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Porcine β-Defensin 2 Attenuates Inflammation and Mucosal Lesions in Dextran Sodium Sulfate–Induced Colitis

Feifei Han,**†,¹ Haiwen Zhang,*,¹ Xi Xia,*,¹ Haitao Xiong,*, Deguang Song,*, Xin Zong,*,¹ and Yizhen Wang*

Intestinal permeability plays a critical role in the etiopathogenesis of ulcerative colitis. Defensins, including porcine β-defensin (pBD2), are crucial antimicrobial peptides for gut protection owing to their antibacterial and immunomodulatory activities. The purpose of this study was to investigate the protective effects of pBD2 on mucosal injury and the disruption of the epithelial barrier during the pathological process of dextran sodium sulfate (DSS)–induced colitis. The effects and mechanism of pBD2 were evaluated both using a DSS-induced C57BL/6 mouse model and, in vitro, using Caco-2 and RAW264.7 cells. DSS-induced colitis was characterized by higher disease activity index, shortened colon length, elevated activities of myeloperoxidase and eosinophil peroxidase, histologic evidence of inflammation, and increased expression levels of TNF-α, IL-6, and IL-8. pBD2 increased the expression of zonula occludens-1, zonula occludens-2, claudin-1, mucin-1, and mucin-2 mRNA and proteins, and it decreased permeability to FITC-D, as well as apoptosis, in DSS-treated mice. pBD2 also decreased inflammatory infiltrates of the colon epithelium. In Caco-2 cells, pBD2 increased transepithelial electrical resistance and mucin mRNA expression, and it decreased the permeability of FITC-D while preserving the structural integrity of the tight junctions. The effects of pBD2 appeared to be through upregulation of the expression of genes associated with tight junctions and mucus, and by suppressing DSS-induced increases in inflammation, inducible NO synthase, cyclooxygenase-2, and apoptosis. These results show that pBD2 improves DSS-induced changes in mucosal lesions and paracellular permeability, possibly by affecting the activation of NF-κB signaling. The present study demonstrates that intrarectal administration of pBD2 may be a novel preventive option for ulcerative colitis. The Journal of Immunology, 2015, 194: 1882–1893.

Ulcerative colitis (UC), a subcategory of inflammatory bowel disease (IBD), is a chronic inflammatory disease characterized by aberrant immune responses to luminal bacteria in genetically susceptible subjects (1). Relapsing-remitting episodes of UC are associated with marked variations in the production of proinflammatory cytokines (2, 3). Various mechanisms have been proposed for UC, including an inappropriate inflammatory response to pathogens, autoimmunity, and an abnormal immune response to microbiota or dietary Ags. Although the exact pathogenesis of IBD is unknown, the initiation of inflammation and relapses of disease activity are associated with engagement of the innate and adaptive immune responses, including increased production of TNF-α and IFN-γ in the intestine (4, 5). These proinflammatory cytokines are potential pathogenic factors that impair mucosal barrier function and intestinal permeability. A disproportionately large number of first-degree relatives of patients with IBD have increased intestinal permeability. This suggests that barrier dysfunction may be an early defect.

Previous studies have demonstrated a decreased expression of junction complex proteins in the intestinal mucosa of patients with IBD (6, 7). Impaired gut epithelial barrier function may lead to persistent immune reactions, thus augmenting gut inflammation (8). The intestinal epithelial barrier consists of epithelial cells and intercellular junctions (9). Tight junctions (TJs) are the most apical intercellular structure in epithelial cells, accounting for cell–cell adhesion, polarity, and a permeability barrier to the paracellular transport of solutes (10). TJs create a semipermeable barrier separating different organ compartments. TJs are composed of a large number of protein components, such as occludins, claudins, and zonula occludens (zo)-1. Both claudins and occludins are coupled either directly or indirectly to cytoskeleton actin filaments with zo-1 and other factors. The distribution of claudins varies along the gut epithelia, and they are differentially expressed in connecting cells through the formation of zipper structures (11, 12). Once the mucosal barrier is breached, the submucosa is exposed to a vast pool of luminal Ags, including food and bacteria, and the innate immune responses are engaged to produce large amounts of cytokines. Therefore, maintaining TJs and barrier function may provide benefits in the adjuvant therapy of many gastrointestinal diseases, including IBD (13, 14).

In the gastrointestinal tract, the first line of defense against invading pathogens is provided by the innate immune system (15).
Part of this immune response is the release of antimicrobial peptides into the lumen of the intestinal tract. These peptides are capable of directly killing a wide variety of bacterial and viral pathogens. As a family of antimicrobial peptides, defensins are secreted by various cells as a component of the innate host defense (16). These peptides have bactericidal activity by forming micropores in the phospholipid bilayer of bacterial membranes, causing the loss of structural integrity and collapse of the bacterial cell (17). This antimicrobial activity allows defensins to protect the host epithelium and stem cells from virulent pathogens, and it also helps to regulate the number and composition of commensal microbiota (18).

