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6-Formylindolo(3,2-*b*)carbazole induced aryl hydrocarbon receptor activation prevents intestinal barrier dysfunction through regulation of claudin-2 expression

Yuanhang Ma[a], Qimeng Wang[a], Kun Yu[a], Xin Fan[a], Weidong Xiao[a], Yujiao Cai[a], Pengyuan Xu[b], Min Yu[a,**], Hua Yang[a,*]

[a] Department of General Surgery, Xinqiao Hospital, Third Military Medical University, Chongqing, China
[b] Department of Gastrointestinal Surgery, The Second Affiliated Hospital of Kunming Medical University, Kunming, China

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ABSTRACT

6-Formylindolo(3,2-*b*)carbazole (FICZ), a high-affinity aryl hydrocarbon receptor (AhR) ligand, plays a protective role in inflammatory bowel disease (IBD) through activation of AhR. Interleukin-6 (IL-6) induced intestinal epithelial barrier dysfunction is involved in the pathological process of IBD. In this study, we investigated the protective effects of FICZ on IL-6 induced intestinal epithelial barrier injury. Our data show that AhR activation by FICZ ameliorated colonic inflammation, decreased IL-6 and claudin-2 expression, and maintained intestinal barrier function in a mouse model of dextran sulphate sodium (DSS)-induced colitis. In Caco-2 and T84 intestinal epithelial cells, FICZ also prevented the increase of intestinal epithelial permeability and claudin-2 expression induced by IL-6. Depletion of AhR expression by small interfering (si)RNA reversed FICZ induced decrease of claudin-2. Furthermore, IL-6 induced upregulation of claudin-2 was required for increased caudal-related homeobox 2 (CDX-2) and hepatocyte-nuclear factor (HNF)-1α. However, FICZ repressed the increase of CDX-2 and HNF-1α expression induced by IL-6. These results reveal the protective effects of FICZ on IL-6 induced disruption of intestinal epithelial barrier function through suppressing the expression of claudin-2. In addition, CDX-2 and HNF-1α are involved in the regulation of claudin-2 after IL-6 and FICZ treatment. Therefore compounds related to AhR ligands may be potential pharmaceutical agents to treat IBD.

1. Introduction

Inflammatory bowel disease (IBD) is a group of chronic, non-specific inflammatory conditions of the gastrointestinal tract. Crohn's disease and ulcerative colitis are the principal types. The defective intestinal barrier function was reported to play an indispensable role in the pathological process of IBD [1]. An intact intestinal epithelium can separate the intestinal contents from the underlying tissue compartments, and this separation depends largely on the tight junction (TJ) [2]. TJs are intercellular junctional complexes that are located in the apical membrane [3]. Transmembrane proteins are the fundamental components of TJs, which establish cell polarity and work as major determinants of epithelial barrier function [4]. There are four families of transmembrane proteins, namely occludins, claudins, junctional adhesion molecules and tricellulins [5].

Accumulating evidence has revealed that the function of claudins can roughly be divided into two types, those involved in the paracellular barrier formation that prevents molecules from passing through epithelial cell sheets, and those involved in the formation of paracellular channels [6,7]. Claudin-2 was known to cause the formation of cation-selective channels sufficient to transform a 'tight' tight junction into a leaky one [8]. The expression of claudin-2 is markedly increased in the colons of patients with IBD, and claudin 2 seems to have an important role in the pathogenesis of intestinal inflammatory disorders [7,9].

Interleukin-6 (IL-6) is a pleiotropic cytokine, which has multiple pro-inflammatory actions and has been reported to play an important role in the pathogenesis of many diseases, including IBD [10–12]. The exploratory clinical trial found that humanized *anti*-IL-6 receptor monoclonal antibody therapy produced a significant clinical improvement in active Crohn's disease [13,14]. Recent evidences demonstrate that IL-6 modulates intestinal epithelial barrier function by stimulating the expression of claudin-2 [15,16].

