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CONTRACTING ORGANIZATION: The University of Texas Medical Branch
Galveston, TX

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14. ABSTRACT

Crohn's disease (CD) is an inflammatory bowel disease (IBD), characterized by recurrent transmural inflammation in the colon or/and ileum. Exclusive enteral nutrition (EEN), involving oral or nasogastric tube feeding of a complete liquid diet with exclusion of normal foods, is a safe and effective treatment for active CD. However, what accounts for the benefits of EEN in CD are not clear. Our project aims to investigate the mechanisms of the benefits of EEN. We hypothesize that: 1) Transmural inflammation in CD causes mechanical stress (MS) in the inflammation and pre-inflammation sites, and the MS induces expression of certain mechanosensitive genes to contribute to the development of inflammation and bowel dysfunctions; 2) EEN treatment is beneficial in CD by preventing MS and blocking the effects of MS-induced gene expression. Accordingly, our Specific Aims are: 1) To determine if MS plays a role in the development of CD by evaluating the effects of EEN on mechanosensitive gene expression, inflammation and bowel dysfunction; 2) To investigate the mechanisms underlying the beneficial effect of EEN on gut inflammation in CD; 3) To investigate the mechanisms underlying the beneficial effect of EEN on bowel dysfunctions, especially motility dysfunction. So far, we have almost completed the study in Aim 1. We found that intracolonic instillation of TNBS induces a localized transmural inflammation (as in CD) in the distal colon, which is associated with mechanical tension in the local inflammation site and lumen distention in the bowel prior to inflammation. These changes represent significant MS in the colon, and induce marked induction of certain pro-inflammatory mediators such as osteopontin (OPN) and cyclo-oxygenase-2 (COX-2) in the inflammation and pre-inflammation sites, which contribute significantly to the development of inflammation and bowel symptoms. EEN treatment decreases feces production, and prevents inflammation-associated MS, and dramatically attenuated expression of OPN and COX-2 in the CD model, and improved inflammation score and motility function. Our study suggests that the benefit of EEN in CD may result from its action in attenuating mechanical stress in transmural inflammation in this disease.

15. SUBJECT TERMS

Inflammatory bowel disease, Crohn's disease, Exclusive enteral nutrition, Mechanical stress, Osteopontin, Cyclooxygenase-2, Yes-associated protein, Th1, Th17 immune response, Gut motility

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TABLE OF CONTENTS

1. INTRODUCTION:

Crohn's disease (CD) is an inflammatory bowel disease (IBD), characterized by recurrent transmural inflammation in the colon or/and ileum. The mechanisms of CD associated inflammation and bowel symptoms are not well understood. Among the therapeutic options, corticosteroids, immune-modulators, and biologic agents all have substantial adverse effects or limitations, whereas exclusive enteral nutrition (EEN) is safe and effective, and now a first line treatment for active CD especially in young patients. EEN involves oral or nasogastric tube feeding of a complete liquid diet with exclusion of normal foods for a defined period (usually 4 to 8 weeks). As a dietary therapy, EEN is found as equivalent as, or superior to corticosteroids in the induction of remission in children (with nearly 80% remission rate). Although EEN is widely used in Europe, Asia, and Australia, it is not well accepted in the US, partly because the exact mechanisms for its therapeutic benefits in CD are not clear. **This project aims to investigate the mechanisms of the benefits of EEN for CD.** Although mechanical stress (MS), associated with inflammatory cell infiltration, edema, fibrosis, and stenosis, are commonly encountered in CD, the role of MS in the development of inflammation and gut dysfunction is largely unrecognized. In a well-established rodent model of CD, we found that intracolonic instillation of a hapten reagent TNBS induces a localized transmural inflammation (as in CD) in the distal colon, which is associated with mechanical tension in the local inflammation site and lumen distention in the bowel prior to inflammation. These changes represent significant MS in the colon. Our preliminary studies demonstrated that MS induces marked induction of certain pro-inflammatory mediators such as osteopontin (OPN) and COX-2 in the inflammation and pre-inflammation sites, which contribute significantly to the development of inflammation and bowel symptoms. We found that EEN treatment dramatically decreases feces production, and prevents inflammation-associated MS. Our **Hypotheses** are: 1) Transmural inflammation in CD causes MS in the inflammation and pre-inflammation sites, and the MS induces expression of mechanosensitive genes OPN and COX-2 to contribute to the development of inflammation and bowel dysfunctions; 2) EEN treatment is beneficial in CD by preventing MS and blocking the effects of MS-induced gene expression. Accordingly, our **Specific Aims** are: 1) To determine if MS plays a role in the development of CD by evaluating the effects of EEN on mechanosensitive gene expression, inflammation and bowel dysfunction; 2) To investigate the mechanisms underlying the beneficial effect of EEN on gut inflammation in CD, by focusing on the role of mechanosensitive expression of OPN in Th1/Th17 immune response; 3) To investigate the mechanisms underlying the beneficial effect of EEN on bowel dysfunctions in CD, by focusing on the role of mechanosensitive expression of COX-2 in motility dysfunction. In **summary**, our project addresses the mechanism of action of EEN, the only established dietary therapy, in CD management, and thus is closely relevant to public health of US general population, our military personnel, their families and children, especially those who suffer from Crohn's disease.

2. KEYWORDS:

Inflammatory bowel disease, Crohn's disease, Exclusive enteral nutrition, Mechanical stress, Osteopontin, Cyclooxygenase-2, Yes-associated protein, Th1 immune response, Th17 immune response, Gut motility.

3. ACCOMPLISHMENTS:

What were the major goals of the project? The major objective of the project is to investigate the mechanisms of the benefit of exclusive enteral nutrition (EEN) for Crohn's disease (CD). Our Hypotheses are: 1) Transmural inflammation in CD causes mechanical stress (MS) in the inflammation and pre-inflammation sites, and the MS induces expression of mechanosensitive genes OPN and COX-2 to contribute to the development of inflammation and bowel dysfunctions; 2) EEN treatment is beneficial in CD by preventing MS and blocking the effects of MS-induced gene expression. Thus, the specific aims are: 1) To determine if MS plays a role in the development of CD by evaluating the effects of EEN on mechanosensitive gene expression, inflammation and bowel dysfunction; 2) To investigate the mechanisms underlying the beneficial effect of EEN on gut inflammation in CD, by focusing on the role of mechanosensitive expression of OPN in Th1/Th17 immune response; 3) To investigate the mechanisms underlying the beneficial effect of EEN on bowel dysfunctions in CD, by focusing on the role of mechanosensitive expression of COX-2 in motility dysfunction.

What was accomplished under these goals?

In the current reporting period (Aug. 1st, 2020 – Jul. 31st, 2021), we have worked mainly on Aim 1. We have established the CD model in rats (TNBS induced Crohn's-like colitis), and accomplished time course study with the rats taking regular pellet foods (mostly Aim 1.1). We have also induced CD but treated rats with EEN (clear liquid diet) (mostly Aim 1.2). Tissue samples were taken from different sites of the colon in CD rats (Site I: inflammation site, Site P: site proximal to inflammation site; Site D: site distal to inflammation) and sham treated rats. Much of the molecular and functional studies has also been completed. The work on mechanical colon obstruction (OB) has also been done (Aim 1.3).

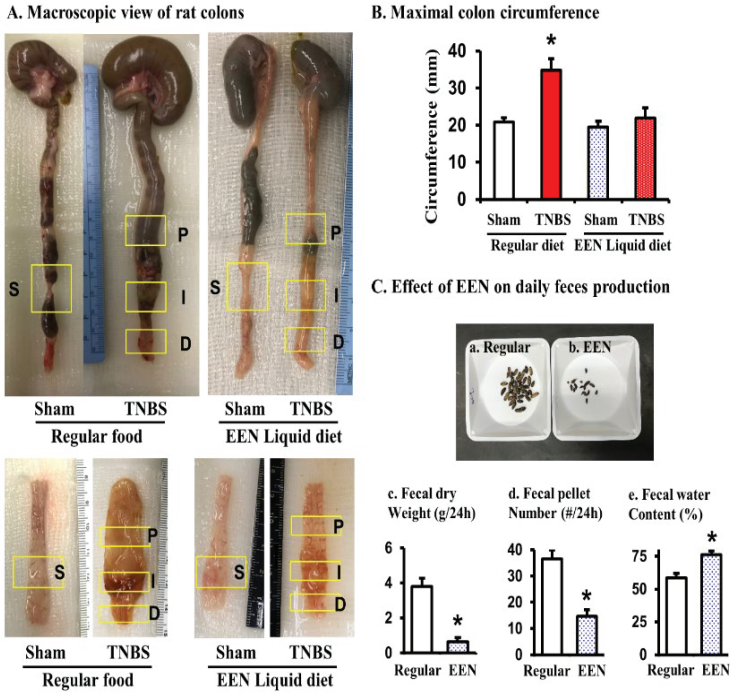

Fig. 1 Rat model of Crohn's-like colitis and effect of liquid diet treatment. (A) Outlook view of colons (top) and macroscopic view of distal colons (bottom). (B) Exclusive enteral nutrition (EEN) prevents colon circumference increase in TNBS colitis rats (7 day after TNBS treatment). (C) EEN treatment in control rats significantly decreased fecal production. Daily fecal production in one rat taking regular diet (a) and EEN for 3 days (b) is shown. Average fecal weight (c), number (d), and % water content (e) are shown (N=4 or 5). *p < 0.05 vs control rats fed with regular diet. The yellow boxes indicate different sites of colon tissue for the study. S, sham control; I, localized inflammation site; P, distended colon site proximal to inflammation site; D, no-distended site distal to inflammation.

We found that intra-colonic instillation of TNBS induced localized transmural inflammation (~ 2 cm length) in the distal colon (site I), with a distended colon segment (site P) prior to, and a non-distended segment (site D) distal to the inflammation site (Fig. 1A). We detected site-

specific changes of inflammation (Fig. 2), gene expression of OPN and COX-2 (Fig. 3a and 3b), and muscle contractility in the rodent model of CD, when rats are fed with regular pellet diet. However, EEN treatment with liquid diet released mechanical stress in the colon (Fig. 1B and 1C), and significantly improved body weight, disease activity, and inflammation in the CD model (Fig. 2. Data on day 7 was shown). The data on mechanosensitive gene expression of OPN and its possible pathogenic significance has been analyzed and reported in Digestive Disease Week 2021 (Reference #1 listed in "**How were the results disseminated to communities of interest?**"). As shown in Fig. 3, OPN mRNA expression was increased 77.8(± 20.6)-fold in site I of CD rats (7 day), compared to controls. Interestingly, OPN expression was also increased in the mechanically distended site P [69(±11.4)-fold], but not in the non-distended site D (Fig. 3a). This data indicates a mechanosensitive mechanism in OPN expression. If rats were treated with EEN (liquid diet), mechanical distention in CD was prevented (Fig. 1), and the increase of OPN mRNA expression was significantly attenuated to 13.2(±5.8)-fold in site I, and completely blocked in site P (Fig. 3). Our studies showed that OPN mRNA was increased mainly in the muscularis externae in the inflamed or distended colons (Fig. 4).

