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RESEARCH ARTICLE

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GPA peptide enhances Nur77 expression in intestinal epithelial cells to exert a protective effect against DSS-induced colitis

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Abstract

Ulcerative colitis (UC) is a widespread inflammatory bowel disease that causes long-lasting inflammation and ulcers in the colon and rectum. In the inflamed tissue of patients with UC, the tight junctions are disrupted and large amounts of pro-inflammatory cytokines are produced, resulting in immune dysregulation. The expression of Nur77 is significantly reduced in the colon of inflammatory bowel disease, while Nur77 deficiency increases the susceptibility to DSS-induced colitis. Here, we report that Gly-Pro-Ala (GPA) peptide isolated from fish skin gelatin hydrolysate can significantly alleviate intestinal inflammation and damage caused by DSS-induced mice colitis. Besides maintaining the intestinal epithelial barrier, GPA alleviates intestinal inflammation and oxidative stress by inhibiting NF-κB activation. Interestingly, GPA binds to the ligand-binding domain of Nur77 and stimulates its autotranscriptional activity to enhance its expression in intestinal epithelial cells. Furthermore, GPA activates the promoter of IκBα to increase its expression, resulting in the abolishment of the NF-κB pathway. In contrast, the inhibitory effects of GPA on colitis are abolished in *Nur77*$^{-/-}$ mice. Our results suggest that as a Nur77 modulator, GPA may be applied to the prevention of intestinal inflammation.

KEYWORDS

GPA, inflammation, Nur77, transcriptionally activate, UC

1 | INTRODUCTION

Ulcerative colitis (UC), an intractable inflammatory bowel disease (IBD), is a chronic and idiopathic inflammatory disease which affects the colon, resulting in weight loss, diarrhea, rectal bleeding, and abdominal pain.[1-4] In the inflamed tissue of patients with UC, the tight junctions of epithelium are disrupted, enabling microbes to migrate from the lumen into the lamina propria. Once the NF-κB pathway is activated, large amounts of pro-inflammatory cytokines will be produced to cause a cycle of uncontrolled inflammation, which results in severe damage to the intestinal wall.[5] Hence,

Abbreviations: ChIP, Chromatin immunoprecipitation; DAMP, damage-associated molecular patterns; GPA, Gly-Pro-Ala; IBD, inflammatory bowel disease; IECs, intestinal epithelial cells; IHC, immunohistochemistry; IL-6, interleukin 6; IL-8, interleukin 8; IL-12, interleukin 12; LPS, lipopolysaccharides; LSPR, Localized surface plasmon resonance; MODE-K, mouse intestinal epithelial cell line; Nur77-LBD, Nur77 ligand-binding domain; PAMP, pathogen-related molecular patterns; TNF-α, tumor necrosis factor-α; UC, ulcerative colitis.

the strategies for treating UC have been focused on regulating immune cells, blocking immune signals, repairing epithelial barrier, or supplementing beneficial microorganisms.[5,6]

The NF-κB signaling pathway plays a predominant role in IBD pathogenesis.[7-11] Stimulation with pathogen-related molecular patterns (PAMP) or host-derived damage-associated molecular pattern (DAMP), including bacterial lipopolysaccharides (LPS) or tumor necrosis factor-α (TNF-α), would result in NF-κB activation, thereby inducing the production of pro-inflammatory cytokines, such as TNF-α, interleukin 6 (IL-6), IL-8, and IL-12.[11] To date, several in vitro and in vivo studies have indicated that anti-NF-κB therapies are effective in reducing the pathology and morbidity of IBD.[12,13]

As a member of the NR4A family of nuclear receptors, the expression of Nur77 (also called TR3, NGFIB, or NR4A1) is significantly reduced in the colon tissues of IBD patients and mice treated with DSS, while the deficiency of Nur77 increases the susceptibility of mice to experimental colitis induced by DSS.[14] However, another study reported that the expression of Nur77 is increased in colon tissues with IBD and mice colitis.[15] Recently, several studies have been focused on the potent anti-inflammatory effects of Nur77.[16-18] For instance, in endothelial cells, Nur77 inhibits NF-κB and the production of pro-inflammatory cytokines via increasing IκBα expression.[19] Similarly, in macrophage, Nur77 inhibits the LPS-induced activation of NF-κB by suppressing the binding of p65 to DNA, reducing the production of pro-inflammatory cytokines.[17] Therefore, we tested whether upregulation of Nur77 expression is an effective way to alleviate intestinal inflammation.

In the present study, we found that GPA peptide isolated from fish skin gelatin hydrolysate[20,21] could increase Nur77 expression and alleviate DSS-induced colitis. In addition to maintaining the intestinal epithelial barrier, GPA alleviates intestinal inflammation and oxidative stress by inhibiting NF-κB pathway in DSS-induced colitis. These anti-colitis effects of GPA are mediated by Nur77. Taken together, our findings may provide an effective way to ameliorate colitis by targeting Nur77.

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

Synthetic GPA peptide was purchased from Top-peptide Biotechnology Co., Ltd (Shanghai, China), GPA was dissolved in PBS. Dextran sulfate sodium (DSS, a molecular weight of 36-50 kDa) was purchased from MP Biomedicals (Irvine, CA, USA). LPS (L6230) was purchase from Sigma-Aldrich (St. Louis, MO, USA). Murine TNF-α recombinant protein (315-01A-20) was purchased from PeproTech (USA). Protease Inhibitor Cocktail (C0001) was purchased from Target Mol (Topscience, Shanghai, China). Cell lysis buffer for Western analysis (P0013), phenylmethanesulfonyl fluoride (PMSF) (ST505) was purchased from Beyotime (Shanghai, China). DAPI (D4054) was purchased from US Everbright®Inc (Suzhou, China). MultiF Seamless Assembly Mix was purchased from Abclonal (Wuhan, China). The antibodies against IκBα (A1187), PCNA (A13336), LC3B (A11282), β-actin (AC026), Cy3 Goat Anti-Rabbit IgG (AS007), 488 Goat Anti-Mouse IgG (AS076), 488 Goat Anti-Rabbit IgG (AS073), and IL-1β (A16288) were purchased from Abclonal (Wuhan, China). The antibodies against ZO-1 (AF5145) and occludin (DF7504) were purchased from Affinity (Cincinnati, USA). The antibodies against IL-6 (GB11117) and GAPDH (GB1102) were purchased from Servicebio (Wuhan, China). The antibodies against Nur77 (ab13851) was purchased from Abcam (Shanghai, China). Dual Luciferase Reporter Gene Assay Kit (11402ES80) was purchased from YEASEN (Shanghai, China).

2.2 | Cell culture

Mouse intestinal epithelial cell line (MODE-K), an intestinal epithelial cell line derived from C3H/HeJ mice,[22] were purchased from BeNa Culture Collection (Beijing, China). The MODE-K cell model served as a system to study the bidirectional signaling responses of gut epithelial cells and to understand the modulating effects of dietary bioactive components.[23] MODE-K cells were cultured in RIPA 1640 (Gibco, San Diego, CA, USA) containing 10% fetal bovine serum (FBS, Gibco, San Diego, CA, USA) and 1% penicillin/streptomycin at 37°C under a 5% CO_2 atmosphere.

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C under a 5% CO_2 atmosphere.

Human acute monocytic leukemia THP-1 cells were obtained from BeNa Culture Collection (Beijing, China) and were authenticated. THP-1 cells were cultured in RIPA 1640 (Gibco, San Diego, CA, USA) containing 10% fetal bovine serum (FBS, Gibco, San Diego, CA, USA) and 1% penicillin/streptomycin at 37°C under a 5% CO2 atmosphere. Differentiation of THP-1 cells was induced by 100nM phorbol 12-myristate 13-acetate (PMA) for 6 hours.

