Paroxetine mediate macrophage polarization by regulating GRK2-EP4-cAMP-pCREB signaling pathway in treating mice with DSS-induced colitis

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Abstract

Objective and design This study aimed to investigate the role of GRK2 in macrophage polarization of ulcerative colitis and to detect whether paroxetine could alleviate the symptoms of DSS-induced colitis in mice by regulating GRK2 translocation to affect macrophage polarization.

Subjects After informed consent, colonic biopsies were obtained from a total of 22 patients with ulcerative colitis group and 22 volunteers who have received colonoscopy as control group. GRK2 heterozygous mice on the C57BL/6J background and WT littermates were used in this study. In vitro experiments were conducted in THP-1 cell.

Methods Lamina propria mononuclear cells (LPMCs) were isolated from surgically resected colonic tissue by using enzymatic technique. THP-1 cells were stimulated by PMA (100 ng/ml) for 48 h to differentiate into macrophages (THP-M). Experimental colitis was induced in mice by administrating 3.5% dextran sodium sulfate for consecutive 7 days. Pathological changes in the colon tissues were assessed by hematoxylin and eosin staining. The levels of inflammatory factors, including PGE2, cAMP, IL-1β, and IL-10 were determined by enzymelinked immunosorbent assay. The expression levels of GRK2, EP4, and pCREB proteins were measured by Western blot analysis. The mRNA levels of IRF5 and IRF4 were measured by real-time quantitative polymerase chain reaction. The levels of CD68, CD86, CD206 and F4/80 were detected by flow cytometric analysis.

Results

UC patients showed higher PGE2 level and higher M1/M2 ratio than control group patients. In mice, the absence of GRK2 prevented higher disease activity index DAI and higher spleen index. In THP-1, the pIRES-EGFP-GRK2 plasmids transfection enhanced the release of IL-1β and increased the ratio of M1/M2. Paroxetine could influence macrophage polarization by down-regulating EP4/cAMP/pCREB-dependent GRK2 translocation, and alleviated the symptoms in mice with DSS-induced colitis.

Conclusion GRK2 mediates the changes of PGE2-EP4-cAMP-pCREB pathway may influence M2 polarization in LPMCs of UC patients. Paroxetine alleviated the symptoms in mice with DSS-induced colitis and maybe a potential target for UC.

Introduction

Ulcerative colitis (UC) was been known as a chronic complex inflammatory bowel disease usually involved the sigmoid colon and rectum with unclear etiology[1]. Together with Crohn's disease (CD) are often identified as inflammatory bowel disease (IBD). In recent decades, the incidence of UC is showing an increasing trend. The highest incidence of UC was 57.9/100,000 person-years in Northern Europe[2]. Meanwhile, the risk of UC has rapidly increased in newly industrialized nations and defined as a substantial burden to the medical system[3]. Until now, the host's microbiome, genetics, and immune response are all been accepted as essential risk factors for the development of UC[4]. Although, the exact
pathogenesis of UC is still an unanswered question. The destruction of the intestinal mucosal barrier maybe one of the main pathogenesis[5]. When the mucosal barrier function had been destroyed, bacterial antigens rapidly invade the colon tissue to activate the mucosal immunity and exacerbates the injury of colon.

Monocytes and macrophages always play important roles in the innate immune response against invade pathogens. They eliminate pathogens by phagocytosis and by releasing inflammatory mediators, such as cytokines, chemokines, and proteases[6]. Although the sources and phenotypes of macrophages are not the same everywhere, they all maintain a dynamic balance. The destruction of balance may cause various diseases. Under the induction of variety factors, polarized macrophages can be form different functional phenotypes: classically M1-type macrophages with proinflammatory functions and M2-type macrophages with anti-inflammatory functions[7]. The M1 macrophages are characterized by the Th1-type immune response and the secretion of pro-inflammatory cytokines, whereas the M2 macrophages would involved in the Th2-type immune response. As a consequence, inappropriate activation of macrophages polarization can induce sustained inflammation resulting in autoimmune and inflammatory diseases. In our previous study, analysis of colonic tissue samples from UC patients showed the infiltration of macrophages. The M1/M2 ratio presented in the intestinal mucosa markedly increased in UC. Thus, the regulation of the balance of M1/M2 may important for the treatment of UC.

G protein-coupled receptor kinases are one large group of kinases that cause rapid desensitization after binding to GPCRs. They belong to the serine/threonine-protein kinase family and is considered to be the important negative regulatory protein in the GPCRs signaling pathway [8]. GRKs are also widely distributed in the digestive tract tissues[9]. Prostaglandin E2 (PGE2) is an inflammatory mediator that plays essential role in the occurrence and development of UC[10]. Study found that PGE2 can increase the level of cAMP in the cytoplasm, further activated CREB, and then promoted the polarization of M2 macrophages[11]. GRK2 is considered to be the important negative regulatory protein in the GPCRs signaling pathway. Due to the influence of the polarization of macrophages, the regulation of GRKs may be the new therapeutic strategy for UC.

