Proton-Pump Inhibitor Exposure Aggravates Clostridium difficile–Associated Colitis: Evidence From a Mouse Model

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Background. Clostridium difficile is currently the leading cause of infectious diarrhea in hospitalized patients. In addition to the infection due to toxigenic C. difficile in the gastrointestinal tract of susceptible hosts, other predisposing factors for C. difficile infection (CDI) are identified, including advanced age, a prolonged hospital stay, and use of acid-suppressive drugs. Of note, exposure to gastric acid-reducing agents, such as H2 blockers and proton pump inhibitors (PPIs), remains a controversial risk factor, and has been associated with CDI in some studies but not in others. A mouse model of antibiotic-associated clostridial colitis was established to examine the role of PPIs for CDI.

Materials and Methods. A mouse model of antibiotic-associated clostridial colitis was set up. NF-κB reporter mice were used to address the in vivo spatial and temporal inflammatory patterns of C. difficile–associated colitis. Serum levels of lipopolysaccharide and dextran-FITC were measured to reflect the barrier permeability of affected intestines.

Results. Mice with CDI that were exposed to PPI exhibited greater losses of stool consistency and body and cecal weights than those that were not exposed to PPI. Further, more neutrophilic infiltrations, epithelial damage, and inflammatory cytokine expression were noted in colon specimens of the mice with PPI exposure. More-evident inflammatory responses were detected by in vivo imaging of NF-κB reporter mice with CDI that were exposed to PPI. Gut barrier permeability was increased to a greater extent, as reflected by higher serum levels of lipopolysaccharide and dextran-FITC in mice with CDI that were exposed to PPI.

Conclusions. Our mouse model demonstrates that PPI exposure increases the severity of intestinal inflammation in mice with C. difficile–associated colitis.

Keywords. C. difficile infection; proton-pump inhibitor; NF-κB reporter mice; gut barrier permeability; mouse model.

Clostridium difficile, an anaerobic, gram-positive, spore-forming bacterium, was initially described in 1935 [1] as part of normal flora in the intestinal tract of newborn infants, but its association with clinical disease was not defined until 1978 [2]. It is now considered to be one of the most important causes of nosocomial infections in hospitalized patients receiving antibiotic treatment [3]. The clinical features of C. difficile infections (CDIs) include asymptomatic colonization, mild diarrhea, and severe infections, including toxic megacolon and pseudomembranous colitis [3, 4]. During the past 3 decades, the incidence and severity of CDI have increased significantly, largely because of epidemic hypervirulent strains causing several CDI outbreaks in the United States, Canada, and Europe [3, 4].

In addition to the presence of C. difficile in the gastrointestinal tract of susceptible hosts, other predisposing factors for CDI include advanced age, prolonged hospital stay, and use of acid-suppressive drugs.
note, proton-pump inhibitors (PPIs), a class of commonly prescribed medications worldwide, can greatly suppress gastric acid secretion and facilitate the survival of *C. difficile* in higher gastric pH levels. Thus, PPI use had been regarded as a risk factor for CDI [5] and recurrent CDI [6]. In our previous study, among patients with toxigenic *C. difficile* colonization, prior PPI exposure was associated with CDI emergence [7]. Therefore, routine use of PPIs for peptic ulcer prophylaxis should be more prudent [8,9] to avoid provoking CDI development in susceptible individuals. However, results from association studies have been extremely varied [10, 11]. Further clinical or animal studies are needed to clarify the pathogenic role of PPIs in predisposing patients to the development of CDI.

Our clinical study in southern Taiwan has reported the interaction between PPI and CDI [12]. The scientific rationale for this association seems strong, especially because gastric acid suppression leads to the loss of an important defense mechanism. Using a recently established mouse model of CDI, we studied whether the disease severity of CDI in mice pretreated with a PPI is higher than in those that were not pretreated with a PPI.

