Lubiprostone Induces Claudin-1 and Protects Intestinal Barrier Function

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Abstract

Introduction: Lubiprostone, a chloride channel activator, is said to reduce epithelial permeability. However, whether lubiprostone has a direct effect on the epithelial barrier function and how it modulates the intestinal barrier function remain unknown. Therefore, the effects of lubiprostone on intestinal barrier function were evaluated in vitro. **Methods:** Caco-2 cells were used to assess the intestinal barrier function. To examine the expression of claudins, immunoblotting was performed with specific antibodies. The effects of lubiprostone on cytokines (IFN γ , IL-6, and IL-1 β) and aspirin-induced epithelial barrier disruption were assessed by transepithelial electrical resistance (TEER) and fluorescein isothiocyanate (FITC) labeled-dextran permeability. **Results:** IFN γ , IL-6, IL-1 β , and aspirin significantly decreased TEER and increased epithelial permeability. Lubiprostone significantly improved the IFN γ -induced decrease in TEER in a dose-dependent manner. Lubiprostone significantly reduced the IFN γ -induced increase in FITC labeled-dextran permeability. The changes induced by IL-6, IL-1 β , and aspirin were not affected by lubiprostone. The expression of claudin-1, but not

claudin-3, claudin-4, occludin, and ZO-1 was significantly increased by lubiprostone. **Conclusion:** Lubiprostone significantly improved the IFN γ -induced decrease in TEER and increase in FITC labeled-dextran permeability. Lubiprostone increased the expression of claudin-1, and this increase may be related to the effect of lubiprostone on the epithelial barrier function.

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Introduction

Irritable bowel syndrome (IBS) is one of the most prevalent functional gastrointestinal disorders, with an estimated prevalence of approximately 10% worldwide [1]. Recent studies with colonic mucosal biopsies have reported that the tissue level of IFN γ and its release were significantly increased in IBS [2]. Pro-inflammatory cytokines including IFN γ , IL-6, IL-8, and TNF α were significantly higher in serum and fecal samples in IBS patients [3, 4]. IFN γ , IL-1 β , and IL-6 cause intestinal epithelial barrier disruption that can be detected by transepithelial electrical resistance (TEER) and transepithelial permeability testing [5–8].

Lubiprostone, an activator of chloride channels-2 and a member of a class of compounds called prostones, is a gas-

trointestinal-targeted bicyclic fatty acid that enhances intestinal fluid secretion and has been shown to be safe and highly effective in treating constipation [9–13]. Furthermore, lubiprostone has been reported to have an effect on the intestinal barrier function, and oral administration of lubiprostone significantly reduced the severity of colitis and reduced intestinal permeability in both dextran sulfate sodium and trinitrobenzene sulfonic acid-induced colitis [14]. Activation of chloride channels-2 with lubiprostone reduced mannitol flux in ischemia-injured intestine [15]. Lubiprostone significantly decreased chronic stress-induced visceral hyperalgesia in rats [16]. The effect of lubiprostone on human intestinal barrier function after administration of diclofenac was assessed by measuring the lactulose-mannitol ratio, and the ratio was significantly lower after lubiprostone treatment than in the untreated group [17].

However, whether lubiprostone has a direct effect on the damaged epithelial barrier function and how it modulates intestinal barrier function remain unknown. Therefore, the effects of lubiprostone on intestinal barrier function were evaluated *in vitro*.

Materials and Methods

Cell Culture

Human Caco-2 intestinal epithelial cells were purchased from ATCC (Rockville, MD, USA). Transwell™-Clear inserts (0.33 cm², 0.4-μm pore size; Costar, Cambridge, MA, USA) were coated with collagen. Caco-2 cells were cultured in Dulbecco's modified Eagle's medium containing 25 mmol/L glucose supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% non-essential amino acid solution. The cells were sub-cultured in Transwell™-Clear wells until reaching approximately 80% confluency. These cells were then used in a short-term cell culture system to establish a model of the small intestinal epithelium, as described in detail previously [18]. Briefly, Caco-2 cells were cultured with basic culture medium for the first day after seeding, and thereafter with differentiation medium (Dulbecco's modified Eagle's medium with MITO™ Serum Extender and butyric acid, DM). DM was exchanged every other day. Differentiated Caco-2 monolayers were ready after 6 days of short-term cell culture. Cells were maintained at 37 °C in a 5% CO₂ incubator.