Paroxetine, a representative of serotonin reuptake inhibitors, is often used for mental disorders such as generalized anxiety disorder, postpartum depression, and also with fewer side effects than the first-generation selective 5-HT reuptake inhibitors [12]. Other researchers have shown that paroxetine could improve cardiac function in heart failure after acute myocardial infarction by inhibiting GRK2 activity[13]. Previous studies demonstrated that selective 5-HT reuptake inhibitors have therapeutic effects for UC patients with severe emotional problems[14]. Gut-brain axis dysregulation has been recognized as a crucial role to the understanding of chronic gastrointestinal disease. Recently, IBD is considered to be a disorder of multi-crosstalk pathway with the gut–brain axis[15]. Some studies showed that paroxetine could alleviate psychological comorbidities to improve IBD patients’ quality of life[16].

The effect of GRK2 in the polarization of macrophages by regulating downstream signaling pathways was still unknown in UC. In this study, we investigated how GRK2 influences the polarization of
macrophages by PGE2-EP4-cAMP-pCREB signaling pathways in UC patients. Using DSS-induced colitis mice, and THP-1 cell line, we demonstrated that GRK2 is involved in the polarization of macrophages and provided evidence for the new mechanism of paroxetine in the treatment of UC.

Materials And Methods

Patients and Clinical score

The present study was approved by the Research Ethics Committee of First Affiliated Hospital of Anhui Medical University (No.5101449, China) and written informed consents were provided by all patients. The study was performed following the Declaration of Helsinki. After informed consent, a total of 22 patients with UC (UC group) and 22 volunteers who have received colonoscopy (Control group) at the First Affiliated Hospital of Anhui Medical University (Anhui, China) between Jan 2016 and Dec 2018 were recruited into this research and had collected complete clinical data. Patients were diagnosed based on endoscopic and histological criteria. Inclusion criteria for the study were: UC diagnosed patients, 18–75 years-old, No history of gastrointestinal surgery. Exclusion criteria for this study were those with non-classifiable IBD, indeterminate colitis, infectious colitis, and patients who have undergone gastrointestinal surgery. Colon specimens and peripheral blood were obtained from UC patients and volunteers. The disease activity index of patients was obtained based on Mayo score for UC.

Materials

Paroxetine was purchased from Abcom (ab120069). Salicylazosulfapyridine (SASP, Lot#: 230014) was purchased from Shanghai XinYi Pharmaceutical Co., Ltd (Shanghai, China). Dextran sulfate sodium(DSS, MW: 36000–50000) was obtained from MP Biomedicals (California, USA). Antibodies for GRK2(80Kd, Lot#: Sc-13143) and EP4(55Kd, Lot#: Sc-55596) were purchased from Santa-cruz (Rosemont, USA). Antibodies for iNOS(131kd, Lot#: GTX130246) was purchased from GeneTex(Rosemont, USA). Antibodies for Arg-1(40Kd, Lot#: 93668), and pCREB (43Kd, Lot#: 9198) were purchased from Cell Signaling Technology Co., Ltd. (USA). cAMP Activity Assay Kit was obtained from BioVision Inc (San Francisco, USA). Antibodies for β-actin (Lot#: E021020), Anti-rabbit IgG (Lot#: E030120) and Anti-mouse IgG (Lot#: E030110) were obtained from EarthOx Life Sciences (California, USA). Alexa Fluor 488- and Alexa Fluor 594-tagged second antibodies were purchased from Proteintech (Chicago, U.S.A.). Serum levels of PGE2, IL-1β IL-10 and IFN-γ were determinated by Elisa following manufacturer's protocol (Enzyme-linked Biotechnology Co., Ltd., Shanghai, China). All other reagents and chemicals were of commercially available analytical grade.

Histological analysis

We used 4% paraformaldehyde to fix colon specimens overnight, and then used paraffin to embed. The tissues were cut into 4μm thick sections for routine hematoxylin and eosin (H&E) staining. Two pathologists who were blinded to this study evaluated all sections, and then we captured the images with an optical microscope (OLYMPUS, Tokyo, Japan).
Immunofluorescence assay of colonic mucosal tissue

We first used fresh colonic mucosal tissue to prepare frozen section (Leica, CM1950, Germany). Then the sections were permeabilized by Triton-X-100 (Beyotime) for 7 min, then blocked with 10% bovine serum (CWBO) for 30 min and finally incubated with monoclonal anti-GRK2 and anti-EP4 receptor antibodies overnight at 4°C in a wet chamber. After being washed by PBS, samples were incubated with Alexa Fluor 488- and Alexa Fluor 594-tagged second antibodies at 37°C for 1 h and subsequently incubated DAPI (10 µg/ml) for 7 min. Images were captured under a TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Preparation of lamina propria mononuclear cells (LPMCs) from colonic mucosa

LPMCs were isolated from surgically resected colonic tissue by using enzymatic technique[17]. Firstly, we use normal saline to remove blood and feces on the colonic tissue surface. Then we dissected mucosa with HBSS (Gibco, Carlsbad, CA, USA) supplemented with 1 mM dithiothreitol (Sigma, St. Louis, MO, USA) to remove mucus. Secondly, we use sterile ophthalmological scissors to cut mucosa into crumbs, and then incubated for 2 h in a PBS culture medium (Thermo, South Logan, UT, USA) with 0.5 mg/mL type IV collagenase and 0.5 mg/mL DNase I (Roche, Indianapolis, IN, USA). Finally, we separated the cellular fraction once through 10% red blood cell lysate solution, and target cells were centrifuged over Ficoll–Hypaque density gradient (GE, Uppsala, Sweden). Target LPMCs population was collected for flow cytometry.

