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Butyrate ameliorated-NLRC3 protects the intestinal barrier in a GPR43-dependent manner

Di Cheng[a,b], Ji-Hao Xu[a], Jie-Yao Li[a], Si-Yi Wang[a], Ting-Feng Wu[a], Qi-Kui Chen[a,b,*], Tao Yu[a,b,*]

[a] Department of Gastroenterology, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, 107 Yan Jiang Xi Road, Guangzhou, Guangdong 510120, People's Republic of China

[b] Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, 107 Yan Jiang Xi Road, Guangzhou, Guangdong 510120, People's Republic of China

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ABSTRACT

Background: Intestinal barrier dysfunctions are related to dysbacteriosis and chronic gut inflammation in type 2 diabetes. Although there is emerging evidence that the chronic gut inflammatory response is stimulated by nucleotide-binding oligomerization domain-like receptors (NLRs), the relationship and precise mechanism between NLRC3 and the colonic epithelial barrier remains largely elusive.

Methods: We investigated the function and mechanism of NLRC3 in the colonic tissues of diabetic mice and colonic epithelial cell lines. The regulatory mechanism between NLRC3, butyrate and tight junctions was elucidated via a transepithelial electrical resistance measurement, transmission electron microscopy, RNA interference and western blotting.

Results: In this study, we found that NLRC3 expression was decreased in the colonic tissues of diabetic mice. NLRC3 over-expression ameliorated colonic epithelial barrier integrity and up-regulated tight junction proteins in colonic epithelial cells. Knockdown of TRAF6 diminished NLRC3-induced ZO-1/occludin expression. In addition, we demonstrated that butyrate could stimulate NLRC3 expression in both diabetic mice and colonic epithelial cells. GPR43 on colonic epithelial cells is involved in the activation of NLRC3 induced by butyrate.

Conclusion: Our findings demonstrated that NLCR3 could ameliorate colonic epithelial barrier integrity in diabetes mellitus in a TRAF6-dependent manner, and NLCR3 was stimulated by butyrate via binding GPR43 on colonic epithelial cells.

1. Introduction

Type 2 diabetes (T2DM), which is clearly associated with both host genetics and environmental factors, has become a common problem worldwide. Recently, several intestinal abnormalities of T2DM have been demonstrated in either laboratory tests or human diabetes, such as a decrease in the diversity of the gut flora and an imbalance in the ratio of bacterial species, which result in chronic gut inflammation and intestinal barrier dysfunctions [1–3].

Most chronic gut inflammatory responses are stimulated by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), which bind to microbe-associated molecular patterns (MAMPs) expressed by gut flora [2]. Gut flora dysbiosis and bacterial products trigger innate NLR signaling activation, such as NOD1 and NLR family pyrin domain-containing 3 (NLRP3), to induce inflammatory responses [2,4]. Furthermore, NLR signaling activation is involved in intestinal epithelial barrier dysfunctions [5,6]. NLR family CARD domain-containing 3 (NLRC3), a cytoplasmic innate immune sensor, is characterized by NOD and leucine-rich repeat configuration [7]. A current study has demonstrated that NLRC3 inhibits innate immunity by down-regulating the Lys63-linked ubiquitination of TNF receptor-associated

Abbreviations: T2DM, type 2 diabetes; PRRs, pattern recognition receptors; TLRs, toll-like receptors; NOD, nucleotide-binding oligomerization domain; NLRs, nucleotide-binding oligomerization domain (NOD)-like receptors; MAMPs, microbe-associated molecular patterns; NLRP3, NLR family pyrin domain-containing 3; NLRC3, NLR family CARD domain-containing 3; SCFAs, short-chain fatty acids; GPR43, G-protein coupled receptor 43; C57, C57BL/6J; db/db, BKS.Cg-Dock7m + / + Leprdb/JNju; shRNA, short hairpin RNA; LPS, lipopolysaccharide; TEER, transepithelial electrical resistance; FITC-D4, fluorescein isothiocyanate-dextran 4 kDa; TEM, transmission electron microscopy; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time PCR; ZO-1, zonula occludens 1; TRAF6, TNF receptor-associated factor 6; GPR109A, G-protein coupled receptor 109A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAB, 3, 30-diaminobenzidine tetrahydrochloride; NF-κB, nuclear factor kappa B; STING, stimulator of interferon genes

