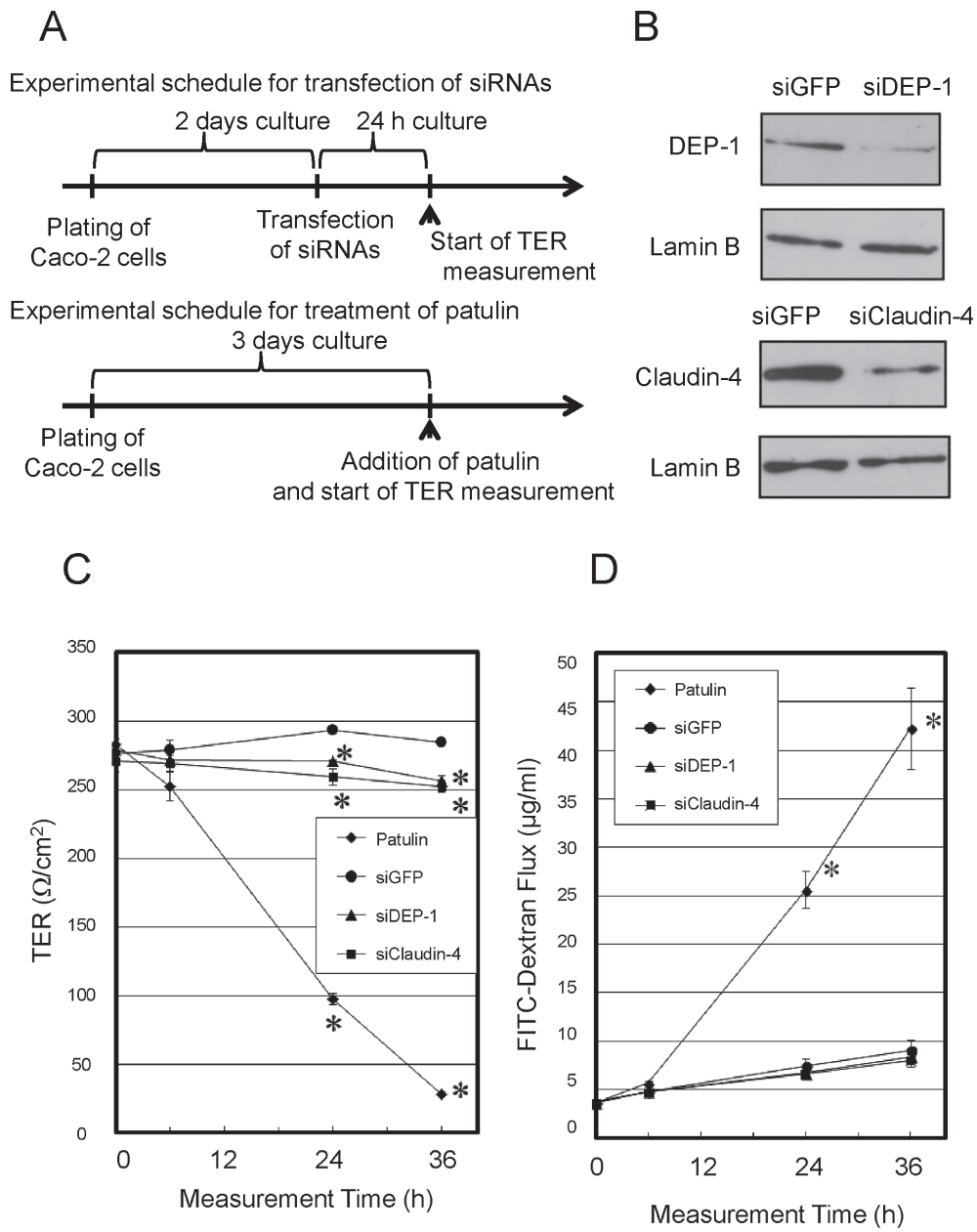

Fig. 2. Effects of patulin and knockdown of DEP-1 on the levels of junction proteins and the cell permeability. (A) Experimental strategy. (B) Western blot analysis of the knockdown of *DEP-1* or *claudin-4* mRNA expression with a respective siRNA at a final concentration of 20 nM. (C) Changes in TER by knockdown of *DEP-1* or *claudin-4*. After treatment of Caco-2 cells with 50 μM patulin or transfection with each siRNA specific for DEP-1 or claudin-4, TER was measured at different times. Caco-2 cells transfected with siGFP were used as a control. (D) Effect on FITC-dextran permeability by knockdown of *DEP-1* or *claudin-4*. Immediately after the addition of 50 μM patulin, or 24 h after the transfection with siRNAs specific for *DEP-1* or *claudin-4* transcripts, 4-kDa FITC-dextran was added to each apical side of the inserts. At each time point, 50 μl of medium was withdrawn from the basal side of the insert (Transwell polycarbonate cell culture, Greiner Bio-One) and its fluorescence was measured using Fluoskan Ascent FL as described in Materials and Methods. In panels C and D, values are means ± SE (n = 3 for each group). An asterisk indicates the mean value of each time point after patulin treatment that is significantly ($p < 0.05$) different from that of the control.

using protein G cross-linked to phospho-Tyr antibody with DMP (Fig. 4B). This means that a detectable amount of claudin-4 was phosphorylated by the decrease of DEP-1, but that DEP-1 might not be a major target of patulin itself. Microscopy of immunostained cells indicated that the decrease of DEP-1 by knockdown did not change the cellular distribution of claudin-4 (Fig. 3B).

Possible involvement of PPARγ in the transcription of DEP-1

As shown in Fig. 1C, treatment of Caco-2 cells for 24 h with patulin reduced levels of *DEP-1* mRNA expression.