Mice

GRK2 heterozygous mice on the C57BL/6J background were obtained from GemPharmatech (Nanjing, China). GRK2+/− heterozygous and WT littersmates (20 ± 2 g) were used for the indicated experiments and were bred at the experimental animal center of Anhui Medical University (Hefei, China). Animals were housed at the temperature of 23 ± 2°C and humidity of 55 ± 10% specific pathogen-free environment with a 12 h light/dark cycle and given standard laboratory diet and water. Experimental protocols were approved by the Animal Experimental Ethics Committee of Anhui Medical University (No. 20220132) and strictly followed the ethical regulation of the Committee for Animal Care and Use at Anhui Medical University and the Guide for the Care and Use of Laboratory Animals (NIH, United States). All efforts were led to minimize animals’ suffering and to reduce the number of animals used. Animals were executed by cervical vertebra luxation, and sealed and stored in animal carcass dedicated freezer.

Establishment of DSS-induced colitis mice model and evaluation of disease activity index (DAI)

After acclimatizing for at least one week, mice were randomly divided into 4 groups (n = 6): GRK2+/+ H2O group, GRK2+/+ DSS group, GRK2+/− H2O group, GRK2+/− DSS group. Experimental colitis was induced in mice by administrating 3.5% dextran sodium sulfate for consecutive 7 days as previously described[18],
while the control mice were given the same volume of distilled water. The experimental time lines of the animal model are described in Fig. 3A. During 3.5% DSS treatment, mice were examined daily and scored for disease activity index under the following criteria (Supplementary Table 1). At the time of harvest, splenic weight and colon length were additionally measured.

**Histopathological assessment**

At harvest, we first removed the fecal content, then colonic tissues were dehydrated and embedded in paraffin after fixed in 4% paraformaldehyde. The tissues were sliced into 4µm thick sections, and stained with H&E. Two pathologists who were blinded to this study performed the evaluation. The degree of damage with colonic samples was quantified according to the scoring system. In colonic tissues, the histological score was calculated by three parts, including the degree of inflammation, the extent of inflammation and the degree of crypt damage (Supplementary Table 2). It resulted in a histological score ranging from 0 (not damaged) to 10 (severe damaged).

**Cells culture and transfections**

Human THP-1 cells were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, and were cultured in RPMI 1640 medium (Gibco, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY) and 1% penicillin/streptomycin (Gibco, USA). Cells were cultured in a humidified 5% (v/v) CO2 atmosphere at 37°C. THP-1 cells were stimulated by PMA (100 ng/ml) for 48 h to differentiate into macrophages (THP-M). pIRES-EGFP-Ctr and pIRES-EGFP-GRK2-WT plasmids were obtained from our lab. THP-M cells were transiently transfected with different plasmids by Lipofectamine 3000 (Invitrogen). We used immunoblot analysis of whole cell lysates by specific antisera to confirm the transient expression. After transfection at 37°C in culture medium without serum, THP-M cells with PGE2 (10 µM) stimulation was performed 30 mins, during the indicated time periods.

**Immunofluorescence assay of cells culture**

We fixed freezing section of colon tissue (6 µm) (Nest) by acetone, washed in 1% PBS-Tween, permeabilized by Triton-X-100 (Beyotime, China) for 7min (F4/80 without this step), then blocked with 10% goat serum (CWBIo, China) for 30min and finally incubated with prime antibody in PBS containing 1% BSA (1:100) overnight at 4°C. Cells were planted in Laser confocal plate. After being washed by PBS (30s×3), samples were incubated with Alexa Fluor 488 or 594 antibody IgG at 37°C for 1h and subsequently incubated DAPI (10 µg/ml) for 5min. Images were acquired by confocal laser-scanning microscope (Olympus, Lake Success, NY).

**Flow cytometric analysis**

Specific antibodies against CD68, F4/80, CD86, CD206 and isotype-matched control antibodies were purchased from e-Biosciences (San Diego, CA, USA). For cell staining, $1 \times 10^6$ freshly isolated cells were incubated with fluorescent-conjugated specific antibodies against CD68, CD86, CD206 and F4/80, or
isotype-matched antibodies for 30 min on ice. Cell surface fluorescence intensity was assessed using a FACS Calibur analyzer and CellQuest software (BD Biosciences, San Jose, CA, USA).

**Western blotting analysis**

Total proteins were extracted from colon tissue or cells with different treatments in lysis buffer (Beyotime, China) containing phosphatase inhibitor cocktail (CWBio, China) and protease inhibitor (Beyotime), and centrifuged at 14,000g for 15 min at 4°C. Collecting the supernatant and added the protein loading buffer (5×), then the sample was boiled for 7 min. Membrane proteins preparation: colon tissue or cells were lysed and centrifuged at 14,000g for 15 min at 4°C. Collecting the supernatant and centrifuged at 100,000g for 1 h at 4°C. After removing the supernatant, the precipitated membrane protein was resuspended by 50µL cell lysis buffer, then the samples were boiled for 7 min with 10 µL protein loading buffer (5×). Protein concentration was determined by using the BCA protein assay (Thermo, Waltham, MA). The denatured protein was separated by 10% SDS-PAGE and then transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA) using a semi-dry transfer system (Bio-rad, Hercules, CA). After blocking with 5% non-fat powdered milk (BBI Life Sciences, Shanghai, China) for 2h at 37°C, proteins were detected by using specific antibodies of GRK2, EP4, pCREB, iNOS and Arg-1 overnight at 4°C followed by HRP-conjugated anti-mouse or anti-rabbit secondary antibodies for 1h at 37°C. The protein was visualized by enhanced chemiluminescent HRP substrate (Millipore) via chemoluminescence and was quantified using Image J software (NIH).

