Acetylcholinesterase Inhibitor Pyridostigmine Bromide Attenuates Gut Pathology and Bacterial Dysbiosis in a Murine Model of Ulcerative Colitis

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Abstract
Background Ulcerative colitis (UC) is a Th2 inflammatory bowel disease characterized by increased IL-5 and IL-13 expression, eosinophilic/neutrophilic infiltration, decreased mucus production, impaired epithelial barrier, and bacterial dysbiosis of the colon. Acetylcholine and nicotine stimulate mucus production and suppress Th2 inflammation through nicotinic receptors in lungs but UC is rarely observed in smokers and the mechanism of the protection is unclear.

Methods In order to evaluate whether acetylcholine can ameliorate UC-associated pathologies, we employed a mouse model of dextran sodium sulfate (DSS)-induced UC-like conditions, and a group of mice were treated with Pyridostigmine bromide (PB) to increase acetylcholine availability. The effects on colonic tissue morphology, Th2 inflammatory factors, MUC2 mucin, and gut microbiota were analyzed.

Results DSS challenge damaged the murine colonic architecture, reduced the MUC2 mucin and the tight-junction protein ZO-1. The PB treatment significantly attenuated these DSS-induced responses along with the eosinophilic infiltration and the pro-Th2 inflammatory factors. Moreover, PB inhibited the DSS-induced loss of commensal Clostridia and Flavobacteria, and the gain of pathogenic Erysipelotrichia and Fusobacteria.

Conclusions Together, these data suggest that in colons of a murine model, PB promotes MUC2 synthesis, suppresses Th2 inflammation and attenuates bacterial dysbiosis therefore, PB has a therapeutic potential in UC.

Keywords Ulcerative colitis · Pyridostigmine bromide · Dextran sodium sulfate · Bacterial dysbiosis · Acetylcholine

Abbreviations
UC Ulcerative colitis
IBD Inflammatory bowel disease
PB Pyridostigmine bromide
DSS Dextran sodium sulfate
nAChR Nicotinic acetylcholine receptors
AChe Acetylcholine esterase
TJ Tight junction
ZO-1 Zona occludin-1

Introduction
Ulcerative colitis (UC) is a chronic idiopathic inflammatory bowel disease (IBD), where inflammation is mainly restricted to colon. The incidence of UC is steadily increasing particularly in the Western world [1]. Although the exact etiology of UC is unclear, increasing evidence suggests that a combination of factors including genetics, environmental agents, immune dysregulation, gut barrier dysfunction, and dysbiosis of the gut microbiota influence the course of the disease [2, 3]. UC is rarely observed in smokers, as nicotine/nicotinic receptor agonists suppress colitis [4]; however, the mechanism by which cigarette smoke/nicotine suppresses the development of UC is unclear. UC is primarily a
Th2-mediated IBD associated with eosinophilic infiltration and overproduction of Th2 chemokines/cytokines including eotaxin (CCL11), IL-5, and IL-13 [5–7]. In animal models, activation of pulmonary nicotinic acetylcholine receptors (nAChRs) moderates eosinophilic infiltration through suppression of Th2 responses [8]. In mammals, acetylcholine (ACh) is the only known biological ligand for nAChRs, and increasing evidence suggests that ACh and PB—the latter of which increases ACh by inhibiting acetylcholine esterase (AChE)—tend to protect against tissue injury [9, 10]. We and others have shown that nicotine, ACh, and the AChE inhibitor neostigmine bromide (similar to PB) promote mucus formation in human lung epithelial cells through α7-nAChRs [11, 12]. MUC2, the predominant mucin in the gut, plays a critical role in gut homeostasis, and MUC2-deficient mice develop colitis spontaneously [13]; however, unlike MUC5AC in bronchial epithelial cells [14], IL-13 does not always stimulate MUC2 [15]. UC also affects gut microbiota and the integrity of the epithelial barrier in the colon [2, 5, 6]; thus normal fecal microbial transplantation is a promising treatment for UC [16, 17]. As DSS has been found to induce UC-like conditions in a mouse model [18, 19], in this study we examined whether PB attenuated DSS-induced colon pathology in C57BL/6 mice.

Materials and Methods

Animals

Pathogen-free 5–6-week-old C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were kept in chambers as described previously [11]. All animal protocols were approved by the Institutional Animal Care and Use Committee.