Increasing evidence suggests that decreased levels or malfunctioning of β-defensins in the intestine might lead to a disturbance of homeostasis, resulting in intestinal diseases such as Crohn’s disease (CD) and UC (19). The expression of human β-defensins (hBD-1 and hBD-2) is altered in patients suffering from UC but not CD (20). Immunohistochemistry showed that hBD-2 is barely present in the uninflamed colon, but it is induced during inflammation. The lower expression of hBD-2 in CD compared with UC indicates different responses of the mucosal innate defense to these two disorders. Furthermore, an upregulation of intestinal defensins is observed as an early response to inflammation. β-Defensins exert their anti-inflammatory effects by inhibiting the production of proinflammatory cytokines (such as TNF-α) and interfering with MAPK and NF-κB signaling pathways (21–23). A previous study tested the influence of β-defensins on ulcer healing in humans. It was discovered that β-defensins were overexpressed in diabetic foot ulcers. This suggested that β-defensins could promote wound healing (24). These findings seem to indicate that β-defensins play a crucial role in the development and healing of mucosal damage and inflammation in UC.

The association of β-defensins with dextran sulfate sodium (DSS)–induced acute colitis is unknown. Thus, the present study sought to elucidate the efficacy of porcine β-defensin (pBD2) in prevention of colitis. pBD2 was administered intrarectally to mice and a parallel treatment starting together with DSS. We hypothesized that pBD2 could exert its anti-inflammatory effect on colitis by inhibiting activation of the NF-κB signaling pathway. The preventive effects, as well as the mechanism of action of pBD2 on colitis, were investigated by assessing alterations in the integrity of the intestinal barrier and the innate immune response during colitis, were investigated by assessing alterations in the integrity and composition of commensal microbiota (18).

The purpose of this experimental design is to investigate the prophylactic effect of pBD2 on inflammation and mucosal lesions in DSS-induced colitis. As for the mode of administration, we have tested the distribution in the mouse body of pBD2 after intrarectal administration of FITC-labeled pBD2 in the preliminary experiment. We found that the FITC-labeled pBD2 was distributed throughout the body 10 min after injection and disappeared practically 24 h after injection (Supplemental Fig. 1). Therefore, rectal administration was selected in this experiment. The mice were divided into four groups. The control and pBD2-treated groups were administered PBS or 5 mg/kg pBD2 rectally without DSS. The DSS and DSS plus pBD2 groups were administered PBS or 5 mg/kg pBD2 rectally, together with oral administration of 3.5% DSS. pBD2 or PBS were administered intrarectally once daily for 7 consecutive days. Mice were kept in an inverted position for 1 min after each administration to prevent leakage from the anus. Body weight was measured at day 7 by cervical dislocation and blood and tissues were collected. Colon sections were separated from the proximal rectum, close to their passage under the pelvis. The colon length, as an index of disease, was measured between the ileocecal junction and the proximal rectum, and the weight was determined (25). Some colonic tissues were excised and homogenized in RIPA lysis buffer. Equal amounts of protein (40 µg/lane) were subjected to Western blotting and ELISA. Some colonic tissues were also used for H&E staining and TUNEL assays as described previously (26). Other tissues were stored at −70°C for further analyses.

**Assessment of disease activity**

The Rachmilewitz disease activity index (DAI) was assessed by an investigator blinded to the protocol according to a standard scoring system (27). Body weight (BW), stool consistency, and occult blood (OB) in the stool were recorded daily. Loss in BW was scored as 0; no weight loss; 1, weight loss between 1 and 5%; 2, weight loss between 5 and 10%; 3, weight loss between 10% and 20%; and 4, >20%. For stool consistency, a score of 0 was assigned for well-formed pellets, 2 for pasty and semiformed stools that did not adhere to the anus, and 4 for liquid stools that adhered to the anus. For OB, a score of 0 was assigned for no blood, 2 for positive OB, and 4 for gross bleeding. These scores are added together and divided by three, resulting in DAI ranging from 0 (healthy) to 4 (maximal colitis activity). BW loss was calculated as the percentage difference between the original BW (day 0) and the BW on any particular day.

**Histology and immunohistochemistry**

The distal colon was taken for histological analysis. Samples were fixed in 10% buffered formalin overnight and kept in 70% ethanol until processed. Tissues were embedded in paraffin and cut into 5-µm-thick sections. For each sample, sections were stained with H&E and mounted in Permount (Fisher Scientific, Philadelphia, PA). The mucus-containing cells were stained purple-red. Morphometric analyses of colons were performed using an image analysis program (28).

**Assessment of apoptosis**

The TUNEL method was used to stain apoptotic cells (29). The number of apoptotic cells was counted in four to six randomly selected fields at ×400 magnification.

**Measurement of Ig concentration**

Concentrations of IgA, IgG, and IgM in sera were determined by ELISA quantitative kits (eBioscience, San Diego, CA) according to the manufacturer’s instructions.

**Enzyme activity measurements**

The activities of myeloperoxidase (MPO) and eosinophil peroxidase (EPO) were assessed in colon samples as described previously (30). Results are expressed in arbitrary units (based on absorbance) per 100 mg tissue.

**Measurement of transepithelial electrical resistance**

Electrophysiological parameters were measured using a multichannel voltage/current clamp (model VCC MC6; Physiologic Instruments). Ussing chambers were equipped with two pairs of Ag/AgCl electrodes connected to the chambers via 3 M KCl/3.5% agar bridges for measuring the potential difference (PD) and for passing current (I). The experiments were performed under open circuit conditions where the current was set to 0 and the natural transepithelial PD could be observed. For each measurement, the PD value was clamped to 20 mV and the necessary current was recorded. Electrical resistance was calculated according to Ohm’s law, that is, RES = (PD − I) / I. The fluid resistance measured prior to mounting the tissue in the diffusion chamber system was subtracted, and the net electrical resistance of the colonic epithelium was multiplied by the apparent exposed tissue area (0.07 cm²) to yield transepithelial electrical resistance.