**Real-time PCR**

Total RNA was extracted from colon tissue and THP-1 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Then Real-time RT-PCR was performed as following protocol: RNA samples were reverse transcribed to cDNA and subjected to quantitative PCR, which was performed with the Light Cycler_ 96 Real-Time PCR System (Roche, Basel, Swiss) using AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China). The program for amplification was 1 cycle of 95°C for 2 min, then 40 cycles of 95°C for 10 s, 60°C for 30 s, and 95°C for 10 s. The PCR data of each gene was normalized to the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression and were quantified by the ΔΔCT method. The primer sequences used in this study were listed as followings(Supplementary Table 3):

**Statistical Analysis**

All results were analyzed by using Prism version 7.00 software (GraphPad Software, USA). A one-way ANOVA with Dunnett’s Multiple Comparison test was applied to analyze the significance between multiple groups and an unpaired Student’s t-test was applied to analyze the significance between two groups. Statistical significance was indicated by a $P$-value of $< 0.05$. Data were expressed as the Mean ± SEM of three independent experiments.

**Results**
Macrophage polarization in UC

From the representative H&E staining images of UC colon tissue, we found damage evidences from destruction of epithelial structures, and surface contour changes, including villiform contours, loss of crypts, and crypt abscess (Fig. 1A). To investigate the density of macrophages in the UC colonic tissue, macrophages were labeled with CD68, and the expression difference between the control group and the UC group was observed using a fluorescence microscope (Fig. 1B). The results showed that the expression of CD68 in the colonic lamina propria of the UC group was significantly stronger than the control group. We then further investigated the proportion of M1 macrophages and M2 macrophages, which have been found to play an important role in the development of colitis. The data showed that in the UC group, the infiltration of M1 macrophages was significantly higher than M2 macrophages in the LPMCs. Compared with the control group, the M1/M2 ratio increased significantly in the UC group (Fig. 1C). Meanwhile, we analyzed the gene expression of M1/M2 specific factors in LPMCs of UC patients. The results showed that mRNA expressions of M1 macrophage-associated factor IRF5, was significantly increased while the levels of M2 macrophage-associated markers IRF4 was markedly decreased in the UC group (Fig. 1D). Western blot (WB) revealed that iNOS expression in the UC group was greatly increased and Arg-1 expression was reduced (Fig. 1E). Taken together, these data imply that the balance between M1 and M2 macrophages was interrupted in UC patients.

GRK2 mediates the activation of PGE2-EP4-cAMP-pCREB pathway in colonic LPMCs of UC patients

Firstly, we investigated the co-localization of GRK2 and EP4 in colon tissue, we used 488 nm green fluorescent labeling EP4, 594 nm red fluorescent labeling GRK2. Immunofluorescence showed that GRK2 and EP4 were mainly distributed on the membrane and cytoplasm, according to the Merge diagram, in the UC group, the co-binding of GRK2 and EP4 was significantly increased on the membrane compared with the control group (Fig. 2A). As shown in Fig. 2D, the membrane expression of GRK2 remarkably increased in colonic LPMCs of the UC group, Conversely, the membrane expression of EP4 decreased, suggesting that increased GRK2 transmembrane lead to excessive desensitization of EP4 receptors. Elisa's results indicate that the level of PGE2 was increased in colonic LPMCs of the UC group, and the trend had a positive correlation with Mayo score significantly ($P < 0.0001$, Fig. 2B). The levels of pro-inflammatory cytokines like IL-1β was also elevated, in contrast the anti-inflammatory cytokine like IL-10 was dropped in UC group(Fig. 2C). To further illuminate whether the cAMP-pCREB signal rebalanced the macrophage polarization, we detected the cAMP in LPMCs by Elisa. The result reported that the cAMP was decreased significantly in the UC group (Fig. 2F). WB showed that the expression of pCREB was also decreased in LPMCs (Fig. 2E). Therefore, we speculated that the high level of PGE2 stimulated the receptor EP4, and enhanced the transmembrane of GRK2 in LPMCs, resulting in EP4 over-desensitization.

GRK2 heterozygous mice are protected from DSS-Induced Colitis
In order to investigate the role of GRK2 in the progression of colitis, we selected GRK2\(^{+/−}\) heterozygous and WT littermates. Then we established the mice model via feeding with 3.5% DSS solution for consecutive 7 days (Fig. 3A). Following 3.5% DSS treatment, GRK2\(^{+/+}\) mice lost significant body weight beginning at day 3 and displayed signs of severe disease such as loose and bloody stool, ruffled hair, hunched posture, etc. Compared with GRK\(^{+/−}\)-DSS group, mice in the GRK2\(^{+/+}\)-DSS group lost their bodyweight significantly (Fig. 3B) and had higher DAI scores (Fig. 3C). DAI is a key indicator to evaluate the severity of colitis, including weight loss, rectal bleeding and stool consistency. Moreover, GRK2\(^{+/−}\)-DSS mice had longer colons and lower spleen-index than GRK2\(^{+/+}\)-DSS mice (Fig. 3D,E). H&E staining of the colons showed that compared with GRK2\(^{+/−}\)-DSS mice, GRK\(^{+/+}\)-DSS mice had the more serious situation of loss of epithelial crypts, structural disorders, extensive infiltration of inflammatory cells in the submucosa (Fig. 3F) and higher pathological score (Fig. 3G). As these results showed that GRK2\(^{+/−}\) Mice were significantly protected from DSS-Induced Colitis.

