Role of ROCK/NF-κB/AQP8 signaling in ethanol-induced intestinal epithelial barrier dysfunction

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Abstract. The present study aimed to investigate the signaling pathways and the underlying molecular mechanisms involved in ethanol-induced intestinal epithelial barrier (IEB) dysfunction. Therefore, an in vitro experimental model of IEB was established using an ethanol-treated Caco-2 intestinal epithelial cell monolayer. The results confirmed that Rho-associated kinases (ROCKs), namely ROCK1 and ROCK2, were involved in the underlying pathway of ethanol-induced IEB dysfunction. Ethanol exposure significantly increased the expression of both ROCK isoforms and the activity of nuclear factor κB (NF-κB). Furthermore, ROCK1- and ROCK2-specific small interfering RNAs (siRNAs), and the NF-κB inhibitor ammonium pyrrolidine dithiocarbamate partially inhibited transepithelial electrical resistance in Caco-2 cells in an in vitro IEB model. In addition, ROCK1- and ROCK2-specific siRNAs inhibited the activity of NF-κB, thereby downregulating the expression of aquaporin 8 (AQP8). Taken together, the results of the present study suggested that ROCK1/ROCK2-mediated activation of NF-κB and upregulation of AQP8 expression levels may represent a novel mechanism of ethanol-induced impairment of IEB function.

Introduction

The human body has multiple barriers between the internal and external environment, including the gastrointestinal barrier, which serves key functions. The intestinal epithelial barrier (IEB) is the line of defense in the intestine that prevents harmful substances from passing through the intestinal epithelium and entering other tissues, organs or the blood stream. The intestinal immune system serves a vital role in maintaining body health; however, it may be dysregulated in response to various diseases (1,2).

Rho is a small G protein with GTPase activity. Rho-associated kinase (ROCK) acts as a downstream effector of Rho signaling and belongs to the serine/threonine protein kinase family (3). The ROCK catalytic center is exposed and activated when the rho-binding domain binds to rho-GTP (4). In addition, our previous study using Caco-2 cells in vitro demonstrated that Rho A is involved in ethanol-induced IEB permeability increase and inhibits tight junction protein (5). The Rho effector molecule ROCK can affect the function of IEB by adjusting the function of tight junctions (6). Dysfunction can occur when IEB is disabled. Based on this research, it was hypothesized that ROCK may participate in the IEB dysfunction process via a signaling pathway.

Currently, two Rho subtypes have been identified, namely ROCK1 and ROCK2. Both isoforms share an amino acid sequence homology of 64-65%, with the highest homology (80-92%) within the kinase domain (7). Although the two ROCK isoforms are structurally similar, they may have different functions. ROCK1 is associated with cell migration, whereas ROCK2 is associated with vimentin and actin tension fibers (8,9). Therefore, it was hypothesized that they serve different roles in IEB dysfunction.

Nuclear factor κB (NF-κB) is an important transcription factor regulating the expression of several inflammatory factors. It has been suggested that ROCK activates the NF-κB signaling pathway (10). Thus, ROCK may accelerate the development and progression of IEB dysfunction by activating NF-κB. ROCK may also activate NF-κB p65 by downregulating NF-κB inhibitor α (IκBa) expression, which in turn mediates the transcription of multiple inflammatory factors.

Aquaporins (AQP)s, a family of homologous water transporters in mammals, mainly mediate the passive trans-biofilm transport of free water and regulate water absorption (11). To date, 13 members of the AQP family have been identified (AQP 0-12) (12). Previous studies have shown that AQP8 is downregulated in the intestinal epithelial cells and is associated with water transport in the human colon (13-15).
The Caco-2 cell model was first proposed by Hidalgo et al. (16) in 1989. Under normal cell culture conditions, Caco-2 cells can differentiate into a polar monolayer on the porous membrane after 21 days and express certain structural and functional characteristics of intestinal epithelial cells. Since then, a number of studies have used this model. Nighot et al. (17) used a Caco-2 monolayer model system to investigate the role of autophagy in regulating the function of the intestinal tight junction barrier. The Caco-2 cell model was also used by Lin et al. (18) and Elamin et al. (19) to study factors that affect intestinal epithelial barrier function. Therefore, the Caco-2 cell model was selected as a normal human intestinal epithelial cell model in the present study.