* Corresponding authors at: Department of Gastroenterology, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, 107 Yan Jiang Xi Road, Guangzhou, Guangdong 510120, People's Republic of China.

E-mail addresses: qkchen2015@163.com (Q.-K. Chen), yutao2014@126.com (T. Yu).

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factor 6 (TRAF6) [8]. However, the correlation between NLRC3 and the colonic epithelial barrier has not yet been described.

Butyrate is a short-chain fatty acid (SCFA) produced by the butyrate-producing bacteria through the fermentation of dietary fiber. It is well known that butyrate has intestinal mucosa, anti-inflammatory, anti-oxidant and anti-tumorigenesis benefits. Currently, butyrate has been reported to protect against gut inflammation and contribute to epithelial integrity via binding G-protein coupled receptor 43 (GPR43) and inducing NLRP3 inflammasome activation [9]. However, although the component of NLRC3 is similar to NLRP3, the relationship between butyrate and NLRC3 has not yet been reported.

In the present study, we examined the differential expression of NLRC3 in the bowel tissues of T2DM mice and control mice. Then, stable overexpressing or knockdown NLRC3 genes in colonic epithelial cell lines were established to determine the correlation between NLRC3 and the colonic epithelial barrier. Furthermore, to illuminate the relationship between butyrate and NLRC3, butyrate was administered to both mice and colonic epithelial cell lines.

2. Materials and methods

2.1. Mice feeding and cell culture

Eight-week-old BKS.Cg-Dock7m + / + Leprdb/JNju (db/db) mice, a well-established animal model of T2DM, and C57BL/6J (C57) mice were obtained from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China) and maintained under a 12 h light/dark cycle in a specific pathogen-free animal facility. The animals were divided into four experimental groups: C57 mice (control, n = 6), C57 mice that were administered butyrate (Sigma-Aldrich, St. Louis, MO, USA; C57 + butyrate, n = 6), db/db mice (db/db, n = 6) and db/db mice that were administered butyrate (db/db + butyrate, n = 6). Sodium butyrate (Selleck, Houston, TX, USA) diluted in normal saline at a dose of 200 mg/kg was perfused into the colon through a polyethylene catheter positioned 5 cm from the anus for 3 consecutive weeks.

Caco-2 and NMC-460 cells (a non-cancerous human colonic epithelial cell) were stored in our laboratory. The cells were grown in Dulbecco's Modified Eagle's Medium with high glucose (4500 mg/L) and L-glutamine, supplemented with 10% (v/v) fetal bovine serum (Gibco, MA, USA) and 100 U/L penicillin-streptomycin (Gibco), and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. In some cases, the cells were treated with varying concentrations of sodium butyrate (2, 10, or 20 mmol/L; Sigma-Aldrich) for 6 h or 1 g/mL lipopolysaccharide (LPS) for 48 h.

2.2. Stable overexpressing NLRC3 cell lines

The full coding sequence of NLRC3 was amplified and then cloned into a pCDH-CMV-MCS-EF1-puro plasmid (designated as pCDH-CMV-NLRC3-EF1-Puro). The pCDH-CMV-NLRC3-EF1-puro or pCDH-CMV-MCS-EF1-puro construct was co-transfected with retroviral packaging plasmids (pPACKH1 Lentivector Packaging Kit; System Biosciences, SF, CA, USA) into HEK293T cells using 10 μg/μL PEI (Sigma-Aldrich) following the manufacturer's protocol. Forty-eight hours after co-transfection, the lentivirus-containing supernatant was collected and passed through a 0.45-μm filter. NMC-460 or Caco-2 cells were transduced with empty or NLRC3-encoding retroviral vectors following the manufacturer's protocol.