**GRK2 regulated macrophage polarization through PGE2-EP4-cAMP-pCREB pathway in DSS-induced colitis model**

To determine the GRK2-dependent regulation of macrophage polarization, we collected fresh mice colon tissue to extract LPMCs when the mice were euthanasia on day 8. Then, we examined the level of protein expression using WB and found that compared with the GRK2\(^{+/+}\)-DSS group, iNOS expression was greatly decreased and Arg-1 expression was increased in GRK2\(^{+/−}\)-DSS group (Fig. 4A). To investigate the impact of GRK2 knockout on the EP4-cAMP-pCREB pathway in the DSS-induced colitis model, the level of cAMP was decreased significantly in the GRK2\(^{+/+}\)-DSS group (Fig. 4D). Further, WB results showed that compared with the GRK2\(^{+/+}\)-DSS group, the EP4 membrane expression and the pCREB expression were increased significantly in the GRK2\(^{+/−}\)-DSS group (Fig. 4B and C). To further investigate the EP4 expression difference between the GRK2\(^{+/+}\)-DSS group and the GRK2\(^{+/−}\)-DSS group was observed using a fluorescence microscope (Fig. 4E). The results showed that the expression of EP4 in the LPMCs of the GRK2\(^{+/−}\)-DSS group was significantly stronger than in the GRK2\(^{+/+}\)-DSS group.

As can be seen from above, GRK2 may be the key factor affecting macrophage polarization, to further explore the impact of altering GRK2 protein activity on GRK2-mediated EP4 desensitization and cAMP-pCREB activation, we chose THP-1 cells model, which was stimulated PMA (100ng/ml) for 48 hours to induce M0 macrophages (Fig. 5B) and then transiently transfected with pIRES-EGFP-ctr and pIRES-EGFP-GRK2-WT plasmids (Fig. 5A). Fluorescence microscopy results showed that GRK2 plasmid was successfully expressed in THP-1 cells. With the stimulation of PGE2 (10 µM) for 30 min, the ratio of M1 (CD86\(^{+}\)CD68\(^{+}\)) / M2 (CD206\(^{+}\)CD68\(^{+}\)) increased significantly in the GRK2 overexpress group comparing with non-overexpress group (Fig. 5C). Meanwhile in the GRK2 overexpress group, the iNOS level increased and Arg-1 and pCREB levels decreased significantly comparing with non-overexpress group (Fig. 5E). After the stimulation of PGE2 (10 µM), EP4 membrane expression decreased significantly in the GRK2 overexpress group compared with non-overexpress group (Fig. 5D). Elisa's results showed that the cAMP
level also has the same trend (Fig. 5F). These results suggest that GRK2 translocation to the membrane is related to EP4 over-desensitization, and GRK2 maybe the key factor affecting macrophage polarization through the PGE2-EP4-cAMP-pCREB pathway.

**Paroxetine alleviated the symptoms of DSS-induced colitis in mice**

The chemical structure of paroxetine is presented in Fig. 6A. We further explored whether paroxetine attenuated DSS-induced colitis in mice. We established the mice model by feeding with 3.5% DSS for consecutive 7 days. Compared with the control group, mice in the DSS group lost bodyweights significantly. While, Paroxetine and SASP as a positive control, rescued the loss of body weights (Fig. 6B). The disease activity index (DAI), which was one important parameter reflecting the severity of colitis, was significantly decreased by the administration of Paroxetine (Fig. 6C). As representative markers of colitis, colonic length (Fig. 6D) and splenomegaly (Fig. 6E) were found in all DSS groups, which were varying degrees rescued by paroxetine. In addition, the histopathological evaluation revealed that DSS elicited colonic inflammation including crypts abscess, infiltration of inflammatory cells and disruption of the mucosal barrier, which all directly led to a higher histological score (Fig. 6F and G). By contrast, either paroxetine or SASP administration improved the pathological destruction and decreased the higher histological scores of DSS-induced colitis mice. Taken together, our data revealed that paroxetine could alleviate the symptoms of DSS-induced colitis in mice.

**Paroxetine inhibited GRK2 translocation to regulate macrophage polarization in mice with DSS-induced colitis**

To confirm whether paroxetine affects GRK2 translocation in mice with DSS-induced colitis, we first detected the expression of GRK2/EP4/cAMP/pCREB in mice’ LPMCs. Compared with the control group, GRK2 membrane expression increased significantly, EP4 membrane expression and pCREB level in cytoplasm decreased in DSS groups, then the expression of pCREB in cytoplasm and EP4 membrane expression also significantly increased after treated with paroxetine. (Fig. 7A, B). Compared with DSS group, elisa’s results showed that the cAMP level increased significantly in the paroxetine treated group (Fig. 7C). Also we noted that the expression of membrane GRK2 in LPMCs was down-regulated, the expression of membrane EP4 was up-regulated and inhibited the membrane association of GRK2 and EP4 in the paroxetine treated group (Fig. 7D).