**Materials and Methods**

**Peptide synthesis**

Linear pBD2 (a 37-aa peptide: DHYICAKGG TCFNSPCPLF NRIE GT CYSG KAKCCIR) was synthesized by standard solid-phase procedures with 9-fluorenylmethoxycarbonyl using an Apex 396 peptide synthesizer (Aappct, Louisville, KY). Ninety-five percent purity of synthetic peptides was achieved and verified using Agilent 1200 series HPLC (Agilent Technologies, Santa Clara, CA) and a Thermo Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). Peptides were dissolved in PBS for rectal administration and in PBS prepared with endotoxin-free water for addition to cultured cells.

**Animal model**

Male 6- to 8-wk-old C57BL/6 mice were obtained from the Laboratory Animal Center of Zhejiang University (Hangzhou, China) and maintained in plastic cages under standard conditions. Standard pelleted diets (Laboratory Animal Center of Zhejiang University, Hangzhou, China) were supplied ad libitum. Animal studies were approved by the Institutional Animal Care and Use Committee of Zhejiang University and performed in accordance with institutional guidelines. Acute colitis was induced through the oral intake of DSS (w/v, molecular mass of 36,000–50,000 Da; MP Biomedicals, Solon, OH) in fresh tap water ad libitum for 7 d (n = 12/group). No major differences in water consumption were detected between groups.
The effective exposed tissue area (the total area of folded mucosa) was unknown, but it was much larger than the apparent exposed tissue area. To ensure comparability of TEER measurements and sufficient data repeatability, the differences between the effective exposed area of the epithelium and the apparent exposed tissue area were normalized by presenting all measurements as a percentage of the TEER value at the end of the equilibration period for each tissue insert. The ratio of normalized TEER of epithelia in different groups was calculated for each time point together with its 90% two-sided confidence interval (corresponding to 95% one-sided confidence interval).

**Cell culture**

RAW264.7 and Caco-2 cells were cultured in RPMI 1640 or DMEM medium, respectively, supplemented with 10% heat-inactivated FBS and glutamine at 37°C under 5% CO₂. For each experiment, cells were detached with a cell scraper. At the cell density used (1 x 10⁶ cells/ml), the proportion of dead cells was <1% according to trypan blue exclusion tests.

**Measurement of NO and PGE₂**

After incubation of RAW264.7 cells (1 x 10⁶ cells/ml) for 24 h, cells were treated with LPS (1 μg/ml) for 12 h and then further incubated with pBD2 (12.5 μg/ml) for 4 h. The effect of this peptide on NO production was determined by analyzing NO levels with the Griess assay. The effect of pBD2 on PGE₂ was determined by treating cells with LPS (1 μg/ml) and pBD2 (12.5 μg/ml) for 24 h and analyzing PGE₂ levels with ELISA as described previously (31).

**Measurement of cytokine concentrations**

Tissue was homogenized and centrifuged for 15 min at 600 g at 4°C. Supernatants were collected for the cytokine assay. The levels of cytokines (TNF-α, IL-6, and IL-8) in supernatants were quantified using ELISA kits according to the protocol supplied by the manufacturer (MultiSciences Biotech, Hangzhou, China). Plates were coated with mAbs overnight and washed. The supernatants were added to the plates and incubated overnight. Biotinylated Abs were then added and incubated for 1 h. The color reaction was developed with o-phenylenediamine and H₂O₂ substrate in sodium citrate buffer. The reaction was terminated by the addition of H₂SO₄. The absorbance was measured at 490 nm using a microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA).

**mRNA analysis by real-time PCR**

Total RNA was isolated from intestinal samples or Caco-2 cells by using TRIzol (Invitrogen) according to the manufacturer’s instructions. One microgram total RNA was reverse transcribed using the GoScript reverse transcription system (Promega, Shanghai, China) following the supplier’s instructions. The reactions were incubated at 25°C for 20 min, then at 42°C for 60 min, and terminated at 70°C for 10 min. Real-time PCR reactions were conducted in triplicate on a StepOne real-time PCR system (ABI Step-OnePlus; Applied Biosystems, Foster City, CA) using the GoTaq qPCR Master Mix kit (Promega). The PCR primer sequences were designed using Primer Premier 5.0 and are listed in Table I. Real-time PCR reactions were performed as follows: a preincubation step at 95°C for 30 s, then 40 cycles of denaturation at 95°C for 10 s and annealing at 60°C for 30 s. Fluorescence was measured at the end of each annealing step, and the melting curves were monitored to confirm the specificity of the PCR products. The 2^(-ΔΔCT) method was used to determine the mRNA expression levels (32).

**Immunofluorescence analysis of TJ proteins**

Colon sections were incubated with a mouse monoclonal anti-claudin-1 Ab and an anti–zo-1 Ab (Santa Cruz Biotechnology, Dallas, TX), then with a tetramethylrhodamine isothiocyanate–conjugated secondary Ab. The sections were then washed, mounted in medium containing 40 μg/ml DAPI, and examined with a Leica fluorescence microscope (Keyence, Osaka, Japan).