Aryl hydrocarbon receptor (AhR), a transcription factor activated by

* Corresponding author. Department of General Surgery, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, China.
** Corresponding author.
E-mail addresses: yu_min_1984@163.com (M. Yu), huayang@tmmu.edu.cn (H. Yang).

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a large variety of natural and synthetic ligands, has recently been reported to dampen inflammatory processes in gut [17,18]. 6-Formylindolo (3,2-*b*)carbazole (FICZ), is a tryptophan photoproduct and formed from tryptophan when exposed to UV-light as well as to visible light [19–22]. It has been reported that FICZ is one of the most potent naturally occurring activators of AhR [20,23–26]. Increasing evidences suggest that FICZ induced AhR activation could inhibit inflammation in the gastrointestinal tract [17,18,27]. In our previous work, we also demonstrated that FICZ could ameliorate epithelial barrier dysfunction induced by intestinal obstruction [28]. However, the effect of AhR on leaky protein claudin-2 and intestinal barrier function in IBD remains unknown. In this study, we used mice model and two *in vitro* intestinal epithelial barrier models, to investigate the role of FICZ induced AhR activation on IL-6 induced increased of claudin-2 and intestinal barrier dysfunction.

2. Materials and methods

2.1. Induction of colitis by dextran sulphate sodium (DSS)

Male, 6-8 week-old, specific pathogen-free C57BL/6J mice purchased from the Laboratory Animal Center (Third Military Medical University, Chongqing, P.R. China) were used in our study. Colitis was induced by the administration of 3% DSS for 7 days. Control mice were given distilled water. FICZ (1 μg/mouse) was administered by intraperitoneal injection daily beginning 2 days after the start of DSS administration. Mice were sacrificed after administration of DSS for 7 days. Intestinal mucosa were collected for histology, and intestinal epithelial cells were collected to detect target RNA and protein expression levels. All procedures were approved by the Laboratory Animal Welfare and Ethics Committee Of the Third Military Medical University, China (approval SYXK-PLA-20120,031).

2.2. Intestinal epithelial cell isolation

Intestinal epithelial cells (IECs) isolation was performed according to our previous study [29]. Briefly, the whole small intestine was removed and placed in tissue culture medium (RPMI 1640, with 10% foetal calf serum). The section was cut into 5-mm pieces, washed in an isolation buffer containing 190 mg ethylenediamine-tetraacetic acid and 80 mg DDT dissolved in phosphate-buffered saline (PBS) 500 ml, and incubated in the same buffer with continuous brisk stirring at 37 °C for 30 min. The supernatant was filtered rapidly through a glass wool column. After centrifugation, the pellets were purified in 40% isotonic Percoll (GE Healthcare Biosciences), and the cells recovered in the suspension were collected for RNA or protein extraction.

2.3. Immunohistochemistry staining

Tissues were fixed with 4% paraformaldehyde, embedded in the paraffin, sliced into 5-μm sections, dehydrated in the gradient ethanol, and treated with 0.3% hydrogen peroxide in methanol for 20 min. Then, the sections were soaked in citrate buffer (pH 6.0) and heated by microwave for 20 min for antigen retrieval. The sections were incubated with *anti*-claudin-2 antibody (Abcam, UK) at 4 °C overnight and then blocked with 5% bovine serum albumin (BSA) for 20 min. These sections were then successively incubated with biotinylated secondary antibody and avidin-biotin complex (Boster, China) at 37 °C for 20 min. Peroxidase activity was detected by diaminobenzidine staining. After counterstaining with haematoxylin, histological evaluation was performed under a light microscope at × 400 magnification.