Therefore, the aim of the present study was to investigate whether AQP8 was involved in the downstream mechanisms of the ROCK/NF-kB pathway in IEB dysfunction using Caco-2 cell model.

Materials and methods

Caco-2 monolayer cell culture. The human colon adenocarcinoma cell line Caco-2 was purchased from the American Type Culture Collection. Caco-2 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone; GE Healthcare Life Sciences) at 37°C (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 5% CO2 atmosphere. When Caco-2 cells had grown to 80% confluence, they were plated onto Transwell filters (Corning Inc.) and the medium was changed every two days. The cells were visually monitored using an inverted microscope and epithelial resistance measurements at 21 days.

Measurement of transepithelial electrical resistance (TEER). TEER values were measured at 21 days when the monolayer formed as previously described (20). The TEER of Caco-2 cell monolayers was measured using a Millicell®-electrical resistance system (EMD Millipore) at 37°C (21). The electrical resistance was expressed in ohm (Ω)·cm² using the surface area of the Transwell insert.

Gene silencing using small interfering RNA (siRNA). For knockdown experiments, Caco-2 cells were transfected with 100 pmol ROCK1- and ROCK2-specific siRNAs (Shanghai GenePharma Co., Ltd.) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 24 h. Subsequent experiments were performed 48 h after transfection. The ROCK1 and ROCK2 siRNA sequences are listed in Table I.

Table I. Sequences of siRNA.

| Gene         | Sequence (5′→3′) |
|--------------|-----------------|
| ROCK1 siRNA-1 | F: UCCACCAAGGAAGUAUAUGTT |
|              | R: CAUUAACCUCUCCUGGUGATT |
| ROCK1 siRNA-2 | F: GCCUGAUAACAAUGCUUGT |
|              | R: CAGCAGCAUGUAUAUGGCTT |
| ROCK1 siRNA-3 | F: GCAUGGUAACGUGUAUAT |
|              | R: UAUCAUGACUGCAUGCTT |
| ROCK2 siRNA-1 | F: AUCAGAGGUGCAACAGAUGAT |
|              | R: UACUGUGAGCCUGAUGATT |
| ROCK2 siRNA-2 | F: CUCAGCAGUGACAAAGCAT |
|              | R: UGUCAUGUACGCUGAAGAT |
| ROCK2 siRNA-3 | F: GUGACUCUCAAUCUGUAGT |
|              | R: CUAACAGUGAGAGAUGACTT |
| Control siRNA | F: UUCUCGGACAGGUGACAGTT |
|              | R: ACGUGACAGGUCCGGAAT |

F, forward; R, reverse; ROCK, Rho-associated kinase; siRNA, small interfering RNA.

NF-kB inhibitor, was added to Transwell chamber medium. The upper chamber medium was removed after 30 min and then ethanol was added. The cells were incubated with DAPI (1:5; Beijing Solarbio Science & Technology Co., Ltd.) at 37°C for 10 min. Finally, the glass slides were observed in all seven groups with three visual fields in each group using immunofluorescence microscopy (Olympus Corporation).

Reverse transcription-quantitative PCR (RT-qPCR). Following Caco-2 cell transfection, the expression levels of ROCK1, ROCK2, NF-kB and AQP8 were determined using RT-qPCR. Briefly, total RNA was extracted from Caco-2 cells with the TRIpure reagent (BioTeke Corporation) and the purity and concentration were measured. Subsequently, RNA was reverse transcribed into cDNA using an RT-PCR kit (v3.0; Takara Bio, Inc.) in a final volume of 20 µl. PCR amplification was performed using cDNA as a template in 50 µl reactions. PCR was performed using SYBR GREEN master kit (Beijing Solarbio Co., Ltd.) running the thermocycling programs: 94°C for 5 min, then 40 two-step cycles of 72°C for 2.5 min, 40°C for 1.5 min and 25°C for 2 min. The specific PCR primers were designed with the Primer Express v2.0 software (Applied Biosystems; Thermo Fisher Scientific, Inc.) and synthesized by Sangon Biotechnology Co., Ltd. The primer sequences are listed in Table II. Relative gene expression levels were analyzed using the 2-ΔΔCq method (22).