2.3 | Animals

Male C57BL/6 mice (5 weeks old) obtained from the Animal Experiment Center at Huazhong Agricultural University (Wuhan, China) were used for the present study. *Nur77*$^{-/-}$ mice were obtained from Cyagen US Inc (Guangzhou, China). The mice were housed under specific pathogen-free conditions in an air-conditioned room

at 23 ± 2°C. Food and water were supplied ad libitum. Animal welfare and experimental procedures were carried out following the criteria outlined in the Guide FOR THE CARE AND USE OF LABORATORY ANIMALS (Eight Edition) and the related ethical regulations of Huazhong Agricultural University. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.4 | Establishment of DSS-induced mice colitis model and treatment

Acute colitis was induced by feeding the mice with 3% (w/v) DSS dissolved in drinking water continuously for 7 days.[24,25] The experiment was randomly divided into five groups: control group, DSS group, and GPA (50, 100, or 150 mg/kg) + DSS groups. GPA (50, 100, or 150 mg/kg) was administered for 7 days before and during DSS treatment via oral gavage once per day. After 14 days, the mice were humanely euthanized, and the colons were excised, measured, and sectioned for further analysis.[26-28]

Subsequently, to further confirm the key role of Nur77 in the effect of GPA, GPA was intragastrically administered daily at 100 mg/kg. Wild-type (WT) or *Nur77*$^{-/-}$ mice were randomly assigned to the control group, DSS group, and GPA (100 mg/kg) + DSS group.

2.5 | Evaluation of colitis severity

The colitis severity was evaluated based on body weight, colon length, and macroscopic and microscopic observations of the stool and colon. The disease activity index (DAI) score was determined by the method reported in previous studies, with five grades of weight change (0, no weight loss or gain; 1, 1%-5% loss; 2, 5%-10% loss; 3, 10%-20% loss; and 4, more than 20% loss), stool consistency (0, normal; 1, mild loose; 2, loose; 3, mild diarrhea; and 4, diarrhea), and stool bleeding (0, negative; 1, light bleeding; 2, mild bleeding; 3, severe bleeding; and 4, complete bleeding). Colon sections were prepared and stained with hematoxylin and eosin (H&E) according to previous research.[26,27,29]

2.6 | MPO assay

Myeloperoxidase activity reflects the number and distribution of neutrophils in the tissues. The colon tissues were weighed and homogenized with phosphate-buffered saline (PBS) [1:9 (w/v)]. The supernatants were collected. The activity of MPO was measured according to the manufacturer's instructions (2M-KMLJM220987m, Nanjing Camilo biological engineering Co.Ltd, Nanjing, China).

2.7 | RNA isolation and quantitative real-time PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen) and transcribed into cDNA using a First-Strand cDNA Synthesis Kit (TOYOBO, Japan). Quantitation of the mRNA levels by quantitative real-time (qPCR) was performed on a real-time PCR system (Bio-Rad, Richmond, CA, USA) using iTaq Universal SYBR Green Supermix (Bio-Rad, Richmond, CA, USA). The mean of the triplicate cycle thresholds (Ct) of the target gene was normalized to the mean of triplicate Ct of the reference β-actin and GAPDH gene using the formula "$2^{-\Delta\Delta Ct}$," which yielded relative gene expression level values. The primers used are listed in Table S1.

2.8 | RNA interference and transfection

Synthetic siRNA oligonucleotides specific for regions in the mice Nur77, TRAF6, and p62 mRNA were designed and synthesized by GenePharma (Shanghai, China). The primers used are listed in Table S3. Cells were transfected at 70%-80% confluence with siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) by the manufacturer's instructions.

2.9 | Plasmid construction

Pgl3-NBRE-Luc-reporter with five NBFI-B response elements (AAAGGTCA) was inserted into the Pgl3-promoter vector (Promega). Pgl3-NurRE-Luc-reporter with three Nur response elements (TGATTT ACCTCCAAATGCCA) was inserted into the Pgl3-promoter vector. Pgl3-NF-kB-Luc-reporter with five NF-kB response elements (GGGAATTTCC) was inserted into the Pgl3-promoter vector.

The human IkB promoter was amplified by PCR on human genomic DNA with primers F: 5-ATTCA GTCCATGGCTTGCAGGCTT-3 and R:5-TGCTTCCTCG CTGGGGCGCT-3 The PCR product was cloned into the pGL3-basic vector (Promega).[19]

The mouse Nur77 promoter was amplified by PCR on mouse genomic DNA with primers F: 5-GATCTGCGATCTAAG TTGCTTCCATCCTGCAGAGTCCTGGGC-3 and R:5-CAGTACCGGAATGCCCCCTGGCGAGCCCG ACCCACATCTT-3. The PCR product was cloned into the pGL3-basic vector (Promega).

2.9.1 | Luciferase reporter assays

The luciferase vector and renilla luciferase-expressing plasmid (pTK) were transfected into HEK293T cells for 6 hours. Then, the cells were treated with GPA for 12 hours. After washing twice with PBS, the cells were lysed using Dual-Glo luciferase reagent (YPH, Beijing, China). The luciferase activity was determined using a dual-luciferase reporter assay system and a luminometer (Dynex Technologies, UK). The luciferase values were normalized to the Renilla values. The transfection experiments were performed in triplicate for each independent experiment.

2.9.2 | Immunohistochemistry

Immunohistochemical (IHC) stains against ZO-1, occluding, and Nur77 were detected using the IHC kit (MaiXin, China). Briefly, paraffin-embedded slides were deparaffinized, rehydrated, and washed in 1% PBS. Afterward, they were incubated with 3% hydrogen peroxide and blocked with 10% goat serum for 1 hour at 37°C. Then, the slides were treated with primary antibodies (1:100) overnight at 4°C. Streptavidin-HRP secondary anti-rabbit antibodies (1:500) were added and incubated at room temperature for 1 hour. Streptavidin-HRP was added, and after 40 minutes the sections were stained with diaminobenzidine as a chromogen and counterstained with hematoxylin. Images at 200× magnification were examined with a microscope (Olympus, Japan).

2.10 | Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using the ChIP Assay Kit according to the manufacturer's instructions (P2078, Beyotime, China). Briefly, after crosslinking (1% formaldehyde) for 10 minutes, the cells were quenched by the addition of 0.125 M glycine and harvested in SDS lysis buffer for sonication digestion. The DNA-protein complexes were immunoprecipitated with ChIP grade antibodies against Nur77 or IgG. DNA obtained from the immunoprecipitation was analyzed by quantitative real-time PCR and normalized to inputs. The primers used are listed in Table S1.

2.11 | Immunofluorescence and confocal imaging

Cells were washed twice with PBS and fixed in 4% paraformaldehyde for 30 minutes at room temperature. 4,6-diamidino-2-phenylindole (DAPI) was used to label DNA. Confocal imaging was performed using a confocal laser scanning microscope (Carl Zeiss, Germany) equipped with an incubation chamber and a motorized table.

2.12 | Localized surface plasmon resonance

To analyze the binding of Nur77 and GPA, Nur77-LBD protein was fixed on the COOH sensor chip by capture-coupling. GPA at the concentrations of 2.5, 5, 10, 20 mmol/L was injected sequentially into the chamber in PBS running buffer, and the interaction of Nur77-LBD with GPA was detected by OpenSPRTM (Nicoya Lifesciences, Waterloo, Canada) at 25°C. The binding time and disassociation time were both 250 seconds; the flow rate was 20 μL/min, and the chip was regenerated with 0.25% SDS. A one-to-one diffusion-corrected model was fitted to the wavelength shifts corresponding to the varied drug concentrations. The data were retrieved and analyzed with TraceDrawer software (Ridgeview Instruments AB, Sweden).