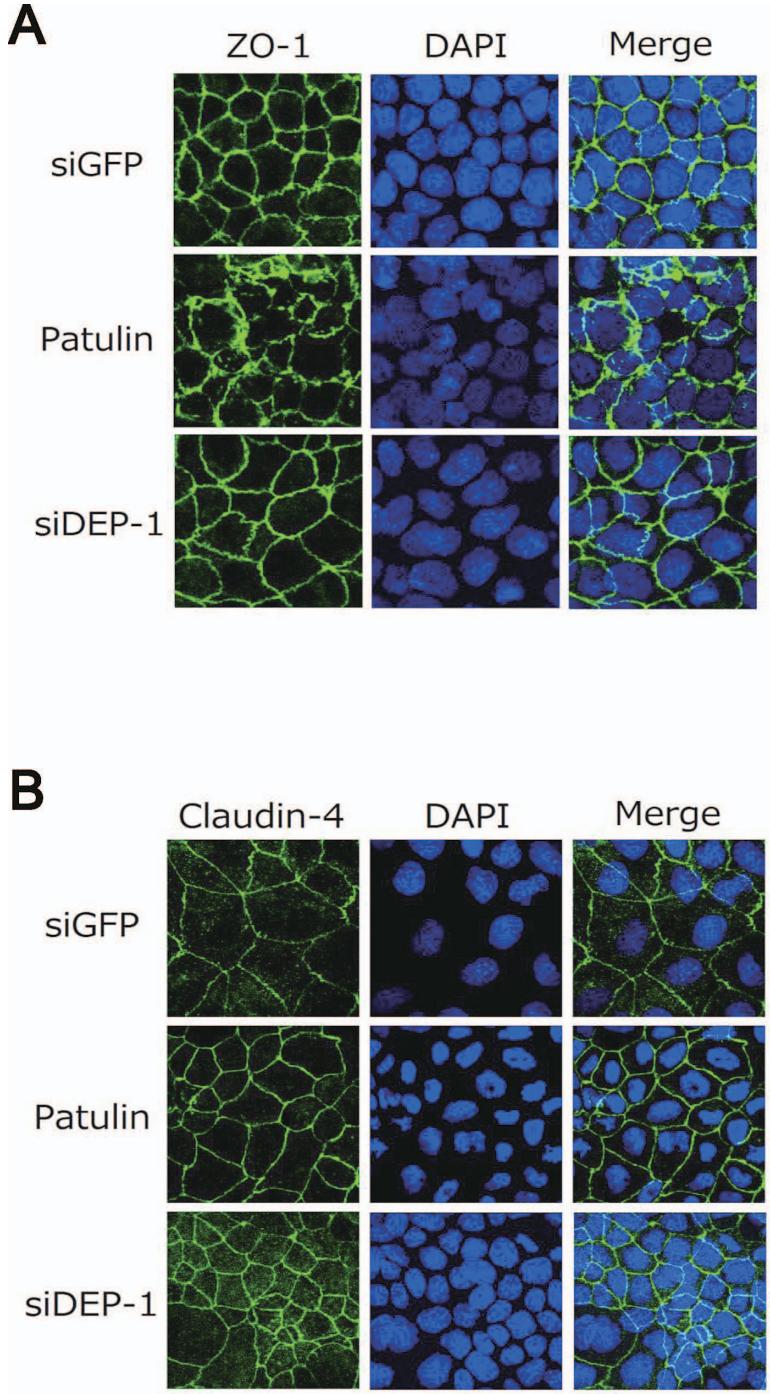

Fig. 3. Effects of patulin and knockdown of DEP-1 on the localization of junction proteins.
Either 24 h after the addition of 50 μM patulin or 72 h after the transfection of siRNA specific for *DEP-1* transcripts, Caco-2 cells were fixed and stained with antibodies against ZO-1 (A) or claudin-4 (B). The nuclei were stained with Hoechst 33258. Cells transfected with siGFP were used as a control.

To address the issue of whether this was caused by a transcription factor(s), we next performed promoter studies, using GeneCards (http://www.genecards.org/) and JASPAR (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl). Sequence analysis of the human DEP-1 promoter revealed at least one putative PPARγ-responsive element (PPRE; AGGTCA) (Takano and Komuro 2009) as well as CP2 and HOXA5 in the region 4 kb upstream of the start codon. We focused on PPARγ because this transcription factor has Cys residues in the DNA binding domain that could be targeted by patulin. The sequence of this PPRE is shown in Fig. 5A. Moreover, we performed an in vitro study using Caco-2 cells. The abundance of PPARγ had decreased about 60% by 6 h after treatment with patulin, as determined by detection with an