Western blot analysis. Caco-2 cells were plated onto glass coverslips and cultured until 70% confluency was reached. Subsequently, cells were washed with PBS containing 0.1 mM EDTA (without calcium and magnesium ions), scraped from the slides using a scraper, homogenized in 1 ml lysis buffer A (2 mM EDTA, 10 mM EGTA, 0.4% NaF, 20 mM Tris-HCl, protease inhibitor, phenylmethylsulfonyl fluoride, protease...
inhibitor cocktail, pH 7.5) and centrifuged at 10,000 x g at 4°C for 10 min. Finally, the supernatant containing the total cellular proteins was collected. Equal amounts of proteins (40 µg) were separated by SDS-PAGE on 8% polyacrylamide gels. After transferring the gel to PVDF membrane, 5% (M/V) skimmed milk powder was used for blocking at 37°C for 1 h. The membrane was incubated with the primary antibodies against ROCK1, ROCK2, NF-κB p65 (all 1:500; cat. nos. WL01761, WL00550 and WL01980, respectively; all Wanleibio co., ltd.) and aQP8 (1:1,000; aB2768409; aBclonal Biotech co., ltd.) overnight at 4°C. Then, the membranes were incubated with β-actin antibody (1:1,000; cat. no. WL01845; Wanleibio Co., ltd.) and Histone H3 antibody (1:1,000; cat. no. WL0984a; Wanleibio Co., Ltd.) overnight at 4°C. Then, the membranes were incubated with a secondary antibody (anti-rabbit; 1:5,000; cat. no. WLA023; Wanleibio Co., Ltd.) at 37 °C for 45 min. The protein bands were visualized using a Gel Imaging Analysis System (cat. no. WD-9413B; Beijing Liuyi Biotechnology Co., Ltd.), and the integrated density values (IDV) for each protein were calculated using the Gel-Pro-Analyzer (v6.0; Media Cybernetics, Inc.) and were normalized to the IDV of β-actin.

Electrophoretic mobility shift assay (EMSA). EMSA binding reactions were performed using a Lightshift Chemiluminescent EMSA kit (Pierce; Thermo Fisher Scientific, Inc.). The double-stranded oligonucleotide probe (5'-AGTTGAGGGGACCTTTCCACACCTCATC-3') (Sangon Biotechnology Co., Ltd) was labeled with biotin. Nuclear proteins were extracted using a Nuclear and Cytoplasmic
Protein Extraction kit (Pulilai Gene Technology Co., Ltd.) according to the manufacturer's protocol, rotated at maximum speed every 2 min for a total of 30 min and centrifuged in 10,000 x g at 4°C for 5 min. The protein concentration was determined by bicinchoninic acid method. The DNA-nucleoprotein binding reaction using a BCA protein concentration determination kit (A:B=50:1; cat. no. Wla004; Wanleibio Co., Ltd.) was carried out for 20 min at room temperature. The DNA-protein complexes were then separated using a 6% non-denaturing polyacrylamide gel by electrophoresis and transferred onto a nylon membrane. Following transfer, the nylon membrane was exposed to 254 nm UV light to cross-link using an ultraviolet lamp in the darkroom for 15 min. The bands were visualized with enhanced chemiluminescence reagents (cat. no. Wla003; Wanleibio Co., Ltd.). Finally, the membrane was exposed to X-ray film and the binding activity of NF-κB was analyzed using a Gel Imaging Analysis System (cat. no. WD-9413B; Beijing Liuyi Biotechnology Co., Ltd.).