2.12.1 | Measurement of ROS

The fluorescent probe DCFH2-DA was used to detect the formation of intracellular ROS (2M-KMLJM219615m, 2H-KMLJh310725, Nanjing Camilo biological engineering Co.Ltd). Briefly, MODE-K cells (5×10^4 cells/well) were seeded in a 24-well plate to reach 80% confluence. After GPA pretreatment for 6 hours followed by LPS incubation for 3 hours, the cells were incubated with 10 μM $DCFH_2$-DA at 37°C for 30 minutes. Finally, cells were washed with PBS for three times, and the fluorescence was quantified on a FACS Calibur cytometry system (BD Biosciences) with excitation at 488 nm and emission at 530 nm. The results were expressed as a percent of control values.[20,30,31]

After treatment, the colon samples were excised and homogenized immediately at 4°C, and the cells were broken by an ultrasonic breaker on the ice. Protein concentration was determined quantitatively with a BCA protein assay kit. The ROS in colonic tissues was measured by chemical chronometry using a relevant assay kit (2M-KMLJM219615m, Nanjing Camilo biological engineering Co.Ltd) based on previous descriptions.[32]

2.13 | Intestinal permeability measurement

Intestinal permeability measurement was performed at 48 hours after NEC induction. The mice were denied access to food for 8 hours before gavage. Mice were orally gavaged with FITC-conjugated 4-kD dextran (FD4, 50 mg/mL, Sigma) (44 mg/100 g) at 4 hours before sacrifice. Blood serum was collected. Then, 50 μL of sample per well was

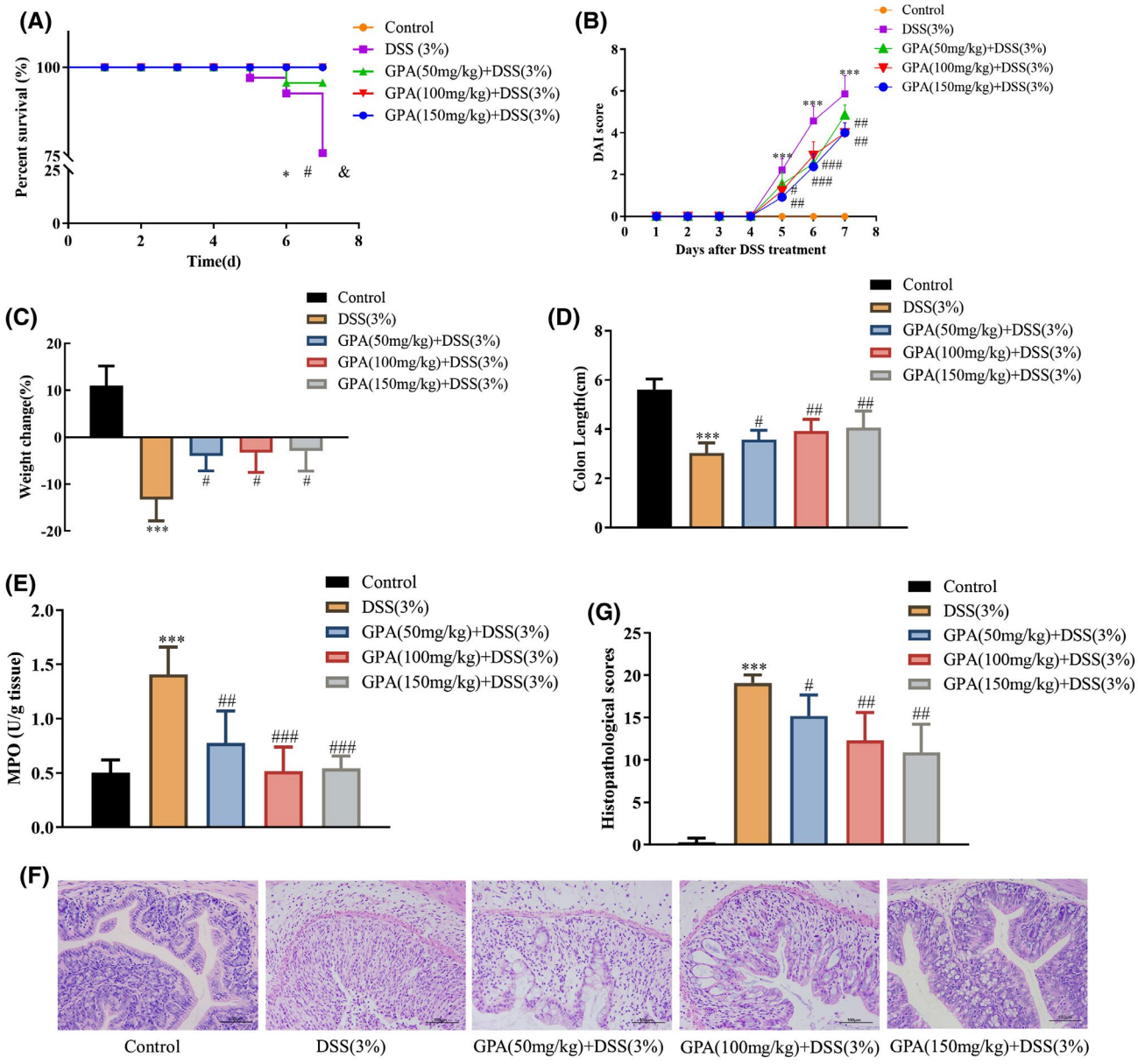

FIGURE 1 Anti-inflammatory effects of GPA in DSS-induced colitis in vivo. GPA (50, 100, 150 mg/kg) was administered for 7 days before and during DSS treatment via oral gavage once per day. Mice (n = 12/group) were sacrificed at day 14 after colitis induction. Survival percentage of mice after DSS treatment (A). Disease activity index (DAI) during the disease process (B). $^{*}P < .05$ vs. the control group. $^{\#}P < .05$ vs. the DSS-treated group, $^{\&}P < .05$ vs. the DSS-treated group. Weight change of mice during the experiment (C). The lengths of colons from each group of mice were measured (D). Myeloperoxidase (MPO) activity in the colonic tissues was detected (E). Histopathological scores of the colon tissues were determined by H&E staining and histopathological scores (FG). The results are expressed as mean ± SD, (n = 12/group). $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ vs. the control group; $^{\#}P < .05$, $^{\#\#}P < .01$, and $^{\#\#\#}P < .001$ vs. the DSS-treated group

added to a 96-well plate and the serum concentration of FD4 was measured using a Synergy HT plate reader (BioTek Laboratories, Inc, WA).

2.14 | Determination of GPA in vitro

After treatment, the cells were lysed with ultra-pure water, broken by ultrasound, added with fold-fold volume of methanol and centrifuged at 12 000 rpm/min for 10 minutes to remove the protein. Then, the GPA concentration was determined by HPLC-ESI-QqQ-MS/MS analysis.[20,21]

2.15 | Statistical analysis

Data were presented as the mean ± standard deviation. Differences between group means were determined by one-way

ANOVA using SAS 8.0 software. The Tukey post hoc multiple comparison test was performed to compare significant variations. Differences were considered as significant at $P < .05$.

3 | RESULTS

3.1 | GPA exerted anti-inflammatory effects in DSS-induced colitis

The anti-inflammatory effect of GPA in vivo was examined with the mice model of colitis. The supplementation of GPA at doses of 50, 100, and 150 mg/kg body weight significantly reduced the DSS-induced morbidity, weight loss, and DAI scores (Figure 1A-C). Besides, DSS typically caused colonic shortening, spleen hypertrophy, and MPO hyperactivity, whereas these symptoms were significantly alleviated in GPA supplementation groups (Figures 1D,E and S1A,B). DSS induced distortion of enterocytes and severe mucosal damage. Importantly, GPA administration on mice with DSS-induced colitis improved the pathological changes in a dose-dependent manner (Figure 1F,G).

Epithelial tight junction (TJ) proteins are essential elements of the mechanical barrier,[33,34] and ZO-1 and Occludin are important epithelial TJ proteins. Thus, we investigated the effect of GPA on epithelial TJ proteins (ZO-1 and Occludin) in colon tissues. The results showed that the expression of ZO-1 and Occludin decreased after DSS administration compared with that in the control group, which was recovered by GPA pretreatment in the colon tissues (Figure S1C-E). Furthermore, we evaluated the intestinal permeability by FITC. The results showed that GPA reduced the amount of the leakage and lowered the circulatory FITC-dextran concentration (Figure S1F). These results indicated that GPA could significantly ameliorate colitis and maintain epithelial barriers.

3.2 | GPA upregulated Nur77 expression to exert anti-colitis effects in the intestine

The expression of transcription factor Nur77 was significantly reduced in the colon tissues of patients with IBD and mice treated with DSS.[14] Interestingly, in the DSS-induced mice colitis, GPA pretreatment for 14 days dramatically increased the expression of Nur77 in the colon tissues (Figures 2A-C and S2A-C), implying that GPA may exert its anti-colitis effects through Nur77.

Next, to identify the role of Nur77 in the anti-inflammatory effect of GPA, we constructed *Nur77*$^{-/-}$ mice by CRISPR-Cas9 (Figure S3A,B) to further determine whether the anti-inflammatory effect of GPA is dependent on Nur77. Again, the administration of GPA (100 mg/kg) resulted in a significant alleviation of DSS-induced colitis in wild-type mice. However, in *Nur77*$^{-/-}$ mice, GPA failed to ameliorate DSS-induced morbidity, DAI scores, spleen hypertrophy, colon pathological changes, and MPO (Figures 2D-J and S3C,D). These results indicated that the anti-colitis effect of GPA was depleted in *Nur77*$^{-/-}$ mice. Thus, Nur77 could be upregulated by GPA and mediate the anti-colitis effect of GPA on intestinal inflammation.