In IBD, the ratio of M1/M2 often associated to the development of inflammatory disorders. To investigate the changes in the ratio of M1/M2, PMs were isolated from the mice with DSS-induced colitis, and evaluated by qRT-PCR. The results indicated that mRNA expressions of M1 macrophage-associated factor IRF5, were significantly increased while the levels of IRF4(M2 macrophage-associated markers) was markedly decreased in the model group (Fig. 7E). Both paroxetine and SASP administration increased the percentage of M2 markedly and rebalanced the ratio of M1/M2 in DSS-induced colitis mice.
In order to detect whether paroxetine can regulate PGE2, INF-γ, IL-1β and IL-10 levels in mice with DSS-induced colitis, elisa was used to examining the level of PGE2, INF-γ, IL-1β and IL-10 in LPMCs. In all the DSS groups, PGE2, INF-γ and IL-1β showed high levels of expression and IL-10 showed a lower level of expression (Fig. 7F). Compared with the model group, PGE2, INF-γ and IL-1β expression levels were reduced, following treatment with paroxetine. These results indicate that paroxetine can increase M2 percentages and regulate corresponding inflammatory cytokines production in mice with DSS-induced colitis.

**Discussion**

Recently, numerous researchs with UC have identified the importance of macrophages in the pathogenesis of UC[19–20]. Macrophages are also widely distributed in the gut, and often play an important role in kinds of physiologic processes, like acute and chronic inflammation, and pathogen defence[21]. Macrophage polarization can be broadly classified into two main groups: classically activated macrophages and alternatively activated macrophages, which induce proinflammatory responses and anti-inflammatory regulation. These macrophages have been called M1 and M2 and induce iNOS or arginase respectively. The M1/M2 imbalance plays a pathogenic role in autoimmune disease[22].

In our study, UC patients’ colon mucosal lesions are characterized by the infiltration of inflammatory cells, which mainly were identified macrophages. It has been reported that the ratio of M1/M2 was increased in UC [23]. In the present study, we found the same trend by flow cytometry. These data showed the imbalance of M1/M2 may be related to the pathogenesis of UC. PGE2 has been proved to be an important immune mediator, and it always had function through autocrine and paracrine. It has been reported that the PGE2/EPs signal is important for M2 polarization. One study indicated that high level PGE2 could promote the M2 polarization through the cAMP-CREB pathway [24]. However, other studies showed an increased level of PGE2 but decreased M2 macrophages in UC patients, the exact reason has not been explained [25,26]. The exact function of PGE2 in inflammatory remains controversial. The previous research of our group showed that the level of PGE2 in the serum of rats with collagen-induced arthritis (CIA) was significantly increased. Constant stimulant of PGE2 on fibroblast-like synovial cell (FLS) of CIA rats resulted in a decreased level of cAMP, which is primarily caused by GRK2-induced EP4 over-desensitization [27]. The decreased level of cAMP inhibited the production of the IRF4 transcription factor, eventually affecting the polarization to M2-type macrophages. From our previous study, the PGE2 treatment of HUVECs increased phosphorylation of GRK2-Ser685, then influenced the membrane (memb) and cytoplasm (cyt) expression of EP4 and GRK2. The results showed that the expression of memb GRK2 was significantly increased, the expression of memb EP4 was decreased, and the combination of EP4 and GRK2 on the membrane was increased significantly. These results indicated that the stimulation of PGE2 can cause GRK2-induced EP4 over-desensitization. [28]

GRK2 had been known as one of serine/threonine protein kinases. After GRK2 specifically recognized GPCR, then mediated the phosphorylation, and regulated GPCR desensitization. GPCR desensitization
could mediate downstream signal transduction to regulate the physiological function. Our group has long been committed to the role of GRK2 in RA and arthritis animal models, we investigated the mechanism of GRK2 on macrophage polarization in RA\[29\]. We found the phenomenon of membrane localization of GRK2 increased in macrophages of UC, as well as increased the combination between EP4 and GRK2. Therefore, GRK2-induced EP4 over-desensitization may be the pathogenesis of ulcerative colitis (Fig. 8).

In this work, we found that GRK2 mediate PGE2-EP4-cAMP-pCREB signaling induced the imbalance of the ratio of M1/M2 in UC for the first time. First, we found that the secretion of PGE2 was increased in LPMCs of the UC group, and the trend had a positive correlation with the Mayo score significantly. Second, to detect the role of GRK2 on EP4 over-desensitization, we used laser confocal microscopy to find that the interaction between GRK2 and EP4 increased significantly in UC group. The result of membrane expression about GRK2 and EP4 are consistent with those of the laser confocal microscopy experiment.

Next, to illuminate the role of GRK2 on macrophages polarization, we choose the THP-1 cell line to be stimulated with PMA 100 ng/ml for 48 hours consecutively, and then transform into M0 macrophages. After the pIRES-EGFP-GRK2 plasmids were successfully constructed and transfected, PGE2 (10µM) was used to stimulate the M0 macrophages transfected with GRK2 overexpression plasmid, and the ratio of M1(CD68\(^{+}\)CD86\(^{+}\))/M2(CD68\(^{+}\)CD206\(^{+}\)) was detected by flow cytometry. The results showed that under the same stimulation conditions, compared with the control group, the ratio of M1/M2 increased significantly in pIRES-EGFP-GRK2 group. Also the expression of iNOS increased, and the level of Arg1 decreased, suggesting that GRK2 is involved in the regulation of macrophage polarization.