**Western blot analysis**

The concentrations of proteins in extracts from RAW264.7 cells were determined using the Coomassie brilliant blue assay. Extracts containing equal quantities of proteins (100 μg) were electrophoresed in polyacrylamide gel. Subsequently, the separated proteins were transferred onto a polyvinylidene difluoride membrane. The membrane was blocked for nonspecific binding for 30 min (5% skimmed milk in PBS) and then incubated overnight at 4°C with Abs for c-Jun, p–c-Jun, NF-κB p65, p-Akt, Akt, p–IκB-α, or p–IκB-β (Santa Cruz Biotechnology or Epitomics, Burlingame, CA). Blots were developed with ECL detection reagents (Santa Cruz Biotechnology), exposed on Kodak Xmat blue XB-1 film, and quantified by BandScan 5.0 software using β-actin as the internal control.

**Statistical analysis**

All data are expressed as means ± SEM. Statistical significance was assessed by one-way ANOVA followed by Duncan’s test using SPSS 18.0. A P value < 0.05 was considered statistically significant.

**Results**

pBD2 ameliorated the clinical symptoms and colonic inflammation during the pathological process of DSS-induced colitis

As expected, DSS damaged the colon’s mucosal barrier, leading to gut inflammation and weight loss. Mice in the DSS-treated group

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The sequences in Table I are available through GenBank (http://www.ncbi.nlm.nih.gov/nuccore/) under the accession numbers listed above. F, forward; R, reverse.
had significantly more weight loss than did those in the control group from days 4 to 7, whereas mice in the pBD2-treated group had less weight loss compared with mice in the DSS-treated group. Mice in the pBD2 plus DSS group rapidly recovered weight from days 4 to 7 (Fig. 1A). Similarly, mice in the pBD2 and pBD2 plus DSS groups had firmer stools and less blood in their stools than did those in the DSS group from days 1 to 7.

Colon shortening was used as a marker of inflammation. Consistent with the DAI, by day 8, the colon length in the DSS group was shorter than that in the control group (4.10 ± 0.22 versus 5.61 ± 0.13 cm, p < 0.05) and in the pBD2 plus DSS group (4.10 ± 0.22 versus 5.05 ± 0.13 cm, p < 0.05) (Fig. 1C). Mice in the DSS group had significantly more macroscopic inflammation than did those in the pBD2 group. Compared to the control group, histological examination of the colon in the DSS group showed extensive ulceration of the epithelial layer, edema, crypt damage of the bowel wall, and infiltration of granulocytes and mononuclear cells into the mucosa. Compared to mice in the DSS group, mice treated with pBD2 plus DSS had reduced histologic evidence of DSS-induced colitis (Fig. 1D).

Although DSS induced histological alterations only in the colon, differences in immune cells and Igs could be detected between the blood of control and DSS groups. To study the immune response in the blood, the concentrations of Igs in blood were analyzed. Treatment with DSS decreased the concentrations of IgA and IgM, but increased the concentration of IgG. There were improvements in these parameters in mice from the pBD2 plus DSS group, returning to levels closer to the control group (Fig. 1E, 1F).

**FIGURE 1.** pBD2 ameliorated clinical symptoms during the pathological process of DSS-induced colitis. (A) BW is shown as a percentage of daily weight change from days 0 to 7. (B) DAI is comprised of weight loss, stool consistency, and OB. (C) Quantitative measurement of the length of the colon. (D) Representative H&E-stained sections from (Da) control, (Dc) pBD2, and (Dd) DSS plus pBD2. Original magnification ×200. (E) Concentrations of IgA, IgG, and IgM in venous blood. Results are given as means ± SEM. Differences between groups were determined by ANOVA followed by Duncan’s test (n = 6). *p < 0.05 compared to control mice, *p < 0.05 compared to DSS-treated mice.
As depicted in Fig. 2A, there was a marked increase in MPO (an indicator of colonic infiltration with polymorphonuclear leukocytes) and EPO (an indicator of colonic infiltration with eosinophils) activities in colon tissue from mice treated with DSS (162 ± 11 and 40.3 ± 3.0 ng/g tissue for MPO and EPO, respectively) compared with control mice (34.9 ± 10.0 and 30.9 ± 1.7 ng/g tissue, respectively). Administration of pBD2 to DSS-treated mice significantly decreased MPO and EPO activities (93.6 ± 5.2 and 22.6 ± 1.2 ng/g tissue, respectively).

As shown by TUNEL staining, <10 apoptotic cells per field were found in the colon from all normal mice, or following treatment with pBD2 alone. This number increased to 50 cells per field in the colon from the DSS-treated mice without pBD2 treatment. The administration of pBD2 significantly reduced the number of apoptotic cells in DSS-treated mice (Fig. 2B).

Infiltration of inflammatory cells (particularly CD177+ neutrophils and F4/80+ macrophages) into the colon is observed in acute colitis. In this study, the infiltration of CD177+ and F4/80+ cells into colonic tissue was detected by immunohistochemistry. There was minimal infiltration of neutrophils (Fig. 2C) and macrophages (Fig. 2D) into the colon of control mice. Compared to control mice, DSS triggered an increased infiltration of CD177+ neutrophils and F4/80+ macrophages into the colonic lesion area. Treatment with pBD2 decreased the infiltration of neutrophils and macrophages compared with the group treated with DSS alone.