3.3 | GPA exerted anti-inflammatory and antioxidant effects in intestinal inflammation through Nur77

The anti-colitis effects of GPA were further confirmed by detection of pro-inflammatory cytokines and ROS. We measured the concentration of tumor necrosis factor-α (TNF-α), Interleukin-6 (IL-6), IL-12, and ROS in the serum and colon tissues. The results showed that GPA significantly inhibited the levels pro-inflammatory cytokines and ROS (Figure 3A,B). Intestinal epithelial cells and macrophages are important component cells in the gut. Hence, we evaluated the anti-inflammatory and antioxidant effects of GPA in intestinal epithelial cells and macrophages. In vitro, HPLC-ESI-QqQ-MS/MS showed that the concentrations of GPA in MODE-K cells and THP-1 cells were 0.1-0.15 μm/10^6 cells after GPA (1 mM) treatment (Figure S4A,B). Then, we assessed the pro-inflammatory cytokines and ROS in MODE-K cells and THP-1 cells. The results showed that GPA significantly inhibited pro-inflammatory cytokines and ROS production (Figure 3C-F). Peptide transporter 1 (pept1) has been shown to transport several different peptides and peptide-derived drugs.[35] To determine whether GPA uptake is mediated by pept1, pept1 was knocked down by siRNA. As a result, the concentration of GPA in MODE-K cells decreased compared with that in the cells treated with GPA alone (Figure S4C,D). Subsequently, we detected inflammatory cytokines. The results showed that the inhibitory effect of GPA on cytokine was abolished after knockdown of pept1 (Figure S4E,F). These results indicated that GPA could enter IECs by pept1 to exert its anti-inflammatory effects.

In *Nur77*$^{-/-}$ mice, we also assessed the pro-inflammatory cytokines and ROS. The results showed that the inhibitory effects of GPA on pro-inflammatory cytokines and ROS were blocked (Figure 3G,H). These results indicated that the anti-inflammatory and antioxidant effects of GPA are dependent on Nur77.

3.4 | GPA blocked NF-κB activation through Nur77

NF-κB pathway plays a central role in inflammatory cytokine production and IBD pathogenesis,[13] and anti-NF-κB therapies

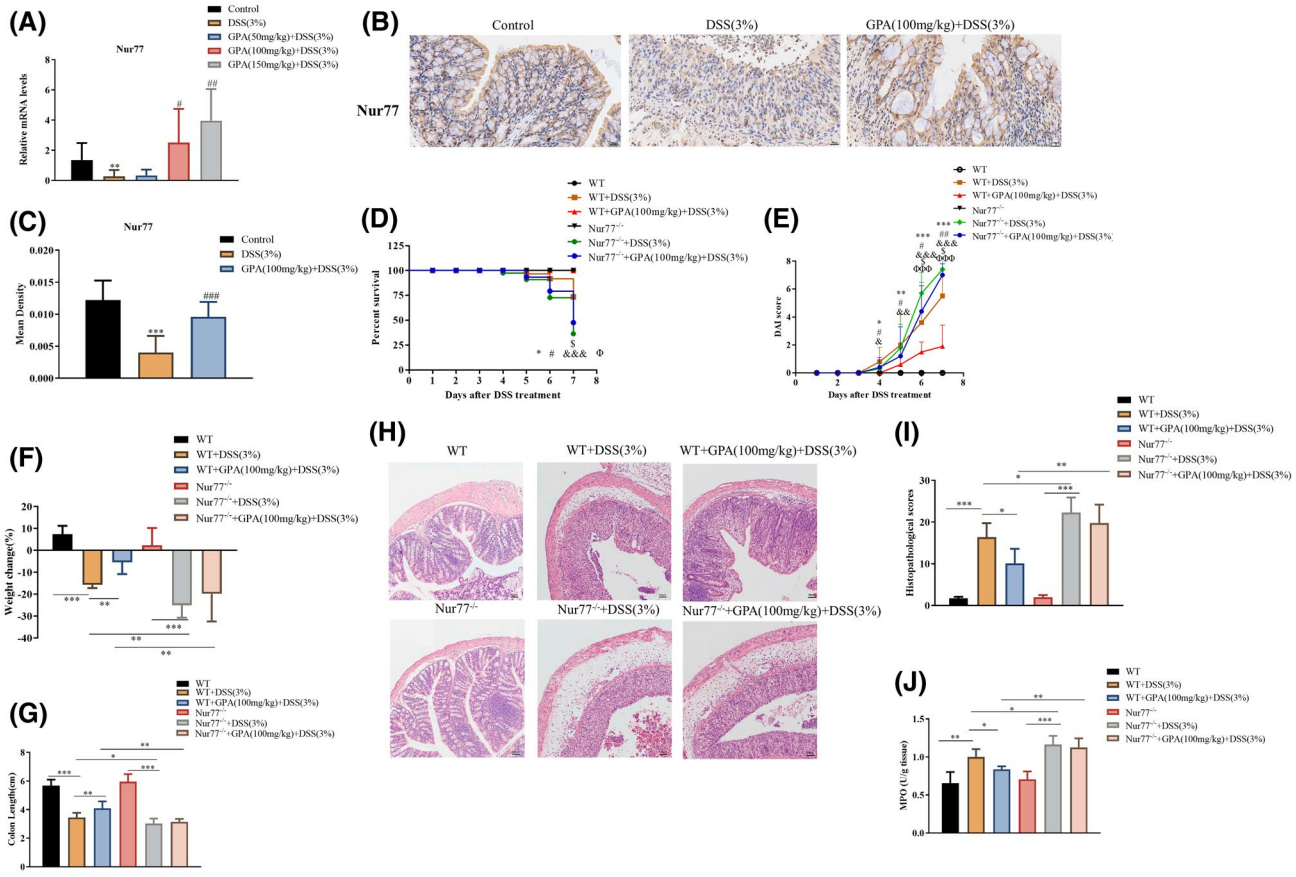

FIGURE 2 GPA promotes Nur77 expression to attenuate colitis in vivo. The levels of Nur77 were determined by qPCR and immunohistochemical analysis in the colon tissues, and the results are expressed as mean ± SD, (n = 12/group). $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ vs. the control group; $^{\#}P < .05$, $^{\#\#}P < .01$ and $^{\#\#\#}P < .001$ vs. the DSS-treated group (ABC). Wild-type (WT) and *Nur77*$^{-/-}$ mice (n = 10/group) were treated with 3% DSS in drinking water for 7 days to induce acute colitis. GPA (100 mg/kg) was administered for 14 days before and during DSS treatment via oral gavage once per day. Mice were sacrificed at Day 14, the survival percentage of mice after DSS treatment (n = 10/group). Survival percentage of mice after DSS treatment (D). Disease activity index (DAI) during the disease process (E). Weight change of mice during the experiment (F). The lengths of colons from each group of mice were measured (G). Histopathological scores of colon tissues were determined by H&E staining (HI). Myeloperoxidase (MPO) activity in the colonic tissues was detected (J). The results are expressed as mean ± SD, (n = 10/group). $^{*}P < .05$, WT + DSS group vs. the WT group. $^{\#}P < .05$, WT + GPA+DSS group vs. the WT + DSS group, $^{\&}P < .05$, *Nur77*$^{-/-}$group vs. the *Nur77*$^{-/-}$ +DSS-treated group. $^{\$}P < .05$, WT + DSS group vs. the *Nur77*$^{-/-}$ +DSS-treated group. $^{\Phi}p < 0.05$, WT + GPA+DSS group vs. the *Nur77*$^{-/-}$ +GPA + DSS group

were reported to be an effective way to reduce the pathology and morbidity in IBD.[12] Therefore, we determined whether GPA could inhibit the NF-κB pathway to reduce inflammatory cytokine production. In vitro, we constructed an intestinal epithelial cells (IECs) inflammation model by LPS, and found that GPA reduced p65 nuclear translocation through western blot and immunofluorescence (Figure 4A-C), which thereby inhibited NF-κB activation in MODE-K cells. A similar anti-inflammatory effect of GPA was also observed to inhibit LPS-induced NF-κB activation HEK293T cells (Figure S5A). Meanwhile, in DSS-mice colitis, GPA suppressed p65 nuclear translocation as indicated by western blot and immunofluorescence in the colon tissues (Figures 4D-F and S5B,C). To test whether GPA suppresses NF-κB activation through Nur77, we examined the NF-κB pathway in mice colon tissues. The administration of GPA (100 mg/kg) resulted in a significant alleviation of DSS-induced p65 nuclear translocation in wild-type mice. However, in *Nur77*$^{-/-}$ mice, GPA failed to ameliorate DSS-induced p65 nuclear translocation (Figure 4G,H). These results indicated that GPA could inhibit the NF-κB activation through Nur77.