**MATERIAL AND METHODS**

**Bacteria Strain**

A toxigenic *C. difficile* strain, VPI 10 463 (CCUG 19 126 or ATCC43255), with ribotype 087 and toxino-type 0 and no gene encoding binary toxin was used in all experiments. *C. difficile* was incubated in CDC anaerobe 5% sheep blood agar (BD, Cockeysville, Maryland) for 2 days at 37°C under anaerobic conditions. A few colonies were transferred by an incubation loop into brain-heart infusion broth (BD), which also included 5 mg/mL yeast extract (MO BIO, Carlsbad, California) and 0.1% L-cysteine (AMRESCO, Solon, Ohio), and incubated anaerobically at 37°C for 2 days. To collect bacteria, bacterial broth was centrifuged at 2000g for 10 minutes. After supernatant was discarded, the bacterial pellet was resuspended with 1 mL of phosphate-buffered saline. The concentrations of resuspended bacteria were adjusted to $3.5 \times 10^6$ colony-forming units (CFU)/mL by measuring light absorbance (600 nm). Serial dilutions and cultures were used for bacterial quantification.

**Animal Model of CDI**

We used a cocktail of antibiotics to disrupt the normal gut microbial communities and to predispose the mice to CDI (Figure 1). Briefly, C57BL/6JNarl mice aged 7–8 weeks were purchased from the National Laboratory Animal Center in Tainan, Taiwan. All mice were maintained and handled according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of National Cheng Kung University (NCKU). All animal studies were performed following a protocol approved by the IACUC of NCKU (approval NCKU-IACUC-102-296). To disrupt the intestinal microbiota, mice were fed drinking water containing an antibiotic mixture [12], which included 0.045 mg/mL vancomycin, 0.215 mg/mL metronidazole, 0.4 mg/mL kanamycin, 0.035 mg/mL gentamicin, and 850 U/mL colistin, from 5 days to 1 day before oral inoculation of vegetative *C. difficile*. All antibiotics were purchased from Sigma-Aldrich. Vancomycin and metronidazole were discontinued to avoid disrupting *C. difficile* colonization from the day before oral inoculation of *C. difficile*. The mice received a PPI, esomeprazole (40 mg/kg/day), or phosphate-buffered saline twice daily for 2 days before oral inoculation of *C. difficile*. Then, $3.5 \times 10^7$ CFU of *C. difficile* VPI10463 vegetative cells were administered orogastrically, and 4 mg/kg clindamycin was injected intraperitoneally. The above experimental scheme is shown in Figure 1. Protocols were approved by the IACUC of NCKU.

**In Vivo Imaging**

To locate the inflammatory site, we used an in vivo luminescence reporter system for NF-κB activation by NF-κB–luciferase

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**Figure 1.** Schematics of the experimental protocol of antibiotic-induced *Clostridium difficile* infection and pretreatment with proton-pump inhibitor (PPI) in mice. Abbreviation: CFU, colony-forming units.
transgenic mice on a FVB/NJNarl genetic background. Following the protocols of animal models of CDI, luciferin (Xenogen, Alameda, California), a luciferase substrate, was intraperitoneally injected at the dose of 150 mg/kg (30 mg/mL) before imaging to demonstrate NF-κB activation–mediated luminescence. Mice were anesthetized with isoflurane/oxygen, and images were collected for 5 minutes by the IVIS Spectrum Imaging system (Xenogen). Data were analyzed by Living-Image software (Xenogen), and in vivo luciferase activity was presented in photons/second/cm²/steradian.

**Phenotypic Analysis of CDI**

Reported signs of colitis in mice included weight loss and diarrhea [13]. Diarrhea severity was scored by stool consistency, as follows: 0, well-formed pellets; 1, semifomed stools that did not adhere to the anus; 2, semifomed stools that adhered to the anus; and 3, liquid stools. Thus, the changes in body weight, stool consistency, gross view of gut, and cecal weight were selected to estimate the severity of CDI in mice.