DSS and PB Treatment

Mice (8–9 weeks old) were divided into three groups (control, DSS, and DSS+PB) with 6–8 animals/group. PB and DSS were purchased from Sigma-Aldrich, USA. Mice were given PB through intraperitoneally implanted Alzet mini-osmotic pumps [20] starting at day 7 prior to DSS treatment and continued until they were euthanized. Mice implanted with mini-osmotic pumps containing sterile saline served as the control. The pumps provided PB at a concentration of 2 mg/day/kg body weight. Colitis was induced by 3.0% (w/v) DSS-containing water. The treatment continued for 7 days; the animals were euthanized and colons harvested on day 8. Animals were weighed daily after DSS treatment. Where indicated, intestines were dissected to obtain ileum, cecum, and colon; tissues were frozen for RNA and protein, and fecal material for bacterial microbiome analysis.

Histochemical Staining and Scoring

Formalin-fixed and paraffin-embedded tissue sections (5 μm) were stained with hematoxylin and eosin (H&E) for general histology. Histochemical staining with Alcian Blue and periodic acid–Schiff reagent (AB–PAS) was carried out as described previously [21]. Eosinophils and goblet cells were enumerated microscopically. Tissue sections were coded and assessed in a blinded manner. Histological damage was scored as described previously [18]. Briefly, sections were scored for eosinophil infiltration (0–3), with 0 representing less than three eosinophils per field of view at 40× magnification in the lamina propria, 1 for greater than three eosinophils per field of view in the lamina propria, 2 representing confluence of eosinophils extending into the submucosa, and 3 representing confluence of eosinophils present in all tissue layers.

Immunofluorescence Imaging

Deparaffinized and hydrated tissue sections were washed in 0.05% v Brij-35 in phosphate-buffered saline (PBS; pH 7.4) and immunostained for antigen expression as described previously [22]. Briefly, the antigens were unmasked and incubated in a blocking solution. The sections were stained with antibodies to ZO-1 (Invitrogen Inc., Carlsbad, CA, USA) or anti-eosinophil antibody (Novus Biologicals, Littleton, CO, USA), or isotype control antibodies. Immunofluorescence images were captured with a BZX700 all-in-one microscope (Keyence Corp., Japan).

qRT-PCR Analysis

Total RNA from frozen mouse colon tissue was isolated using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using the StepOnePlus detection system (Applied Biosystems, Foster City, CA, USA) and the TaqMan One-Step RT-PCR kit containing AmpliTaq Gold® DNA polymerase [23]. Specific primers and probes were obtained from Applied Biosystems. Fold changes in qPCR expression were calculated by the $2^{-\Delta\Delta CT}$ method [24].

Western Blot Analysis

Western blot analysis of eotaxin, IL-5, IL-13 and MUC2 in mice colon homogenates was performed as described previously [23]. Briefly, colon homogenates (70 μg) were fractionated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for eotaxin, IL-5, and IL-13, and 5% SDS-PAGE for MUC2. After transfer to
nitrocellulose membranes, blots were probed with antibodies to eotaxin, IL-5, IL-13, and MUC2 (Abcam), and after stripping were re-probed with anti-β actin antibody.

Histological Grading

The H&E-stained sections were analyzed for histological grading to evaluate the severity of inflammation. The total score ranged from 0 to 14, representing the sum of scores from the following: (1) severity of inflammation, with 0 = none, 1 = mild, 2 = moderate, and 3 = severe; (2) mucosal damage, with 0 = none, 1 = mucosal layer, 2 = submucosa, and 3 = transmural; (3) crypt damage, with 0 = none, 1 = basal one-third damage, 2 = basal two-thirds damage, 3 = crypt lost with intact epithelium, and 4 = crypt and epithelium lost; and (4) percentage involvement, with 0 = none, 1 = ≤25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100%, as previously described [25].