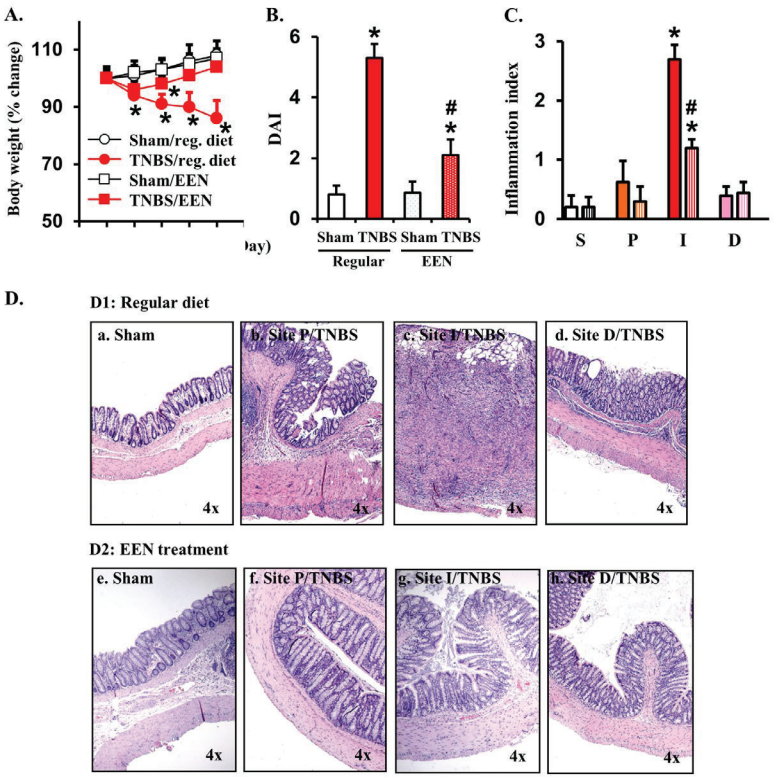

Fig. 2 Beneficial effect of EEN in a rodent model of CD. (A) Body weight change for 7 days, (B) Disease activity index (DAI) (day 7 data), (C) Inflammation index in sham (S) and different sites (P, I, and D) of TNBS colitis (day 7) after regular diet (solid bar) and EEN treatment (streaked bar). (D) Representative microscopic views of sham and different sites of TNBS colitis (day 7) after regular diet (a-d) and EEN treatment (e-h). S, sham colon; I, inflammation site; P, distended colon site proximal to inflammation; Site D, non-distended site distal to inflammation. N=5, or 6 in each group. *p < 0.05 vs sham group control. #p < 0.05 vs. TNBS/regular diet.

In a separate model, where mechanical distention, but no inflammation, was induced by an obstruction band in the distal colon, OPN mRNA expression was increased significantly in the distended segment prior to obstruction, but not in the segment distal to obstruction (Fig. 5). Plasma OPN level was increased significantly in rats with mechanical obstruction (608±116 vs. 222±39 ng/mL in sham, 7 day), and in TNBS induced colitis when rats were fed with regular pellet diet (424±118 ng/mL), but not in colitis rats when colon distention was prevented with clear liquid diet (Fig. 4B; Fig. 5A).

We then compared smooth muscle contractile function in wild-type and OPN$^{-/-}$ mice, and found that circular muscle contractility was significantly lower in the OPN$^{-/-}$ mice comparing to WT (Fig. 6). Colon inflammation led to decreased muscle contractility in WT mice, but not in OPN$^{-/-}$ mice.

Thus, our preclinical studies suggest that mechanical stress plays a critical role in the up-regulation of OPN and COX-2 in CD. EEN treatment reduces mechanical stress in the gut and blocks up-regulation of OPN, suggesting that the benefit of EEN in CD may result from its effect to reduce mechanical stress in the gut. Increased plasma OPN in gut inflammation may indicate obstruction (i.e. stenosis in CD). We found that OPN, like COX-2, may also contribute to motility dysfunction in CD. Some of these findings have already been presented in Digestive Disease Week 2021 (Please see below of results dissemination: Ref #1 and Ref #2). We are analyzing more data for more presentations and publications in the next year. Some of the data on COX-2 studied in Aim 1 will be reported together with the work proposed in Aim 3 (e.i. significance of COX-2 expression in gut motility dysfunction in CD, and the impact of EEN treatment on COX-2 expression and function).

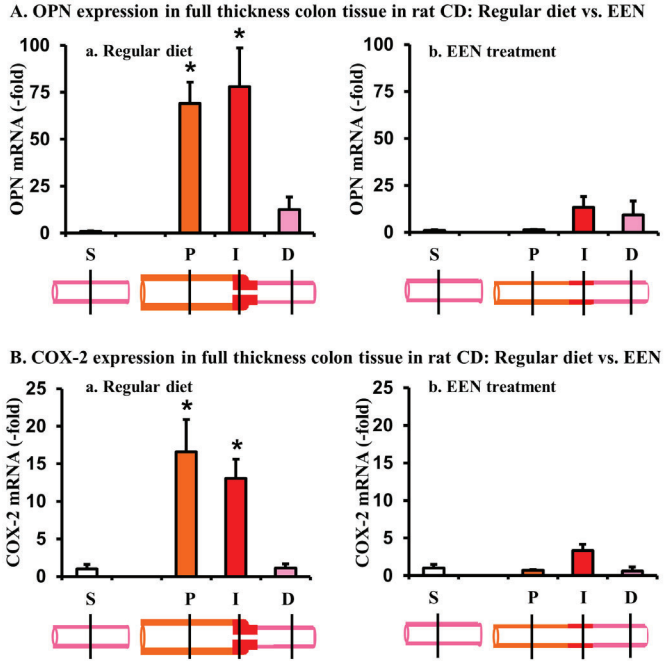

Fig. 3 Effect of EEN on mechanosensitive gene expression. OPN (A) and COX-2 (B) mRNA expression was markedly induced in sites I and P, but not D in TNBS colitis rats (7 day) when fed with regular diet (a). However, EEN treatment almost completely blocked up-regulation of OPN and COX-2 (b). N=5 in a, N=4 in b. *p < 0.05 vs S (sham).

We have just started to determine the Th1 and Th17 immune response in rat CD fed with pellet (regular) and liquid diet (EEN) (Aim 2.1).

In addition, we have taken some effort to finish an on-going study of EEN effect on opioid-induced bowel dysfunction in a rat model. That work has just been published in American Journal of Physiology (Please see below of results dissemination: Ref #3).

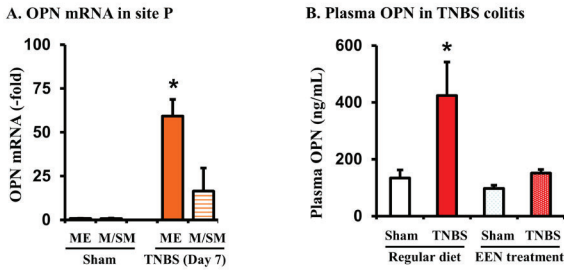

Fig. 4 OPN mRNA was increased mainly in the muscularis externa (ME), not in the mucosa/submucosa (M/SM) of the TNBS colitis model (A). Plasma OPN level was significantly increased in TNBS colitis rats when fed with regular pellet diet (B), but not when treated with EEN. N= 4 or 5. *p < 0.05 vs S (sham).

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Some of the work resulted from the Aim 1 study of the project has been presented as two abstracts (one was recognized as abstract of distinction) in the Digestive Disease week (DDW) in May 21~23, 2021. These abstracts were published in Gastroenterology [Ref #1, Ref #2].

We have published a peer-reviewed full-length research article in American Journal of Physiology in June, 2021, which is very relevant to the funded DoD project and acknowledges the support of the DoD award [Ref. #3]. This paper describes the effect of EEN treatment with liquid diet in another gastrointestinal disorder – opioid-induced bowel dysfunction. The EEN treatment described in the study was an inspiration for us to test the mechanism of EEN in obstructive Crohn's disease (DoD project).

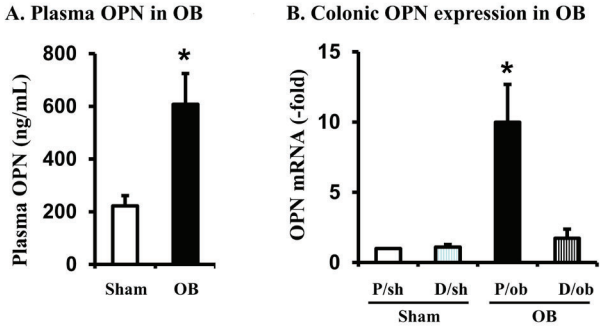

Fig. 5 Plasma and colon OPN levels in mechanical obstruction. Partial colon obstruction was induced with an obstruction band in the distal colon of rats. Tissue was taken from the sites proximal (P, with mechanical distention) and distal (D, without distention) to obstruction band 7 days after obstruction. Plasma OPN level was increased significantly in rats with mechanical obstruction (A). This is associated with increased OPN expression only in the distended proximal segment of the colon, not in the un-distended distal segment. N= 5. *$p < 0.05$ vs S (sham).

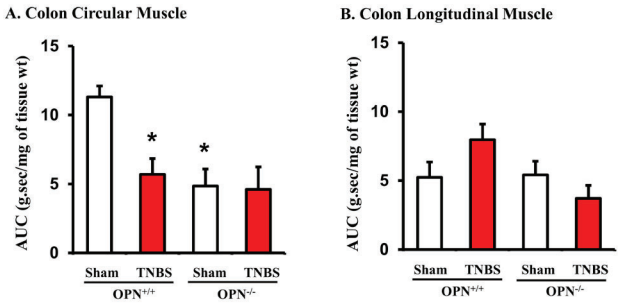

Fig. 6 Colonic smooth muscle contractility in TNBS-induced colitis in wild-type ($OPN^{+/+}$) and $OPN^{-/-}$ mice. Mice were treated intra-colonically with saline or TNBS (1 mg/kg in 40% ethanol). Contractile response of colon circular (A) and longitudinal smooth muscle (B) to acetylcholine (10^{-7} to 10^{-3} M), cumulative) was determined in muscle bath recordings 7 days after induction of inflammation. N= 4 or 5. *$p < 0.05$ vs sham wild-type mice group.

References (a copy of the publications are attached in Appendices):

1. Lin YM and **Shi XZ**. PRECLINICAL STUDIES OF PATHOPHYSIOLOGICAL ROLE AND DIAGNOSTIC POTENTIAL OF OSTEOPONTIN IN CROHN'S DISEASE. *Gastroenterology* 160 (6), S-626-S-627, 2021.

2. Lin YM, Qiu SM, M'Koma AE, Powell DW, Cohn S, **Shi XZ**. MECHANICAL STRESS PLAYS A CRITICAL ROLE IN INTESTINAL FIBROSIS AND SMOOTH MUSCLE HYPERPLASIA IN A RODENT MODEL OF CROHN'S DISEASE. *Gastroenterology* 160 (6), S-431, 2021

3. Lin YM, Tang Y, Fu Y, Hegde S, Shi DW, Huang LM, **Shi XZ**. An opioid receptor-independent mechanism underlies motility dysfunction and visceral hyperalgesia in opioid-induced bowel dysfunction. *Am J Physiol Gastrointest Liver Physiol.* 2021 Jun; 320(6): G1093-G1104. Epub 2021 Apr 28. doi: 10.1152/ajpgi.00400.2020. PMID: 33908261.

What do you plan to do during the next reporting period to accomplish the goals?

In the next reporting period, we will work mainly on Specific Aim 2 to investigate the mechanisms underlying the beneficial effect of EEN on gut inflammation in CD by focusing on the role of

mechanosensitive expression of OPN in Th1/Th17 immune response. We will work on not only rat model of CD, but also mice model of CD; to study the effect of EEN on Th1/Th17 response and determine if mechanosensitive expression of osteopontin plays a role in the EEN effects.