**Bacterial Burden**

Bacterial loads of *C. difficile* in feces were measured to correlate with disease progression. To quantify bacterial loads, feces specimens were collected and cultured. CFU were counted on cycloserine-cefoxitin-fructose agar (CCFA; Creative Microbiologicals, Taiwan) in 37°C incubator anaerobically for 2 days.

**Histopathologic Examination**

Colon was cut longitudinally and fixed by 10% formaldehyde (Mallinckrodt, Hazelwood, Missouri). Samples were embedded in paraffin, sectioned (5 µm), deparaffinized, and prepared with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS) stains for microscopic examination. For immunohistochemical staining, sections were deparaffinized, blocked, and incubated overnight with primary antibodies against ZO-1 (Invitrogen, Camarillo, California). Secondary antibody staining was performed using a kit (Vector Laboratories, Burlingame, California), and detection was performed using a 3, 3′-diaminobenzidine substrate chromogen solution (Dako, Carpinteria, California). The stained areas were quantified using ImageJ software version 1.42q (NIH, Bethesda, Maryland).

**RNA Analysis**

Colonic RNA was extracted with REzol isolation reagent (Protech Technology Enterprise, Taipei, Taiwan). Samples of messenger RNA were analyzed with SYBR Green, using real-time quantitative reverse transcription–polymerase chain reaction, with the housekeeping gene, GAPDH, as the reference gene in each reaction.

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**Figure 2.** Severity of *Clostridium difficile* infection in different experimental groups. Mice were treated with an antibiotic cocktail with or without a proton-pump inhibitor (PPI) and then challenged by *C. difficile* or reverse osmosis water for 2 days. Body weight change (A), stool consistency (B), gross views of cecum and colon (C), and colon length (D; n = 23–25 mice/group), as well as *C. difficile* counts in feces on day 2 (E; n = 4 mice/group), were assessed. Values are expressed as means ± standard errors of the means. *P < .05, **P < .01, and ***P < .001. Abbreviation: CDI, *Clostridium difficile* infection.
Cytokine and Chemokine Measurement

The concentrations of cytokines and chemokines in mouse colon homogenates were measured by the Luminex assays (Millipore, St. Charles, Missouri), according to the manufacturer’s protocol. Samples were measured in duplicate and analyzed by Beadview software (Millipore).

In Vivo Intestinal Permeability Measurement

Epithelial barrier permeability was assessed by oral inoculation of high-molecular-weight dextran-FITC and measuring its serum concentration. In brief, dextran-FITC at a dose of 60 mg/100 g of body weight (Sigma-Aldrich) was given by oral gavage. Blood specimens were obtained by cardiac puncture and serum dextran-FITC levels were measured by a Modulus II Microplate Multimode Reader (TurnerBioSystems, California), in which emission and excitation wavelengths were 490 nm and 520 nm, respectively. Serum lipopolysaccharide (LPS) levels were determined by the PyroGene Recombinant Factor C Endotoxin Detection Assay kit (Lonza, Walkersville, Maryland), in accordance with the manufacturer’s instructions.

Data Analysis

Values are reported as the means ± standard errors of the mean. The Student t test was used for comparisons between groups, and the differences were considered to be statistically significant with a P value of <.05.

RESULTS

PPI Aggravates CDI Colitis

To evaluate the effects of PPIs in an experimental clostridial mouse model, we first assessed the colitis phenotype of the mice. There were 3 study groups: the vehicle group, the CDI group, and the PPI+CDI group. PPI was administrated orally twice daily for 2 days before oral inoculation of C. difficile. All infected mice in the latter 2 groups showed symptoms of CDI, including loss of body weight and cecal weight and loss of stool consistency, at the acute stage of colitis (ie, day 2). The mice in the PPI+CDI group lost more body weight (Figure 2A). Simultaneously, stool consistency scores for the PPI+CDI group were higher than those for the CDI group (Figure 2B). Gross views of colon and cecum indicated greater severity of colitis in the PPI+CDI group than in the CDI group (Figure 2C). Mice in the PPI+CDI group displayed greater cecal weight loss than those in the CDI controls (Figure 2D). To further test the effects of spores in our PPI model, the mice were fed spores of VPI10463 (10⁶ CFU) as the infectious agent. We also found that receipt of the PPI and C. difficile spores caused more-severe symptoms of CDI, including loss of body weight and cecal weight and loss of stool consistency, at the acute stage of colitis than receipt of C. difficile spores only (Supplementary Figure 1). In the sporulation experiment, VPI10463 produced significantly fewer spores than BAA1805, a ribotype 027 strain (Supplementary Figure 2).
PPI Increases C. difficile Colonization

