Endoplasmic reticulum stress contributed to inflammatory bowel disease by activating p38 MAPK pathway

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Recent evidence suggests that endoplasmic reticulum (ER) stress plays a vital role in inflammatory bowel disease (IBD). Therefore, the aim of this study was to investigate the mechanism by which ER stress promotes inflammatory response in IBD. The expression of Gro-α, IL-8 and ER stress indicator Grp78 in colon tissues from patients with Crohn’s disease (CD) and colonic carcinoma was analyzed by immunohistochemistry staining. Colitis mouse model was established by the induction of trinitrobenzene sulphonic acid (TNBS), and the mice were treated with ER stress inhibitor tauroursodeoxycholic acid (TUDCA). Then the body weight, colon length and colon inflammation were evaluated, and Grp78 and Gro-α in colon tissues were detected by immunohistochemistry. Epithelial cells of colon cancer HCT116 cells were treated with tunicamycin to induce ER stress. Grp78 was detected by Western blot, and chemokines were measured by PCR and ELISA. The expression levels of Grp78, Gro-α and IL-8 were significantly upregulated in intestinal tissues of CD patients. Mice with TNBS induced colitis had increased expression of Grp78 and Gro-α in colonic epithelia. TUDCA reduced the severity of TNBS-induced colitis. In HCT116 cells, tunicamycin increased the expression of Grp78, Gro-α and IL-8 in a concentration-dependent manner. Furthermore, p38 MAPK inhibitor significantly inhibited the upregulation of Gro-α and IL-8 induced by tunicamycin. In conclusion, ER stress promotes inflammatory response in IBD, and the effects may be mediated by the activation of p38 MAPK signaling pathway.

Key words: Inflammatory bowel disease; endoplasmic reticulum stress; IL-8; Gro-α; p38 MAPK.

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Introduction

Inflammatory bowel disease (IBD) is a chronic gastrointestinal inflammatory disorder. Crohn’s disease (CD) and ulcerative colitis (UC) are the two common types of IBD. Despite well-defined clinical manifestations, the pathogenesis of IBD has not been fully elucidated. It is generally accepted that gut microbiome dysbiosis, host immune response, environmental and genetic factors play key role in the initiation and progression of IBD.1-3

Recent studies have shown that endoplasmic reticulum (ER) stress is associated with the susceptibility to IBD.4 Various cellular stress conditions can result in the accumulation of unfolded or misfolded proteins in the ER lumen. The accumulation of unfolded and misfolded proteins in the ER lumen activates unfolded protein response (UPR) to repair protein folding defect and restore homeostasis. UPR signaling is mainly mediated by three protein sensors on the ER membrane: inositol-requiring transmembrane kinase/endonuclease 1 (IRE1), pancreatic ER eIF2α kinase (PERK), and activating transcription factor 6 (ATF6). Upon ER stress, activated IRE1α cleaves a 26-base intron at the dual stem loop structure of an mRNA that is then translated to produce X-box-binding protein 1 (XBP1). XBP1 then induces the expression of genes that regulate protein folding, maturation, transport, phospholipid biosynthesis, and ER expansion to alleviate ER stress. Activated PERK phosphorylates α subunit of eukaryotic translation initiation factor 2 (eIF2α) on Ser51, which then suppresses phosphorylation of genes that regulate protein folding, maturation, transport, phospholipid biosynthesis, and ER expansion to alleviate ER stress.

Chemokines play a central role in the pathogenesis of both CD and UC, and their expression is consistently increased during the active phases of the diseases. In particular, interleukin 8 (IL-8) and its receptor are upregulated in intestinal mucosa.9-12 IL-8 is one of the members of the CXC chemokine family which recruits neutrophil to inflamed sites. Previous studies have confirmed that IL-8 was expressed in the intestinal mucosa of CD, and IL-8 expression level was correlated with disease activity.13,14 Gro-α is another member of CXC family and promotes chemotaxis to neutrophil. Gro-α secretion in the serum increased in patients with UC and CD and then decreased after treatment.15 Gro-α may be an important factor aggravating IBD inflammation, but its expression in IBD intestinal mucosal is still unknown.16 Therefore, the aim of this study was to investigate the mechanism by which ER stress promotes inflammatory response in IBD focusing on the expression of chemokines.

Materials and Methods

Clinical samples

Colonic mucosal tissues were obtained from surgical specimens from 10 patients with CD. Control normal colonic mucosal specimens were obtained from pericarcinomatous tissue 8-10 cm away from the lesion location from 5 patients with colonic carcinoma. The surgical specimens were fixed with formalin, then dehydrated in alcohol, embedded in paraffin, sectioned, stained with hematoxylin-eosin, and mounted.