Then we generated GRK2 heterozygous mice (GRK\(^{+/−}\)) to explore the role of GRK2 in experimental colitis. Compared with GRK\(^{+/+}\) mice, the membrane localization of EP4 significantly increased in GRK\(^{+/−}\) mice. At the same time, the ratio of M1/M2 in GRK\(^{+/−}\) mice is decreased, and the degree of pathological damage of colon tissue is lower. All these results indicated that the reduction of GRK2 contributed to restoring the sensitization of EP4 and then rebalancing the ratio of M1/M2 by the cAMP-pCREB signal.

Symptoms of generalized anxiety disorder and various degrees of depression are very common in IBD. Different antidepressants are taken by approximately 30% of people with IBD\[30\]. However, it remains unknown whether antidepressants have other therapeutic effects in IBD, except in managing physical symptoms. Previous studies have found that selective serotonin reuptake inhibitors (SSRI) play a protective role in severe UC patients, but many data are low certainty evidence\[31, 32\]. Paroxetine, an FDA-approved SSRI, was identified as a potent GRK2 inhibitor with higher selectivity for GRK2 over other GRKs both in vivo and in vitro\[33\]. Paroxetine binds to the active site of GRK2 to inhibit GPCR phosphorylation and over-desensitization. Paroxetine can inhibit GRK2 membrane recruitment to recover the balance in GPCR signaling(Fig. 8).

In conclusion, we provide new evidence demonstrating that paroxetine attenuates symptoms of the DSS-induced colitis mice model. The group of paroxetine treated had less weight loss, lower disease activity index and pathological score. Also pro-inflammatory cytokines in peripheral blood are significantly
reduced. Our results in mice peritoneal macrophages showed that paroxetine could block GRK2-EP4 interaction to adjust the ratio of M1/M2 by influencing the level of cAMP and p-CREB. These evidence implicate GRK2 may as a novel therapeutic target for UC by rebalancing the macrophage polarization.

**Abbreviations**

cAMP, cyclic adenosine monophosphate; CD, Crohn's disease; CIA, collagen-induced arthritis; CREB, cyclic AMP responsive element binding; DAI, disease activity index; EP4, Prostaglandin E2 receptor 4; FBS, fetal bovine serum; GPCRs, G protein-coupled receptors; GRK2, G protein-coupled receptor kinase 2; HBSS, Hank's salt solution; H&E, hematoxylin and eosin; IBD, inflammatory bowel disease; IL, interleukin; LPMCs, lamina propria mononuclear cells; memb, membrane; pae, paeoniflorin; PBS, phosphate-buffered saline; PGE2, prostaglandin E2; PVDF, polyvinylidene fluoride; SASP, Salicylazosulfapyridine; SSRI, selective serotonin reuptake inhibitors; THP-1, Human acute monocytic leukemia cells; TNF-α, tumor necrosis factor α; UC, ulcerative colitis.

**Declarations**

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Author Contribution**

Wei Wei and Jiawei Zhang conceived and designed the study. Jiawei Zhang performed most of the experiments, analyzed data, writing- original draft preparation and wrote the manuscript. Xianzheng Zhang: Validation, Formal analysis, Investigation. Mingdian Lu and Yan Chang: Formal analysis, Investigation. Qingtong Wang: Formal analysis, Investigation. Jiajie Tu: Software, Formal analysis, Investigation. Huaxun Wu: Investigation. Chun Wang: Investigation. Zhongyang Hong: Data curation, Investigation. Maoming Xiong: Funding acquisition, Supervision. Lihua Song: Funding acquisition, Supervision. Wei Wei: Funding acquisition, Writing – review, Supervision. All authors read and approved the final version to be published.

**References**


**Figures**
Figure 1

Macrophages polarization in UC. (A) Representative H&E staining images of colon tissue (scale bar, 200 μm). (B) Confocal imaging of CD68 in colon tissue by fluorescence microscope (scale bar, 50 μm). (C) The ratio of M1/M2 in LPMCs was determined by flow cytometry (M1: CD68+/CD86+; M2: CD68+/CD206+). (D) mRNA expression of IRF5 and IRF4 were detected by RT-PCR. (E) The protein level of
iNOS and Arg1 were determined by Western blot. Data are represented as mean ± SEM (n=22). Significant differences were indicated as *P<0.05, **P<0.01 versus control group using t test.

**Figure 2**

GRK2 mediates the changes of PGE2-EP4-cAMP-pCREB pathway in colonic LPMCs of UC patients.(A) Confocal imaging and co-localization of GRK2 and EP4 in colon tissue (scale bar, 50 μm). (B) The level of...
PGE2 in LPMCs and Spearman correlation between Mayo score and PGE2 level. (C) The level of IL-1β and IL-10 in LPMCs was determined by Elisa. (D) The membrane protein level of GRK2 and EP4 in LPMCs were determined by Western blot. (E) Representative band of pCREB. (F) The level of cAMP in LPMCs was determined by Elisa. Data are represented as mean ± SEM (n=22). Significant differences were indicated as *P<0.05, **P<0.01 versus control group using t test.