Statistical analysis. All data are expressed as mean ± SD of at least three independent experiments. Multiple comparisons between groups were performed using one-way ANOVA followed by post hoc analysis (Bonferroni). All statistical analyses were performed with SPSS v22.0 (IBM Corp.). P<0.01 and P<0.05 were considered to indicate a statistically significant difference.

Results

Selection of ROCK1/ROCK2 siRNAs and their transfection efficiency by RT-qPCR and western blotting. The mRNA and protein expression levels of ROCK1 and ROCK2 were measured by RT-qPCR and western blot analysis, respectively, following siRNA transfection. ROCK1 and ROCK2 expressions were reduced following transfections, indicating the transfection was successful. ROCK1 mRNA and protein expression levels were significantly decreased in the ROCK1 siRNA-1 group, with the reduction in expression up to 71 and 70%, respectively (Fig. 1). ROCK2 mRNA and protein expression levels were significantly decreased in the ROCK2 siRNA-2 group, with the reduction in expression up to 80 and 78%, respectively (Fig. 2). As the results showed significantly downregulated protein and mRNA expressions in the ROCK1 siRNA-1 group and ROCK2 siRNA-2 group compared with expressions in the control group, these siRNAs were chosen for use in subsequent experiments.

Effects of ROCK1 and ROCK2 knockdown or PDTC treatment on ethanol-induced IEB permeability in Caco-2 cells.
Treatment of Caco-2 cells with 5% ethanol for 1 h significantly reduced TEER values compared with the control group (Fig. 3). Furthermore, TEER values were partially recovered in the ethanol + ROCK1 siRNA and in the ethanol + ROCK2 siRNA groups, and significantly affected in the ethanol + ROCK1 + ROCK2 siRNA group. When compared with the ethanol + ROCK1 siRNA (P=0.002) and ethanol + ROCK2 siRNA (P=0.004) groups, TEER values of ethanol + ROCK1 + ROCK2 siRNA group were more significantly recovered. In addition, compared with the ethanol-treated group, treatment of Caco-2 cells with PDTC also restored the decreased TEER values induced by ethanol.

Effects of ethanol exposure on ROCK isoform expression in Caco-2 cells. The mRNA and protein expression levels of ROCK1 and ROCK2 in Caco-2 cells from control, ethanol-treated and ROCK siRNA groups were determined by RT-qPCR and western blot analysis, respectively (Fig. 4). The results showed that exposure of intestinal epithelial cells to ethanol significantly increased ROCK1 and ROCK2 mRNA and protein expression in the ethanol-treated group compared with the control group. ROCK1 and ROCK2 siRNAs, as well as PDTC treatment, partially restored AQP8 mRNA and protein expression levels in the ethanol-treated group compared with the ethanol-only group. Furthermore, AQP8 expression levels were also recovered in ethanol-treated cells transfected with the combination of ROCK1 + ROCK2 siRNAs. The distribution of AQP8 in the IEB model from each group was detected using immunofluorescence (Fig. 7), which showed that the expression level was recovered in the ethanol-treated group compared with the ethanol-only group and that the AQP8 cell distribution pattern was consistent with the aforementioned results.

Discussion

The effects of Rho/ROCK pathway in IEB dysfunction have already been reported in the literature. Mihaescu et al. (23), demonstrated that the ROCK signaling pathway was involved in the IEB dysfunction in an experimental C57BL/6J mouse model of radiation enteritis. In addition, our previous studies confirmed that the Rho/ROCK signaling pathway was associated with ethanol-induced IEB dysfunction (5,24). The effects of Rho/ROCK have also been found to be reversed following treatment with the ROCK inhibitor Y-27632 (25).