In addition, we will start the motility study proposed in Aim 3 and finish residual work of Aim 1. Will also complete data analysis and prepare full length manuscripts based on the study of Aim 1.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Our presentations based on the Aim 1 study at DDW, the largest scientific gathering on digestive diseases in the world, demonstrated that mechanical stress plays a critical role in the up-regulation of OPN and COX-2 in CD. EEN treatment reduces mechanical stress in the gut and blocks up-regulation of OPN, suggesting that the benefit of EEN in CD may result from its effect to reduce mechanical stress in the gut. Unfortunately, these were virtual presentations in the Covid-19 pandemic time (May, 2021). However, the findings published in Gastroenterology shall help the Crohn's Disease field to realize that the benefits of EEN may rely on its capability to reduce mechanical stress in the gut, thus raising physicians and CD patients' awareness of the importance of bowel luminal load in CD.

What was the impact on other disciplines?

We have published a full-length research article in American Journal of Physiology in June, 2021, which is very relevant to the funded DoD project. This paper describes the beneficial effect of EEN treatment with liquid diet in another gastrointestinal disorder – opioid-induced bowel dysfunction. This pre-clinical study calls for clinical trials to test EEN treatment in opioid-induced bowel dysfunction, which is a refractory condition secondary to repeated opioid use.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS: Changes in approach and reasons; Problems or delay; ……

Nothing to report.

6. PRODUCTS:

Publications, conference papers, and presentations

Journal publications.

- Lin YM, Tang Y, Fu Y, Hegde S, Shi DW, Huang LM, **Shi XZ**. An opioid receptor-independent mechanism underlies motility dysfunction and visceral hyperalgesia in opioid-induced bowel dysfunction. *Am J Physiol Gastrointest Liver Physiol.* 2021 Jun; 320(6): G1093-G1104. Epub 2021 Apr 28. doi: 10.1152/ajpgi.00400.2020. PMID: 33908261. (Copy attached in Appendices)

Other publications, conference papers, and presentations.

- 1. Lin YM and **Shi XZ**. PRECLINICAL STUDIES OF PATHOPHYSIOLOGICAL ROLE AND DIAGNOSTIC POTENTIAL OF OSTEOPONTIN IN CROHN'S DISEASE. Accepted and presented as an abstract of distinction at the Digestive Disease Week (DDW) 2021 on May 21~23, 2021. Published in: *Gastroenterology* 160 (6), S-626-S-627, 2021. (Copy attached in Appendices)

- 2. Lin YM, Qiu SM, M'Koma AE, Powell DW, Cohn S, **Shi XZ**. MECHANICAL STRESS PLAYS A CRITICAL ROLE IN INTESTINAL FIBROSIS AND SMOOTH MUSCLE HYPERPLASIA IN A RODENT MODEL OF CROHN'S DISEASE. Accepted and presented at the Digestive Disease Week (DDW) 2021 on May 21~23, 2021. Published in: *Gastroenterology* 160 (6), S-431, 2021. (Copy attached in Appendices)

Other Products. Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	*Xuan-Zheng Shi, MD, MS*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*ORCID 0000-0002-9014-853X*
Nearest person month worked:	*4.2*
Contribution to Project:	*As PI, Dr. Shi oversees the project, establishes animal models, and helps on bench work, data analysis, writing, and reporting.*
Funding Support:	*NIH in addition to DoD.*

Name:	*You-Min Lin, MD, Ph.D*

Project Role:	*Research Scientist*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Dr. Lin has performed mostly on the animal work, tissue processing, molecular and functional studies, statistics and data analysis.*
Funding Support:	*NIH in addition to DoD.*

Name:	*John Winston, Ph.D.*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	ORCID 0000-0003-4220-2236
Nearest person month worked:	1.2
Contribution to Project:	*Dr. Winston contributed to the animal model and animal protocol establishment, and performed some histology work.*
Funding Support:	*NIH in addition to DoD*

Name:	*Ramasatyaveni Geesala, Ph.D.*
Project Role:	*Post-doctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	ORCID 0000-0002-5520-2457
Nearest person month worked:	2
Contribution to Project:	*After a national search, Dr. Geesala was selected in June, 2021 to join the lab to work mainly on the DoD project. She has been working on some of the molecular work of Aim 1 and started to work on Th1/Th17 immune response in Aim 2.*
Funding Support:	

Name:	*Ke Zhang, MS*
Project Role:	*Research Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*7*
Contribution to Project:	*Mr. Zhang joined the lab in Jan, 2021 to help mainly on the DoD project, especially some animal work and molecular and histological detections.*
Funding Support:	

Changes in active other support

Nothing to report.

Other organization involvement

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

9. APPENDICES:

Please see the following attachments, which acknowledged DoD award support.

- Copy of the peer-reviewed publication in *American Journal of physiology*;

- Copy of two conference abstracts presented at Digestive Disease week 2021 and published in *Gastroenterology*.

AMERICAN JOURNAL OF PHYSIOLOGY
GASTROINTESTINAL AND LIVER PHYSIOLOGY

Am J Physiol Gastrointest Liver Physiol 320: G1093–G1104, 2021.
First published April 28, 2021; doi:10.1152/ajpgi.00400.2020

RESEARCH ARTICLE

Neurogastroenterology and Motility

An opioid receptor-independent mechanism underlies motility dysfunction and visceral hyperalgesia in opioid-induced bowel dysfunction

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[1]*Department of Internal Medicine, The University of Texas Medical Branch, Galveston, Texas;* [2]*Department of Gastroenterology, The First Affiliated Hospital, Guangxi University of Science and Technology, Guangxi, China;* [3]*College of Science, Texas A&M University, College Station, Texas; and* [4]*Department of Neuroscience and Cell Biology, The University of Texas Medical Branch, Galveston, Texas*

Abstract

Constipation and abdominal pain are commonly encountered in opioid-induced bowel dysfunction (OBD). The underlying mechanisms are incompletely understood, and treatments are not satisfactory. As patients with OBD often have fecal retention, we aimed to determine whether fecal retention plays a pathogenic role in the development of constipation and abdominal pain in OBD, and if so to investigate the mechanisms. A rodent model of OBD was established by daily morphine treatment at 10 mg/kg for 7 days. Bowel movements, colonic muscle contractility, visceromotor response to colorectal distention, and cell excitability of colon-projecting dorsal root ganglion neurons were determined in rats fed with normal pellet food, or with clear liquid diet. Morphine treatment (Mor) reduced fecal outputs starting on *day 1*, and caused fecal retention afterward. Compared with controls, Mor rats demonstrated suppressed muscle contractility, increased neuronal excitability, and visceral hypersensitivity. Expression of cyclooxygenase-2 (COX-2) and nerve growth factor (NGF) was upregulated in the smooth muscle of the distended colon in Mor rats. However, prevention of fecal retention by feeding rats with clear liquid diet blocked upregulation of COX-2 and NGF, restored muscle contractility, and attenuated visceral hypersensitivity in Mor rats. Moreover, inhibition of COX-2 improved smooth muscle function and fecal outputs, whereas anti-NGF antibody administration attenuated visceral hypersensitivity in Mor rats. Morphine-induced fecal retention is an independent pathogenic factor for motility dysfunction and visceral hypersensitivity in rats with OBD. Liquid diet may have therapeutic potential for OBD by preventing fecal retention-induced mechanotranscription of COX-2 and NGF.

NEW & NOTEWORTHY Our preclinical study shows that fecal retention is a pathogenic factor in opioid-induced bowel dysfunction, as prevention of fecal retention with liquid diet improved motility and attenuated visceral hyperalgesia in morphine-treated animals by blocking expression of cyclooxygenase-2 and nerve growth factor in the colon.

constipation; fecal retention; mechanical stress; narcotic bowel syndrome; visceral sensitivity

INTRODUCTION

Opioid analgesics are the mainstay for treating moderate-to-severe pain, especially cancer pain (1–4); their use has escalated over the past 2 decades (5–8). The number of prescriptions for opioids has increased from 76 million in 1991 to 207 million in 2013 in the United States alone (1, 4). The United States consumes more than 80% of the world's total opioid supplies (3, 4). Unfortunately, the use of opioids is commonly associated with opioid-induced bowel dysfunction (OBD), including constipation, nausea, bloating, and abdominal pain (9–11). Among these, opioid-induced constipation (OIC) is the most common; it is present in up to 90% of opioid users (7, 8). Moreover, repeated use or large doses of opioid analgesics may result in opioid-induced abdominal pain (OAP) or hyperalgesia. Tuteja et al. (11) reported that chronic abdominal pain is experienced in 58% of patients who took opioids for 10 days or longer. When abdominal pain becomes severe, and worsens with continued or escalating dosages of narcotics, it is diagnosed as narcotic bowel syndrome (NBS) (11–13). Current treatments such as laxatives, secretogogues, antidepressants, and anxiolytics for OIC and OAP are not very satisfactory (9–11, 13–15). Although peripheral opioid antagonists were highly anticipated for their utility in OBD, recent studies showed that a majority of patients remain constipated despite treatment with methylnaltrexone or naloxegol (16, 17). Because of

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intolerable OBD, one-third of patients eventually choose to discontinue or decrease the use of opioid analgesics for pain management (1, 7, 10–12). To develop effective or alternative therapies for OBD, further investigation into the underlying mechanisms of constipation and abdominal pain in OBD is needed.

Current theories for mechanisms of OIC focus on opioid receptors [i.e., μ-opioid receptor (MOR)] in the enteric nervous system (ENS) and the enteric neuronal-mediated blockade of secretomotor function in the gut (9, 15, 18). Activation of MOR was shown to inhibit enteric neurotransmitter release to slow intestinal transit and decrease mucosa secretion (15, 18). These changes may account for the initiation of constipation in OIC. However, constipation persists long after tolerance to opioid-associated analgesia and other effects have been developed (5, 7, 15). What accounts for the sustained effect on constipation is not clear. On the other hand, central nervous system is the focus of putative mechanisms for opioid-induced hyperalgesia and abdominal pain (3, 13, 14). Grunkemeier et al. (13) proposed that activation of excitatory antianalgesic pathways within a bimodal opioid regulation system, descending facilitation of pain at the rostral ventral medulla, and glia cell activation in the dorsal horn may account for the enhanced pain perception in chronic opioid users. However, preclinical studies found that sensitization of peripheral sensory neurons is associated with opioid-induced visceral hyperalgesia (19, 20). The mechanisms underlying peripheral sensitization in opioid-induced visceral hyperalgesia are not well understood.

It is noteworthy that OBD is a functional condition, as it is not associated with any well-recognized physical abnormalities. However, fecal retention with bowel distention is an obvious symptom in OIC (5, 7). Clinical evidence showed that bowel distention associated with fecal retention, partial obstruction, and pseudo-obstruction is also very common in OAP and NBS (12, 13). In the original description of NBS, Sandgren et al. (12) reported that all five patients with NBS demonstrated features of intestinal pseudo-obstruction with prolonged use of opioids. In the clinical observation by Grunkemeier et al. (13), three out of five NBS cases had objective evidence (radiographic) or clinical symptoms of bowel distention or pseudo-obstruction. Importantly, chronic lumen distention, i.e., fecal retention, partial obstruction, or pseudo-obstruction, represents a circumferential mechanical stress to the gut wall (21–23), and has been proposed as a common cause in functional bowel disorders (24). Previous studies, in vitro and in vivo, demonstrated that mechanical stress is a potent stimulus to induce expression of proinflammatory and pain mediators such as cyclo-oxygenase-2 (COX-2) and nerve growth factor (NGF) in gut smooth muscle cells (SMC) (22, 25). We hypothesize that fecal retention (mechanical stress)-induced upregulation of COX-2 and NGF may play a critical role in neuromuscular dysfunctions in the colon, contributing to constipation and abdominal pain in OBD. We found that morphine treatment led to marked fecal retention in a rodent model of OBD, when rats were fed with regular pellet food. However, fecal retention was prevented if rats were fed exclusively with clear liquid diet. We determined colonic motor function, visceral sensitivity, and colon-projecting sensory neuron excitability in vehicle-treated and morphine-treated rats fed with either pellet food or liquid diet. We also sought to determine the pathogenic role of fecal retention-induced COX-2 and NGF in the development of motility dysfunction and visceral hypersensitivity in the OBD model.