Figure 3
**GRK2 heterozygous mice are protected from DSS-induced colitis.** (A) Experimental design for DSS-induced colitis in WT littermates and GRK2 heterozygous mice. (B) Body weight changes and (C) Disease activity index (DAI). (D) Macroscopic photographs of the colon and length of the colons. (E) Macroscopic photographs of the spleen and spleen index. (F) Representative H&E staining images of colon tissue (scale bar, 100 μm). (G) Histological scores of colon tissue. Data are represented as mean ± SEM (n=6). Statistical analysis was performed using one-way ANOVA with Dunnett’s Multiple Comparison test. Significant differences were indicated as *P<0.05 versus GRK2+/+DSS group; *P<0.05, **P<0.01 versus GRK2+/−H2O group; and #P<0.05, ##P<0.01, GRK2+/+DSS group versus GRK2+/−H2O group.
Figure 4

GRK2 regulates macrophage polarization through PGE2-EP4-cAMP-pCREB pathway in DSS-induced colitis model. (A) The protein level of iNOS and Arg1 in LPMCs were determined by Western blot. (B) The membrane protein level of GRK2 and EP4 were determined by Western blot in LPMCs. (C) Representative band of pCREB. (D) The level of cAMP in LPMCs was determined by Elisa. (E) Confocal imaging of EP4 (red) in colon tissue by fluorescence microscope (scale bar, 50 μm). Data are represented as mean ± SEM.
Statistical analysis was performed using one-way ANOVA with Dunnett’s Multiple Comparison test. Significant differences were indicated as *P<0.05, *P<0.01 versus GRK2+/+DSS group; *P<0.05, **P<0.01 versus GRK2+-H2O group; and #P<0.05, ##P<0.01, GRK2+/+-DSS group versus GRK2+/+H2O group.

Figure 5
Macrophage polarization in THP-1 differentiated macrophages (THP-Ms) after transfected with pIRES-EGFP-GRK2-WT. (A) Experimental design for THP-1 differentiated macrophages (THP-Ms) and then transfected with plasmid. (B) Microscopic image of THP-1 cells treated with PMA (100ng/ml) for 48h to differentiate into macrophages (THP-Ms) (scale bar, 50 μm). (C) The ratio of M1/M2 in THP-Ms was determined by flow cytometry (M1: CD68+/CD86+; M2: CD68+/CD206+). (D) The membrane protein level of GRK2 and EP4 were determined by Western blot. (E) Representative bands and the protein levels of pCREB, iNOS and Arg1 were detected by Western blot. (F) The level of cAMP in THP-1 differentiated macrophages (THP-Ms) was determined by Elisa. Data are represented as mean ± SEM (n=6). Statistical analysis was performed using one-way ANOVA with Dunnett's Multiple Comparison test. Significant differences were indicated as *$P<0.05$, **$P<0.01$ versus GRK OE− group with the stimulation of PGE2 (10 μM); *$P<0.05$, **$P<0.01$ versus GRK OE+ group without the stimulation of PGE2 (10 μM); and #$P<0.05$, GRK OE+ group without the stimulation of PGE2 (10 μM) versus GRK OE− group with the stimulation of PGE2 (10 μM).
Figure 6

**Paroxetine alleviated DSS-induced colitis in mice.** (A) Chemical structure of paroxetine. (B) Body weight changes and (C) Disease activity index (DAI) evaluations during the disease process. (D) Macroscopic photographs and length of the colons. (E) Macroscopic photographs of the spleen and spleen index. (F) Representative H&E staining images of colon tissue (scale bar, 100 μm), and (G) Histological scores of colon tissue. The data are presented as the means ± SEM (n = 6) and statistical analysis was performed using one-way ANOVA with Dunnett’s Multiple Comparison test. Significant differences were indicated as \(^{#}P<0.05\), \(^{##}P<0.01\) vs. Control group; \(^{*}P<0.05\) vs. paroxetine group; \(^{*}\ P<0.05\) vs. SASP group.
Figure 7

Paroxetine down-regulated EP4/cAMP/pCREB-dependent GRK2 translocation in mice with DSS-induced colitis. (A) The membrane protein level of GRK2 and EP4 in mice' LPMCs were analyzed by Western blot. (B) The protein level and representative band of pCREB. (C) The level of cAMP in LPMCs was determined by Elisa. (D) Confocal imaging and co-localization of GRK2 (green) and EP4 (red) in LPMCs by fluorescence microscope (scale bar, 20 μm). (E) mRNA expression of IRF5 and IRF4 were detected by RT-
PCR. (F) The level of PGE2, IL-1β, IFN-γ and IL-10 in LPMCs was determined by Elisa. The data are presented as the means ± SEM (n = 6) and statistical analysis was performed using one-way ANOVA with Dunnett’s Multiple Comparison test. Significant differences were indicated as #P < 0.05, ##P < 0.01 vs. Control group; *P < 0.05 vs. paroxetine group; *P<0.05 vs. SASP group.

Figure 8

Proposed model shows some of the potential translocation activity-dependent mechanisms by which GRK2 may modulate EP4-cAMP-pCREB signal to regulate macrophage polarization in colitis and the alleviation of paroxetine. Under the condition of inflammation, PGE2 binds to ligand EP4 receptor, mediating cAMP/pCREB signaling activation to enhance M2 polarization. Similarly, membrane targeting of over-activated GRK2 to associate with EP4 receptor and maintain persistent EP4 receptor desensitization to influence M2 polarization. Paroxetine can inhibit GRK2 membrane recruitment to recover the balance in GPCR signaling and thus alleviate inflammatory response in colitis.

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