Bacterial Microbiome Analysis: Sequencing and 16S DNA Analysis

Fecal contents were collected from the gut encompassing distal cecum and distal sigmoid colon and frozen on dry ice. The fecal matter was lysed using glass beads in a MagNA Lyser tissue disruptor (Roche Diagnostics, Indianapolis, IN, USA) and total DNA was isolated using the DNeasy Powersoil® or AllPrep PowerFecal® DNA isolation kits (MO BIO Laboratories, Carlsbad, CA, USA) and quantified as described elsewhere [26]. Briefly, the bacterial 16S V4 rDNA region was amplified using fusion primers, and each sample was PCR-amplified with two differently barcoded V4 fusion primers and loaded into the MiSeq cartridge (Illumina Inc., San Diego, CA, USA). The amplicons were sequenced for 250 cycles with custom primers designed for paired-end sequencing. Using the QIIME platform, sequences were quality-filtered and demultiplexed using exact matches to the supplied DNA barcodes. The sequences were searched against the Greengenes reference database and clustered at 97% by UCLUST (closed-reference operational taxonomic unit [OTU] picking). The relative abundance of OTUs constituting a single phylum in treated animals was normalized with the OTUs in controls. The resulting ratios were analyzed using Prism software (GraphPad Software, LA Jolla, CA, USA) to identify changes between groups in the five major bacterial phyla in the microbiome.

Results and Discussion

PB Ameliorates the DSS-Induced Increase in IL-5 and Eotaxin

While the exact pathophysiology of UC is unknown, several immune cell types, including neutrophils, eosinophils, and lymphocytes, have been implicated in the disease process [27, 28]. Similar to allergic asthma in the lungs, UC is a Th2 disease where eosinophils and eosinophil-associated proteins play a significant role in the disease pathology [29], and eosinophil granulocyte infiltration is seen in UC-associated inflammation [5, 30] and implicated in the pathophysiology of UC in humans [31, 32]. Moreover, eosinophils are a therapeutic target for gut inflammation [33]. IL-5 drives the growth of eosinophils, and eotaxin is the most potent chemokine that stimulates eosinophilic infiltration, while IL-13 affects the gut epithelial barrier; these factors are elevated in UC [27, 32], and interestingly, eosinophilic granules contain IL-5, IL-13, and eotaxin [34, 35]. Eotaxin has been established as the chemokine that drives eosinophilic infiltration in UC. We analyzed colonic tissues from control, DSS-treated, and DSS+PB-treated mice for eosinophil infiltration of the tissue. Eosinophils were visualized by immunohistochemistry using an eosinophil-specific antibody that showed that DSS stimulated eosinophilic infiltration of the colon (Fig. 1a, left panel), with a pathology score of > 3.0, which was significantly reduced by PB treatment (Fig. 1a, right panel), indicating that inhibition of AChE partially protects the colon from infiltration by eosinophils. Accumulation of eosinophils at the site of inflammation requires cooperation between IL-5 and eotaxin [36], where IL-5 promotes proliferation and differentiation of eosinophils, and eotaxin is a potent chemoattractant for eosinophils that is increased significantly in UC [5, 32, 33]. We determined the expression of IL-5 and eotaxin by Western blot and/or RT-qPCR analyses. The results showed that DSS doubled the amount of IL-5 protein in the colon, which was significantly reduced by the PB treatment (Fig. 1b). DSS-induced IL-5 levels were comparable to IL-5 mRNA levels observed in rectal biopsy samples from UC patients [5]. Similarly, PB reduced eotaxin protein (Fig. 1c) and eotaxin mRNA (Fig. 1d) levels. DSS treatment has also been found to cause body weight loss in mice [4]. Following DSS treatment, we weighed mice daily. On day 4, DSS-treated animals began to exhibit loss of body weight, and the decrease was statistically significant compared with control mice on day 5. However, on day 5 there was no significant difference in body weight between the control and DSS+PB groups (Fig. 1E). This difference in body weight became significant on day 7/8, but the reduction was lower than in the animals treated with DSS alone (Fig. 1e). These results suggest that PB slightly but
significantly moderates DSS-induced weight loss in mice. Thus, PB appears to attenuate the pro-eosinophilic effects of DSS in the mouse colon through downregulation of IL-5 and eotaxin expression and moderates the DSS-induced loss of body weight.