Animals

Six-week-old female BALB/c mice were purchased from Hunan Slake Jingda Laboratory Animal Co., Ltd (Changsha, China). The mice were acclimatized for 1 week before the experiment and housed individually in a room maintained at 22°C under a 12-h day/night cycle. All animal experiments were reviewed and approved by the Institutional Animal Care Committee of Changsha Central Hospital (Approval No. 20200512). Mouse IBD model was established by the induction with trinitrobenzene sulfonic acid (TNBS) following previous protocol. Briefly, 75 µL of TNBS (5% w/v) and 75 µL of ethanol absolute were fully mixed to form 130 µL of TNBS enema. After fasting for 24 h, the mice were anesthetized by intraperitoneal injection with 10% chloral hydrate (3 mL/kg). Next, Intragastric needle was slowly inserted into the intestinal cavity through the anus and TNBS enema was injected. The mice were inverted for about 5 min to ensure that the enema fluid was fully dispersed in the intestinal cavity. The model mice then received intraperitoneal injection of tauroursodeoxycholic acid (TUDCA) at dose of 50 mg/kg or normal saline as the control every day from the 2nd day after enema. Disease activity index (DAI) score was calculated as the sum of the weight loss score, the diarrhea score and the hematochezia score following previous protocol.17 At the end, all mice were killed by cervical dislocation.

Cell culture and treatment

Epithelial cells of colon cancer HCT116 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) and high glucose supplemented with 10% fetal bovine serum (FBS),10,000 U/mL penicillin and 10,000 µg/mL streptomycin in a humidified incubator with 5% CO2 at 37°C. Cells were treated with 5 µg/mL of tunicamycin (Cell Signaling, Danvers, MA, USA) (1:2,000) in PBST overnight at 4°C. Cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature for 1 h, incubated with 1% BSA (Beyotime Biotechnology) and 1% goat serum (Abcam) (1:100). Then, the cells were incubated with primary antibodies (rabbit polyclonal antibody for Grp78 (1:1,000) and GAPDH (Beyotime Biotechnology, Suzhou, China) (1:2,000) in PBST overnight at 4°C. After washing with PBS, the cells were incubated with secondary antibodies (Abcam) (1:200) at room temperature for 1 h, followed by 10 min of incubation with DAPI (Sigma-Aldrich). Cells were washed with PBS and observed under a fluorescence microscope (Leica, Mannheim, Germany).

Immunohistochemical staining

Paraffin-embedded colonic or ileal sections of patients and mice were cut at a 5 µm thickness. The sections were boiled in 10 mM sodium citrate buffer for antigen retrieval. After blocking endogenous peroxidase with 3% hydrogen peroxide, sections were blocked for 30 min with 3% BSA, and then incubated with primary antibodies for Grp78 (Abcam, Cambridge, UK) (1:500), Gro-α (R&D Systems, Minneapolis, MN, USA) (1:4,000) and IL-8 antibody (R&D Systems) (1:25) for 24 h at 4°C. Phosphate buffered saline was used instead of primary antibodies as negative control. After incubation with horseradish peroxidase labeled secondary antibodies (Abcam) (1:200) at room temperature for 1 h, the sections were visualized with DAB, and counterstained with hematoxylin.

Western blot analysis

Cells were collected and lysed in 150 µL of lysis buffer containing 1% phenylmethylsulfonyl fluoride (PMSF) for 30 min on ice, and centrifuged at 12,000 rpm for 5 min at 4°C. Next, protein samples were separated in 8% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis and transferred onto polyvinylidene fluoride membrane. The membrane was blocked with 3% skim milk solution in phosphate buffered saline containing 0.1% (v/v) Tween-20 (PBST) for 1 h at room temperature, and incubated with primary antibodies for Grp78 (1:1,000) and GAPDH (Beyotime Biotechnology, Suzhou, China) (1:2,000) in PBST overnight at 4°C. The membrane was further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Beyotime Biotechnology) (1:4,000) at room temperature for 1 h, incubated with enhanced chemiluminescence (ECL) solution, and exposed to X-ray films. The protein bands were quantified and analyzed using Image Pro Plus 6.0 software.

