Original Article
Guanylate cyclase-C signaling pathway regulates intestinal inflammatory injury and epithelial barrier function in Caco-2 cells

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Abstract: Background: Guanylate cyclase-C (GC-C) signaling pathway via guanylin (Gn) and uroguanylin (Ugn) activation regulates intestinal fluid and electrolyte homeostasis. It is down-regulated in the patients of inflammatory bowel diseases (IBD). However, studies on the role of GC-C signaling in murine models of colitis are controversial. Here, we investigated the effect of GC-C on intestinal inflammation using Caco-2 cells. Methods: Caco-2 monolayers grown on Transwell filters were stimulated with IL-1β to model the inflammatory cells of intestine. The activity of GC-C signaling was regulated by transfection with Gn overexpression vector or GC-C shRNA plasmid. After different treatment with cells, the levels of Gn, Ugn, GC-C, paracellular permeability, superoxide dismutase (SOD), pro-inflammatory cytokines (IL-8 and TNF-α) and tight junction proteins (occludin, claudin-1 and ZO-1) were detected. Results: The expression of Gn, Ugn and GC-C was all significantly reduced after stimulation with IL-1β. Relative to the empty vector controls, IL-1β-treated cells transfected with Gn overexpression vector had significantly increased levels of Gn, GC-C, SOD, claudin-1 and ZO-1 as well as decreased levels of IL-8, TNF-α and permeability. Conversely, GC-C-silencing cells had significantly increased levels of IL-8, TNF-α and permeability as well as decreased levels of Gn, Ugn, GC-C, SOD, claudin-1 and ZO-1 induced by IL-1β compared with the corresponding empty plasmid controls. Conclusions: GC-C signaling pathway plays a protective role in the intestinal inflammatory injury and epithelial barrier function in Caco-2 cells. These observations further support the possible pathogenetic role of GC-C and clinical therapeutic potential of GC-C agonists in IBD.

Keywords: Guanylate cyclase-C, inflammatory bowel disease, caco-2 cells

Introduction

Inflammatory bowel diseases (IBD) are chronic, relapsing inflammatory disorders that primarily affect the small intestine and colon, including two main types of ulcerative colitis (UC) and Crohn’s disease (CD). The etiology of IBD is still not well established. The prevailing understanding is that the interaction between environmental and microbial triggers in the gut lumen and a dysfunctional intestinal barrier lead to an inappropriate immunological response in genetically susceptible individuals [1, 2]. Although current treatment outcome for IBD has improved with the use of anti-TNFα agents such as infliximab, about 30% of patients with severe UC are resistant to pharmacological therapy and require colectomy [3]. Therefore, a continuous search for novel therapeutic targets is needed.

In recent years, Guanylate cyclase-C (GC-C) signaling pathway has received increasing interest in IBD. GC-C is a transmembrane enzyme expressed primarily on intestinal epithelial cells (IECs) and serves as the receptor for the peptides guanylin (Gn) and uroguanylin (Ugn), and also the receptor for heat-stable enterotoxins (STa) produced by enterotoxigenic Escherichia coli [4, 5]. The binding of these ligands (Gn/Ugn/STa) to GC-C results in the conversion of guanosine triphosphate (GTP) to cyclic-guanosine-3’, 5’-monophosphate (cGMP). cGMP as a second messenger activates the cGMP-depen-
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dient protein kinase II (PKG II), cross-activates cAMP-dependent protein kinase (PKA) and inhibits a cAMP-specific phosphodiesterase (PDE III). Inhibition of PDE III regulates the action of the apical Na+/H+ exchanger 3 (NHE3), which causes the decreased absorption of Na⁺. Moreover, PKGII and PKA phosphorylate the cystic fibrosis transmembrane conductance regulator (CFTR), increasing its chloride-secreting activity, exchanging Cl for HCO₃⁻, and inducing an increase of bicarbonate secretion into the lumen. Consequently, this physiological activation of GC-C regulates intestinal fluid and electrolyte homeostasis, preventing dehydration and intestinal obstruction [6-8].

Our previous study found that Gn, Ugn and GC-C of GC-C signaling pathway were down-regulated in the inflamed colonic mucosa of UC patients, and this downregulation is more significant with the increase of disease activity index [9]. Recent studies have revealed that GC-C signaling plays a protective role in the integrity of intestinal mucosal barrier [10, 11]. However, studies using murine models of colitis show that the role of the GC-C signaling in intestinal inflammatory injury is controversial. Steinbrecher KA et al. demonstrated that dextran sodium sulfate (DSS)-induced clinical disease and histological damage to the colonic mucosa were significantly less severe in GC-C⁻/⁻ mice and moderately reduced in Gn⁻/⁻ mice [12]. Oppositely, Lin et al. indicated that GC-C⁻/⁻ mice have increased susceptibility to colonic inflammatory injury by DSS [11]. The study by Harmel-Laws E et al. confirmed that GC-C⁻/⁻ mice had increased proinflammatory gene expression in whole colon tissue and more severe spontaneous colitis when intraperitoneally injected with lipopolysaccharide [13]. These apparently conflicting findings may be because the use of laboratory animals cannot provide the most accurate means to model human IBD.