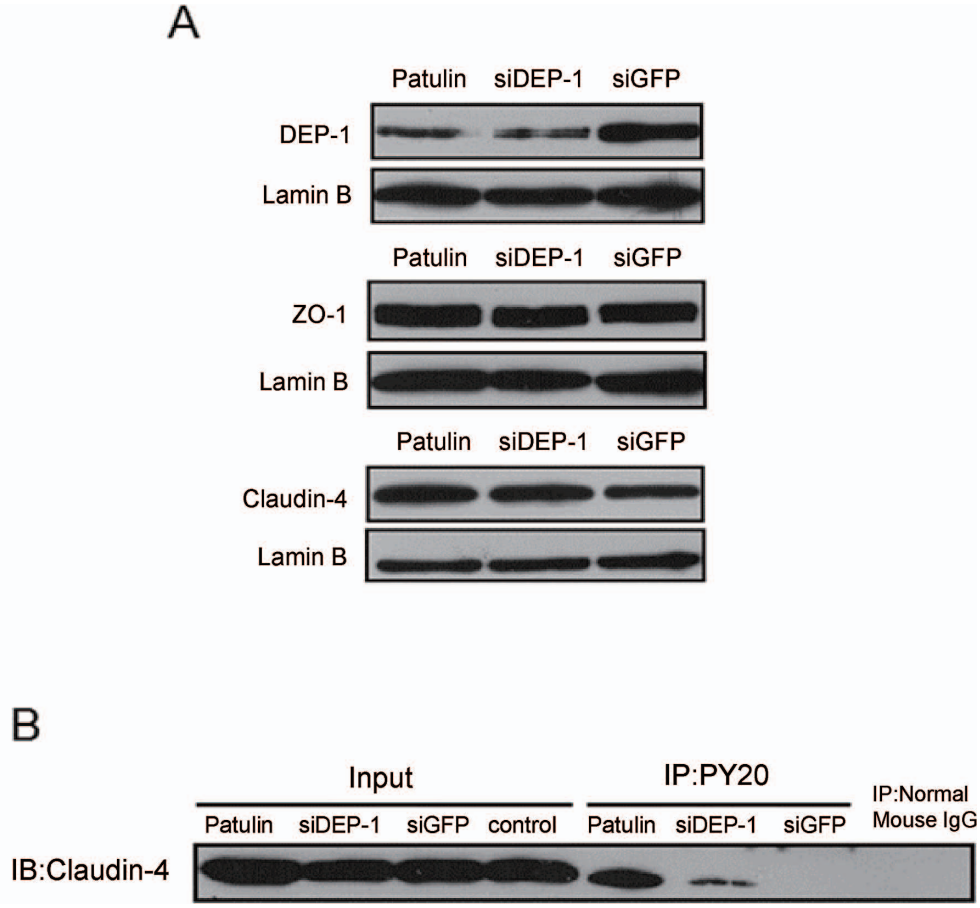

Fig. 4. The effects of patulin and the knockdown of DEP-1 on the levels and phosphorylation of junction proteins. Either 24 h after the addition of 50 μM patulin or 72 h after transfection with siRNA specific for *DEP-1* transcripts, Caco-2 cells were harvested and subjected to western blot analysis. Thereafter, DEP-1, ZO-1, or claudin-4 was detected. Lamin B was also detected as a loading control (A). Either 24 h after the addition of 50 μM patulin or 72 h after the transfection of siRNA specific for *DEP-1* transcripts, Caco-2 cells were harvested and immunoprecipitated with mouse anti-phosphotyrosine antibody PY20. Thereafter, immunoblotting was used to detect levels of claudin-4 (B).

antibody against it (Fig. 5B and C). Furthermore, we observed a higher level of DEP-1 mRNA in Caco-2 cells after 24 h of treatment of the cells with troglitazone, a ligand for PPARγ, compared with that in the control group (vehicle alone; Fig. 5D); this indicated that the effect of patulin on DEP-1 expression was mediated by PPARγ.

Discussion

Investigations of the mechanism behind the toxicity of patulin have focused primarily on its effects on the stability of TJs (McKinley et al. 1982; Mahfoud et al. 2002; Kawauchiya et al. 2011). Given that the stability of TJ proteins is regulated by their phosphorylation on tyrosine residues, decreased phosphatase activity might account for patulin-mediated increases in the phosphorylation of TJ proteins. The