NF-κB is a nuclear transcription factor that regulates the expression of several genes. In addition, several studies have shown that Rho/ROCK signaling pathway may also activate NF-κB pathway (26). Segain et al (27) and Anwar et al (28) demonstrated that Rho/ROCK signaling served a role in the activation of NF-κB in peripheral blood mononuclear cells and endothelial cells, respectively. Other studies in endothelial cells also confirmed that the Rho/ROCK pathway could mediate NF-κB p65 activation (29). Furthermore, Rodriguez et al. (10) showed that Rhoc activated NF-κB via upregulating ROCK1 expression. Therefore, it was hypothesized that ROCK activated NF-κB p65, thereby resulting in IEB dysfunction.

The ROCK protein family comprises two members, ROCK1 and ROCK2. Both members share an overall 64-65% homology
Figure 4. ROCK1 and ROCK2 expression levels in Caco-2 cells treated with ethanol, ROCK1 or ROCK2 siRNAs or the NF-κB inhibitor PDTC. (A) Increased ROCK1 mRNA expression levels in ethanol-treated Caco-2 cells were determined by reverse transcription-quantitative PCR. (B) Representative image and (C) semi-quantification of western blotting showed significantly increased protein expression levels of ROCK1 in ethanol-treated Caco-2 cells. (D) Increased ROCK2 mRNA expression levels in ethanol-treated cultured Caco-2 cells were determined by reverse transcription-quantitative PCR. (E) Representative image and (F) semi-quantification of western blotting showed significantly increased protein expression levels of ROCK2 in ethanol-treated Caco-2 cells. All results were compared with the ethanol group and expressed as the mean ± SD; n=3; *P<0.01, EtOH, ethanol; ROCK, Rho-associated kinase; siRNA, small interfering RNA; PDTC, ammonium pyrrolidine dithiocarbamate; IDV, integrated density values.
Figure 5. NF-κB p65 expression levels in Caco-2 cells treated with ethanol, ROCK1 or ROCK2 siRNAs or the NF-κB inhibitor PDTC. (A) No statistically significant changes were observed in the total NF-κB p65 mRNA expression in the ethanol, ROCK1 siRNA, ROCK2 siRNA, ROCK1 + ROCK2 siRNAs or PDTC treatment groups. (B) Reduced NF-κB p65 protein expression levels in the cytoplasm of ethanol-treated Caco-2 cells were detected using western blot analysis. (C) Increased activity of NF-κB p65 in cultured ethanol-treated Caco-2 cells was evaluated using EMSA. (D) Increased NF-κB p65 protein expression levels in the nuclei of cultured ethanol-treated Caco-2 cells were detected using western blot analysis. All results were compared with the ethanol group and expressed as mean ± SD; n=3; *P<0.01. EtOH, ethanol; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor κB; PDTC, ammonium pyrrolidine dithiocarbamate; ROCK, Rho-associated kinase; siRNA, small interfering RNA; IDV, integrated density values.
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Although ROC1 and ROC2 are structurally similar, it is hypothesized that both isoforms may exhibit different functions. However, their functions have not yet been reported. In the present study, ROC1 and ROC2 siRNAs and a NF-κB inhibitor, PdTc, were used to investigate the expression levels of ROC1, ROC2, NF-κB and aQP8 in Caco-2 cells. Consistent with our previous study (5), the results demonstrated that ethanol treatment reduced the TEER values in the in vitro IEB model, which were restored following transfection of cells with ROC siRNAs and then ethanol treatment, thus suggesting a recovered intestinal epithelial permeability. Furthermore, ROC siRNAs and PdTc downregulated the expression of NF-κB p65 in the cell nucleus, while its expression was increased in the cytoplasm. This finding indicated that both treatments affected the distribution and activity of NF-κB in Caco-2 cells.

Figure 6. AQP8 expression levels in Caco-2 cells treated with ethanol, ROCK1 or ROCK2 siRNAs or the NF-κB inhibitor PdTc. (A) Reduced AQP8 mRNA expression levels in cultured ethanol-treated Caco-2 cells were detected by reverse transcription-quantitative PCR. (B) Representative image and (C) semi-quantification of western blotting showed significantly reduced AQP8 protein expression levels in ethanol-treated Caco-2 cells. All results were compared with the ethanol group and expressed as the mean ± SD (n=3). **P<0.01. EthOH, ethanol; ROCK, Rho-associated kinase; PdTc, ammonium pyrrolidine dithiocarbamate; AQP8, aquaporin 8; siRNA, small interfering RNA.