Thus, in this study, we used the human colon adenocarcinoma cell line, Caco-2 cells. This is an ideal cell line to research the functions of intestinal epithelial as this cell line imitates inflammatory status following stimulation with IL-1β [14, 15]. Furthermore, these cells cultured on permeable membranes (Transwell) form a confluent monolayer of polarized columnar cells with tight junctions and the majority of intestinal enzymes and transporters are expressed [16]. We employed the genetic approaches to regulate the activity of GC-C signaling. We also tested the levels of Gn, Ugn, GC-C, paracellular permeability, activity of superoxide dismutase (SOD), pro-inflammatory cytokines (IL-8 and TNF-α) and tight junction proteins (TJPs) (occludin, claudin-1 and ZO-1). To clarify the effect of GC-C signaling pathway on intestinal inflammatory injury and epithelial barrier function in Caco-2 cells.

Methods

Cells culture and monolayer preparation

The Caco-2 cell line was obtained from the Cell Bank of the Kunming institute of zoology of the Chinese Academy of Sciences (Kunming, China). Briefly, Cells were grown at 37 °C in 5% CO₂ in high glucose DMEM supplemented with 100 μg/mL streptomycin, 100 IU/mL penicillin, and 10% fetal bovine serum. To prepare Caco-2 monolayers, cells were plated at 5×10⁴/cm² on collagen-coated permeable polycarbonate membrane Transwell supports with 0.4 mm pores (Corning, NY) and grown as monolayers for 20 days prior to experiments. The integrity of Caco-2 monolayer was determined by measuring the transepithelial electrical resistance (TER) of the cell monolayer. At each time point point of 0, 10, 15, 20 days, TER was determined with a Millicell-ERS-2 volt-ohmmeter (Millipore, Bedford, MA). Each TER measurement was calculated by subtracting the resistance value of the filters and fluids. Results were expressed as Ω·cm² monolayer surface area. Based on the literature, a resistance reading of 200-250 Ω·cm² was considered as indicative of a confluent Caco-2 monolayer with tight junctions. At 20 days during the experiment, the TER of Caco-2 monolayers was all 250 Ω·cm² approximately. So it could be used for the following experiments.

Transfection of cells with Gn overexpression vectors

Caco-2 cells were treated with 10 ng/mL of human recombinant IL-1β (Miltenyi Biotec, USA) from the apical side for 48 h [15]. Untreated Caco-2 monolayers served as the blank controls. After 48 h stimulation with IL-1β, Caco-2 cells were transfected with Gn overexpression vectors. Liposome-mediated transfection was performed using Lipofectamine 2000 reagent (Invitrogen, USA) on cells with a control empty vector, as well as an expression vector for Gn cDNA (GeneCopoeia, China). Briefly, according
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to the manufacturer’s instructions, Lipofectamine 2000 and plasmid DNA were diluted in 375 µl of Opti-MEM medium (Gibco, USA) separately and incubated at room temperature for 10 min after mixing. The lipofectamine-DNA complex was added to cells and incubated at 37°C for 6 h in an incubator containing 5% CO₂ and full humidity. Cells were then washed with phosphate-buffered saline (PBS) and replenished with high glucose DMEM. At 48 h after transfection, the permeability of Caco-2 monolayers was determined, the culture supernatant was collected and the cells were harvested for the following investigations. IL-1β-treated cells transfected with the corresponding empty vectors served as the empty vector controls.

Transfection of cells with GC-C shRNA plasmids

GC-C short hairpin RNA (shRNA) plasmids were used to silence GC-C gene in Caco-2 cells. The four GC-C shRNA expression vectors were shRNA21, shRNA22, shRNA23 and shRNA24 (GeneCopoeia, China). A pervasive disturbing sequence was also designed as the empty plasmid controls (GeneCopoeia, China). The screening experiment was done to pick up the GC-C shRNA expression vector with the best gene silencing results. The transfection was performed using Lipofectamine 2000 reagent following the manufacturer’s instruction. Briefly, both plasmid DNA and Lipofectamine 2000 reagent were diluted in 500 µl of Opti-MEM medium separately and incubated at room temperature for 10 min after mixing. After incubation, the lipofectamine-DNA complex was added to each well containing cells and medium. Cells were incubated at 37°C for 6 h in an incubator containing 5% CO₂ and full humidity. Cells were then washed and replenished with high glucose DMEM. After transfection with GC-C shRNA plasmid for 48 h, cells were stimulated with IL-1β as mentioned above. Caco-2 monolayers transfected with the corresponding empty plasmid served as the empty plasmid controls.