METHODS

Rodent Model of OBD and Experimental Protocols

Sprague-Dawley male rats aged 8–10 wk (Harlan Sprague-Dawley, Indianapolis, IN) were used for the study. The rats were housed in a controlled environment (22°C, 12-h light-dark cycle) and always allowed food and water ad libitum unless stated otherwise. The Institutional Animal Care and Use Committee at the University of Texas Medical Branch approved all procedures performed on the animals.

A rat model of OBD was established as previously described by daily subcutaneous injection (sc) of morphine sulfate (Mor, Hikma, Eatontown, NJ) in slow-release emulsion at 10 mg/kg for 7 days (19) (Fig. 1). Vehicle control rats (Veh) were treated only with the emulsion (50% liquid paraffin and 50% Arlacel A, Sigma, St. Louis, MO) (19). Rodent pellet food LM-485 (Harlan, Indianapolis, IN) was used as a regular food. For the groups fed a clear liquid diet, the regular pellet food was removed 1 day before Veh or Mor treatment, and rats were given bowel cleanser PEG 3350 (GoLYTELY, Braintree, MA) for overnight, and then were fed ad lib with liquid diet Ensure Clear (Abbott Nutrition, Lake Forest, IL), and kept in wire-bottomed cages. All rats had free access to water at all time. In some experiments involving in vivo inhibition of COX-2 activity, Veh or Mor rats were treated with COX-2 inhibitor NS-398 (Cayman Chemical, Ann Arbor, MI) at 10 mg/kg intraperitoneally (ip) in 250 μL of 20% DMSO (22, 26). In experiments involving neutralization of NGF, rats were treated with anti-NGF antibody (R&D Systems, Minneapolis, MN) at 20 μg/kg ip daily (25).

Tissue Isolation

The 5 cm-long distal colon (starting 2 cm from the anus) was collected and placed immediately in carbogenated Krebs buffer (in mmol/L: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 NaH_2PO_4, 1.2 $MgCl_2$, 11 D-glucose, and 25 $NaHCO_3$). The colonic mucosa/submucosa (M/S) and muscularis externa (ME) layers were separated by microdissection as described previously (22, 27–29). The tissues were snap-frozen in liquid nitrogen and stored at −80°C until further work. In some experiments, the fresh ME tissues were used for the measurement of muscle contractility.

RNA Preparation and Quantitative RT-PCR

Total RNA was extracted from the colon muscularis externae samples using the Qiagen RNeasy kit (Qiagen, Valencia, CA), and reverse transcribed with SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) (23, 25, 29, 30). Real-time quantitative RT-PCR was performed using the Bio-Rad CFX96 Real-Time PCR system (Hercules, CA), as described previously. The TaqMan probes for detection of rat COX-2 (Rn00568225-m1) and NGF (Rn01533872m) mRNAs were purchased from Invitrogen (23, 25, 29, 30). The fold-change relative to control was calculated with the

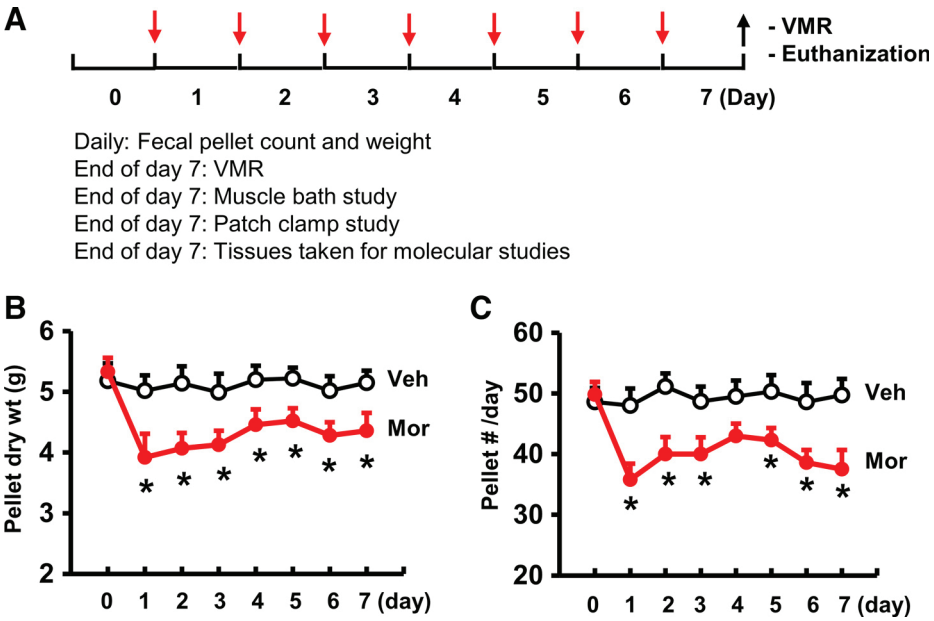

Figure 1. Rodent model of opioid-induced bowel dysfunction. *A*: experimental protocol. Red arrows indicate daily vehicle or morphine treatment (10 mg/kg sc daily). Fecal pellet count and weight were measured daily starting 1 day before vehicle or morphine treatment. At the end of *day 7* (black arrows), some rats were tested for visceromotor response (VMR), and others were euthanized for muscle bath, patch-clamp, and molecular studies. *B* and *C*: opioid-induced constipation. Morphine treatment reduced fecal outputs immediately and led to constipation afterward. Both daily fecal weight (*B*) and pellet number (*C*) were decreased in morphine-treated rats (Mor) compared with vehicle control rats (Veh). $N = 6$ rats in each group. $^*P < 0.05$ vs. Veh.

comparative C_T ($\Delta\Delta CT$) method with endogenous reference 18S rRNA (Part no. 4352930E, Applied Biosystems) as the normalizer.

Protein Extraction and Western Blotting

Whole cell protein was extracted from the colon ME samples. The tissues were homogenized on ice in lysis buffer (Cat. No. 9806S, Cell Signaling Technology, Danvers, MA) supplemented with protease inhibitor cocktails (Sigma Aldrich, St. Louis, MO). After spinning at 12,000 *g* at 4°C for 15 min, the supernatant proteins were collected and resolved by a standard immunoblotting method (22, 25, 29, 31). In brief, protein samples in equal quantity (20 μg) were run on premade 4%–12% Bis-Tris SDS-PAGE (Invitrogen, Carlsbad, CA). The proteins were transferred from the gel to the membrane. After being blocked with the Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE), the membrane was incubated with primary antibodies to COX-2 (1:1,000, Cayman Chemical, Ann Arbor, MI) (22, 23), or β-actin (loading control, 1:5,000, Sigma, St. Louis, MO) (22, 23) at 4°C overnight. Then, the membrane was incubated with secondary anti-rabbit antibody (1:2,000, Invitrogen, Carlsbad, CA) and anti-mouse antibody (1:10,000, Invitrogen, Carlsbad, CA) for detection of COX-2 and β-actin, respectively, at room temperature for 1 h (22). The imaging detection and analysis were done using Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Enzyme Immunoassay

Colonic ME tissue was homogenized in cold PBS buffer (in mmol/L: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, KH_2PO_4, pH 7.4) supplemented with protease inhibitors. NGF or prostaglandin E_2 (PGE_2) in the extraction was measured with the enzyme immunoassay kits purchased from R&D Systems (Minneapolis, MN) and Cayman Chemical (Ann Arbor, MI), respectively, by following the manufacturers' protocols (22, 25, 32).

Immunohistochemistry Study

Immunohistochemical staining of COX-2 and NGF protein was performed on formalin-fixed, paraffin-embedded colon segments (3 to 4 cm from the anus) isolated from rats with sham and morphine treatment (7 day), as described previously (22, 25). Sections at 4 μm thickness were blocked with 5% normal goat serum in PBS for 20 min at room temperature, and incubated with the rabbit anti-COX-2 antibody (1:200, Cayman Chemical, MI) or anti-NGF antibody (1:200, Santa Cruz Biotech, CA), and a biotin-conjugated anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) (22, 25). After being incubated with avidin-biotin complex (Vector kit, Vector Laboratories), the sections were stained in diaminobenzidine tetrahydrochloride with 0.03% hydrogen peroxide. As negative controls, sections of the same specimens were processed by the same method but omitting the anti-COX-2 or anti-NGF primary antibody.

Measurement of Visceromotor Response

Visceral sensitivity was measured by electromyographical (EMG) measurements of visceromotor response (VMR) to colorectal distention (CRD) as described previously (23). Briefly, two electrodes were implanted in the external oblique muscle and externalized behind the head. Rats were allowed 1 wk to recover from the surgery. Under mild sedation with 2% isoflurane, a balloon (5 cm) was inserted 7 cm into the distal colon via the anus and held in place by taping the tubing to the tail. Rats were placed in a container and allowed to adapt for 30 min, and then CRD was performed by rapidly inflating the balloon to constant pressure. Pressure was measured via a sphygmomanometer connected to a pressure transducer. The balloon was inflated to various pressures (20, 30, 40, 50, 60, and 80 mmHg) for a 20-s stimulation period followed by a 2-min rest. EMG was recorded continuously during the experiment with a Biopac System EMG 100 C (Biopac Systems, Goleta, CA). EMG signals were amplified (5,000×), filtered with a 1-Hz high-pass filter and a

500-Hz low-pass filter, and digitized by use of Acknowledge (Biopac Systems). The area under the curve (AUC) for the EMG signal during each 20 s of distention was calculated by use of an in-house-written computer program (23). The net value for each distention was calculated by subtracting the baseline value derived from the AUC for the 20 s predistention period.

Labeling of Colon-Specific Sensory Neurons in DRG

Colon-specific neurons in the dorsal root ganglia (DRG) were labeled for patch-clamp recordings by injecting 1,1′-dioleyl-3,3,3′,3-tetramethylindocarbocyanine methane sulfonate (DiI, invitrogen, Carlsbad, CA) into the colon wall as described previously (23, 25, 29, 30). In brief, animals were anesthetized by 2% isoflurane with an E-Z anesthesia vaporizer. After a midline laparotomy, 1 μL of DiI (50 mg/mL in methanol) was injected into 10 sites on the exposed distal colon (~5 cm in length). Animals were returned to normal housing and were treated for Mor or Veh before euthanasia for patch-clamp recordings ~7–10 days after DiI injection.

DRG Neuron Dispersion and Patch-Clamp Study

Isolation of DRG neurons from adult rats has been described previously (23, 25, 29, 30). Briefly, rats were euthanized by decapitation. The spinal column was removed and transferred to ice-cold, oxygenated fresh dissecting solution containing (in mmol/L): 130 NaCl, 5 KCl, 2 KH_2PO_4, 1.5 $CaCl_2$, 6 $MgSO_4$, 10 glucose, and 10 HEPES, pH 7.2 (osmolarity, 305 mosM). Thoracolumbar DRG (T13–L2) were obtained bilaterally. The ganglia were digested in dissecting solution containing collagenase D (~1.5 mg/mL; Roche, Indianapolis, IN) and trypsin (~1.2 mg/mL; Sigma, St. Louis, MO) at 34.5°C for 1.5 h. The DRG samples were washed in enzyme-free solution and triturated repetitively with glass pipettes to obtain single-cell suspension. Cells were plated onto acid-cleaned glass coverslips and perfused with normal external solution containing (in mmol/L): 130 NaCl, 5 KCl, 2 KH_2PO_4, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose, pH adjusted to 7.4 (osmolarity, ~295–300 mosM). DiI-labeled neurons (bright red) were identified by the fluorescence microscope (Olympus, Tokyo, Japan) with a rhodamine filter (excitation 546 mm, barrier filter at 580 mm). Whole cell current and voltage were recorded by a Dagan 3911 patch-clamp amplifier (Dagan, Minneapolis, MN) (23, 25, 29, 30). The currents were filtered at ~2–5 kHz and sampled at 50 or 100 μs per point. To obtain rheobase values, a series of stimulation currents (300 ms in duration, 5 pA in steps) were injected to induce action potentials. Data were acquired and analyzed by pCLAMP 9.2 (Axon Instruments, Sunnyvale, CA).