Figure 7. Distribution of AQP8 in the intestinal epithelial barrier model from each group detected using immunofluorescence. Cell nuclei were stained with DAPI. Scale bar, 100 µm. AQP8, aquaporin 8; PdTc, ammonium pyrrolidine dithiocarbamate; ROCK, Rho-associated kinase; siRNA, small interfering RNA.

in amino acid sequence and 80-92% homology in their kinase domains (30). Although ROC1 and ROC2 are structurally similar, it is hypothesized that both isoforms may exhibit different functions. However, their functions have not yet been reported. In the present study, ROC1 and ROC2 siRNAs and a NF-κB inhibitor, PdTc, were used to investigate the expression levels of ROC1, ROC2, NF-κB and AQP8 in Caco-2 cells. Consistent with our previous study (5), the results demonstrated that ethanol treatment reduced the TEER values in the in vitro IEB model, which were restored following transfection of cells with ROC siRNAs and then ethanol treatment, thus suggesting a recovered intestinal epithelial permeability. Furthermore, ROC siRNAs and PdTc downregulated the expression of NF-κB p65 in the cell nucleus, while its expression was increased in the cytoplasm. This finding indicated that both treatments affected the distribution and activity of NF-κB p65 in Caco-2 cells. In addition, the results revealed that both ROC isoforms had the same effects on NF-κB expression in ethanol-treated Caco-2 cells.

It has also been reported that NF-κB inhibitors upregulate AQP1, AQP3 and AQP8 expression in irritable bowel syndrome (IBS) (13). This finding suggested that the NF-κB pathway regulated the expression of AQP1, AQP3 and AQP8, and that abnormal water metabolism and intestinal permeability could mediate IBS pathogenesis. However, NF-κB inhibition in IBS model rats did not fully restore the intestinal epithelium (13). Furthermore, Duan et al (31) found that the early acute receptor-interacting protein kinase 1
(RIPK1)/NF-κB/AQP8 axis mediated the RIPK1-dependent acinar cell necrosis. Consistent with this, the present study suggested that the NF-κB-mediated regulation of AQP8 expression was likely to be involved in ethanol-induced IEB dysfunction. Following treatment of Caco-2 cells with ROCK siRNAs or PDTC, the expression of AQP8 was significantly increased, indicating that ROCK1, ROCK2 and NF-κB were involved in the regulation of the ethanol-induced increase in IEB permeability. These findings were consistent with the results observed in the IBS model (13). Taken together, the aforementioned results indicated that ROCK may regulate the NF-κB pathway, which in turn mediates the activation AQP8, resulting in the regulation of ethanol-induced IEB dysfunction. However, further research is required to determine the exact molecular mechanism.

There were some limitations in the present study. For example, it could not be verified whether NF-κB was the only intermediate pathway between ROCK and AQP8. In addition, the application of ROCK siRNAs or PDTC did not completely restore the ethanol-induced IEB dysfunction, thus indicating that there are some unknown factors involved in this process. Furthermore, the involvement of a feedback pathway must be investigated. Therefore, the authors aim to explore the role of ROCK/NF-κB/AQP8 signaling pathway and its mechanism of action in future experiments.

In the present study, Caco-2 monolayers were used as a model of the IEB. The study of the role of ROCK and its downstream targets, NF-κB and AQP8, in regulating IEB may lay the foundation for the treatment of ethanol-induced IEB dysfunction.

Taken together, results from the present study suggested that the activation of ROCK/NF-κB/AQP8 signaling pathway in Caco-2 cells may be involved in ethanol-induced IEB dysfunction.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

HZ and JT designed the experiment together. HZ conducted experiments, collected and analyzed data, and wrote the manuscript. XS provided technical support for experiments. JT helped analyze results and gave useful suggestions for modification. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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