**PB Inhibits DSS-Induced IL-13 Expression and Improves Colonic Epithelial Integrity and MUC2 Mucin Expression**

UC is predominantly a Th2 inflammatory disease and IL-13 is the key effector cytokine in UC pathogenesis [6, 37], which in excessive concentrations impairs the integrity of colonic epithelium [38–40]. In the lung, IL-13 induces airway mucin (MUC5AC) formation [11]; however, unlike the lung, IL-13 does not regulate mucin (MUC2) production in the intestinal epithelial cells [41]. The lamina propria of UC patients produces substantially more IL-13 than normal individuals [28, 42] and neutralization of IL-13 prevents experimental UC and attenuates human UC [37, 43]. Therefore, we analyzed the effects of PB on the IL-13 expression in gut tissues. Western blot analysis indicated that DSS upregulates the expression of IL-13 protein and PB significantly downregulates
this response (Fig. 2a). The intestinal barrier consists of epithelial columnar cells connected by intracellular junctions that are covered by mucus. The epithelium and the mucus layer inhibit the invasion of gut mucosa by luminal microbes. Impaired colonic barrier promotes gut inflammation and the thickness of the mucus layer is decreased in active UC patients [44, 45]. Mucus is made by goblet cells, and the major gel-forming mucin in the gut is MUC2 [46]. MUC2 synthesis is defective in UC patients, and variants of MUC2 gene are associated with increased incidence of UC [47]. In UC patients and experimental animals, bacteria penetrate the normally impenetrable colonic mucus layer and MUC2 expression is strongly downregulated in UC [48]. MUC2 provides protection against colitis, and MUC2-deficient mice exhibit increased susceptibility to colitis [49, 50]. We compared the expression of MUC2 protein in DSS and DSS+PB colons by Western blot analysis. The results presented in Fig. 2B show that compared to normal colonic tissues, MUC2 protein expression is significantly decreased \((p < 0.05)\) by DSS; however, PB treatment strongly upregulates the expression of MUC2 protein in DSS-treated animals \((p < 0.01)\), and MUC2 expression is even higher \((p < 0.05)\) than the control group. These results suggest that loss of MUC2 protein expression is associated with UC inflammation, and PB promotes MUC2 expression.

Normal functioning of the gut epithelium requires tight junction (TJ) proteins, which provide structural integrity to the tissue by forming a highly polarized barrier with selective paracellular permeability [51]. Histopathologically, UC is associated with disruption of TJ proteins and the TJs are composed of proteins that include occludin, claudins, junctional adhesion molecules, and intracellular scaffold proteins such as zonula occludens (ZO), including ZO-1 [52]. ZO-1 is a scaffolding linker protein that interacts with occludin and is reduced in UC patients [53]. To determine the effects of PB on colonic architecture, colon tissues were stained with H&E and analyzed for histological grading to evaluate the severity of UC. We based the disease severity primarily on histopathological changes in the colon on a scale of 0–14 (see “Materials and methods” section). As assessed by these criteria, Fig. 3a shows that DSS-induced disease severity of \(6.33 \pm 0.42\), which was significantly moderated by PB to \(2.67 \pm 0.33\). Moreover, as seen by immunofluorescence/immunohistochemistry analysis, the DSS-induced reduction in colonic ZO-1 was attenuated by PB treatment (Fig. 3b, in green). These results suggest that PB partially protected mice from DSS-induced disruption of the gut epithelial architecture.

**PB Attenuates DSS-Induced Dysbiosis of Microbiota in the Colon**

The intestines, particularly the colon, are inhabited by a large number of microorganisms that normally provide symbiotic benefits to the host; however, barrier dysfunction in UC causes unfavorable changes in the composition of gut microbiota (dysbiosis) that facilitates the penetration of colonic mucus layers by these bacteria to induce gut inflammation [13, 38, 54]. Normal gut bacterial microbial community is typically dominated (nearly 95%) by phyla Firmicutes, Bacteroidetes, and Proteobacteria [55], and each phylum has members that are beneficial or pathogenic in the gut. Imbalances in gut microbiota are seen in the DSS-induced UC in mice [56], and recent results suggest that fecal transplants containing normal bacterial microbiota attenuate UC symptoms in patients [17, 57] and in experimental animal models [58].

To determine whether DSS-induced changes in the bacterial composition of the colon are attenuated by PB, we analyzed the colonic 16S V4-rDNA. Figure 4a shows that in a normal mouse colon (CON), Erysipelotrichia, Clostridia, Flavobacteria, and Fusobacteria represent approximately