Permeability measurement

After apical treatment, permeability of Caco-2 monolayers was determined by measuring the paracellular passage of the fluorescein isothiocyanate (FITC)-dextran (4 kDa, Sigma) from apical to basolateral compartments of the chamber culture as described elsewhere [17, 18]. Briefly, monolayers were gently washed with HBSS and transferred to 500 µl HBSS. DMEM media in apical chamber were gently aspirated and replaced with 100 µl of 1 mg/ml FITC-dextran in HBSS. Then, monolayers were incubated for 2 hours at 37°C. After this time, the basolateral medium was withdrawn and the fluorescence was determined using a fluorescent plate reader (Thermo, Vantaa, Finland) with an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Results were expressed as the percentage of the FITC-dextran flux of basal chamber relative to the total flux.

Superoxide dismutase (SOD) assay

The SOD activity of Caco-2 cells was evaluated using commercially available kits (Beyotime, Jiangsu, China). This assay was based on the reduction of nitroblue tetrazolium (NBT) to water insoluble blue formazan. Briefly, the cells were cultured in twelve-well plates. After different treatments for cells and incubation at 37°C for 30 min, the absorbance at 560 nm was monitored using a Beckman DU530 UV-visible (UV-Vis) spectrophotometer. One unit of SOD was defined as the amount of enzyme inhibiting the reduction of NBT by 50%. Results were expressed as U/mg protein.

Quantitative real-time PCR analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to assess the mRNA expression of Gn, Ugn, GC-C, occludin, claudin-1, ZO-1, IL-8 and TNF-α. The total RNA of Caco-2 cells was extracted using Trizol reagent (Qiagen, Hilden, Germany). The complementary DNA was synthesized using SYBR PrimeScript RT reagent kits (TaKaRa, Dalian, China) according to the manufacturer’s instructions. qRT-PCR was performed in an ABI prism 7900 HT sequence detector (Applied Biosystems, Foster City, CA, USA) using the SYBR green methodology. The specific primers of Gn, Ugn, GC-C, occludin, claudin-1, ZO-1, IL-8, TNF-α and GAPDH were purchased from GeneCopoeia (Guangzhou, China). Briefly, in a 20 µl reaction volume, 1 µl of cDNA was added to 10 µl of SYBR green Master mix (Darmstadt, Germany) and 0.3 µmol/L of each primer. The condition of PCR reactions was identical and the following: 95°C 10 min; 95°C 10 sec, 60°C 20 sec, 72°C 30 sec, 40 cycles; 60°C 30 sec. The lengths of expected products were as fol-
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Figure 1. Expression of GC-C in the screening experiment of GC-C shRNA plasmid. Expression of GC-C was determined in cells transfected with GC-C shRNA21, GC-C shRNA22, GC-C shRNA23, GC-C shRNA24 and the corresponding empty plasmids respectively. Control: cells without transfection. A. The level of GC-C mRNA was detected by qRT-PCR. Gene expression was normalized to GAPDH mRNA levels in each sample. B. The level of GC-C protein was detected by Western blotting. An unrelated protein β-actin was used as the internal control. *P<0.05, **P<0.01 versus the empty plasmid controls.

Table 1. mRNA expression of Gn, Ugn and GC-C in cells

<table>
<thead>
<tr>
<th></th>
<th>Gn</th>
<th>Ugn</th>
<th>GC-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty vector</td>
<td>0.562 ± 0.214</td>
<td>0.488 ± 0.132</td>
<td>0.909 ± 0.351</td>
</tr>
<tr>
<td>Gn overexpression</td>
<td>59.536 ± 0.483**</td>
<td>0.504 ± 0.164</td>
<td>1.875 ± 0.312**</td>
</tr>
<tr>
<td>Empty plasmid</td>
<td>0.553 ± 0.268</td>
<td>0.492 ± 0.151</td>
<td>0.896 ± 0.273</td>
</tr>
<tr>
<td>GC-C shRNA</td>
<td>0.203 ± 0.036a</td>
<td>0.113 ± 0.012a</td>
<td>0.014 ± 0.009**</td>
</tr>
<tr>
<td>Blank controls</td>
<td>0.548 ± 0.164</td>
<td>0.459 ± 0.372</td>
<td>0.912 ± 0.285</td>
</tr>
<tr>
<td>IL-1β stimulation</td>
<td>0.107 ± 0.054AA</td>
<td>0.055 ± 0.026AA</td>
<td>0.263 ± 0.097AA</td>
</tr>
</tbody>
</table>