potential effects of an increase in the level of phosphorylation of TJ proteins might be offset by reduced overall abundance of TJ proteins as a result of their proteolytic degradation (Staddon et al. 1995; Gonzalez-Mariscal et al. 2000). Occludin and ZO-1 are dephosphorylated by DEP-1 in MCF10A cells treated with ochratoxin A, another type of mycotoxin (Sallee and Burridge 2009). Here, we report that DEP-1 protein decreased within 24 h after patulin treatment (Fig. 1A and B), whereas previously we reported that patulin increased the level of phosphorylation of ZO-1 (Kawauchiya et al. 2011). Although the patulin treatment of Caco-2 cells destroyed the function of TJs completely, the knockdown of DEP-1 partly reduced (by 15%) the function of TJs, as determined by the TER method (Fig. 2C). This means that patulin must have other target molecules besides DEP-1 that enable it to destroy TJs completely.

The patulin-induced decrease in DEP-1 occurred at both the protein (Fig. 1A) and mRNA (Fig. 1C) levels. Investigation of the transcriptional mechanism of DEP-1 regulation revealed it to be under the control of PPARγ (Fig. 5D). Patulin treatment of Caco-2 cells decreased the level of PPARγ (Fig. 5B). From these observations, we propose a hypothetical model of patulin toxicity (Fig. 6). Patulin could have multiple target molecules inside the cell because of its reactivity with the Cys residues of proteins. DEP-1 is one of the molecules targeted by patulin. Part of the TJ

