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The effect of serine phosphorylated claudin-7 on the epithelial barrier and the modulation by transient receptor potential vanilloid 4 in human colonic cells

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ABSTRACT

Abnormal phosphorylation of claudins changes the interaction and aggregation of tight junction proteins, affecting the intestinal epithelial barrier. Selective blockade of transient receptor potential vanilloid 4 (TRPV4) alleviated experimental colitis. Whether TRPV4 affects the intestinal epithelial barrier and the relationship to claudin-7 phosphorylation remain unknown. In the present study, we investigated the TRPV4 expression in human colonic tissues and colonic cells. Using the site-directed mutagenesis approach, we also identified the roles of claudin-7 phosphorylation in the epithelial barrier and the relationship between TRPV4 and claudin-7 phosphorylation. Increased TRPV4 expression was found in the colonic mucosa from IBD patients. In colonic cells, the mutation of claudin-7 at position 204 decreased the TRPV4 expression. Mutation of claudin-7 at position 204 significantly decreased the FD20 permeability in monolayer colonic cells, while mutations of claudin-7 at positions S206 and S207 increased the FD20 permeability. Meanwhile, mutations of claudin-7 at positions S204 and S207 increased the TER in monolayer colonic cells. TRPV4 agonist GSK1016790 A increased the FD20 permeability in the control group, cld7-wild group, cld7-S206A group and cld7-S207 A group, while the TRPV4 antagonist HC067047 decreased the FD20 permeability in the same groups. HC067047 treatment increased the TER in vector cells, cld7-wild cells and cld7-S206 A cells compared to the respective cells in GSK1016790A-treated groups. HC067047 treatment decreased the migration in vector cells, cld7-wild cells and cld7-S206 A cells compared to the respective cells in the GSK1016790A-treated groups. These results indicated that TRPV4 might be a target for the maintenance of the intestinal epithelial barrier and indicated the mechanism involved in the modulation of serine phosphorylated claudin-7.

1. Introduction

Inflammatory bowel disease (IBD) is a gastrointestinal disorder characterized by inflammation and ulceration in the intestine. Damage to the intestinal epithelial barrier is an important step in the development of IBD, which increases the intestinal permeability, facilitating the movement of bacteria and toxins through the mucosa into the submucosa and leading to immunologic disorders and pathological damage in the intestine [1,2].

Tight junctions (TJs) are the most important connections between the intestinal epithelium. TJs are composed of transmembrane proteins, such as claudins and occludin, and cytosolic proteins, such as ZO-1. Previous studies have demonstrated that the differential expression of some TJ proteins was related to the impairment of intestinal barrier

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function [3,4]. However, in the early stage of some damaged barriers, the expression of TJ proteins was not altered compared with the control group, but the cellular allostery was activated and cellular polarity changed [5–7]. Recently, several studies showed that the abnormal phosphorylation of claudins activated cellular allostery and changed the interaction and aggregation of TJ proteins, thus increasing the gap permeability and decreasing the transepithelial impedance [8–10].

Claudin-7 has a strong basolateral membrane distribution, which localizes primarily to apical TJs in the intestinal epithelium. The *Cldn7*$^{-/-}$ mice had severe intestinal defects, including mucosal ulcerations, epithelial cell sloughing, and inflammation, suggesting important roles of claudin-7 in the intestinal barrier and homeostasis [11]. In DSS-induced experimental colitis, phosphorylated claudin-7 levels were increased at days 6 and 8 compared with the control groups [12]. The role of claudin-7 phosphorylation in the intestinal barrier is largely unknown.

TRPV4, a member of the transient receptor potential (TRP) superfamily of nonselective cation channels, has been found in many different epithelial cells. TRPV4 was expressed in intestinal epithelial cells, and the activation of TRPV4 in the gastrointestinal tract caused chemokine release and colitis [13]. Activation of TRPV4 also caused the down-regulation of the tight junction proteins claudin-1, claudin-3, claudin-4, claudin-5, claudin-7, and claudin-8 and dramatic changes in tight junction morphology, which then affected the epithelial permeability [14]. Selective blockade of TRPV4 in the 2,4,6-trinitrobenzenesulfonic acid animal model alleviated colitis and pain associated with intestinal inflammation, which indicated that TRPV4 might be an attractive target for topical anti-inflammatory treatment in patients with IBD [15]. Whether TRPV4 affects the intestinal epithelial barrier and the relationship to claudin-7 phosphorylation remain unknown.

In the present study, we first investigated the TRPV4 expression in human colonic tissues and colonic cells. Using the site-directed mutagenesis approach, we also identified the roles of claudin-7 phosphorylation and TRPV4 in the epithelial barrier and the relationship between TRPV4 and claudin-7 phosphorylation.