To examine whether PPI therapy promotes colonic colonization of C. difficile in mice, quantitative cultures of feces were performed in CCFA plates. At day 2, there was a significant increase (near 10-fold) in the number of C. difficile in feces specimens from the PPI+CDI group (3.9 × 10⁴ CFU/g feces), compared with that in the CDI group (4.1 × 10³ CFU/g feces; Figure 2E).

Increased Histopathological Severity in the Colon of PPI-Administrated Mice With CDI

All the clinical assessments were validated by histological examinations of representative colonic tissues. In agreement with previous studies [12, 14], we observed marked histopathological changes in HE-stained colonic specimens from the CDI and PPI+CDI groups, as characterized by epithelial damage, and infiltrating inflammatory cells, including macrophages, lymphocytes, and mainly neutrophils (Figure 3B and 3C). In contrast, colonic sections for the PPI+CDI group displayed severe epithelial damage, transmural inflammation with extensive crypt abscess, and submucosal edema (Figure 3C).

Upregulation of Proinflammatory Cytokines in Mice with CDI

Consistent with the enhanced infiltration of inflammatory cells in the infected mice, the expression of proinflammatory cytokines, such as interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), macrophage inflammatory protein 2 (MIP-2), interleukin 17A (IL-17A), and monocyte chemoattractant protein 1 (MCP-1), was significantly increased in the colons of the PPI+CDI group, compared to that in the vehicle group (ie, the mice without CDI). Colons from the CDI group exhibited increased levels of TNF-α, IL-1β, IL-6, MIP-2, and IL-17A, compared with those from the vehicle group. Compared with mice in the CDI group, expression of TNF-α and MIP-2 RNA was significantly increased in the PPI+CDI group (Figure 4A). In addition to gene expression, proinflammatory cytokines and chemokines of colon homogenates were analyzed. Levels of IL-1β, TNF-α, IFN-γ, and MCP-1 were increased in colons from the PPI+CDI group, compared with those from the vehicle group. Compared with IFN-γ levels in the CDI group, levels in the PPI+CDI group were significantly increased (Figure 4B).

PPI Enhances Clostridial Colitis in NF-κB Reporter Mice

A central mediator of inflammatory and stress responses is the NF-κB family of transcription factors. To directly address the difference of NF-κB–dependent inflammatory responses between the CDI and PPI+CDI group in vivo, we assessed NF-κB reporter gene activity by noninvasive imaging of

Figure 4. Expression of inflammatory genes in colons from Clostridium difficile–infected mice. A, Levels of RNA expression for proinflammatory cytokines and chemokines in the vehicle group, C. difficile infection (CDI) group, and proton-pump inhibitor (PPI)+CDI group (n = 18 mice/group), as measured by real-time polymerase chain reaction. B, Tissue levels of proinflammatory cytokines and chemokines measured by Luminex assay in the 3 groups (n = 6 mice/group). Values are expressed as means ± standard errors of the means. *P < .05, **P < .01, and ***P < .001. Abbreviations: IFN-γ, interferon γ; IL-1β, interleukin 1β; IL-6, interleukin 6; MCP-1, monocyte chemoattractant protein 1; MIP-2, macrophage inflammatory protein 2; TNF-α, tumor necrosis factor α.
NF-κB reporter mice. Ventral assessment of photon flux of luminescence signals from the abdomen showed that NF-κB activity in the PPI+CDI mice was significantly higher than that in the CDI mice (Figure 5A). Quantification of photon flux showed the significant increased luminescence intensity in the abdomen of mice in the PPI+CDI group (Figure 5B). Mice were euthanized 10 minutes after luciferin injection, and their gut was dissected to measure NF-κB activation. The NF-κB–dependent luminescence signals were more distinct in the colon and cecum, compared with the small intestine, in both the CDI group and the PPI+CDI group (Figure 5C). Photon flux from the intestinal tract in both infected groups was significantly higher than that in the vehicle group. Furthermore photon flux from the intestinal tract in the PPI+CDI group was significantly higher than that in the CDI group (Figure 5D).