Empty vector: cells transfected with the corresponding empty vectors, Gn overexpression: cells transfected with the Gn overexpression vectors, Empty plasmid: cells transfected with the corresponding empty plasmids, GC-C shRNA: cells transfected with the GC-C shRNA plasmids, Blank controls: cells without treatment, IL-1β stimulation: cells stimulated with IL-1β. Gene expression was normalized to GAPDH mRNA levels in each sample. *P<0.05, **P<0.01 versus the empty vector controls; *P<0.05, **P<0.01 versus the empty plasmid controls; ΔΔP<0.05, ΔΔΔP<0.01 versus the blank controls. Data are expressed as mean ± SD. The data are a representative of three independent experiments.

Western blotting analysis

Western blotting analysis was performed to determine the protein levels of Gn, Ugn, GC-C, occludin, claudin-1, ZO-1, IL-8 and TNF-α. Briefly, the cells were washed with PBS and dissolved in lysis buffer that contained protease inhibitor. Equal amounts of protein were loaded, and electrophoresis was applied on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5% fat-free milk at room temperature for 1 h. Then, the membranes were incubated with primary antibody (1:250 for rabbit polyclonal anti-Gn; 1:500 for goat polyclonal anti-Ugn; 1:1000 for mouse monoclonal anti-GC-C; 1:1000 for rabbit monoclonal anti-claudin-1; 1:2000 for rabbit monoclonal anti-ZO-1; 1:1000 for mouse monoclonal anti-IL-8; 1:1000 for rabbit monoclonal anti-TNF-α; respectively). After three washes in TBST, the membranes were incubated with Horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-mouse IgG) for 1 h at room temperature. After washing, the protein expression was visualized using enhanced chemiluminescence (ECL kit) and X-ray film (Kodak, USA). β-actin served as the internal control.

ELISA assay

Cell culture medium were diluted with assay diluent. IL-8 (sensitivity: 1 pg/mL) and TNF-α
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(sensitivity: 30 pg/mL) levels in cell culture medium were measured with commercially available ELISA kits (RayBiotech, Minneapolis, MN, USA). Kits are specific for human IL-8 and TNF-α respectively.

Statistical analysis

Experiments were performed at least three times. The data are expressed as the mean ± standard deviation (SD). Data among groups were analyzed using one-way analysis of variance (ANOVA) followed by LSD-t post hoc tests or the independent sample t-test, as appropriate. Similarly, P<0.05 was regarded as being statistically significant. All statistical analyses were performed by the SPSS 17.0 software (Chicago, USA).

Results

Changes of Gn, Ugn and GC-C in cells with different treatment

The screening experiment was done to pick up the best GC-C shRNA plasmid. After transfection...
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significantly reduced expression of Gn and Ugn except the reduction of GC-C (P<0.05, respectively). After exposure to 10 ng/ml IL-1β for 48 h, IL-1β-treated cells had significantly reduced expression of Gn, Ugn and GC-C (P<0.01, respectively).

GC-C signaling regulates the activity of SOD and the production of IL-8 and TNF-α in Cells

As shown in Figures 3-5, IL-1β-treated cells had decreased SOD activity and increased expression of IL-8 and TNF-α (P<0.01 compared with.
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 incorporator for 48 h, we found that the activity of SOD was elevated and expression of IL-8 and TNF-α was reduced (P<0.01 compared with the empty vector controls). In addition, GC-C-silencing cells had significantly decreased SOD activity as well as increased expression of IL-8 and TNF-α (P<0.01 compared with the empty plasmid controls). Similar alterations of IL-8 and TNF-α were observed in the cell culture medium using ELISA Assay.

GC-C signaling regulates paracellular permeability and TJPs assembly in Caco-2 monolayers

As shown in Figures 6-8, paracellular permeability of Caco-2 monolayers was significantly increased after stimulation with IL-1β. IL-1β-treated cells had decreased expression of claudin-1 and ZO-1 (P<0.01 compared with the blank controls). After transfection with Gn overexpression vector, the permeability in the Gn overexpression group was significantly decreased compared to the empty vector group. IL-1β-treated cells transfected with Gn overexpression vector had significantly increased expression of claudin-1 and ZO-1 (P<0.01 compared with the empty vector controls). Additionally, GC-C-silencing cells had significantly higher permeability and lower expression of claudin-1 and ZO-1 than the empty plasmid.
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controls (P<0.01, respectively). But expression of occludin had no significant difference among different groups (P>0.05, respectively).