3.5 | Nur77 mediated GPA-induced IκBα expression to suppress NF-κB activation

GPA treatment increased the level of IκBα (Figure 5A,B). Since IκBα is a target gene of Nur77 and negatively regulates NF-κB pathway, this result suggested that GPA may increase the level of IκBα through Nur77 to abolish NF-κB activation in IECs. Subsequently, we determined the level of IκBα using an in vitro model of LPS induced inflammation

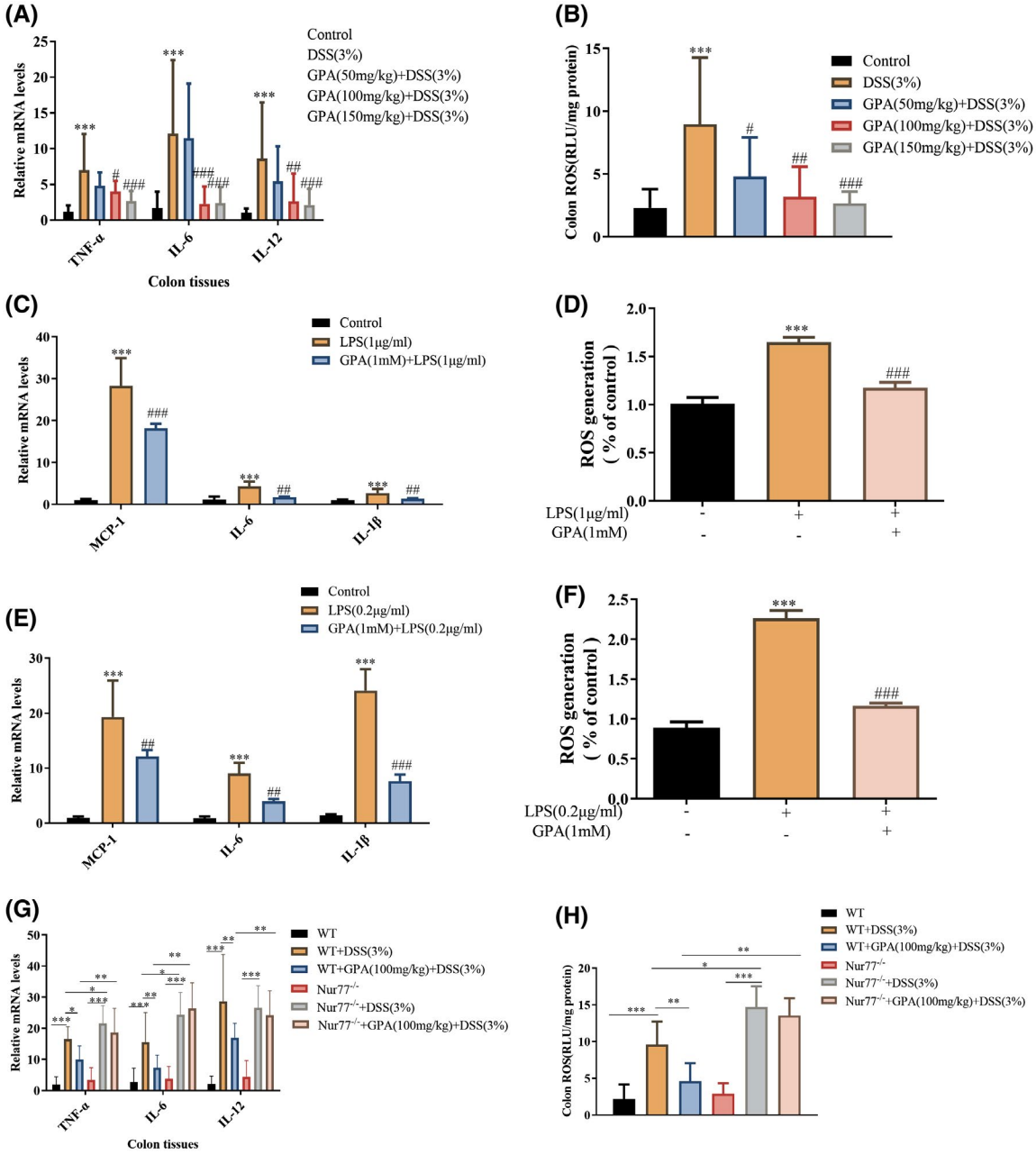

FIGURE 3 GPA exerts anti-inflammatory and antioxidant effects in intestinal inflammation through Nur77. The mRNA levels of TNF-α, IL-6, and IL-12 in the colon tissues were measured by quantitative real-time PCR (A). ROS content in the colon tissues was measured (B). The results are expressed as mean ± SD, (n = 12/group). $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ vs. the control group. MODE-K cells pre-treated with different concentrations of GPA for 6 h were exposed to LPS (1 μg/ml) for 3 h. The levels of pro-inflammatory cytokines and ROS were measured (CD). THP-1 cells pre-treated with different concentrations of GPA for 6 h were exposed to LPS (0.2 μg/ml) for 3 h. The levels of pro-inflammatory cytokines and ROS were measured (EF). The values presented are the means ± SD of three independent experiments (n = 4/group). $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$ vs. the control group. In WT and *Nur77*$^{-/-}$ mice colon tissues, the mRNA levels of TNF-α, IL-6, and IL-12 in the colon tissues were measured by quantitative real-time PCR (G). ROS content in the colon tissues was measured (H). The results are expressed as mean ± SD, (n = 10/group). $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ vs. the control group

in MOCE-K cells and an in vivo model of DSS-induced colitis in mice. The results showed that GPA increased the level of IκBα (Figures 5C,D and S5C,D). In the in vitro model of TNF-α induced inflammation in MODE-K cells, GPA also increased the level of IκBα (Figure S5F,G).

Promoter assays confirmed that GPA transcriptionally activated IκBα promoter activity in HEK293T cells (Figure 5E). Accordingly, ChIP-qPCR assays showed that GPA treatment promoted the binding of Nur77 to the promoter region of IκBα in MODE-K cells (Figure 5F). To test whether GPA