Muscle Bath Experiments

Distal colon was opened along the mesenteric border, and pinned flat in a Petri dish with Sylgard base in carbogenated Krebs buffer. The mucosa/submucosa layers were separated and discarded by microdissection. The smooth muscle strips (3 mm × 10 mm) were mounted along the circular muscle orientation in individual muscle baths (Radnoti Glass, Monrovia, CA) filled with 10 mL of carbogenated Krebs solution at 37°C. The contractile activity was recorded as previously described (22, 27, 32, 33) with isometric force transducers and amplifiers (Grass Instruments) connected to Biopac data acquisition system (Biopac Systems, Goleta, CA). The muscle strips were equilibrated in the muscle bath under 1 g tension for 60 min at 37°C before they were tested for contractility. Muscle contractility was tested in response to acetylcholine (ACh; 10^{-6} to 10^{-2} M) and to KCl (62.5 mM). The strips were washed after the test of each concentration of ACh and KCl, and left to equilibrate for 15 to 20 min before another addition of different concentration of ACh or KCl. When the contractile response to KCl was tested, the concentration of KCl (62.5 mM) in the Krebs buffer was increased by the equimolar replacement of NaCl. The contractile response was quantified as the increase in area under contractions (AUC) during 4 min after the addition of ACh or KCl over the baseline AUC during 4 min before the addition of ACh or KCl.

Statistical Analysis

Data points are expressed as means ± SE, unless otherwise specified. Statistical analysis was performed by analysis of variance with nonrepeated measures by Student–Newman–Keuls test for comparisons of multiple groups, or by Kruskal–Wallis test followed by Dunn's for nonparametric multiple comparisons, if data in a group does not follow normal distribution. Student's t test was used for comparisons of two groups. A P value of ≤ 0.05 was considered statistically significant.

RESULTS

Morphine Treatment Led to Fecal Retention in the Distal Colon

Morphine treatment significantly reduced fecal outputs in rats (Fig. 1), consistent with previous findings in humans (6, 9) and rodents (34, 35). The daily fecal dry weight and pellet number decreased dramatically starting on *day 1*, and throughout the 7-day period of morphine treatment (Fig. 1, *A–C*). The reduction of fecal output was associated with apparent fecal retention (Fig. 2*A*). As measured on *day 7*, the fecal content in the distal colon (5 cm) was significantly increased and maximal colon circumference was enlarged (Fig. 2, *B* and *C*). These changes indicate that the distal colon was subject to significant mechanical stress in the morphine-treated (Mor) rats, as in patients (5, 7, 12).

Morphine Treatment Led to Impairment of Colon Smooth Muscle Contractility

It is known that morphine treatment slows colon transit via opioid receptors on the enteric nervous system (15, 18). Whether gut smooth muscle function is affected by chronic treatment of morphine is not well known. We determined the contractility of circular smooth muscle isolated from the distal colon as previously described (22). The contractile response of colon circular muscle to cholinergic activation by acetylcholine (ACh; 10^{-7} to 10^{-2} M) was decreased significantly in Mor rats, with the maximal response of 3,185 ± 408 AUC/mm^2 in Mor compared with 5,254 ± 590 AUC/mm^2 in vehicle controls (Veh; $P < 0.05$) (Fig. 3*A*). It seems that morphine treatment did not change the potency of cholinergic receptors, as the EC_{50} values were not significantly changed

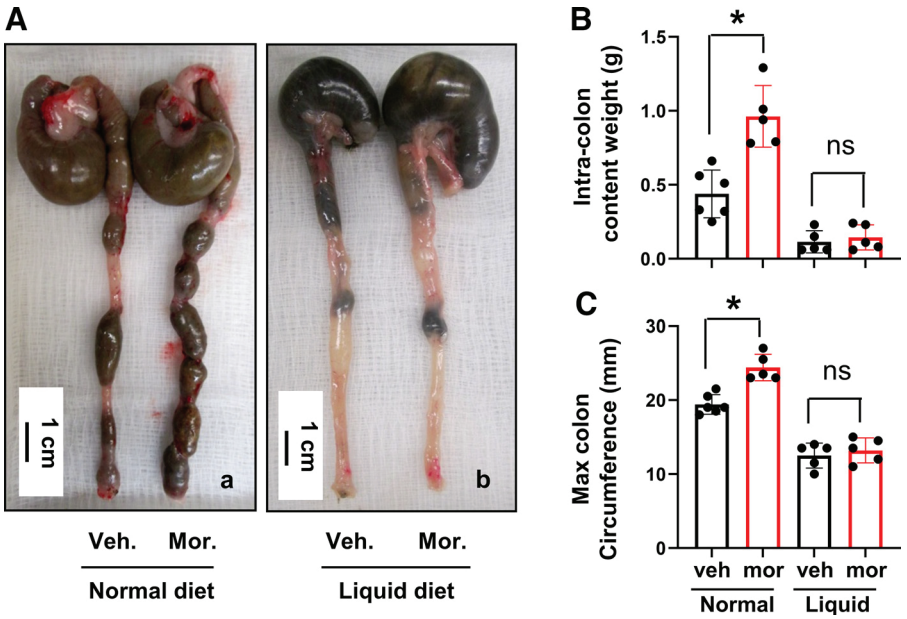

Figure 2. Morphine-induced fecal retention in the distal colon: effect of colon cleansing. Although morphine treatment reduced fecal outputs, it increased fecal retention in the colon, leading to colon distention (*A*a). The fecal content in the distal colon (5 cm) was significantly increased (*B*) and maximal colon circumference was enlarged (*C*). However, when normal pellet food was removed, and rats were treated with colon cleanser on *day 0* and fed exclusively a clear liquid diet afterward (liquid diet), fecal retention (*Ab*) and fecal retention-associated mechanical stress in the colon (*B* and *C*) were prevented. Data are represented as means ± SD. $N = 5$ or 6 rats in each group. $^*P < 0.05$ vs. Veh. ns: $P > 0.05$. ns, nonsignificant; Veh, vehicle control rats.

between Veh [2.8 (±1.0) × 10^{-5} M] and Mor rats [3.8 (±1.3) × 10^{-5} M, $P > 0.05$]. The smooth muscle contractile response to cell membrane depolarization with a high concentration of KCl (62.5 mM) was also decreased significantly by nearly 50% in the Mor rats (Fig. 3*B*). Earlier studies by others and ourselves (36–38) revealed that ACh-induced and KCl-induced contractile response mainly tests gut smooth muscle contractility, as blocking ENS activity with tetrodotoxin (TTX) pretreatment does not significantly change ACh-induced or KCl-induced response. These data suggest that the contractility of colonic smooth muscle is suppressed by chronic treatment of morphine. Impairments of colonic smooth muscle contractile activity may well contribute to motility dysfunction (32, 39) in OIC.

Morphine Treatment Increased Visceral Sensitivity

We next assessed the effect of chronic morphine treatment on visceral sensitivity by recording visceromotor response (VMR) of the rats in response to colorectal distention (23). Electromyogram recording of the abdominal muscle contractions found that morphine-treated rats showed a significantly heightened visceromotor response to graded colorectal distensions compared with controls (Fig. 4*A*).

Furthermore, patch-clamp studies (23, 25) found that the colon-projecting DRG neurons (colon neurons, ~20–28 μm in diameter) demonstrated an abnormal hyperexcitability in Mor rats with decreased rheobase (0.22 ± 0.02 nA vs. 0.16 ± 0.01 nA, $P < 0.01$) and increased number of action potential in response to 2× rheobase (1.25 ± 0.07 vs. 1.56 ± 0.09, $P < 0.01$) (Fig. 4*C*). The resting membrane potential (RP) changed from 54.9 ± 0.23 mV in Veh to 51.8 ± 0.46 mV in Mor rats ($P < 0.05$). The cell capacitance (43.4 ± 1.16 pF and 44.1 ± 0.44 pF in Veh and Mor, respectively, $P > 0.05$) and input resistance (548 ± 22.5 MΩ vs. 488 ± 20.1 MΩ, $P > 0.05$) were not significantly changed between Veh and Mor groups. These data suggests that visceral hypersensitivity in the OBD model is associated with peripheral sensitization of the colon neurons.

Liquid Diet Prevented Fecal Retention and Mechanical Stress in Morphine-Treated Rats

To determine if morphine-treatment-associated fecal retention plays an independent role in neuromuscular dysfunction in the rodent model of OBD, we developed the following

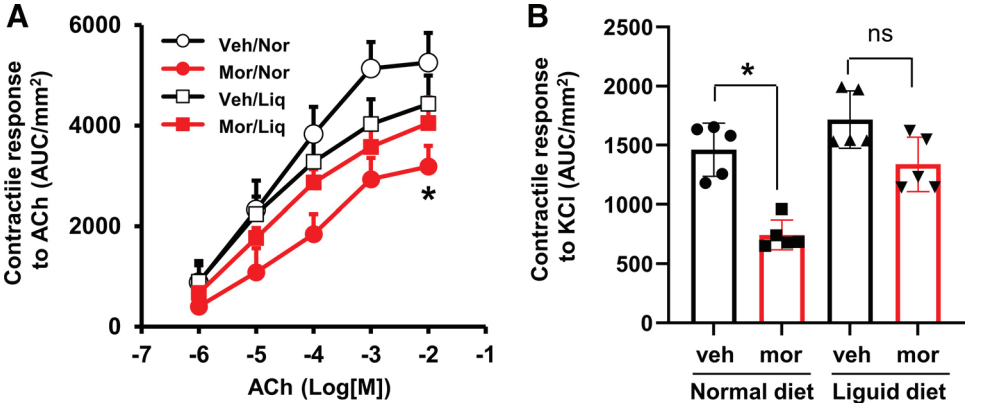

Figure 3. Effect of morphine treatment on contractile response of colon circular smooth muscle to acetylcholine (*A*) and to membrane depolarization by KCl (62.5 mM) (*B*) in rats fed with normal pellet diet and clear liquid diet. When rats were fed normal pellet food, the contractility of colon circular muscle decreased significantly ($^*P < 0.05$ vs. Veh) in Mor rats (*day 7*). However, when rats were fed a liquid diet and the colon was cleansed, the contractile response was not different between Veh and mor rats. $N = 5$ rats in each group. Liq, liquid diet; Mor, morphine-treated rats; Nor, normal pellet diet; Veh, vehicle control rats.