To determine the anti-inflammatory effect of pBD2 on DSS-induced colitis, the expression and secretion of the inflammatory markers TNF-α, IL-6, and IL-8 were evaluated in colon tissues. Compared with the normal control group, there was a higher expression of TNF-α, IL-6, and IL-8 mRNA in the DSS-treated group. However, the levels of TNF-α, IL-6, and IL-8 mRNA were significantly lower in both the pBD2- and pBD2 plus DSS–treated groups compared with the DSS-treated group (p < 0.05) (Fig. 3A). There was no significant difference between the pBD2- and pBD2 plus DSS–treated groups.

Administration of DSS caused a significant elevation of colonic levels of the proinflammatory cytokines TNF-α, IL-6, and IL-8 as compared with those in the colons from the normal control group (Fig. 3B). Treatment of mice with pBD2 significantly reduced the DSS-induced production of TNF-α, IL-6, and IL-8 compared with that in the DSS alone group (Fig. 3B). Taken together, these data indicated that treatment with pBD2 ameliorated the colonic inflammation caused by DSS.

pBD2 prevented the DSS-induced disruption of intestinal TJ structure and function

To investigate the effect of pBD2 on intestinal structure and function, intestinal permeability was assessed using FITC-dextran permeability. Mice in the pBD2 plus DSS–treated group had a lower intestinal permeability to 4-kDa FITC-dextran when compared with that in the DSS alone–treated group (Fig. 4A). To evaluate the
functional viability and integrity of mouse intestinal epithelium under ex vivo conditions, TEER measurements were performed for 60 min. As shown in Fig. 4B, in the DSS–treated group, the TEER values declined significantly, indicating an increase in permeability. In contrast, DSS-treated mice given pBD2 showed a significant recovery in TEER. These data support a significant role for pBD2 activation in minimizing DSS-induced intestinal epithelial hyperpermeability.

The expression of zo-1, zo-2, claudin-1, mucin-1, and mucin-2 was monitored and compared between groups using real-time PCR. As shown in Fig. 4C, all genes were downregulated in mice treated with DSS alone, compared with normal animals. Administration of pBD2 increased the expression of all genes up to 90% in DSS–treated mice. To further evaluate the protective effect of pBD2 on the disruption of TJs by treatment with DSS, the TJ markers zo-1 and claudin-1 were detected by immunofluorescence on the colon epithelium. In the control group, zo-1 and claudin-1 proteins (red) were seen on the cellular membrane of the epithelial cells, mostly in the spinous and granular layers. In the pBD2 alone or with DSS–treated group, the expression of zo-1 and claudin-1 increased in both the cellular membrane and cytoplasm of spinous and granular layers in the mucosa. However, in the DSS–treated group, the expression of zo-1 and claudin-1 decreased in individual cells of the spinous and granular layers (Fig. 4D, 4E).

pBD2 attenuated the DSS-induced paracellular permeability in vitro

To further address the molecular mechanisms of the protective role of pBD2 in DSS-induced changes to intestinal barrier function, we used an established Caco-2 cell culture system and the TEER assay. When a monolayer of Caco-2 cells was incubated with 3.5% DSS, the TEER values rapidly decreased by 2 h and reached their lowest level (70% of the baseline value) after 4 h. However, in the pBD2 plus DSS–treated cells, the average TEER value was only moderately reduced by 2 h (Fig. 5A) and increased by 6 h, reaching >80% of the baseline value. At the end of the experiment (24 h), the average TEER value of the DSS-treated Caco-2 monolayer remained significantly below the value of the control cells. In contrast, the TEER value of Caco-2 cells treated with pBD2 plus DSS was not significantly different from the baseline value or the value of the control cells.

Caco-2 cells form TJs when grown to a monolayer. To determine whether pBD2 could prevent the DSS-induced reduction in TJ expression, real-time PCR analysis was performed for message levels of TJ markers (Fig. 5B). DSS caused a significantly reduced expression of zo-1, claudin-1, and occludin, compared with control (0.49 ± 0.08 versus 1.00 ± 0.30 ± 0.12 versus 1.00 ± 0.05, 0.61 ± 0.03 versus 1.00 ± 0.01, respectively; p < 0.05). The expression of TJ markers in the pBD2 plus DSS group were significantly higher than that in the DSS alone group (zo-1, 0.75 ± 0.06; claudin-1, 0.76 ± 0.15; occluding, 0.93 ± 0.07; p < 0.015) (Fig. 5B). This supports the importance of pBD2 in maintaining the integrity of the TJs.

pBD2 inhibited the production of inflammatory mediators

The effect of pBD2 on the proinflammatory mediators inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) is presented in Fig. 6A and 6B. Colonic iNOS and COX-2 levels in the DSS alone group were significantly higher (4.07 ± 0.08 μM/100 mg and 3.06 ± 0.09 U/100 mg tissue, respectively) than the corresponding values in control animals (1.68 ± 0.07 μM/100 mg and 1.19 ± 0.08 U/100 mg tissue, respectively). Treatment with pBD2 alone produced iNOS and COX-2 levels similar to the control group. The administration of pBD2 to DSS–treated mice significantly decreased these inflammatory mediators (2.06 ± 0.08 μM/100 mg and 1.31 ± 0.04 U/100 mg for iNOS and COX-2, respectively).
DSS caused a significant increase in the concentration of NO in the supernatant of RAW264.7 cells. pBD2 significantly inhibited this DSS-induced increase in NO production (Fig. 6C). Similarly, DSS significantly increased the production of PGE2 in RAW264.7 cells and 12.5 μg/ml pBD2 blocked this effect (Fig. 6D).