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**Fig. 3** PB ameliorates the colonic epithelial integrity loss and inflammation. a Representative H&E stained colon sections and the histological scoring of pathological severity. The lower panel shows the enlarged images of the inset drawn on upper panels (scale, 50 microns). b Representative colonic expression of ZO-1 as assessed by immunofluorescence analysis. Images show ZO-1 (in green) and DAPI-stained nuclei (in blue). Lower panels show the enlarged images of the inset drawn on upper panels. Image magnification 100× for upper panel and 400× for lower panel. Figures are representative of two separate experiments \((n = 4–5/group)\); where \(* \leq 0.01, ** \leq 0.001, \text{ and } *** \leq 0.0001\).
69%, 14%, 13%, and 4% of all sequences, respectively, and DSS treatment alters this composition to 82%, 3%, 7%, and 8%, respectively. Thus, DSS increases Erysipelotrichia and Fusobacteria and decreases Clostridia and Fusobacteria in the gut. PB treatment nearly restored the normal microbiota composition of Erysipelotrichia, Clostridia, Flavobacteria, and Fusobacteria to about 73%, 12%, 11% and 4%, respectively. The weighted Unifrac principal coordinate analysis of the microbiome data shows that the microbiome from the control animals cluster distinctly compared to the DSS-treated animals (Fig. 4b).

Clostridia difficile infection is known to recur in UC patients and is associated with increased morbidity and mortality in this population [59]; however, organisms within Clostridia genera are higher in normal gut flora and normal fecal microbiota is used to treat recurrent Clostridia difficile infection [60]. Both Clostridia and Erysipelotrichia belong to phylum Firmicutes, but commensal Clostridia maintains gut homeostasis and moderates Clostridia difficile infection [60, 61]. Commensal Clostridia promotes the development of anti-inflammatory IL-10-producing Fox3p+ T-reg cells in the gut [62–64], and defects in the IL-10 or IL-10 receptor are known to promote early onset of UC [65]. On the other hand, Erysipelotrichia increases mucosal permeability and stimulates inflammatory immune responses in the gut [66]. The role of Flavobacteria in the human gut has not been clearly defined; however, Flavobacteria are less abundant in human IBD [67]. Similarly, the function of Fusobacteria in the gut are unknown, but their numbers increase in colorectal cancer [68], and UC increases susceptibility to colon cancer [69]. Therefore, it is likely that PB, through increased acetylcholine levels, (a) increases mucus formation and (b) inhibits the DSS-induced loss of the epithelial barrier function by suppressing IL-13 production. Together, this inhibits the DSS-induced migration of pathogenic bacteria and bacterial dysbiosis in the gut. Thus, PB has the potential to stabilize gut flora and attenuate inflammation associated with UC.

Cholinergic stimuli involving ACh are important in the regulation of gut function, and ACh regulates both motility and mucosal responses in the gut. Recent evidence suggests that ACh and PB tend to be protective against experimental tissue injury. PB restored cardiac autonomic balance in mice and rats after experimental myocardial infarction [9, 70, 71], and heart-specific overexpression of the ACh-synthesizing enzyme choline acetyltransferase was found to protect the myocardium against ischemia-induced injury in mice [72]. PB has also been shown to help some patients with spinal cord injuries [73] and diabetic patients with gastrointestinal disorders [74]. Moreover, release of ACh through electrical stimulation of the vagus nerve ameliorates gut inflammation through nAChRs [75]. We have shown that ACh and neostigmine bromide induce mucus production in airway epithelial cells through α7-nAChRs [11], and nicotine suppresses both inflammatory and adaptive immune responses [76, 77]; interestingly, tobacco smokers were found to be very resistant to UC [78]. In mammals, ACh is the only known biological ligand that reacts with nicotinic as well as muscarinic receptors.
receptors, and nAChRs on non-neuronal cells respond to very low concentrations of nicotine [11, 79]. Therefore, it is likely that at lower concentrations, ACh reacts primarily with nAChRs thereby protecting the gut from colitis and bacterial dysbiosis and has the potential to suppress UC. Because nAChRs are present on many different cell types, including T cells, monocytes/macrophages, and epithelial cells, at present the identity of the cell type(s) that is/are the primary target of acetylcholine in the gut is unclear. In the lung, bronchial epithelial cells express nAChRs, and acetylcholine, neostigmine bromide, and nicotine promote mucus production from these cells [11, 80]; however, nicotine is known to inhibit Th2 immune responses through its effects on T cells and macrophages [8, 81]. It is therefore likely that the effects of acetylcholine/PB on UC severity involve multiple cell types. Further experiments are needed to identify the target cell type(s) that is/are critical in moderating UC in this model.

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Compliance with Ethical Standards

Conflict of interest The authors have no financial conflicts of interest.

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