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[Supplementary Information]

[Figures and Tables]
PCR

Total RNA from HCT116 cells was extracted using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized for real-time PCR using RevertAid 1st cDNA Synth kit (Thermo Fisher Scientific). PCR primers were synthesized by Sangon (Shanghai, China) with the following sequences: GAPDH forward 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse 5'-GAAGATGGTGATGGGATTTC-3'; Gro-α forward 5'-TCACCCCAAGAACATCCAAA-3' and reverse 5'-TCCTAAGCGATGCTCAAACA-3'; IL-8 forward 5'-TTGGCAGCCTTCCTGATTT-3' and reverse 5'-TCAAAAACTTCTCCACAACC-3'. The amplification protocols were as follows: 95°C for 10 min, 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. GAPDH was used to normalize gene expression. Relative mRNA levels were calculated using the 2−ΔΔCt method.

ELISA

The supernatants were collected from cells, and IL-8 levels in the supernatants were determined using Human IL-8/RANTES DuoSet kit (4A Biotech Co. Ltd., Beijing, China) following the manufacturer’s instructions.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8.0, and all values were presented as means ±SD. Student’s t-test were used to compare differences between groups, and p<0.05 was considered significant.

![Figure 1](image)

**Figure 1.** ER stress marker Grp78 and chemokines IL-8 and Gro-α were upregulated in the inflamed mucosa of CD patients. A) Representative staining of Grp78 in intestinal sections from colon mucosa of control (left) and CD patients (right). B) Representative staining of Gro-α in intestinal sections from colon mucosa of control (left) and CD patients (right). C) Representative staining of IL-8 in intestinal sections from colon mucosa of control (left) and CD patients (right). Staining intensity of positive area was quantified by image J software and illustrated as bar charts (n=5); *p<0.05 compared to control.
Figure 2. Treatment of TUDCA alleviated TNBS-induced colitis in mice. A) Colon length; a, control; b, TNBS; c, TNBS+ TUDCA. B) Disease activity index score (DAI). C) Histological scores based on hematoxylin and eosin (H&E) staining of colon sections. Values were expressed as means ± SD (n=6); *p<0.05 vs control; #p<0.05 vs TNBS treated mice.
Results

Upregulation of ER stress marker and chemokines in intestinal tissues of CD patients

To determine whether ER stress and chemokines are involved in CD, we performed immunohistochemistry staining for ER stress marker Grp78 and two chemokines IL-8 and Gro-α. Grp78, Gro-α and IL-8 were mainly localized to the epithelial lining of the gut and in Paneth cells, and were also localized in inflammatory cells. Optical density analysis showed that the positive rates of Grp78, Gro-α and IL-8 staining in inflamed intestinal tissues of CD patients were higher compared with pericarcinomatous tissue (Figure 1 A-C).

TUDCA alleviated TNBS-induced colitis in mice

To confirm the involvement of ER stress and chemokines Gro-α and IL-8 in the development of IBD, we established mouse colitis model induced by TNBS. TNBS treated mice had significantly lower body weight, decreased colon length (Figure 2A) and higher disease activity index score (DAI) than control mice (Figure 2B). Histological analysis revealed that TNBS treated mice developed serious colitis (Figure 2C). However, ER stress inhibitor TUDCA improved colon shortening in TNBS induced mice (Figure 2A). Moreover, signs of colitis were markedly ameliorated, as evidenced by decreased DAI and histological score (Figure 2 B,C).

TUDCA inhibited the upregulation of Grp78 in colonic epithelia of mice

Furthermore, immunohistochemical staining of the colon tissues of the three groups of mice showed that the expression of Grp78 in TNBS model group was significantly higher than that of vehicle control group, but the expression of Grp78 in TNBS+TUDCA group was significantly lower than that of the TNBS model (Figure 3 A-D). These data indicated that TUDCA relieved ER stress in mouse colitis model.

Tunicamycin induced ER stress and increased Gro-α and IL-8 expression in HCT116 cells

To reveal the relationship of ER stress and chemokines in intestinal inflammation, human epithelial cells of colon cancer were cultured and treated with different concentrations of tunicamycin to induce ER stress. PCR analysis showed that tunicamycin increased the expression of Gro-α and IL-8 at mRNA levels in HCT116 cells (Figure 4 A,B). Western blot analysis of ER stress marker Grp78 confirmed that tunicamycin treated cells had higher expression of Grp78 compared to vehicle treated control cells (Figure 4C). Furthermore, ELISA showed that IL-8 levels in the medium harvested from HCT116 cells treated with tunicamycin were higher compared to vehicle treated HCT116 cells (Figure 4D). Collectively, these data demonstrated that tunicamycin induced ER stress and increased expression of chemokines Gro-α and IL-8 in vitro.