Effect of pBD2 on the in vitro activation of upstream signaling molecules for NF-κB translocation

Immunohistochemistry results showed that LPS elevated the expression of phosphorylated p65 NF-κB, IκB-α, and Akt compared with the control group (Fig. 7). Treatment with 12.5 μg/ml pBD2 effectively inhibited the phosphorylation of these proteins. The suppressive effect of pBD2 appears to involve NF-κB translocation. Indeed, pBD2 suppressed the transcriptional regulation of inflammatory genes caused by LPS by inhibiting the activation of p65, a major subunit of NF-κB. However, pBD2 did not suppress the phosphorylation of c-Fos (data not shown), a member of the AP-1 family of proteins. This indicated that the inhibition was selective to NF-κB.

Discussion

Murine models of IBD have been used to investigate the regulatory mechanisms that reduce inflammation and restore intestinal homeostasis (33). We referenced the previous studies to determine the induction method of the colitis model (34–36) and a rough dose range of peptide first (36–42). We found that providing drinking water containing DSS induces colitis was one of the well-established experimental models for studying IBD (43). The present study focused on studying the effects of pBD2 on DSS-induced experimental colitis. We demonstrated that administration of pBD2 effectively attenuated colonic inflammation in mice with DSS-induced colitis. After 1 wk of treatment, BW loss, DAI, colonic MPO activity, and macroscopic and histo-
logical changes were significantly improved in mice treated rectally with 5 mg/kg/d pBD2. The present study also demonstrated the anti-inflammatory and intestinal TJ regulatory activities of pBD2 against DSS-induced colitis. Additionally, we have demonstrated that the molecular mechanisms involved in the therapeutic effects of pBD2 on colitis involved an inhibition of NF-κB signaling pathways. Finally, we showed that pBD2 reduced the increased serum and colon levels of TNF-α, IL-6, and IL-8 caused by DSS. All of these findings suggest that pBD2 is protective in a mouse model of DSS-induced colitis.

Colitis induced by DSS has characteristics similar to human UC, such as signs of diarrhea, gross rectal bleeding, weight loss, shortening of the colorectum, histological features of multiple erosions, and inflammatory mucosal changes occasionally in-

FIGURE 5. Effect of pBD2 on DSS-induced paracellular permeability in vitro. (A) Effect of pBD2 (12.5 μg/ml) on TEER of a Caco-2 cell monolayer. Changes in TEER induced by DSS were prevented with pBD2. (B) Expression of zo-1, claudin-1, and occludin mRNA was assessed by real-time PCR. Treatment with DSS markedly decreased the expression of zo-1, claudin-1, and occludin mRNA compared with control. pBD2 prevented the reduction in TJ expression induced by DSS. Results are given as means ± SEM. Differences between groups were determined by ANOVA followed by Duncan’s test (n = 6). *p < 0.05 compared to control cells, #p < 0.05 compared to DSS-treated cells.

FIGURE 6. pBD2 inhibited the production of inflammatory mediators. Effects of pBD2 (12.5 mg/kg BW) on colonic iNOS (A) and COX-2 (B) in mice with DSS-induced colitis. Treatments were administered once daily for 7 consecutive days simultaneously with the induction of colitis. (C) Levels of NO determined by the Griess assay in culture supernatants of RAW264.7 cells treated with LPS and/or pBD2 for 24 h. (D) Levels of PGE2 determined by ELISA in culture supernatants of RAW264.7 cells treated with LPS and/or pBD2 for 24 h. Data are expressed as means ± SEM of six independent assays. Differences between groups were determined by ANOVA followed by Duncan’s test. *p < 0.05 compared to control cells, #p < 0.05 compared to DSS-treated cells or LPS-treated cells.
phosphorylation of p65 NF-κB. However, 1 μg/ml LPS decreased the expression of phosphorylated p65 NF-κB and Akt. Treatment with pBD2 (12.5 μg/ml) inhibited the protein phosphorylation of p65 NF-κB, IkB-α, and Akt.

FIGURE 7. Effect of pBD2 on the in vitro activation of upstream signaling molecules for NF-κB translocation. Phosphorylated and total protein levels of p65 NF-κB (A), IkB-α (B), Akt (C), and β-actin from RAW264.7 cell lysates were determined using Abs recognizing phospho-specific or total protein. Total protein levels of p65 NF-κB, IkB-α, Akt, and β-actin were not changed by treatment with LPS, pBD2, or pBD2 plus LPS, compared with control. However, 1 μg/ml LPS increased the expression of phosphorylated p65 NF-κB, IkB-α, and Akt. Treatment with pBD2 (12.5 μg/ml) inhibited the protein phosphorylation of p65 NF-κB, IkB-α, and Akt.

cluding crypt abscess. The major causative factors in the initiation of human colitis include enhanced vasopermeability and upregulation of inflammatory mediators and some cytokines. Prolonged neutrophil infiltration is also involved in this animal model of colitis (44, 45). Initiation and progression of intestinal inflammation, including IBD, requires the presence of bacterial pathogens (46, 47). To maintain normal homeostasis, the host has evolved several antibacterial defense systems, including antimicrobial peptides such as defensins, to defend against microorganisms (20). Endogenous antimicrobial peptides play an important role in innate immunity. An important advance in the defensins field has been evidence indicating that defensins may play a role in innate immunity. Welkmamp et al. (48) reported an increased expression of colonic hBD2 mRNA in UC patients but not in CD patients. Altered defensin production was thus suggested to be an integral element in the pathogenesis of IBD.