2. Materials and methods

2.1. Materials

NCM460 colonic cells were obtained from the American Tissue Type Culture Collection (Rockville, MD, USA). Fluorescein isothiocyanate-dextran (FD20, 20,000 Daltons) and GSK1016790 A (TRPV4 agonist) were purchased from Sigma (MO, USA). HC067047 (TRPV4 antagonist) was purchased from MedChem Express (New Jersey, USA). Phosphorylated rabbit anti-claudin-7 (Phospho-Tyr210) antibody was purchased from Assay Biotechnology (CA, USA). Non-phosphorylated rabbit anti-claudin-7 antibody, Fluo-8/AM and pluronic F-127 were purchased from Invitrogen (Carlsbad, CA, USA). Rabbit anti-TRPV4 antibody was purchased from Abcam (OFW, UK). Rabbit anti-GAPDH was purchased from Santa Cruz Biotechnology (CA, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Kangchen Biotech (Shanghai, China). Chemiluminescent HRP substrate was purchased from Millipore (MA, USA).

2.2. Human tissues

Patients with IBD were seen at the Nanjing General Hospital of Nanjing Military Region (China) and had established and well-characterized (both endoscopically and microscopically) Crohn's disease or ulcerative colitis. Human colonic mucosa was obtained from the descending and ascending colon of patients by colonoscopy biopsy. Non-IBD patients underwent surgery for cancer tissue resection, and only healthy colonic mucosa (non-cancer) was considered a control in the present study (from the First Affiliated Hospital of Anhui Medical University, China). Informed consent from patients and approval from the Ethics Committees were obtained. Tissues were immediately frozen and stored in liquid nitrogen for subsequent western blot analysis.

2.3. Generation of claudin-7 mutations by serine phosphorylation site mutagenesis and cell culture

The human Claudin-7 cDNA was purchased from Vazyme (Nanjing, China), and the full length Claudin-7 cDNA was used as a template to generate Claudin-7 mutations. Claudin-7 S204 A, S206 A, and S207 A mutants were generated by replacing serine with alanine at positions 204, 206, and 207 in antisense primers using Phanta Super-Fidelity DNA Polymerase (S204A: ATTTAAATTCGAATTCACATACTCCTTGGA AGAGTTGG- CCTTAG, S206A: ATTTAAATTCGAATTCACATACTCCTT GGACGCGTTG, S207A: ATTTAAATTCGAATTCACATACTCCTTGGCAG AGTTG). All mutations of Claudin-7 were confirmed by sequencing (Sangon Biotech, Shanghai, China). The cld7, cld7-S204 A, cld7-S206 A, and cld7-S207 A were sub-cloned into PCDH-CMV-EF1-puro-GFP vector at the EcoRI site using the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China).

The human normal colonic epithelium cell line NCM460 was grown in RPMI 1640 culture medium with 10% FBS, 1% penicillin and 1% streptomycin. NCM460 cells were cultured to 80% confluence and harvested using 0.25% trypsin and 0.02% EDTA. To generate the stable cell lines, 1×10^6/100 μl cell suspensions in 6-well cell culture clusters were transfected with 4 μg of plasmid DNA using PEI and cultured in RPMI 1640 with 2 μg/ml puromycin for approximately two weeks to select for stably transfected cells. GFP-positive colonies of the stable NCM460 cell lines were selected during the subsequent culture. Cells were grown in selection medium until the density of GFP-positive cells was 80%. Finally, stable NCM460 cell lines with vector, wild type Claudin-7, and Claudin-7 mutants at serine phosphorylation sites were obtained using the same methods.

2.4. Detection of claudin-7 location by confocal microscope

In order to investigate the location of claudin-7 mutation, green fluorescence in NCM460 cells was observed by confocal microscope. The stable cells were seeded onto glass discs one day before the experiment. Cells were washed with normal physiological saline solution (NPSS, containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$,1 mM $MgCl_2$,10 mM glucose, and 5 mM HEPES, pH 7.4) two times and mounted onto the chamber with NPSS. To observe how GSK1016790 A and HC067047 affected the motion of the claudin-7 mutation, the images were obtained every 15 s and lasted for 10 min. GSK1016790 A and HC067047 were added into NPSS in chamber until the fourth 15seconds. In the TRPV4 antagonist group, cells were pre-treated with HC067047 for 10 min, and then, GSK1016790 A was added for later detection.

2.5. Monolayer permeability assays

Permeability studies were performed using confluent monolayers 14 days after seeding. The integrity of the confluent monolayers was verified by measuring transepithelial electrical resistance (TER) at different time intervals. TER of > 450 Ω/cm^2 monolayer was achieved as measured using a Millicell-ERS voltmeter (Millipore, USA). The intestinal epithelial monolayers were treated with TRPV4 agonist (GSK1016790 A, 50 nM) or TRPV4 antagonist (HC067047, 10 μM). In the TRPV4 antagonist group, cells were pre-treated with HC067047 for 10 min, and then, GSK1016790 A was added for later detection. TER (Ω/cm^2 monolayer) = (Total resistance − Blank resistance) (Ω) × Area (cm^2 monolayer) [16].

The integrity of the confluent monolayers was also checked by measuring the permeability of fluorescein. The stock solution of the permeability probe FD20 (25 mg/ml) was prepared by dissolving the

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