PPI Increases the Disruption of Colonic Integrity
To investigate the gut barrier integrity after infection, we evaluated the serum LPS levels and the permeability to dextran-FITC (average molecular weight, 4000 kDa). Both infected groups had significantly higher serum concentrations of LPS than the vehicle group (Figure 6A and 6B). The gut barrier permeability, as reflected by serum FITC-dextran levels, was greater in the PPI+CDI group, compared with the CDI group (Figure 6B). To further determine whether the increased gut permeability was due to loss of functional tight junction protein, the apical distribution of ZO-1, a tight junction maker, was analyzed by immunofluorescence staining on colon sections. Intercellular levels of ZO-1 proteins were decreased in infected mice, compared with the vehicle group (Figure 6C), suggesting that epithelial integrity was damaged in the infected mice, with greater severity in the PPI+CDI group.

Decreased Mature Goblet Cells in the PPI+CDI Group
To assess the number of goblet cells, goblet cell mucin was stained with PAS/AB. In the vehicle group, PAS/AB staining showed abundant presence of mucus within the goblet cells in the colon (Figure 7A). The number of PAS-positive goblet cells and the intracellular granules were reduced in infected mice (Figure 7B and 7D). These results indicate that the number of mature goblet cells with mucus was significantly decreased in the infected mice, especially those in the PPI+CDI group.

Figure 5. Colonic NF-κB activation in mice with Clostridium difficile infection (CDI). A, In vivo luminescence images of NF-κB reporter (NF-κB-RE-luc) mice in the vehicle group, the CDI group, and the proton-pump inhibitor (PPI)+CDI group. B, Quantification of luminescence in mouse abdomen was compared in the 3 groups (n = 5 mice/group). C, Luminescence images of the resected intestines of FVB mice in three groups. D, Quantification of luminescence of the resected colons from mice in the 3 groups was compared (n = 10–12 mice/group). Values are expressed as means ± standard errors of the means. *P < .05, **P < .01, and ***P < .001.
Our study presented an animal model of CDI induced by antibiotic cocktail treatment that mimicked clinical symptoms and was easy to manipulate. There are several host factors evidently associated with CDI, including age, comorbidities, length of hospital stay, and prior exposure to antibiotics or PPIs [15]. Prophylactic use of PPIs against stress ulcers in hospitalized patients is common. To ascertain the association between PPI therapy and CDI, it is important to translate this phenomenon by experimental designs. Here, we demonstrated that the administration of PPI increased the severity of CDI symptoms, including weight loss, decreased stool consistency, increased inflammatory responses, decreased colonic integrity, and impaired the mucus secretion of goblet cells.

Administration of PPI to the antibiotic cocktail–treated mice is akin to the clinical scenario in which prophylactic PPI is prescribed to critically septic patients receiving broad-spectrum antibiotics. Once C. difficile vegetative cells and spores are ingested, they pass through the stomach, and spores germinate into vegetative cells in the colon upon exposure to bile acid and then multiply [16]. Although C. difficile spores are resistant to acid, the loss of acidity due to acid suppressant use may predispose patients to CDI because of the shorter time needed for
It is well accepted that gastric acid suppression increases the risk of CDI [17–19]. Of all medications used in suppressing gastric acid secretion, PPIs had stronger effects, resulting in more-rapid gastric ulcer healing and symptom relief than H2 blockers in the clinical setting [20, 21]. Nevertheless, in the systematic review and meta-analysis by Tleyjeh et al [22], evidence for the association of PPI use and the development of CDI was of low quality and did not support a causal relationship. However, a study conducted among hospitalized adults showed that receipt of PPIs concurrent with _C. difficile_ treatment was not associated with CDI recurrence [23]. Although the causal effect of PPIs in CDI is controversial, our translational animal model supports the contributory role of PPIs in aggravating the severity of CDI.