Consistent with these findings, the present study provides evidence for the involvement of defensins in the pathological process of colitis in DSS-exposed mice. β-Defensins comprise a major subclass of the defensins (49) and play an important role in both innate and adaptive immune defense (50, 51). They are chiefly expressed in a variety of epithelial cells (e.g., airway epithelia, urogenital tissues, nasolacrimal duct, and mammary gland) and sometimes immune cells (e.g., dendritic cells and macrophages) (50, 52, 53). Both humans and mice express a relatively large number of β-defensins, of which six human (hBD1–6) and five mouse (mBD1–5) defensins have been isolated and characterized. hBD1 and its murine ortholog mBD1 are expressed constitutively. Other β-defensins, including hBD2 and its murine ortholog mBD3, are induced by bacteria and cytokines. mBD3, similar to certain human β-defensins, possesses the ability to attract immune cells chemotactically (54). To date, 12 pBDs have been identified, and the expression of pBD1 and pBD2 in the small intestine of the pig has been described recently (55, 56).

From an immunological point of view, the epithelial lining of the intestine has been long considered as solely a physical barrier to protect the underlying mucosa. Intestinal epithelial cells produce cytokines and chemokines that are crucial for the recruitment and activation of immune cells. Additionally, they produce and secrete effector molecules, such as β-defensins, that can directly affect bacterial populations in the gut (57). The intestinal mucosa is a physical and metabolic barrier against toxins and pathogens in the lumen. The barrier regulates macromolecule trafficking between the lumen and the internal milieu, and it protects the host by preventing harmful solutes, microorganisms, toxins, and luminal Ags from entering the body (58). Compromising the barrier function of the intestinal mucosa can result in an increased exposure of the host to luminal Ags and pathogens, leading to inflammation (59), and is a major contributing factor in multiple pathological conditions of the gastrointestinal tract. The epithelial barrier function is largely determined by intercellular TJs. The TJs are responsible for restricting paracellular movement of compounds across the intestinal mucosa (60, 61). An increased permeability in the TJ may provide a major site for both infection and the establishment of inflammation in the gut (62, 63). Bacterial translocation is thought to occur via a paracellular pathway through the epithelial cells. Our data show that pBD2 might have a protective effect on barrier integrity by maintaining the expression of TJs and mucin proteins, thereby reducing the severity of gut inflammation. The results of our study suggest that the bioavailability of pBD2 is an important contributing factor for determining epithelial integrity.

Once the mucosal barrier is breached, the submucosa is exposed to a vast pool of luminal Ags, including food and bacteria, thereby engaging the innate immune responses, including increased production of proinflammatory cytokines. We showed that the addition of pBD2 ameliorated the increased expression in TNF-α and IL-6 induced by DSS. The reduction in proinflammatory cytokines by pBD2 may be either due to a direct suppressive effect on their expression or effects on the maintenance of epithelial barrier function, leading to a reduction in foreign luminal antigenic load and full activation of the innate immune system.

A major function of defensins is thought to be protection of the adjacent epithelial stem cells at the base of the crypt (64). Therefore, continual expression of defensins allows constant protection of the integrity of the epithelial barrier. However, as has been described in numerous studies, the expression of defensins can vary in the presence of disease (20). In the present study, the administration of pBD2 ameliorated signs and symptoms of inflammation, such as colon shortening, weight loss, and increased DAI.

The infiltration of activated neutrophils is one of the most prominent histological features observed in IBD (34, 65). Neut-
trophils produce superoxide anions and other reactive species leading to the formation of the very reactive hydroxyl radical that may contribute significantly to the tissue necrosis and mucosal dysfunction of IBD (66). In the present study, histological analysis showed an increase in the colonic submucosal infiltration of neutrophils and macrophages 7 d after mice were treated with DSS. Pretreatment with pBD2 in DSS-induced colitis significantly reduced the DAI, the wet weight of the colon (a reliable and sensitive indicator of the severity and extent of inflammatory response) (67), and loss of BW.

MPO is a peroxidase enzyme found in the azurophilic granules of neutrophils and monocytes. The level of MPO activity is directly proportional to the neutrophil concentration in the inflamed tissue and is thus an index of neutrophil infiltration and inflammation (68). Therefore, the measurement of MPO activity has been considered a quantitative and sensitive assay for assessing acute intestinal inflammation. Our findings show that colonic MPO activity was markedly increased in DSS-treated mice, and that 7 d of treatment with pBD2 significantly reduced this effect. This suggests that pBD2 has an anti-inflammatory effect that parallels the histological evidence of protection.

Apoptosis is one of the ulcerogenic processes in the gastric mucosa (69). Increasing evidence shows that dysregulation of this process in the colonic mucosa is involved in the pathogenesis of IBD (70). As shown by TUNEL staining, DSS markedly increased the number of apoptotic cells in the colonic mucosa. Administration of pBD2 decreased the extent of apoptosis with no effect in the normal mucosa. Thus, one of the mechanisms for pBD2 to prevent DSS-induced colitis may be through an inhibition of apoptosis in the colonic mucosa.