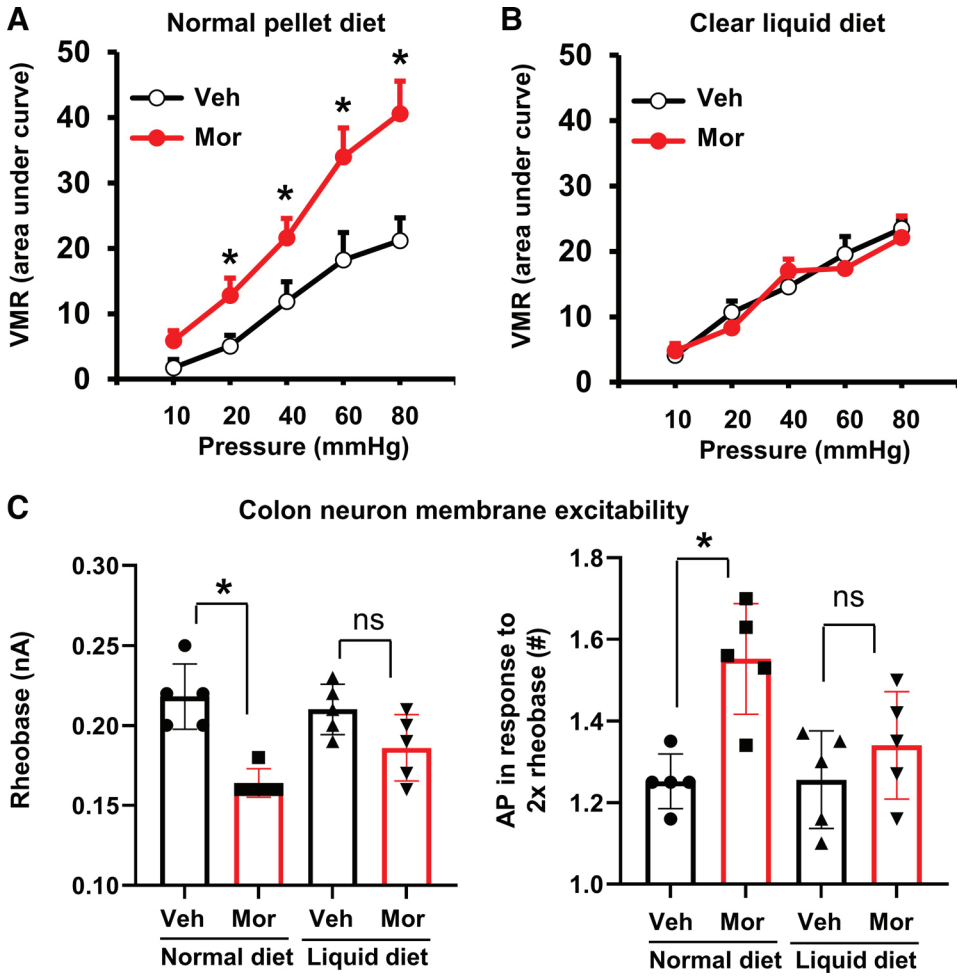

Figure 4. Opioid-induced visceral hypersensitivity in rats: effect of colon cleansing. The visceromotor response (VMR) was increased significantly by morphine treatment (7 days) in rats when fed a normal pellet food diet (*A*), but not in rats fed a clear liquid diet (*B*). Patch-clamp study (*C*) found that the colon-projecting DRG neurons demonstrated hyperexcitability in Mor rats with decreased rheobase (*left*) and increased number of action potential in response to 2× rheobase (*right*), when rats were fed normal pellet food. However, colon cleansing attenuated morphine-induced neuronal hyperexcitability. $N = 5$ rats in each group, with ~17–20 neurons recorded for each group in patch-clamp study. *$P < 0.05$ vs. normal/Veh. $P > 0.05$. DRG, dorsal root ganglia; Mor, morphine-treated rats; NS, no significant difference; Veh, vehicle control rats.

procedure to cleanse the colon and prevent fecal retention in rats. Regular pellet diet (LM-485, Harlan, Indianapolis, IN) was removed 1 day before Veh or Mor treatment, and rats were given bowel cleanser GoLYTELY overnight. Rats were then fed ad lib a liquid diet of Ensure Clear and kept in wire-bottomed cages with free access to water throughout the 7-day experimental period. Liquid diet has been used effectively as exclusive enteral nutrition in the management of Crohn's disease (40, 41). Indeed, this procedure of colon cleansing left fewer or no residue feces in the colon in both Veh and Mor rats (Fig. 2*A*). It effectively prevented fecal retention and colon distention in the morphine-treated rats, as both the intracolon content and max colon circumference were decreased to nearly the same level as in the control rats (Fig. 2, *B* and *C*).

Liquid Diet Restores Smooth Muscle Contractility and Visceral Sensitivity Affected by Morphine Treatment

We then measured colon smooth muscle contractility in the liquid-diet-treated rats and found that the muscle contractility to ACh or KCl was not significantly different between Mor and Veh rats when the bowel was cleansed (Fig. 3, *A* and *B*). The maximal response to ACh (4,432 ± 562 AUC/mm^2 and 4,047 ± 510 AUC/mm^2 for Veh and Mor, respectively, $P > 0.05$) and EC_{50} values [2.2 (±1.1) × 10^{-5} M and 2.9 (±1.7) × 10^{-5} M, $P > 0.05$] were not significantly different in the Veh and Mor groups when rats were fed with liquid diet.

Colon cleansing also attenuated visceral hypersensitivity associated with chronic morphine treatment. As shown in Fig. 4*B*, morphine treatment did not significantly increase visceromotor response to colon distention when rats were kept in clear liquid diet. Furthermore, when the colon is cleansed, the colon-projecting DRG neurons of the morphine-treated rats demonstrated a nearly similar level of membrane excitability as in the vehicle-treated rats (Fig. 4*C*).

Effect of Liquid Diet on Expression of COX-2 and NGF in the Colon

COX-2 and NGF are well recognized in suppressing gut smooth muscle contractility and increasing visceral sensitivity, respectively, in inflammatory or obstructive disorders in the gut (22, 25, 42–44). More importantly, expression of COX-2 and NGF in gut smooth muscle are highly sensitive to mechanical stress in vivo and in vitro (22, 25). To determine specifically if mechanotranscription of COX-2 and NGF is involved in mediating neuromuscular dysfunction in chronic use of morphine, we then determined mRNA and

protein expression of COX-2 and NGF in the colon of control and Mor rats fed with either regular pellet food or liquid diet.

When rats were fed a regular pellet diet, COX-2 mRNA expression was significantly increased in the colonic muscularis externae in Mor rats (Fig. 5*A*). COX-2 expression was upregulated by 3.2 (± 0.53)-fold ($P < 0.05$) in the muscularis externae of distended colon in Mor rats compared with Veh controls. This is associated with increased PGE_2 in the tissue. However, the myeloperoxidase (MPO) levels in colonic muscularis externae were not changed, indicating that there was no clear inflammation in the Mor tissue (Fig. 5*A*). Further immunohistochemical study found that COX-2 expression was increased mainly in the smooth muscle cells of the distended colon in Mor tissue (Fig. 5*B*). Expression of NGF mRNA and protein was also increased in the colonic muscularis externae in the morphine-treated rats (Fig. 6, *A* and *B*), with the NGF mRNA level increased by 1.9 (± 0.38)-fold compared with controls. Normally, NGF is detectable only in the myenteric plexus, but not in smooth muscle cells (Fig. 6*Ca*). However, its expression was increased in the colonic smooth muscle cells in morphine-treated rats (Fig. 6*Cb*). Negative control without primary antibody shows no immunostaining in either myenteric plexus or smooth muscle cells (Fig. 6*Cc*).

When the colon was cleansed with liquid-diet treatment, morphine-induced upregulation of COX-2 protein and mRNA (Fig. 5*C*) and NGF expression (Fig. 6*D*) was almost completely blocked. These results suggest that upregulation of COX-2 and NGF in the colon of Mor rats is a mechanosensitive process and is induced by fecal retention-associated mechanical stress.

Inhibition of COX-2 and NGF Attenuated the Impacts of Morphine Treatment on Colonic Motor Function and Visceral Sensitivity

To further determine if fecal retention-associated upregulation of COX-2 contributes to the sustained motility dysfunction in morphine-treated rats, we administered COX-2 inhibitor NS-398 (22, 26) in control and Mor rats fed regular pellet food. Figure 7, *A* and *B*, shows the daily fecal output data with the use of COX-2 inhibitor in the vehicle control and morphine-treated rats. NS-398 did not affect morphine-induced decrease of fecal output on *day 1*. However, it almost completely blocked the fecal output changes with morphine-treatment afterward (Fig. 7, *A* and *B*) and improved fecal accumulation in the colon (Fig. 7*C*). Studies of the colon circular muscle strips in

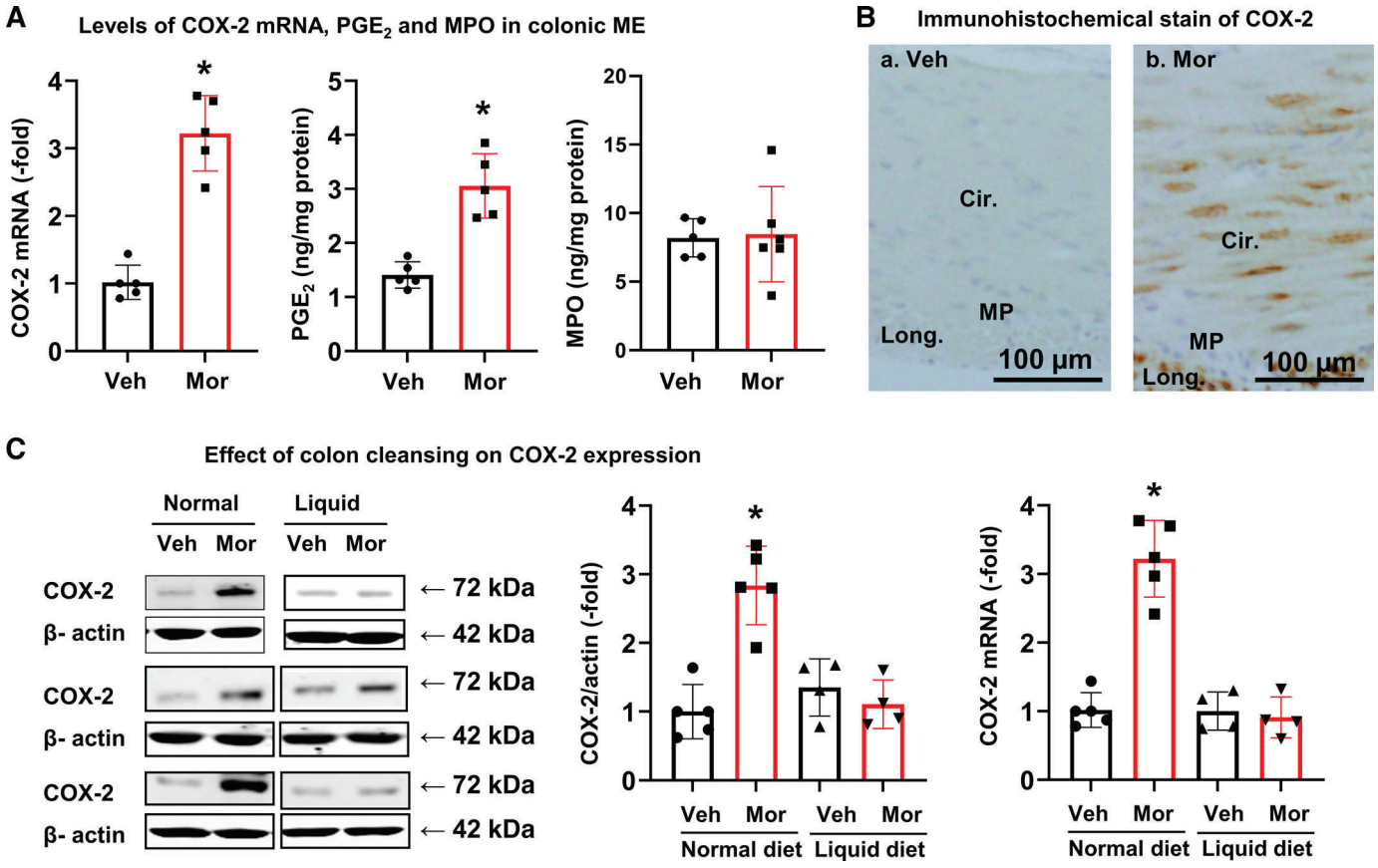

Figure 5. Expression of COX-2 in colonic smooth muscle in morphine-treated rats. *A*: COX-2 mRNA expression and PGE_2 production are increased in muscularis externae of the distended distal colon in Mor rats compared with Veh. However, MPO levels are not different between Veh and Mor groups. *B*: immunohistochemical stain showed that expression of COX-2 (stained in brown) is increased in SMC in Mor rats. *C*: however, when colon was cleansed (rats on a clear liquid diet), the expression of COX-2 protein and mRNA was not different between Mor and Veh rats. Data are expressed as means ± SD. $N = 4$ or 5 rats in each group. *$P < 0.05$ vs. Veh. Cir, circular smooth muscle; COX-2, cyclo-oxygenase-2; Long, longitudinal smooth muscle; Mor, morphine-treated rats; MP, myenteric plexus. MPO, myeloperoxidase; NS, no significant difference; SMC, smooth muscle cells; Veh, vehicle control rats.