Discussion

Recent studies have shown that the aberrant of GC-C signaling pathway is implicated in several gastrointestinal (GI) disorders [19]. GC-C is a transmembrane receptor that is expressed primarily on IECs. Endogenous ligands for GC-C are the peptides Gn and Ugn, and are highly expressed in the GI epithelium [5]. In the present study, expression of Gn, Ugn and GC-C was all significantly decreased in Caco-2 cells after stimulation with IL-1β (Table and Figure 2C), which indicates that the activation of GC-C signaling pathway is down-regulated in the intestinal inflammatory status. As was previously found, the crucial mediators of the GC-C signaling pathway are down-regulated in both IBD patients and in rats with chemically induced colitis [9, 20, 21]. In addition, cells transfected with Gn overexpression vector had increased expression of GC-C (Table 1 and Figure 2A). GC-C-silencing cells also had reduced expression of Gn and Ugn except for GC-C (Table 1 and Figure 2B). These results suggest that the regulation of the ligands Gn, Ugn and their corresponding receptor GC-C is combined, the dormant GC-C signaling pathway can be restored by the agonists of GC-C.

As shown in our study, IL-1β-treated cells had significantly increased levels of IL-8 and TNF-α as well as decreased level of intracellular SOD activity, but after transfection with Gn overexpression vector, the levels of IL-8 and TNF-α decreased and activity of SOD increased significantly. Reversely, GC-C-silencing cells had...
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more IL-8 and TNF-α levels as well as less SOD activity than the empty plasmid controls induced by IL-1β (Figures 3-5). IL-8, the neutrophil chemoattractant and activator, is increased in inflamed intestinal mucosa of IBD, and is associated with the macroscopic grade of local inflammation in mucosal tissue of patients with UC [22, 23]. TNF-α is an important proinflammatory cytokine involved in inflammatory responses and increased in intestinal tissues of patients with IBD [24]. During inflammation, high levels of reactive oxygen species (ROS) can decrease the production and activity of SOD [25]. SOD is an antioxidant enzyme that counteracts the harmful effects of ROS [26]. Thus, our findings confirm that the inflammatory injury induced by IL-1β in Caco-2 cells can be mitigated by restitution of the dormant GC-C pathway. Loss of GC-C signaling could in turn exacerbate the inflammatory injury of intestine.

Additionally, our studies found that the paracellular permeability of IL-1β-treated cell monolayers was reduced and expression of Claudin-1 and ZO-1 was increased after transfection with Gn overexpression vector. In the effect of IL-1β, GC-C-silencing cells had elevated permeability and decreased expression of Claudin-1 and ZO-1 compared to the empty plasmid controls. But expression of occludin had no significant difference among groups (Figures 6-8). TJPs regulate the barrier function of IECs, which control the penetration of pathogens and allergens to the submucosa and beyond [27, 28]. Amongst many components of TJPs, occludin, Claudins and ZO-1 are membrane proteins that connect adjacent IECs and build the epithelial barrier [29, 30]. Similar results have been described in the previous studies, showing that elimination of GC-C or Ucn in mice increased the intestinal permeability, with reduced expression of junctional proteins, activation of GC-C signaling in mice decreased the barrier permeability associated with increased junctional proteins [10, 11]. Our findings demonstrated that GC-C signaling defends the epithelial barrier function of intestine by regulating tight junctions’ assembly. The precise mechanisms elucidating how GC-C signaling is involved in the regulation of TJPs is the basis for ongoing investigation.

Figure 8. Immunoblot pictures of occludin, Claudin-1 and ZO-1 in different groups. The levels of proteins for occludin, Claudin-1 and ZO-1 were determined in the blank controls and IL-1β stimulation group (A), IL-1β+empty vector and IL-1β+Gn overexpression group (B). Empty plasmid+IL-1β and GC-C shRNA+IL-1β group (C). Groups are the same as described in Figure 3. β-actin was used as the internal control. The immunoblot pictures are a representative of each group.
Conclusions

GC-C signaling pathway plays a protective role in the intestinal inflammatory injury and epithelial barrier function in Caco-2 cells. In light of the important role of the intestinal barrier dysfunction in the genesis of human IBD, these observations further support that GC-C signaling pathway might be implicated in the pathogenesis of IBD and the emerging clinical development of oral GC-C agonists might be used for IBD.

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Disclosure of conflict of interest

None.

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