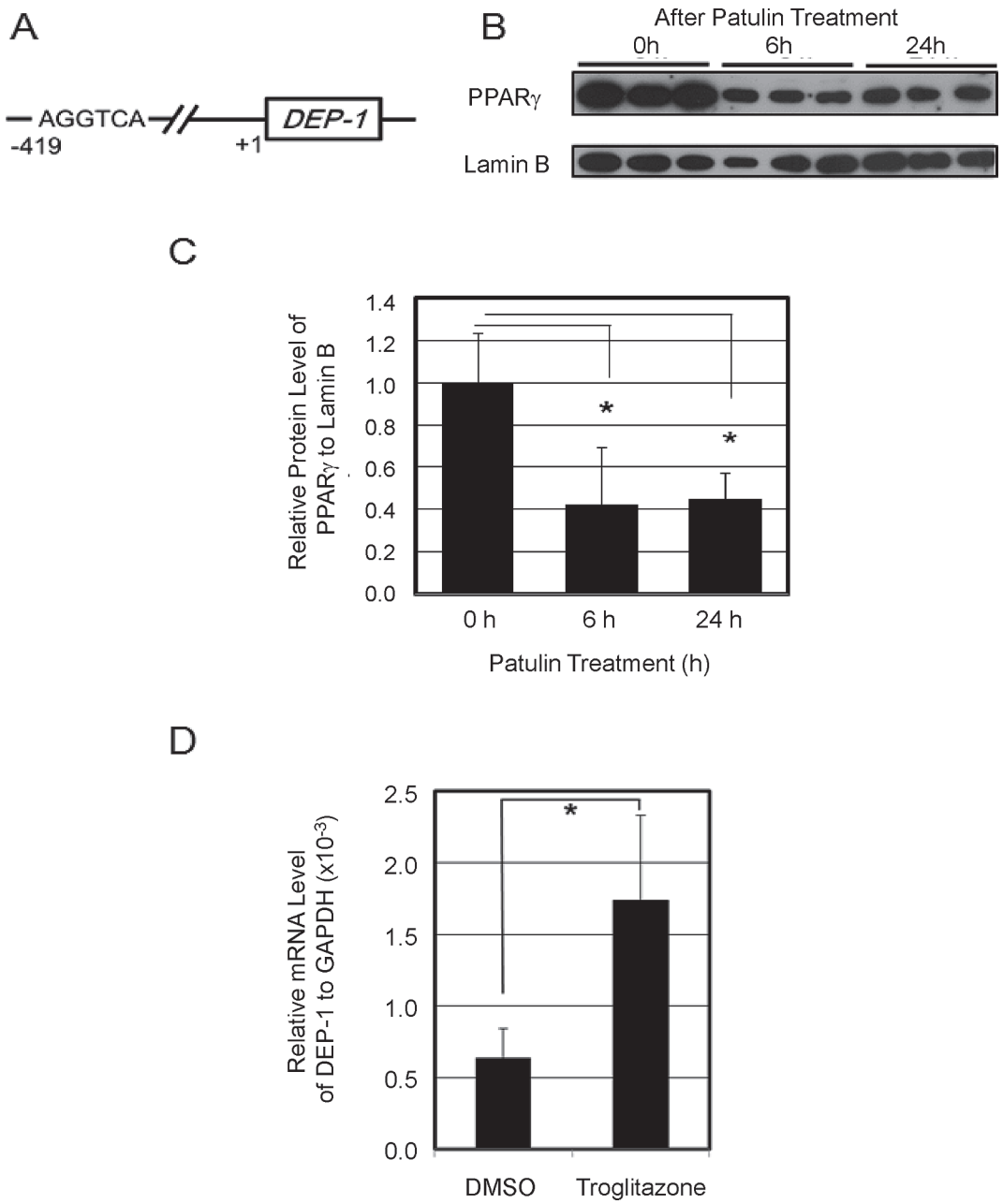

Fig. 5. PPARγ regulates the expression of *DEP-1*.
(A) The *DEP-1* promoter contains one PPARγ responsive element (–419 bp). Caco-2 cells were treated with 50 μM patulin for 0, 6, or 24 h. The cells were then harvested and subjected to western blot analysis to detect the levels of PPARγ and lamin B (loading control) (B). The level of PPARγ protein was quantified using ImageJ 1.44 (C). (D) Effect on the level of DEP-1 by troglitazone. Caco-2 cells were treated with 50 μM troglitazone for 24 h. Total RNA was isolated and reverse-transcribed to cDNA. Levels of DEP-1 and GAPDH mRNA were determined by qPCR using specific primer sets. In panels C and D, values are means ± SE (n = 3 for each group). An asterisk indicates the mean value of each time point after patulin treatment that is significantly ($p < 0.05$) different from that of the 0-h control (C) or DMSO control (D).