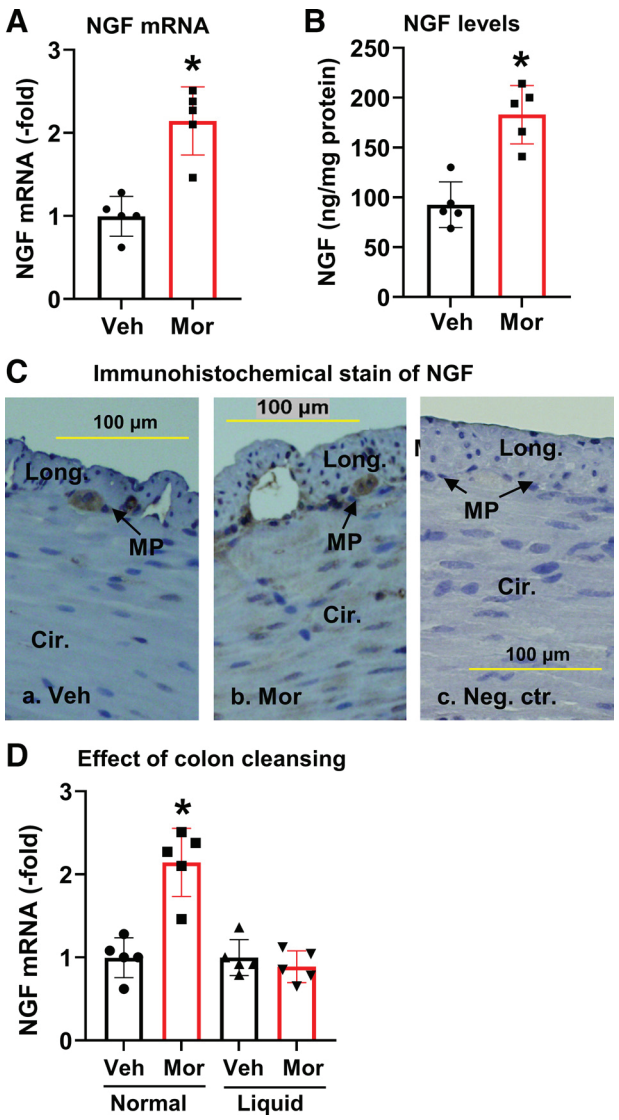

Figure 6. Expression of NGF in colonic smooth muscle in morphine-treated rats. Morphine treatment increased NGF mRNA expression (*A*) and protein levels (*B*) in muscularis externae tissue of the distal colon in Mor rats compared with Veh. *C*: immunohistochemical stain of colonic muscularis externae showed that NGF expression (stained in brown) is detectable in MP in Veh rats (*a*), and expression of NGF is increased in SMC in Mor rats (*b*). In the negative control, where no primary antibody was added, no immunoreactivity was detected in either MP or SMC (*c*). *D*: colon cleansing with liquid-diet treatment blocked morphine-induced upregulation of NGF expression. Data are expressed as means ± SD. $N = 5$ rats in each group. $^*P < 0.05$ vs. Veh. Cir, circular smooth muscle; Long, longitudinal smooth muscle; Mor, morphine-treated rats; MP, myenteric plexus; NGF, nerve growth factor; SMC, smooth muscle cells; Veh, vehicle control rats.

vitro found that the smooth muscle contractility was largely restored with COX-2 inhibition in the morphine-treated rats (*day 7*) (Fig. 7*D*). The maximal contractile response was 5,425 ± 562 and 4,767 ± 512 AUC/mm^2 ($P > 0.05$), and the EC_{50} values were 3.7 (±1.5) × 10^{-5} M and 2.2 (±1.1) × 10^{-5} M ($P > 0.05$) in Veh and Mor rats, respectively, when administered with NS-398.

Finally, to determine if fecal retention-associated expression of NGF is involved in visceral hypersensitivity in morphine-treated rats, we administered anti-NGF antibody (25, 43) in Veh and Mor rats. Quantitative measurements of the visceromotor response to colorectal distention showed that anti-NGF treatment significantly attenuated morphine-induced visceral hypersensitivity in the rats (Fig. 8), suggesting that fecal retention-associated induction of pain mediators such as NGF in the colon may contribute to visceral hyperalgesia in OBD.

DISCUSSION

Bowel dysfunctions are among the most common adverse effects associated with chronic use of opioids (3–7). Although the mechanisms for constipation and abdominal pain in OBD are not clear, research has been focused on the enteric and central nervous systems (6, 9, 13, 15, 18). However, the present study suggests that a peripheral mechanism involving the distal colon may play a critical role in the development of sustained constipation and visceral hyperalgesia. We found that treatment with morphine leads to profound fecal retention in the distal colon. The fecal retention-associated mechanical stress causes upregulation of COX-2 and NGF in the colon smooth muscle, and suppression of muscle contractility and increase of visceral sensitivity. Interestingly, when fecal retention is prevented, morphine treatment does not lead to suppression of muscle contractility or increase of visceral sensitivity. Expression of COX-2 and NGF in the colon smooth muscle is also blocked when fecal retention is prevented. Our study thus reveals a previously unrecognized peripheral mechanism in OBD that fecal retention, a common phenomenon in opioid users, serves as an independent pathogenic factor in motility dysfunction and visceral hyperalgesia in OBD. Intervention studies found that COX-2 inhibitor improves smooth muscle contractility and bowel movement in morphine-treated rats. Anti-NGF treatment attenuated visceral hypersensitivity in the OBD model. Thus, mechanical distention-induced expression of COX-2 and NGF in the colon may play a critical role in the development of opioid-induced constipation and abdominal pain.

Constipation is a sustained adverse effect in chronic opioid users (5–7). Although tolerance to analgesia and other effects are commonly present with chronic use of opioids, constipation still persists. In fact, tolerance to the effects of morphine or opioid receptor stimulation occurs in all gastrointestinal organs, except in the colon (15, 45). In search for the mechanisms underlying OIC, investigators have focused mainly on opioid receptors in the ENS (6, 15, 18, 45). The μ-opioidreceptor (MOR) is known to affect ENS to inhibit enteric neuron excitability, and to reduce release of excitatory neurotransmitters, i.e., acetylcholine (ACh), thus slowing intestinal transit and reducing mucosal secretion (6, 15, 18). These opioid receptor-dependent effects may well be the primary reason for initial stage of constipation (Fig. 9). However, constipation consequently leads to fecal retention in the distal bowel. In fact, we found that constipation and fecal retention start on the very first day of morphine treatment and remain throughout the 7-day treatment of morphine.

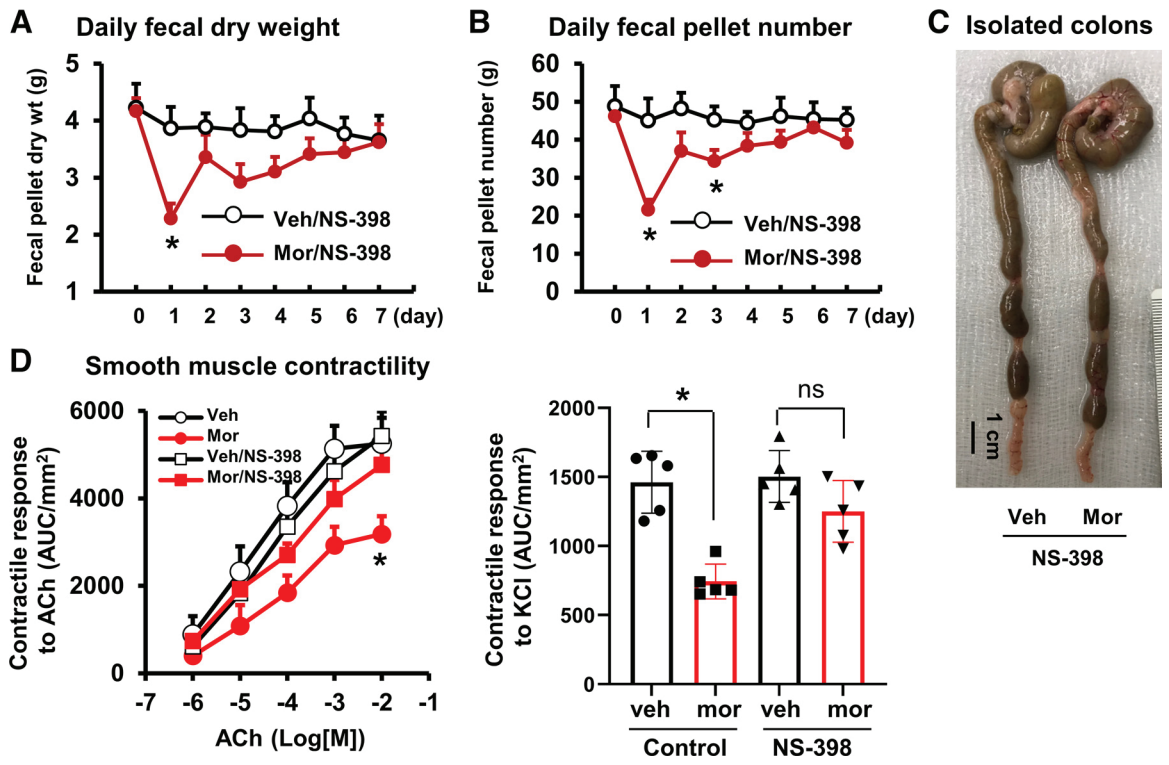

Figure 7. Inhibition of COX-2 improves colon motor function in morphine-treated rats. Daily administration of COX-2 inhibitor NS-398 (10 mg/kg ip) did not affect morphine-induced decrease of fecal outputs on *day 1* (*A* and *B*), but improved fecal outputs afterward (*A*–*C*). The colonic smooth muscle contractility in response to ACh (*left*) and KCl (*right*) was also improved with NS-398 treatment in the morphine-treated rats (*day 7*) (*D*). $N = 5$ or 6 rats in each group. $^*P < 0.05$ vs. Veh. ACh, acetylcholine; COX-2, cyclo-oxygenase-2; Veh, vehicle control rats.