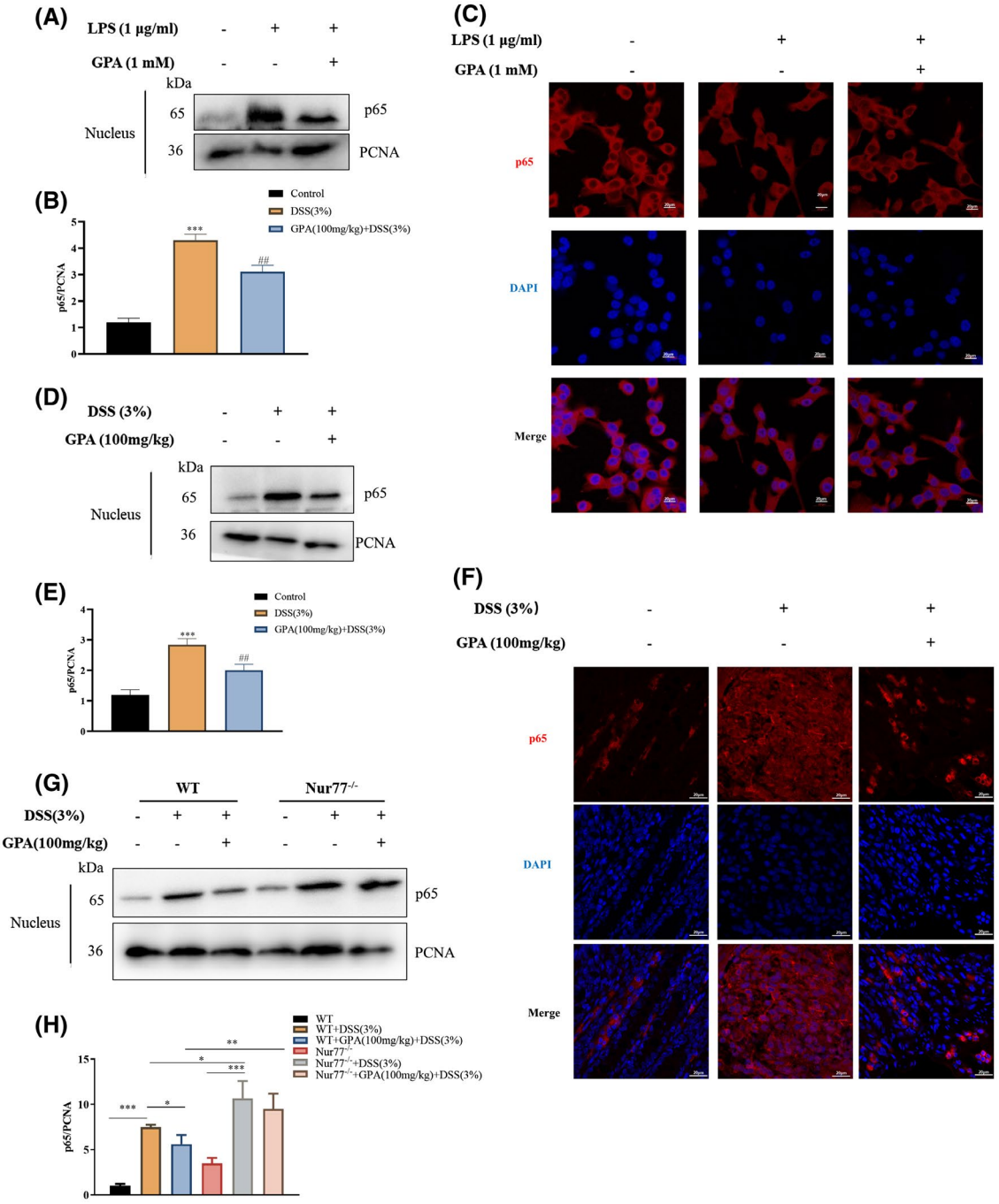

FIGURE 4 GPA blocks NF-κB activation through Nur77. MODE-K cells were stimulated with GPA (1 mM) for 6 h, followed by LPS (1 μg/mL) for 3 h. The NF-κB p65 entering the nucleus was detected by western blot through extracting nucleus, and immunofluorescence in MODE-K cells (ABC). P65 (red) and DAPI (blue) scale bar, 20 μm, three independent experiments. In mice colon tissues, the p65 entering the nucleus was detected by western blot through extracting nucleus, and immunofluorescence, p65 (red), and DAPI (blue) scale bar, 20 μm (n = 5/group) (DEF). In WT and *Nur77*$^{-/-}$ mice colon tissues, the NF-κB p65 entering the nucleus was detected by western blot through extracting nucleus (GH). The results are expressed as mean ± SD, (n = 5/group). $^{*}P < .05$, $^{**}P < .01$ and $^{***}P < .001$ vs. the control group

has anti-inflammatory effects and targets IκBα by Nur77, *Nur77* was knocked down in HEK293T cells and knocked out in mice.

The results showed that the activation effect of GPA on IκBα promoter activity was reduced by siRNA against Nur77 (Figure 5G), and the regulation effect of GPA on IκBα was abolished at the absence of Nur77 (Figure 5H,I). As expected, the inhibitory effect of GPA on NF-κB activation was abolished when *IκBα* was knocked down in MODE-K cells (Figure 5J,K). These results demonstrated that GPA exerts its anti-inflammatory effects through regulating Nur77 and further increasing IκBα expression.

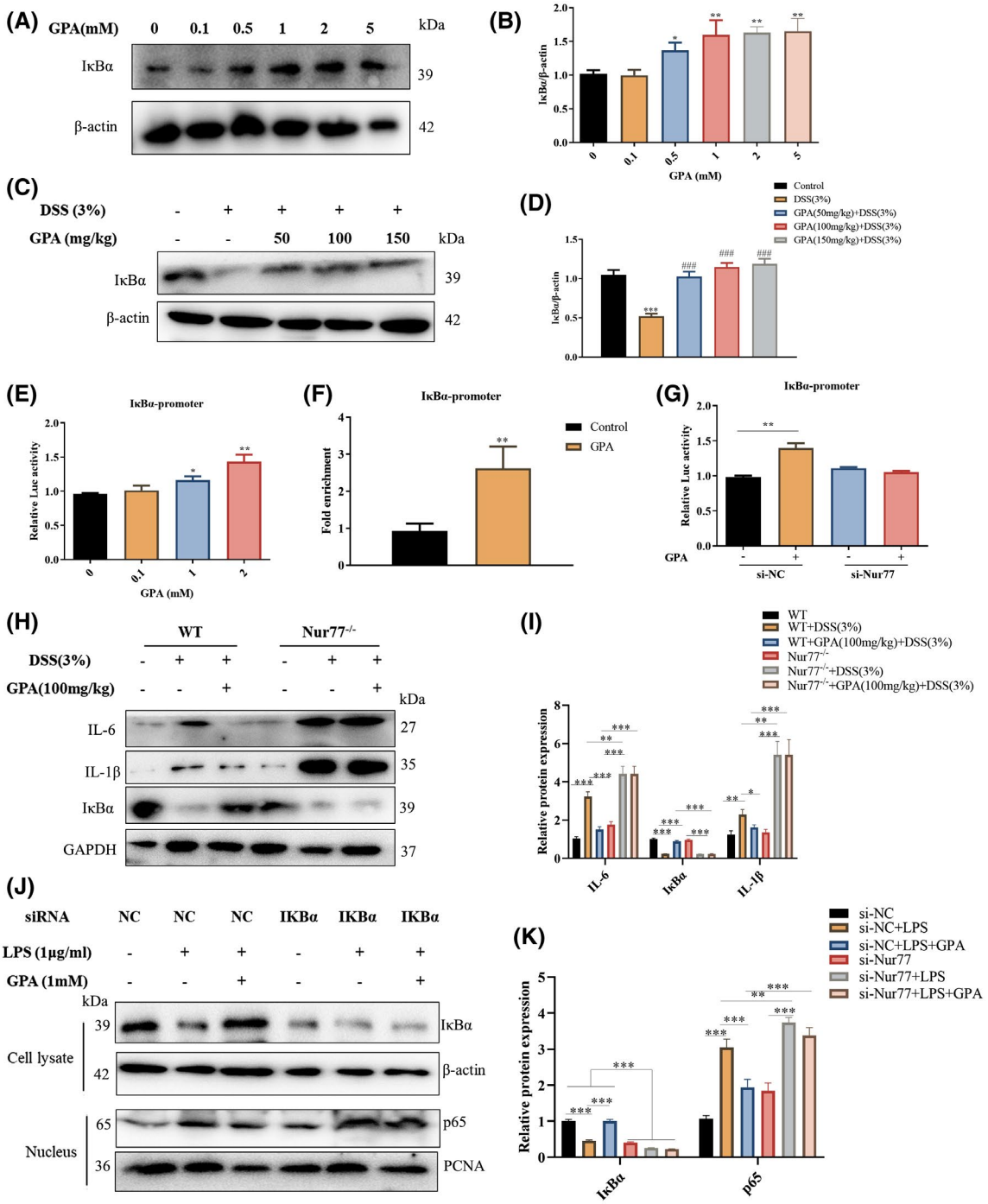

FIGURE 5 Nur77 mediates GPA-induced IκBα expression to suppress NF-κB activation. MODE-K cells were treated with different concentrations of GPA for 12 h, and then the level of IκBα was determined by western blot in three independent experiments (AB). Protein levels of IκBα in the mice colon tissues in DSS-induced colitis were analyzed by western blotting (n = 5/group) (CD). The dual-luciferase activity of IκBα promoter was measured following GPA treatment in HEK293T cells in three independent experiments (E). ChIP-qPCR assay was used to measure the binding of Nur77 to IκBα promoter in MODE-K cells with or without the treatment of GPA (1 mM) in three independent experiments (F). HEK293T cells were transfected with si-Nur77 or si-NC (scrambled siRNA), together with IκBα-promoter-reporter and pTK. After 16 h of transfection, the cells were treated with GPA (1 mM) for 12 h, and then the dual-luciferase activity was measured in three independent experiments (G). The levels of IκBα, IL-1β, and IL-6 were determined by western blot in the colon tissues of WT and *Nur77*$^{-/-}$ mice (n = 5/group) (HI). MODE-K cells were transfected with control or IκBα siRNA and further stimulated with GPA (1 mM) for 6 h, followed by LPS (1 μg/mL) for 3 h. The NF-κB p65 entering the nucleus was detected by western blot through extracting nucleus in three independent experiments (JK). The values presented are means ± SD. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ vs. the control group