destruction may thus be attributable to a decrease of DEP-1 activity. Through changes in signal transduction (Wu et al. 2005; Liu et al. 2007) or reduced stability of PPARγ caused by patulin treatment, the level of *DEP-1* mRNA might be decreased by the decreased amount of PPARγ binding to a *cis*-element upstream of the coding region of the *DEP-1* gene. PPARγ could be a target of patulin, because it is a member of zinc-finger family, the members of which use Cys and His residues to chelate a zinc metal-ion. If that hypothesis is correct, it might account for the acute toxicity of patulin in terms of the decreases in the levels of DEP-1 (Fig. 1B) and PPARγ proteins (Fig. 5C) during the first 6 h. The decrease in the transcription factor activity of PPARγ might be sufficient to sustain the continuous decrease in

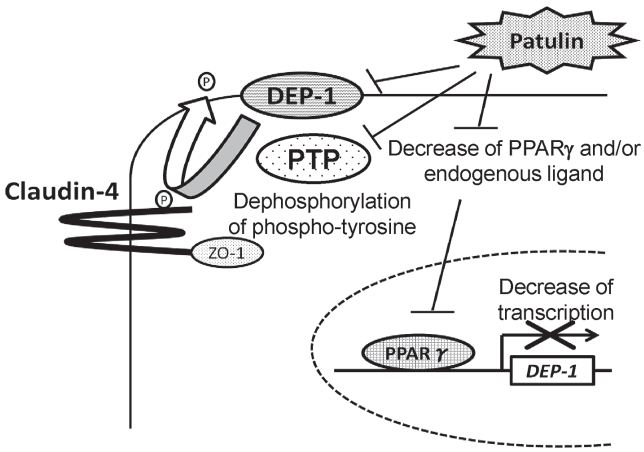

Fig. 6. Hypothetical mechanisms.
The decrease of DEP-1 by the treatment of patulin occurred at both the levels of protein and mRNA. Through some signal transduction or decrease of stability of PPARγ by patulin treatment, the transcription of DEP-1 may be suppressed by the decreased binding of PPARγ to a *cis*-element upstream of the gene. The decrease of DEP-1 and inhibition by direct attack of patulin to unidentified PTP(s) might cause hyper-phosphorylation of claudin-4. The decrease in the interaction between ZO-1 and claudin-4 as a result of this phosphorylation might lead to the release of claudin-4 from TJs. The destruction of TJs would increase paracellular permeability and hence lower the TER.

DEP-1 mRNA level (Fig. 1C). However, chromatin immunoprecipitation assays have yet to establish whether PPARγ binds to the *cis*-element upstream of *DEP-1* gene. Furthermore, the involvement of the endogenous ligand for PPARγ (Kim and Surh 2008) or non-ligand activation of PPARγ (Al-Rasheed et al. 2004) in this process remains to be clarified.

Transfection with siDEP-1 decreased TER without altering the flux of FITC-dextran across cell membranes (Fig. 2C and D). These results suggested that the knockdown of DEP-1 affects the permeability of membranes to small entities (such as sodium ions), but not macromolecules. Therefore, we focused on the function of claudin-4, which has been reported to regulate the permeability of TJs to sodium ions (Krug et al. 2012). However, the transfection of siDEP-1 did not seem to alter the localization of ZO-1 (Fig. 3A) or claudin-4 (Fig. 3B). These results suggest that the depletion of DEP-1 is insufficient to change the localization of ZO-1 and claudin-4 or the function of TJs, although patulin treatment causes significant destruction of TJs. Mohan and colleagues (2012) reported that the high concentration of patulin (500 μM) was required to produce the similar effect of patulin on intestinal permeability or ion transport in isolated colonic mucosa, compared with that in culture cell experiments as shown in this report (50 μM). Future experiments need to elucidate the meaning of the sensitivity difference to patulin between the colonic mucosa and culture cells.

In the present study, we used patulin at the concentration of 50 μM (7.7 μg/ml), as reported in the previous reports (Mahfoud et al. 2002; Liu et al. 2007). Such a high concentration may not happen on commercially available apple juice (detected at 5.7-26.0 μg/l) (Rychlik and Schieberle 1999). Destruction of TJ by patulin is considered acute effect on digestive tracts. The allowable level in most country is 50 μg/l in apple juice, and the upper limit was decided based on the experiments of chronic effect on tumor genesis. However, there has been no report about the effect of sub-toxic dose of patulin on intestinal permeability. Here we have proposed that the possible target molecule might be not only PTPs but also PPARγ and the binding reaction is irreversible. Further studies should be done to clarify these points.

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Conflict of Interest

The authors declare no conflict of interest.

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