Reagents

Lubiprostone was purchased from Abcam plc (Cambridge, UK). IFN γ , IL-6, and IL-1 β were purchased from R&D Systems (Minneapolis, MN, USA). Aspirin was purchased from Tocris Bioscience (Bristol, UK). IFN γ , IL-6, IL-1 β , and aspirin were used at concentrations of 2–50 ng/mL, 5 ng/mL, 1.25 ng/mL, and 1 mmol/L, respectively according to the previous reports [6, 8, 19].

Construction of Experimental Model and Treatment Regimen

Each well of the Transwell™ plate had 2 compartments: an apical compartment that represented the luminal side of the small intestine and a basolateral compartment that represented the sub-

epithelial side. Differentiated Caco-2 cells were stimulated from the basolateral compartment with IFN γ , IL-6, and IL-1 β , and stimulated from the apical compartment with aspirin. Cells were pretreated with lubiprostone at the apical side for 1 h before cytokine or aspirin stimulation.

Measurement of TEER

Resistance across the epithelial monolayer was measured using a MILLICELL-ERS (Millipore Corporation, Bedford, MA, USA) with “chopstick” electrodes, according to a previously reported method [20]. The values obtained from blank inserts were subtracted to give the net resistance, which was multiplied by the membrane area to give the resistance in area-corrected units ($\Omega\cdot\text{cm}^2$). The change in electrical resistance was represented by percentage baseline resistance, which was calculated as follows: % baseline resistance = $([\text{resistance from each time point}] - [\text{resistance from a blank insert}]) / ([\text{baseline resistance}] - [\text{resistance from a blank insert}]) \times 100$, where baseline resistance was the resistance at time point 0. TEER values were recorded at 4, 8, 24, 48, 72, and 96 h. Relative electrical resistance was expressed as follows: (% baseline resistance at 96 h) / (average of % baseline resistance from controls at 96 h).

Evaluation of Paracellular Permeability

Fluorescein isothiocyanate-labeled dextran (FITC-dextran; FD4, Sigma, St. Louis, MO, USA) was used as a permeable tracer that passes across the epithelial monolayer [21]. Caco-2 cells were grown on the Transwell™ culture inserts. Differentiated Caco-2 cells were pretreated with 500 nmol/L lubiprostone and/or 50 ng/mL IFN γ for 96 h. HBSS containing 10 mg/mL FITC-dextran was put into the luminal chamber. After 2-h incubation, the medium was collected from the basolateral chamber, and the absorbance of FITC-dextran was determined at an excitation wavelength of 485 nm and emission wavelength of 530 nm using a microplate fluorometer (Infinite M200, Tecan Trading AG, Männedorf, Switzerland). The data were expressed as follows: permeability index (%) = $([\text{experimental clearance}] - [\text{spontaneous clearance}]) / ([\text{clearance of filter alone}] - [\text{spontaneous clearance}]) \times 100$, where experimental clearance was the absorbance from the lower chamber in untreated control monolayers, and clearance of filter alone was the absorbance from a filter alone without cells [21].

Western Blot Analysis

Cells were collected after stimulation for 96 h. Total protein was obtained under reducing conditions. Protein concentrations were measured with a protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Equal quantities of protein were separated by electrophoresis on 12% or 4–20% SDS-PAGE gels. After electrophoresis, proteins were transferred to PVDF membranes (Bio-Rad Laboratories Inc.). Blocking was then performed by placing each membrane in PBS-T buffer containing 5% skim milk at room temperature for 60 min. After blocking, membranes were exposed to claudin-1 (1:1,000), -2 (1:250), -3 (1:500), 4 (1:500), occludin (1:1,000), and ZO-1 antibodies (1:500, Cell Signaling Technology, Inc., Danvers, MA, USA) at 4 °C overnight. Membranes were then washed with PBS-T buffer and exposed to horseradish peroxidase-labeled horse anti-mouse or goat anti-rabbit IgG antibody (1:1,000, Cell Signaling Technology, Inc.). Membranes were again washed with PBS-T buffer and then treated with Western blotting detection reagent (ECL Prime; GE Healthcare, Little Chalfont, UK). Next the intensity of each band was scanned and quantified using

Fig. 1. Effect of IFN γ on Caco-2 TEER. Confluent Caco-2 monolayers were incubated with medium only or IFN γ for 96 h. **a** The time course effect of IFN γ (50 ng/mL). Data are presented as means $\pm$ SD; * p < 0.05, *** p < 0.001 vs. Control. **b** Stimulation with IFN γ at concentrations of 2, 10, 50 ng/mL. * p < 0.05, ** p < 0.01 vs. Control (IFN γ , 0 ng/mL).