The colonic epithelium is composed of trefoil peptides such as mucin-1 and mucin-2, in which mucin-2 is the predominant secretory mucin in the colon (71). The present study demonstrated that mucin-1 and mucin-2 genes were markedly downregulated during DSS-induced colitis. A dose of 5.0 mg/kg/d pBD2 significantly upregulated the expression of these genes at the mRNA level. A study with a mucin-2 knockout mouse model confirmed that mucin-2 deficiency caused abnormal morphology, ulceration of epithelial cells, and a mild increase of inflammatory cells in the colon (72). The knockout animals developed more serious colitis symptoms by feeding 2.5% DSS for 2 d, suggesting that mucin-2 is critical for colonic protection. The mucus layer in the colon of patients with UC was thinner than in healthy subjects (73), and a reduction of the mucus layer in UC patients was linked to a decrease in mucin-2 expression (74). Taken together, pBD2 may be useful in treating colitis patients by boosting mucin gene expression and increasing mucus synthesis in the colonic mucosa, which is perturbed in UC patients.

UC is a nonspecific inflammatory disorder involving primarily the mucosa and submucosa of the colon. Although the precise etiopathogenesis of UC remains unknown, there is accumulating evidence that an increase of proinflammatory mediators leads to an inflammation cascade and tissue damage (75). TNF-α, which is a pleiotropic cytokine, plays a fundamental role in inflammatory conditions such as colitis by triggering the accumulation and activation of leukocytes (76). Therefore, TNF-α is an important therapeutic target and several engineered Abs against TNF-α are beneficial in the treatment of IBD (77). IL-6 is produced by various cell types and exerts pleiotropic effects on different organ systems. Alterations in the production of IL-6 have been found in inflammatory states such as rheumatoid arthritis (78), CD, UC (79), mesangial glomerulonephritis (80), and sepsis (81). IL-6 can stimulate neutrophil chemotaxis and lead to tissue destruction in the colon. In the present study, the levels of TNF-α and IL-6 were elevated in the serum from mice with DSS-induced colitis. Importantly, the administration of pBD2 significantly reduced the serum levels of TNF-α and IL-6. These results indicated that pBD2 ameliorates DSS-induced colitis by suppressing proinflammatory mediators such as TNF-α and IL-6. NO is generated enzymatically in a variety of cells by three isoforms of NO synthase. Increased expression of iNOS leads to the synthesis of micromolar quantities of NO that can damage cells through the formation of NO-reactive products and can be measured as a toxicity marker (82). The activation of COX-2 and iNOS produces excessive inflammatory mediators that may contribute to the development of intestinal damage. Additionally, iNOS acts in synergy with COX-2 to promote the inflammatory response (83). In the present study, NO and COX-2 were significantly increased in LPS-treated RAW264.7 cells. Treatment with pBD2 decreased NO production through downregulation of iNOS and also significantly diminished COX-2 expression. These effects may account for the protective role of pBD2 in the colitis model. Similarly, earlier studies revealed that there was a reduced risk of gastrointestinal ulcer complications using COX-2 inhibitors (84).

Proinflammatory cytokines such as IL-1β, IL-6, and TNF-α, as well as COX-2, iNOS, and adhesion molecules, are regulated through the NF-κB pathway (85). This suggests that blocking these inflammatory mediators en masse by inhibiting NF-κB might be more effective in the treatment of colitis than suppressing individual factors such as TNF-α and IL-6. The present study revealed that the anti-inflammatory effect of pBD2 may be linked with the downregulation of p65 NF-κB in DSS-induced colitis in mice. Western blot analysis indicated that the phosphorylation of p65 NF-κB was significantly increased by LPS in RAW264.7 cells and was decreased after treatment with pBD2. Similarly, the levels of TNF-α and IL-6 in the DSS-treated group were increased more than in the normal control group in response to p65 NF-κB.

Because the upstream signaling events for NF-κB activation have been well established, we examined specific targets of pBD2 for the inhibition of NF-κB activity. As Fig. 7 depicts, pBD2 suppressed the phosphorylation of IkB-α. The activation of upstream enzymes for IkB-α phosphorylation were also suppressed. Thus, pBD2 reduced the phosphorylation of Akt. Because the promoter regions of TNF-α and IL-6 contain consensus binding motifs for NF-κB, our data strongly suggest that pBD2 can suppress the production of inflammatory mediators such as NO and PGE2 by suppressing NF-κB–mediated transcriptional activation of inflammatory gene expression.

In summary, our data confirm that pBD2 prevents DSS-induced colitis in mice and that this protective effect may be due to maintaining the integrity of the intestinal mucosal barrier and regulating innate immunity. These effects are mediated via modulation of TJ proteins in the colonic tissue, inhibiting the production of inflammatory mediators (including iNOS, COX-2, TNF-α, and IL-6) and inhibiting NF-κB signal transduction pathways. Numerous efforts have focused on developing new anti-inflammatory drugs targeted to NF-κB (86). The present results support previous research suggesting that targeting NF-κB may be a new and effective treatment in IBD patients.

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Disclosures
The authors have no financial conflicts of interest.
References