Although _C. difficile_ is primarily transmitted by the ingestion of acid-resistant spores, vegetative cells are killed during their route to the colon [24]. Administration of _C. difficile_ spores was an alternative method to enhance susceptibility to _C. difficile_ colonization and infection. A delayed progression of CDI might be expected with oral inoculation of _C. difficile_ spores, due to the lapse of spore germination in the mice. Thus, the advantages of rapid disease progression and easy preparation make vegetative cells of _C. difficile_, as our mouse model, more suitable for animal experiments. Although some reports suggest that sporulation rates of hypervirulent _C. difficile_ strains may contribute to the severity of the CDI epidemic [25], it is now accepted that varied sporulation rates exist among different _C. difficile_ strains and that such rates do not correlate with certain hyper-virulent subtypes [26, 27]. However, our _C. difficile_ study strain, VPI10463 (ribotype 087), produced significantly fewer spores than BAA1805 (ribotype 027). This finding indicates that PPIs may play a more important role for _C. difficile_ strains with lower sporulation capacity in the establishment of _C. difficile_-associated diarrhea and warrants more experimental and clinical research.

We compared the degrees of loss of body weight, loss of cecal weight, and stool consistency as markers of CDI severity in mice. Although these markers they may not be fully representative of the clinical manifestations of CDI in human, they were objective and comparable among affected mice. In addition, serum LPS levels could be used to reflect the degree of gut leakage in mice models [28, 29]. Increased serum levels of LPS and FITC-dextran and loss of ZO-1 tight junction proteins in the CDI mice represented the increased permeability of colon epithelia. This phenomenon closely resembles the situation in human CDI, in which _C. difficile_ toxins induce intestinal epithelial cell damage and increased mucosal permeability [30]. Because of epithelial

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**Figure 7.** Depleted goblet cells and mucin release in mice with _Clostridium difficile_ infection (CDI). A–C, Periodic acid-Schiff (PAS) and mucus staining show PAS-positive goblet cells in colons from mice in the vehicle group (A), the CDI group (B), and the proton-pump inhibitor (PPI)+CDI group (C). The scale bar represents 20 µm (original magnification ×400). Quantification of mucin-containing goblet cells was compared in 3 groups (D). Values are expressed as means ± standard errors of the means. *P < .05, **P < .01, and ***P < .001.

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damage and leakage, bacterial translocation from the intestine to distant organs should be further examined.

In our study, inflammatory responses in NF-κB reporter mice clearly showed in vivo spatial and temporal patterns. Transgenic mice expressing luciferase under the control of NF-κB enable real-time and noninvasive imaging of NF-κB activity [31]. As a result, this NF-κB reporter mouse model can be used to screen anticancer drugs, with the advantage of instant and accurate monitoring of NF-κB activation [32, 33]. CDI, either in mice or humans, is a dynamic inflammatory response, ranging from mild inflammation to pseudomembranous formation or colon perforation [16]. Detection of inflammatory gene expression or protein production at specific time points only demonstrates the static profile of CDI, not the dynamic course of CDI. Our NF-κB reporter mouse model provides a platform for disclosing the dynamic changes of inflammation in the intestines of C. difficile–infected mice and can serve as a tool to screen for therapeutic compounds.

In conclusion, this is the first mouse experiment to demonstrate that PPI administration can increase the severity of C. difficile–associated colitis induced by an antibiotic cocktail. Clinical use of PPIs should be prudently evaluated for their risk-benefit balance.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by authors that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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