ImageJ software (National Institutes of Health, Bethesda, MD, USA). GAPDH (1:1,000, Cell Signaling Technology, Inc.) was analyzed to assess the amount of loaded protein.

Statistical Analysis

All data are expressed as means $\pm$ SD. Data were analyzed using unpaired t test for comparison of 2 groups, and one-way ANOVA followed by Sidak's test for multiple comparisons. All tests were two-sided with a significance level of p < 0.05. GraphPad Prism6 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis.

Results

Effect of IFN γ on Caco-2 TEER

Differentiated Caco-2 cells were exposed to different concentrations of IFN γ (0, 2, 10, and 50 ng/mL) on the basolateral side for 96 h. IFN γ significantly decreased TEER. IFN γ stimulation tended to show a difference from control TEER after 48 h (Fig. 1a), and the reduction rate was dose-dependent (Fig. 1b).

Effects of Lubiprostone on Intestinal Barrier Function

Differentiated Caco-2 cells were exposed to pro-inflammatory cytokines or aspirin with or without lubiprostone for 96 h. Confluent Caco-2 monolayers were pretreated with 500 nmol/L lubiprostone for 1 h from apical side. Pro-inflammatory cytokines were added from the basolateral side, and aspirin was added from the apical side. Lubiprostone treatment did not change the relative electrical resistance. IFN γ (50 ng/mL), IL-6 (5 ng/mL), IL-1 β (1.25 ng/mL), and aspirin (1 mmol/L) significantly decreased TEER at 96 h. The addition of lubiprostone significantly improved the IFN γ -induced decrease in TEER (Fig. 2a), but not the IL-6, IL-1 β , and aspirin-induced decreases in TEER (Fig. 2b–d).

Lubiprostone improved the IFN γ -induced decrease in TEER at 96 h in a concentration-dependent manner (Fig. 3a). After stimulation with IFN γ , the permeability was evaluated by flux of FITC-dextran from the apical to the basolateral compartments. Lubiprostone significantly reduced the IFN γ -induced increase in FITC-dextran permeability (Fig. 3b).

Tight Junction Protein Expression after IFN γ and Lubiprostone Stimulation

After stimulation with IFN γ (50 ng/mL, 96 h), proteins were extracted, and the expression of tight junction proteins was assessed using Western blot analysis. IFN γ did not change the expression levels of claudin-1, claudin-2, claudin-4, occludin, and ZO-1. The expression of claudin-1 was significantly increased by the administration of lubiprostone (Fig. 4a), but claudin-3, claudin-4, occludin, and ZO-1 (Fig. 4b–e) expressions were not increased.

Discussion

Lubiprostone significantly improved the IFN γ -induced decrease in TEER and increase in FITC-dextran permeability. However, IL-6, IL-1 β , or aspirin-induced changes were not modified by lubiprostone. Lubiprostone increased the expression of claudin-1, and this increase may be related to the effect of lubiprostone on intestinal epithelial barrier function.

IFN γ has been reported to cause a time-dependent decrease in TEER and a time-dependent increase in transepithelial permeability [6, 8, 22]. The data in the present study were consistent with the previous data, but the effect of IFN γ on TEER needed a longer time to see the change in TEER. These differences might be related to the

Fig. 2. Effect of Lub on the decrease of TEER. Confluent Caco-2 monolayers were incubated with pro-inflammatory cytokines or aspirin with or without Lub for 96 h. **a** The effect of Lub was evaluated after stimulation with IFN γ ($n = 11$). **b** The effect of Lub was evaluated after stimulation with IL-6 ($n = 6$). **c** The effect of Lub was evaluated after stimulation with IL-1 β ($n = 4$). **d** The effect of Lub was evaluated after stimulation with aspirin ($n = 4$). Data are presented as means $\pm$ SD; * $p < 0.05$, *** $p < 0.001$ vs. Control, ## $p < 0.01$ vs. IFN γ . Lub, lubiprostone; Asp, aspirin; IL-6, Interleukin 6.