p38 MAPK signaling contributed to tunicamycin-induced ER stress and increased expression of GRO-α and IL-8 in HCT116 cells

To identify signaling pathway responsible for the upregulation of Gro-α and IL-8 by tunicamycin, we used p38 MAPK kinase inhibitor SB203580. HCT116 were treated with different concentrations of SB203580 (5, 20, 50 µM) in the presence or absence of tunicamycin, and supernatants were harvested 24 h after treatment. PCR analysis showed that SB203580 significantly decreased the...
expression of Gro-α and IL-8 at mRNA levels in HCT116 cells treated with TM (Figure 5 A,B). Western blot analysis of ER stress marker Grp78 confirmed that SB203580 inhibited TM induced expression of Grp78 compared to TM treated cells (Figure 5C). Furthermore, ELISA showed that IL-8 levels in the medium harvested from HCT116 cells treated with SB203580 were significantly lower compared to TM treated HCT116 cells (Figure 5D). Collectively, these data suggested that p38 MAPK signaling may mediate ER stress induced Gro-α and IL-8 expression in intestinal epithelial cells.

Discussion

In this study, we provide evidence that chemokines and ER stress played significant role in intestinal inflammation. A variety of chemokines have emerged as essential molecules in the pathogenesis of IBD. Multiple chemokines including Gro-α (CXCL1), ENA-78 (CXCL5), GCP2 (CXCL6) and IL-8 (CXCL8) are positively correlated with the inflammatory state in IBD patients. IL-8, a classical pro-inflammatory chemokine, plays a role in the recruitment of neutrophils from the vasculature to the sites of infection or tissue injuries, and it has been shown that CXCL1 and CXCL8 were upregulated in colonic IBD. By immunohistochemical staining we demonstrated that the expression of Grp78, Gro-α and IL-8 significantly increased in intestinal tissues of CD patients, compared with pericarcinomatous tissues.

Tunicamycin, a common inducer of ER stress, was previously shown to activate ER stress in intestinal epithelial cells. In contrast, TUDCA can significantly reduce ER stress in colonic epithelial cells, and inhibit DSS-induced colon inflammation in mice. Our results showed that the expression of Grp78 was upregulated by tunicamycin in a concentration dependent manner. In a mouse model of IBD induced by TNBS, inhibition of ER stress by TUDCA could reduce DAI score, increase survival rate, increase the colon length, maintain body weight, reduce the infiltration of inflammatory cells in the intestinal tissue, and alleviate TNBS-induced enteritis in mice. In addition, TUDCA inhibited the upregulation of Grp78 induced by tunicamycin in the colonic epithelium of mice.
Recent evidence suggests the role of MAPK signaling in the response to ER stress. For example, p38 was shown to promote ER stress induced apoptosis in fibroblasts and inflammation in macrophages. In addition, p38 can activate the transcription of CHOP through ATF6. During inflammation, IL-17 enhanced TNF-α induced CXCL8 expression through p38 MAPK pathway, while p38 inhibitor SB203580 reversed the effect of IL-17 on CXCL8 mRNA, indicating that p38 MAPK signaling mediates the stabilization of CXCL8 mRNA by IL-17. In this study, we found that SB203580 significantly inhibited the expression of ER stress marker Grp78 and chemokines Gro-α and IL-8 as well as the secretion of IL-8 in HCT116 cells treated with tunicamycin. Our findings suggested that p38 MAPK signaling may mediate ER stress induced inflammation in intestinal epithelial cells.

In conclusion, ER stress promotes inflammatory response in IBD, and the effects may be mediated by the activation of p38 MAPK signaling. Targeting p38 MAPK signaling may be a novel approach to the treatment of IBD.