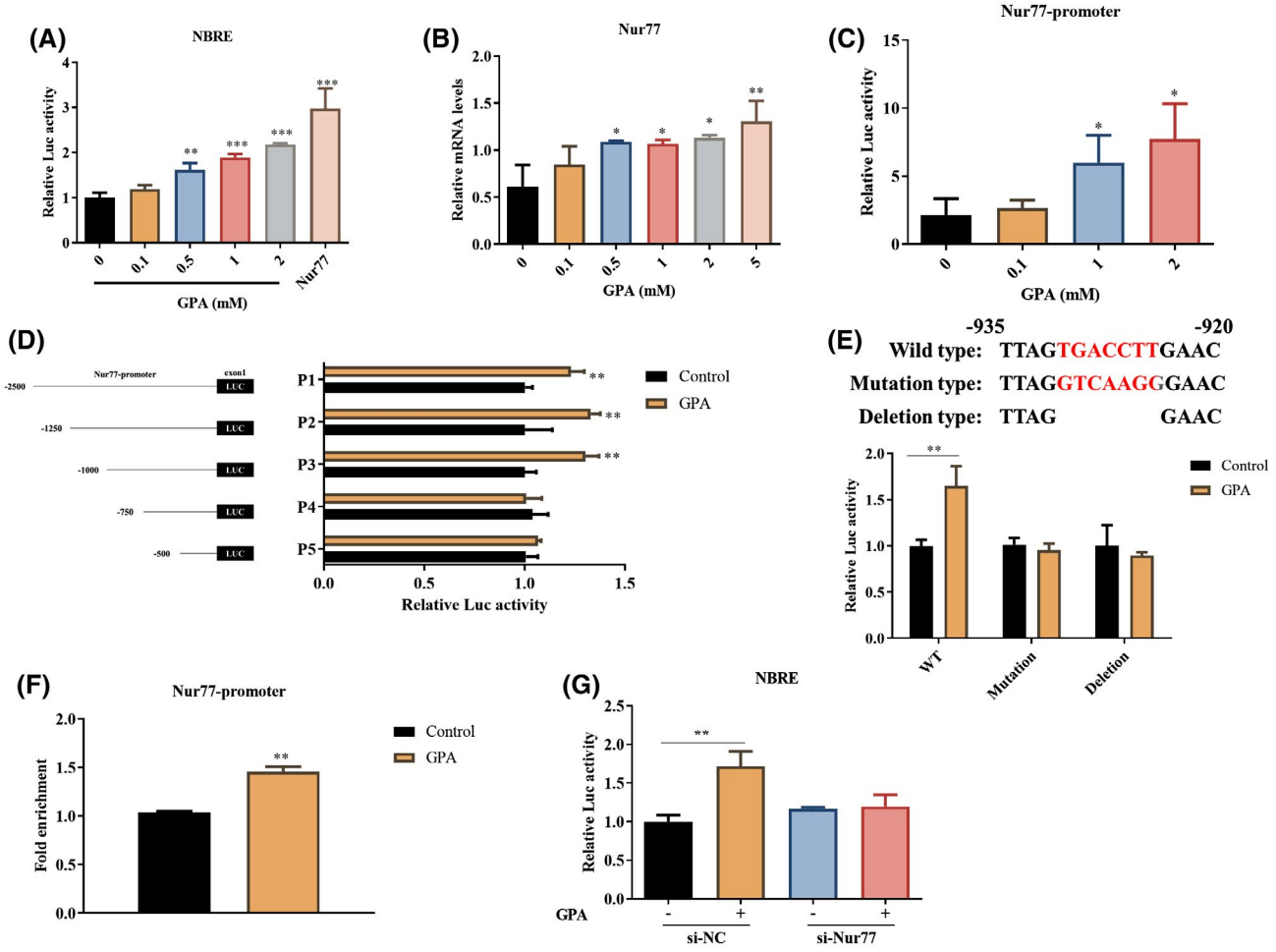

FIGURE 6 GPA mediates Nur77 autoregulation and promotes its expression. GPA stimulates Nur77-dependent transcription. Reporter genes for Nur77 (NBRE) together with renilla luciferase-expressing plasmid (pTK) were transiently transfected into HEK293T cells. After 16 h of transfection, the cells were treated with GPA for 12 h at different concentrations as indicated, and the activities of the reporter gene were determined by luciferase assay and normalized to the pTK activity (A). MODE-K cells were treated with different concentrations of GPA for 12 h, and then the level of Nur77 was detected by qPCR in three independent experiments (B). The dual-luciferase activity of the Nur77 promoter was measured following GPA treatment in HEK293T cells (C). The truncated Nur77 promoter reporters (P1-P5) and pTK were transiently transfected into HEK293T cells. After 16 h of transfection, the cells were treated with GPA (1 mM) for 12 h, and then the dual-luciferase activity was measured (D). Wild-type (WT) promoter, mutation or deletion of Nur77, and pTK were transiently transfected into HEK293T cells. After 16 h of transfection, the cells were treated with GPA (1 mM) for 12 h, and then the dual-luciferase activity was measured (E). ChIP-qPCR assay was used to measure the binding of Nur77 on the Nur77 promoter in MODE-K cells with or without treatment of GPA (1 mM) (F). Effect of siRNA against Nur77 on GPA-induced transactivational activity. HEK293T cells were transfected with si-Nur77, or si-NC (scrambled siRNA), together with NBRE-luciferase reporter and pTK. After 16 h of transfection, the cells were treated with GPA (1mM) for 12 h, and then the dual-luciferase activity was measured (G). The values presented are the means ± SD of three independent experiments. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ vs. the control group

3.6 | GPA induced Nur77 autoregulation and promoted its expression in IECs

As described above, GPA administration significantly enhanced the expression of Nur77 in the colon tissues of mice with DSS-induced colitis (Figure 2). Next, to dissect the mechanism underlying GPA-induced Nur77 expression, we evaluated the effect of GPA on the transcriptional activity of Nur77 using Nur77 specific luciferase reporter (NurRE-Luc and NBRE-Luc) in HEK293T cells. The results showed that GPA treatment increased the activity of the two reporters (Figures 6A and S6A). Moreover, the mRNA and protein levels of Nur77 were also upregulated by GPA in MODE-K cells (Figures 6B and S6B,C). Promoter assays confirmed that GPA transcriptionally activated Nur77 promoter activity in HEK293T cells (Figure 6C). Sequence analysis of the Nur77 promoter revealed that it contained conserved NBRE binding sites (Table S2), implying the possibility of the autoregulation of Nur77 on its transcriptional activity in the presence of GPA. To test the hypothesis, we generated

a series of constructs containing fragments of the mouse Nur77 promoter (P1-P5). As shown in Figure 6D, GPA treatment significantly enhanced the activity of P1, P2, and P3, but not that of P4 and P5. Moreover, when the NBRE in the Nur77 promoter was mutated or deleted, the transactivation of promoter activity by GPA was not observed (Figure 6E). Accordingly, the ChIP-qPCR assay results showed that GPA treatment promoted the binding of Nur77 to its promoter region in MODE-K cells (Figure 6F).

The Nur77 ligand-binding domain (Nur77-LBD) was reported to bind to small molecules.[36] Here, we purified the recombinant mice Nur77-LBD protein from *Escherichia coli*. It was found that GPA could directly bind with the purified Nur77-LBD at a K_d of 7.53 mM by a Localized Surface Plasmon Resonance (LSPR)-based assay in vitro (Figure S6D). To further explore the effect of GPA treatment on Nur77, we carried out an in vitro knockdown experiment by siRNA against Nur77 in HEK293T cells. The GPA-induced transcriptional activity of Nur77 was attenuated by siRNA against Nur77 (Figures 6G and S6E). Then, we overexpressed Nur77 and transfected HEK293T cells with NurRE-Luc. It was found that GPA treatment enhanced the transcriptional activity of Nur77 (Figure S6F). Finally, the effects of different peptides or amino acid on Nur77 promoter activity were compared. The results showed that only GPA enhanced Nur77 promoter activity, while Leu-Pro-Gly (LPG) did not affect Nur77 promoter activity, and Pro-Hyp (PH) and Glycine decreased its activity (Figure S6G). These results indicated that GPA could bind to Nur77, and induce Nur77 autoregulation to promote its expression in IECs.