Fig. 3. Effects of Lub on intestinal barrier function after IFN γ stimulation. **a** The effect of Lub (0, 250, 500 nmol/L) on the IFN γ -induced decrease in TEER was evaluated at 96 h ($n = 3$). **b** The effect of Lub on the permeability of FITC-dextran was evaluated after stimulation with IFN γ ($n = 6$). Data are presented as means $\pm$ SD; *** $p < 0.001$ vs. Control, # $p < 0.05$, ### $p < 0.001$ vs. IFN γ . Lub, lubiprostone.

dose of IFN γ and cell conditions. Aspirin, IL-6, and IL-1 β are known to have direct effects on gastrointestinal epithelial cells and disrupt the epithelial barrier function [5, 19, 23]. Although aspirin, IL-6, and IL-1 β decreased TEER in the present study, lubiprostone did not modulate the epithelial barrier function.

Although IFN γ reduced the endothelial barrier and the occludin expression [24], occludin was not affected by IFN γ or lubiprostone in this study. Regarding the expression pattern of claudins in the gastrointestinal tract, previous data in inflammatory bowel diseases were inconsistent. Downregulation of claudin-3 and -4 was reported in ulcerative colitis (UC) [25]. Claudin-2, but not claudin-1 and -3, expression increased in active UC [26]. Increased expression of claudin-1 was reported in UC and Crohn's

disease [25, 27]. Furthermore, in IBS patients, occludin and claudin-1 expressions decreased in IBS with predominant diarrhea (IBS-D) patients, but not in IBS with predominant constipation (IBS-C) and IBS with constipation alternating with diarrhea (IBS-A) patients [28]. Claudin-2 mRNA increased in IBS-D patients [29]. In the present study, treatment with lubiprostone increased the expression of claudin-1, but not claudin-3 and claudin-4, though they were not affected by IFN γ stimulation. Claudin-1 is widely expressed in the intestinal epithelium and is known for its barrier-forming abilities. Therefore, it has been proposed to have an important role in tight junction integrity [25, 30, 31], and increased expression of claudin-1 by lubiprostone may be involved in the improvement of the intestinal epithelial barrier function. Even in

Fig. 4. Tight junction protein expression after IFN γ and lubiprostone stimulation. The effects of Lub on IFN γ -induced changes in (a) CLDN1, (b) CLDN3, (c) CLDN4, (d) occludin, and (e) ZO-1

expressions were evaluated by Western blotting ($n = 4$). GAPDH was used as a loading control. Data are presented as means $\pm$ SD; ^{##} $p < 0.01$ vs. IFN γ . CLDN1, claudin-1; Lub, lubiprostone.

a recent report, cortisol-induced decreases in barrier function and claudin-1 were recovered by lubiprostone [16]. An IFN γ -induced increase in claudin-1 has been reported, and the changes in the levels of claudins during the inflammatory response may not only result in changes in the barrier properties, but also in the protective response to host defense [8, 25].

IFN γ -induced increases in epithelial paracellular permeability are reported to be critically dependent on phosphatidylinositol 3-kinase (PI3-K) activity [32]. IFN γ induces endocytosis of epithelial TJ proteins via RhoA/Rho-associated, kinase-mediated, myosin II-dependent formation of vacuolar apical compartments. Direct phosphorylation of myosin light chain (MLC) by Rho kinase represents the major mechanism underlying the activation of myosin II in IFN γ -treated T84 cells [33]. Even in our preliminary experiments, the plan was to assess the blocking effect of lubiprostone on PI3-K or MLC activa-

tion. However, phosphorylation of PI3-K (p85) or MLC (Ser19) could not be detected after IFN γ stimulation in our model (data not shown).

The present study has several limitations. First, the study was done only in vitro. However, the results of this study proved the direct effect of lubiprostone on barrier function in vitro. Second, only the level of claudin-1 was examined by Western blotting, and the expression pattern of claudin-1 was not evaluated in this study. Since the level of tight junction proteins is not always related to increased barrier function [34], further studies are needed to elucidate the detailed mechanisms of lubiprostone-induced intestinal epithelial barrier protection. However, it is important to emphasize in the present study that lubiprostone at least has a direct effect on the epithelial barrier function. Third, the mechanisms of how IFN γ induces barrier dysfunction and what TJ proteins are involved in the dysfunction are not clear. Lubiprostone may improve the barrier

dysfunction caused by factors other than the decrease of claudin-1 by increasing the expression of claudin-1.

In summary, lubiprostone improved the IFN γ -induced decrease in TEER and increase in permeability. Lubiprostone increased the expression of claudin-1, and this increase may be related to the effect of lubiprostone on the intestinal epithelial barrier to protect against intestinal inflammatory diseases.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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