References

1. Kaser A, Zeissig S, Blumberg R S. Inflammatory bowel disease. Annu Rev Immunol 2010;28:573-621.
2. Ananthakrishnan AN. Epidemiology and risk factors for IBD. Nat Rev Gastroenterol Hepatol 2015;12:205-27.
3. De Souza HSP, Fiocchi C, Iliopoulos D3 The IBD interactome: an integrated view of aetiology, pathogenesis and therapy. Nat Rev Gastroenterol Hepatol 2017;14:739-49.
4. Cao SS. Endoplasmic reticulum stress and unfolded protein response in inflammatory bowel disease. Inflamm Bowel Dis 2015;21:636-44.
5. Ma X, Dai Z, Sun K, Zhang Y, Chen J, Yang Y, et al. Intestinal epithelial cell endoplasmic reticulum stress and inflammatory bowel disease pathogenesis: An update review. Front Immunol 2017;8:1271.
6. Cao SS. Epithelial ER stress in Crohn's disease and ulcerative colitis. Inflamm Bowel Dis 2016;22:984-93.
7. Luo K, Cao SS. Endoplasmic reticulum stress in intestinal epithelial cell function and inflammatory bowel disease. Gastroenterol Res Pract 2015;2015:328791.
8. Kulkarni N, Pathak M, Lal G. Role of chemokine receptors and intestinal epithelial cells in the mucosal inflammation and tolerance. J Leukoc Biol 2017;101:377-94.
9. Wu M, Sun M, Lai Q, Lu Y, Fu Y, Peng Y, et al. Chemokine ligand 13 expression is abundant in the tumor microenvironment and indicates poor prognosis of kidney clear cell carcinoma. Biocell 2021;45:589-97.
10. Danese S, Gasbarrini A. Chemokines in inflammatory bowel disease. J Clin Pathol 2005;58:1025-7.
11. Uguccioni M, Gionchetti P, Robbiani DF, Rizzello F, Peruzzi S, Campieri M, et al. Increased expression of IP-10, IL-8, MCP-1, and MCP-3 in ulcerative colitis. Am J Pathol 1999;155:331-6.
12. Katsuta T, Lim C, Shimoda K, Shibuta K, Mitra P, Banner BF, et al. Interleukin-8 and SDF1-alpha mRNA expression in colonic biopsies from patients with inflammatory bowel disease. Am J Gastroenterol 2000;95:3157-64.
13. Ina K, Kusugami K, Yamaguchi T, Imada A, Hosokawa T, Ohsuga M, et al. Mucosal interleukin-8 is involved in neutrophil migration and binding to extracellular matrix in inflammatory bowel disease. Am J Gastroenterol 1997;92:1342-6.
14. Mahida YR, Ceska M, Effenberger F, Kurlak L, Lindley I and Hawkey CJ. Enhanced synthesis of neutrophil-activating peptide-1/interleukin-8 in active ulcerative colitis. Clin Sci (Lond) 1992;82:273-5.
15. Alzoghaibi MA, Al-Mofleh IA, Al-Jebreen AM. Neutrophil chemokines GCP-2 and GRO-alpha in patients with inflammatory bowel disease. J Dig Dis 2008;9:144-8.
16. Mitsuyama K, Tsuruta O, Tomiyasu N, Takaki K, Suzuki A, Masuda J, et al. Increased circulating concentrations of growth-related oncogene (GRO)-alpha in patients with inflammatory bowel disease. Dig Dis Sci 2006;51:173-7.
17. Shen YM, Xie YH, Zhao Y, Zeng Y. Pim-1 inhibitor attenuates trinitrobenezene sulfonic acid induced colitis in the mice. Clin Res Hepatol Gastroenterol 2018;42:382-6.
18. da Paz Martins AS, Campos SBG, Goulart MOF, Moura FA. Extraintestinal manifestations of inflammatory bowel disease, nitrooxidative stress and dysbiosis: What is the link between them? Biocell 2021;45:461-81.
19. Neurath MF. Cytokines in inflammatory bowel disease. Nat Rev Immunol 2014;14:329-42.
20. Puleston J, Cooper M, Murch S, Bid K, Makh S, Ashwood P, et al. A distinct subset of chemokines dominates the mucosal chemokine response in inflammatory bowel disease. Aliment Pharmacol Ther 2005;21:109-20.
21. Akiyama T, Oishi K, Wullaert A. Bifidobacteria prevent tunicamycin-induced endoplasmic reticulum stress and subsequent barrier disruption in human intestinal epithelial caco-2 monolayers. PLoS One 2016;11:e0162448.
22. Cao SS, Zimmermann EM, Chuang BM, Song B, Nwokoye A, Wilkinson JE, et al. The unfolded protein response and chemical chaperones reduce protein misfolding and colitis in mice. Gastroenterology 2013;144:989-1000.
23. Darling NJ, Cook SJ. The role of MAPK signaling pathways in the response to endoplasmic reticulum stress. Biochim Biophys Acta 2014;1843:2150-63.
24. Lee JW, Wang P, Kattah MG, Youssuf S, Steinman L, DeFea K, et al. Differential regulation of chemokines by IL-17 in colonic epithelial cells. J Immunol 2008;181:6536-45.