2.4. Detection of intestinal permeability

The *trans*-epithelium electrical resistance (TER, Ω·cm^2), which indicates tissue viability and intestinal epithelial barrier function, was determined according to Ohm's law. The permeability of the small bowel was assessed by TER. Tissues were placed in a modified Ussing chambers (Physiologic Instruments, San Diego, CA, U.S.A.) and bathed with 5 ml Krebs buffer (110.0 mM NaCl, 3.0 mM CaCl2, 5.5 mM KCl, 1.4 mM KH2PO4, 29.0 mM NaHCO3, 1.2 mM MgCl2, pH 7.4) on both the mucosal and serosal sides. The buffer was continuously oxygenated with 5% CO_2 in O_2 and maintained at 37 °C by water-jacketed reservoirs. After a 20-min equilibration period, the detections were performed for up to 90 min in the Ussing chambers. TER was calculated by the injected short-circuit current and spontaneous potential difference.

2.5. Cell culture and treatments

Caco-2 and T84 intestinal epithelial cells were grown under standard cell culture conditions and maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gibco, U.S.) with 10% foetal bovine serum (Life Technologies, Gibco, U.S.) in the presence of 100 units/ml streptomycin and 100 units/ml penicillin (Beyotime Biotechnology, China). The culture medium was changed every two day. The cells were subcultured successively when they reached approximately 80% confluence. Cells were plated on Transwell filters (Corning, USA) with 0.4 mm pore size at 10^5 cells/cm^2 and incubated with IL-6 (10 ng/ml) or FICZ (100 nM) for 48 h.

2.6. Measurement of TER

The integrity of the confluent Caco-2 cells was assessed by measuring the TER. Caco-2 cells were seeded on Millicell filters (0.33 cm^2 area, 0.4 μm pore diameter, and 6.5 mm diameter) at a density of 0.5×10^5 cells/cm^2 as monolayers and used in experiments after achieving confluence. The TER of the treated monolayers was detected by a Millicell ERS-2 volt·ohmmeter (Millipore, U.S.) according to ohm's law: TER (Ω·cm^2) = (Total resistance-Blank resistance) (Ω)·Area (cm^2).

2.7. Quantitative real-time polymerase chain reaction (QRT-PCR)

Total RNA was extracted from cells and colon mucosa of mice using TRIzol (TaKaRa Bio Inc. Japan) reagent according to the manufacturer's protocol (Sigma Chemical Co.). The total RNA concentration was determined by spectrophotometry. For reverse transcription-PCR, 1 μg of total RNA was converted to cDNA using a reverse transcription kit (Takara, Japan) in a 20-μl volume. Then, SYBR green-based real-time PCR was used to measure the levels of IL-6, cytochrome P450 (CYP) 1A1, claudin-2, and β-actin mRNAs. The primer sequences were designed using online tools and are as follows:

β-actin (mouse)	forward, 5′-CTTCTTTGCAGCTCCTTCGTT-3′
	reverse, 5′-AGGAGTCCTTCTGACCCATTC-3′
IL-6 (mouse)	forward, 5′-CTTCCAGCCAGTTGCCTTCTTG-3′
	reverse, 5′-GGTCTGTTGTGGGTGGTATCCTC-3′
CYP1A1 (mouse)	forward,
	5′-CAATGAGTTTGGGGAGGTTACTG-3′
	reverse, 5′-CCCTTCTCAAATGTCCTGTAGTG-3′
Claudin-2 (mouse)	forward, 5′-ATGGCATCCAGCAGAATACA-3′
	reverse, 5′-ACCCACAAATGTACGGGAAT-3′
β-actin (human)	forward, 5′-CCACGAAACTACCTTCAACTCC-3′
	reverse, 5′-CGTGATCTCCTTCTGCATCCTG-3′
Claudin-2 (human)	forward 5′-CGGGACTTCTACTCACCACTG-3′
	reverse 5′-GGATGATTCCAGCTATCAGGGA-3′

2.8. Small interfering (si)RNA transfection

For transient knockdown of AhR, hepatocyte-nuclear factor (HNF)-1α and caudal-related homeobox 2 (CDX-2) expression, cells were