2.3. Stable knockdown NLRC3 cell lines

Permanent knockdown of NLRC3 has been performed using RNA interference technology that employs a short hairpin RNA (shRNA) targeting NLRC3. The lentivirus vector containing shNLRC3 (hU6-NLRC3-Ubiquitin-EGFP-IRES-puromycin) and non-targeting scrambled shRNA plasmid were purchased from Genechem (Shanghai, China). The shNLRC3 target sequence on its mRNA was 5'- GGGAACACGACTTCA CACA-3'. NMC-460 or Caco-2 cells were transduced with control or shNLRC3-containing retroviral vectors following the manufacturer's protocol.

2.4. RNA interference

The siRNAs were designed in accordance with Reynolds et al. [10]. The target sequences of siRNAs (siTRAF6, siGPR43 and siGPR109A) are shown in Supplementary Table 1. Non-targeting scrambled siRNA (siNC) was used to control for non-specific effects. The siRNAs were synthesized by Invitrogen Co, Ltd. The synthetic siRNAs were transfected into cells using Lipofectamine 3000 reagent (Invitrogen), according to the manufacturer's instructions. Total RNA was isolated at 48 h post-transfection, and protein lysates were prepared 72 h post-transfection.

2.5. Transepithelial electrical resistance (TEER) measurement

The TEER assay was performed in collagen-coated permeable polycarbonate filters with a surface area of 0.33 cm^2 and a pore size of 0.4 mm (Corning, NY, USA). Additionally, 5×10^4 stable overexpressing or knockdown NLRC3 cell lines and control cells were seeded separately with 0.2 mL of culture medium in each Transwell (Corning). In some experiments, the cell monolayers were incubated with 1 g/mL LPS (Sigma-Aldrich) or siTRAF6 (GenePharma, Shanghai, China) for 48 h. The TEER of epithelial monolayers was measured using an Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL, USA) in each insert, multiplied by the membrane surface area (0.33 cm^2), and corrected by subtracting the background resistance of the blank membrane.

2.6. Determination of the paracellular permeability

Colonic epithelial permeability was assessed using fluorescein isothiocyanate-Dextran 4 kDa (FITC-D4; Sigma-Aldrich) as a paracellular tracer. Before the assay, eight-week-old C57 and db/db mice fasted without water for 4 h. The mice were then orally gavaged with FITC-D4 (50 mg per 100 g of body weight). Two hours after the gavage, blood was collected from the facial vein, and the serum was prepared for fluorescence measurements (excitation, 490 nm; emission, 520 nm).

At the end of the monolayers' TEER assay, 1.0 g/L FITC-D4 was added to the upper chamber and incubated for 1 h at 37 °C. FITC-D4 containing medium from the apical and basal chamber were collected separately in 96-well plates. Then, FITC-D4 was measured spectrophotometrically at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Monolayer permeability was quantified as the percentage of FITC-D4 permeating from the apical to the basal chamber.

2.7. Transmission electron microscopy (TEM)

The colonic tissues were cut into sections of approximately 1 mm^3 thickness on ice. These sections were fixed overnight at 4 °C with 2.5% glutaraldehyde and 2.0% paraformaldehyde. Next, the samples were washed three times for 10 min each time with 0.1 mol/L phosphate-buffered saline (PBS) and then post-fixed with 1% osmic acid for 2 h at 4 °C. After fixation, the samples were dehydrated in graded acetone and embedded with Epon 812. The semi-thin sections were then cut and double-stained with 2.0% uranyl acetate and 2.0% lead citrate. The colonic epithelial tight junction ultrastructure was observed and photographed under a TEM (JEM-100CXII; JEOL, Japan) and operated at an accelerating voltage of 100 kV.

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