4 | DISCUSSION

In the past decade, UC has become a globally widespread disease to affect millions of people.[2,4] Although the etiology and pathogenesis of UC are complicated and remain poorly understood, it is generally believed that the pathology of UC involves environmental, genetic and lifestyle factors, as well as host immune system, intestinal microflora, altered intestinal barrier function, aggravated oxidative stress, and inflammatory response.[37-39]

In clinical trials, the strategies for treating UC have been focused on four main aspects, including limiting the access of immune cells to the lamina propria, selective disruption of the key inflammatory signaling pathways,[5,40] repair of the epithelial barrier,[5,41] and regulation of intestinal microbiome using existing probiotics.[5,42] Among the inflammatory signaling pathways, the NF-κB pathway plays a predominant role in IBD pathogenesis.[13] As a transcriptional factor, NF-κB regulates the transcription of some pro-inflammatory cytokines such as TNF-α, IL-6, IL-8, and IL-12.[43] To date, several in vitro and in vivo studies have indicated that anti-NF-κB therapies, such as corticosteroids, could reduce the pathology and morbidity in IBD.[12] These studies were focused on the inhibition of NF-κB itself or downstream inflammatory signal pathways. However, these drugs are associated with certain side effects and dependency.[5] Different from previous studies, this study shows that GPA blocks NF-κB activation through Nur77 in IECs. It was also found that Nur77 is an inhibitor upstream of NF-κB pathway by upregulating IκBα, which might be a more effective block of NF-κB activation and limit the overproduction of pro-inflammatory cytokines and ROS, due to the upstream signal transduction of NF-κB activation. These results suggest that GPA can inhibit intestinal inflammatory signaling pathways and simultaneously repair the epithelial barrier, and thus may make a dual-effective agent for the remission of colitis.

Nur77 plays an important role in the early development of embryos, thymocyte selection, and immune-inflammatory response. Besides, it is implicated in several diseases such as pulmonary arterial hypertension (PAH), fibrotic skin, and IBD.[14,18,44] In IBD, the expression of Nur77 is significantly reduced in colon tissues, and loss of Nur77 contributes to the development of colitis.[14,15] These results may be due to

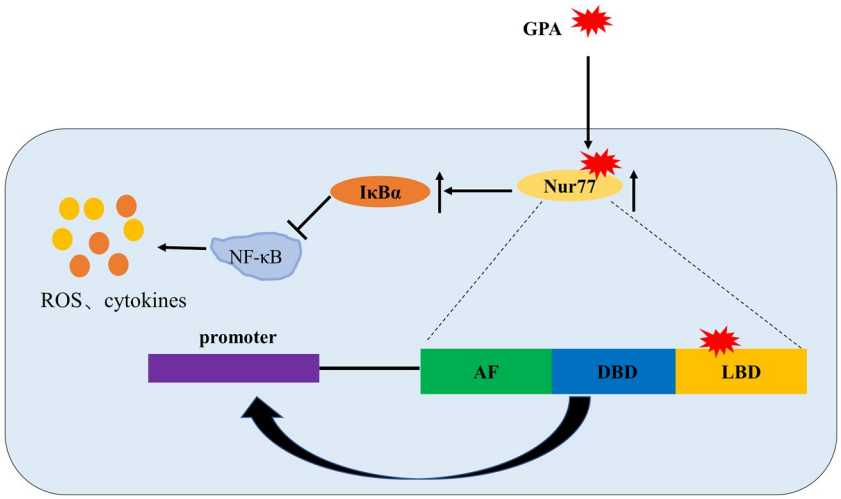

FIGURE 7 Putative mechanism for the anti-inflammatory effect of GPA in IECs. GPA transcriptionally activates Nur77 to target its own promoter, resulting in enhanced expression of Nur77. Subsequently, GPA increases the level of IκBα, a target gene of Nur77, resulting in the abolishment of the NF-κB pathway

different cell types: one study was focused on the whole colon tissues,[14] while the other study was focused on IECs and macrophages.[15] However, it remains undetermined whether upregulating Nur77 expression is an effective way to treat intestinal inflammation. Interestingly, our results showed that pretreatment with GPA for 2 weeks increased Nur77 expression in the colon tissues of mice with DSS-induced colitis, resulting in alleviation of the colitis. Moreover, Nur77 was found to mediate the protective effect of GPA against DSS-induced colitis. These results suggest that the upregulation of Nur77 expression, may be a potential target for the remission of colitis.

In addition, our results show that GPA could transcriptionally activate Nur77 to target its promoter, resulting in enhanced expression of Nur77 in IECs. Nur77 induced IκBα expression and autophagy to contribute to the protective effect of GPA against DSS-induced colitis, which was mostly dependent on the transcriptional activation of Nur77. In IECs, it was found that GPA could enter intracellular to exert anti-inflammatory and antioxidant effects through pept1. Several studies have reported that peptides could alter colonic microbes, and could be modified by bacterial or proteases.[45,46] Here, we evaluated the effects of GPA in vitro to exclude the microbial and cellular interference. In a previous study, peptides were detected in the colon tissues.[45] These results indicate that peptides, as a nutrient, could enter IECs and colon tissues to exert certain anti-colitis effects. Since peptides are broken down in the upper intestinal system, it is vital to reduce microbial degradation of peptides by decreasing microbial and cellular interference with material encapsulation or structural modification. For the intestinal microbiome, there were several studies reported that peptide could alter colonic microbes, and peptide could be modified by bacterial.[45] And regulation of the intestinal microbiome is an effective way to treat colitis.[5] Therefore, GPA may exert anti-colitis effects in other ways, that is, microbial metabolism. In the present study, pretreatment with GPA for 2 weeks might provide the basic conditions, including sufficient GPA concentration and high Nur77 expression, for Nur77-mediated inhibition of inflammatory signaling pathways and repair of the epithelial barrier in the colon tissues. Therefore, the application of GPA may provide a prevention strategy for IBD.

Intestinal epithelium plays a vital role in separating intestinal contents and lamina propria and also is the first barrier to prevent bacteria, endotoxin and other harmful substances from entering the intestinal lamina propria and blood.[47] Then, the epithelial barrier ameliorates inflammatory response by preventing local inflammation from becoming systemic inflammation.[47] In the present study, GPA was found to remove pro-inflammatory cytokines to enhance the tight junctions. In our study, GPA peptide plays an anti-inflammatory and antioxidant roles in IECs and macrophages, and we find GPA could increase the expression of Nur77 in IECs, IECs are the first barrier of mucosal immunity; meanwhile, GPA would be first transported and absorbed by IECs after entering the intestine, then we focus on the anti-inflammatory effects and mechanisms of GPA in IECs. And in the gut, IECs could crosstalk with macrophages, GPA would block the inflammatory signaling pathway in IECs, inhibit the secretion of cytokines and reactive oxygen species, and prevent the disorder of macrophages in the lamina propria.

In summary, GPA could significantly attenuate DSS-induced colitis. GPA binds to Nur77-LBD, and then transcriptionally activates Nur77 to target its promoter, resulting in enhanced expression of Nur77 in IECs. Subsequently, GPA increases the level of IκBα, a target gene of Nur77, resulting in the abolishment of the NF-κB pathway. Ultimately, GPA alleviates intestinal inflammation and maintains the intestinal barrier (Figure 7). Our results suggest that as a Nur77 modulator, GPA may be applied to the prevention of colitis with Nur77 as a candidate target.

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CONFLICT OF INTEREST

The authors have declared that no conflicts of interest exist.

AUTHOR CONTRIBUTIONS

Z. Deng conducted almost experiments and wrote the manuscript; L. Zheng designed the experiment; X. Xie completed the statistical analysis of the data; H. Wei modified the manuscript; J. Peng designed the experiment and provided funding.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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