Our data suggests that fecal retention itself may play a critical role in contractility changes of colon smooth muscle, which may well contribute to the sustained motility dysfunction and constipation (32, 39), as seen in chronic use of morphine. As summarized in Fig. 9, we propose that an opioid receptor-dependent mechanism to inhibit enteric neural activity may be the initial cause of fecal retention, and that an opioid receptor-independent mechanism, i.e., fecal retention-initiated mechanotranscription (i.e., COX-2), may account for gut smooth muscle dysfunction, contributing to sustained constipation in OBD. This is supported by multiple lines of evidence. First, although analgesic tolerance to opioids is well documented, there is no or least tolerance to opioid-induced constipation with extended use of opioids (15, 18, 45). This indicates that an opioid receptor-independent mechanism may likely be involved. Second, peripheral opioid receptor antagonists are not very effective in releasing OIC, especially after long-term use of opioids (7, 8, 16, 17). More importantly, our results in the present study found that inhibition of COX-2 does not affect opioid-induced constipation on *day 1* of morphine treatment, indicating that early phase of constipation may not be due to mechanotranscription of COX-2, but most possibly due to opioid receptor-dependent mechanism as previously proposed (9, 15, 18). However, COX-2 inhibitor effectively blocked morphine-associated reduction of feces production for almost all other days except *day 1*, and restored smooth muscle function. Given that prevention of fecal retention blocked COX-2 expression and also restored smooth muscle function, these data suggest that mechanotranscription of COX-2 may play a critical role in sustained motility dysfunction and chronic constipation. In fact, COX-2 expression in gut SMC is highly sensitive to mechanical stress, as previously demonstrated in mechanical distention model in vivo (22, 23) and in cultured SMC in vitro (22, 27). Although COX-2 is often considered a proinflammatory mediator, we found no detectable

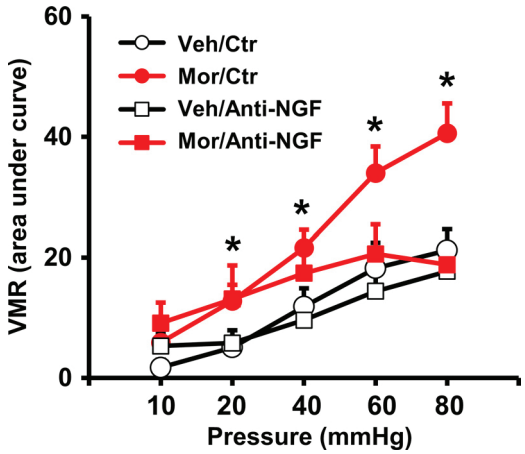

Figure 8. Effect of anti-NGF treatment on morphine-induced visceral hypersensitivity. The visceromotor response to colorectal distention was increased by chronic use of morphine (7 days). However, anti-NGF treatment (20 μg/kg ip daily) attenuated morphine-induced visceral hypersensitivity. $N = 5$ or 6 rats in each group. $^*P < 0.05$ vs. Veh. NGF, nerve growth factor; Veh, vehicle control rats.

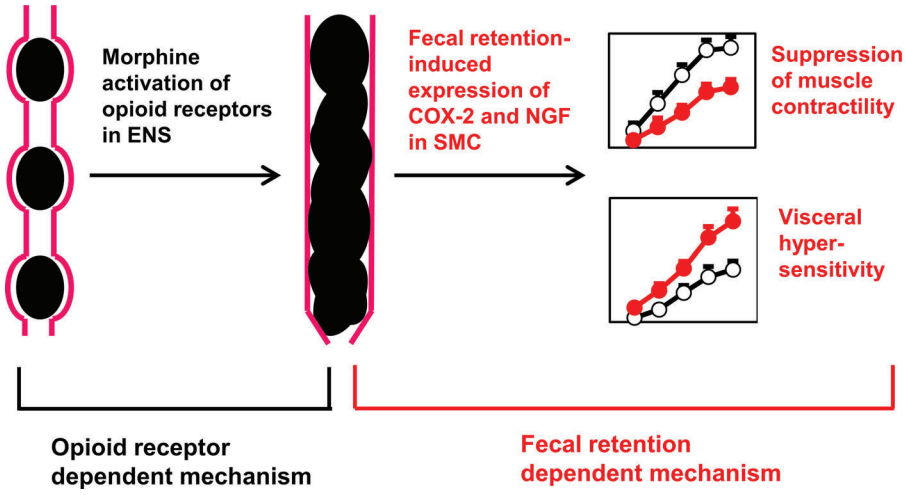

Figure 9. Proposed mechanisms underlying OBD. Opioid receptor-dependent mechanism via enteric nervous system (ENS) leads to fecal retention in the distal colon, which induces expression of COX-2 and NGF in the colonic smooth muscle. The fecal retention-dependent expression of COX-2 and NGF may underlie motility dysfunction and visceral hyperalgesia in prolonged use of opioids. Our study shows that prevention of fecal retention by colon cleansing with clear liquid diet eliminates the effect of opioid receptor-dependent mechanism and blocks the initiation of fecal retention-dependent mechanism. COX-2, cyclo-oxygenase-2; NGF, nerve growth factor; OBD, opioid-induced bowel dysfunction.

colonic inflammation in our OBD model. Our study thus suggests that peripheral opioid receptor antagonists may be useful to prevent initiation of OIC, and that mechanotranscription-dependent process shall be explored as a novel therapeutic target for the management of sustained OIC.

More than half of chronic opioid users experience abdominal pain in addition to constipation and fecal retention (3, 11). In fact, when abdominal pain becomes a predominant symptom in chronic use of opioids, the condition is defined as NBS, a subset of OBD (3, 11–13). Clinical and preclinical studies found that visceral hyperalgesia or hypersensitivity, as a widely recognized mechanism for abdominal pain (19, 23, 46), is present in chronic use of opioids (3, 11, 19, 20). Mechanisms of visceral hyperalgesia in OAP and NBS are incompletely understood. Current theories are largely based on reports in broader fields of opioid-induced hyperalgesia and addiction, but not on visceral pain (3, 13, 14). However, not any therapeutic agent for OAP or NBS have been developed around these concepts. The current treatment for NBS consists of opioid withdrawal and nonspecific treatments such as antidepressants and other psychosocial interventions (3, 13, 14). This so-called "detoxification management" led to only 35% reduction of abdominal pain after a 3-mo treatment (14). Half of the patients returned to using narcotics with recurrent NBS (14). Nevertheless, bowel distention or fecal retention is a distinctive feature in OAP and NBS (12, 13). In the present study, we found that chronic use of morphine leads to visceral hypersensitivity and sensory neuron hyperexcitability in rats fed with regular pellet food. These rats demonstrated apparent fecal retention, and upregulation of pain-mediator NGF in the muscle tissue of the distended colon. Interestingly, colon cleansing not only prevented fecal retention and NGF upregulation but also attenuated visceral hypersensitivity and sensory neuron hyperexcitability. To determine visceral sensitivity, we measured viscero-motor response to colorectal distention via a balloon inserted into the distal colon. This measurement is a widely used assessment of visceral sensitivity of the colon (19, 23). However, it is not known if morphine-treatment-associated fecal retention affects colon compliance and visceromotor response results. Thus, we also measured cell excitability of isolated colon-specific DRG neurons, and found that these neurons were highly excited in the morphine group, suggesting that peripheral visceral hypersensitivity is present in the OBD model. Taken together, we propose a peripheral mechanism in OAP that opioid-initiated fecal retention triggers mechanotranscription of pain mediators, such as NGF in colonic SMC, which sensitizes primary afferent neurons to contribute to visceral hyperalgesia (Fig. 9). As processes such as mechanotranscription of pain mediators and sensitization of afferent neurons may take days, visceral hyperalgesia may not be present immediately, but after prolonged use of narcotics (11–13). The fecal retention-dependent mechanism of peripheral sensitization represents a novel pathway in the development of visceral hyperalgesia in OBD.

To prevent fecal retention in opioid treatment, we removed normal pellet food from rats and applied bowel cleanser the day before morphine treatment and kept rats exclusively in liquid diet throughout the 7-day period of morphine treatment. This protocol almost completely cleansed the colon in vehicle-treated and morphine-treated rats, and prevented fecal retention-associated mechanotranscription and neuromuscular changes associated with morphine treatment. These results suggest that colon cleansing could be an effective treatment for opioid bowel dysfunction. Notably, colon cleansing has been tried as an alternative treatment for constipation, especially neurogenic and idiopathic constipation (47–51). When first-line constipation treatments failed, colon cleansing (i.e., colonic irrigation) was found to be effective in 65%–90% of the patients (47–50). Unfortunately, these studies did not further investigate the possible mechanisms underlying the efficacy of colon cleansing. Based on our results, we believe that the benefits of colon cleansing in persistent constipation may be a result of inhibition of mechanotranscription of mediators such as COX-2. In fact, Cong et al. (52) reported significant increase of COX-2 expression and prostaglandin production in colonic smooth muscle of patients with chronic constipation.

To the best of our knowledge, the utility of colonic irrigation as a way to cleanse bowel has not been tried for patients with OBD. In fact, colonic irrigation may have safety and compliance concerns (50, 53). Our regimen with liquid diet

after bowel cleanser may offer a better choice to achieve colon cleansing, as it is very efficient in keeping the colon from fecal retention for extended time. Exclusive enteral nutrition (EEN), involving oral or nasogastric tube feeding of liquid diet for 6–8 wk, is an effective therapy in the management of Crohn's disease, especially in pediatric patients (40, 41). Our study suggests that EEN may also be useful for the management of OBD. We found that liquid diet not only physically preempts fecal accumulation in the colon but also attenuates fecal retention-induced gene expression of proinflammatory and pain mediators such as COX-2 and NGF. Consequently, it helps to mitigate COX-2-mediated and NGF-mediated motility dysfunction and visceral hyperalgesia in OBD. EEN may affect gut microbiota composition and diversity (40, 54), though these changes do not account for the benefits of EEN treatment for Crohn's disease. In an attempt to minimize the effect of possible liquid-diet-associated microbiota changes, we have always included vehicle control along with morphine treatment in each diet group (either normal chow or liquid diet). Liquid-diet treatment left fewer or no residual feces in the colon in both Veh and Mor rats, indicating that the effect of microbiota changes, if any, would be similar in the Veh and Mor rats. It will be interesting to determine in the future if microbiota may play any role in the improvement of bowel function by liquid diet in OBD.

In summary, opioids may initially act on opioid receptors on the enteric nervous system to cause fecal retention. However, we found that fecal retention, as a mechanical stress in the distal bowel, subsequently induces mechanotranscription of COX-2 and NGF in colonic smooth muscle, which contributes to sustained motility dysfunction, constipation, and visceral hypersensitivity in the rodent model of OBD (Fig. 9). Thus, fecal retention, may play an independent pathogenic role in OBD. As liquid diet prevents fecal retention, it may be useful for the management of OBD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

X-Z.S. conceived and designed research; Y-M.L., Y.T., Y.F., S.H., D.W.S., and X-Z.S. performed experiments; Y-M.L., Y.T., Y.F., S.H., D.W.S., L-Y.M.H., and X-Z.S. analyzed data; Y-M.L., Y.T., Y.F., L-Y.M.H., and X-Z.S. interpreted results of experiments; Y-M.L., Y.F., and X-Z.S. prepared figures; Y-M.L. and X-Z.S. drafted manuscript; Y.T., S.H., D.W.S., L-Y.M.H., and X-Z.S. edited and revised manuscript; Y-M.L., Y.T., Y.F., S.H., D.W.S., L-Y.M.H., and X-Z.S. approved final version of manuscript.

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over mice for studying naturally occurring disorders, including gastrointestinal diseases, and for pharmaceutical drug development, as they share similar genetic, environmental, genomic, anatomical, and physiologic features with humans. Importantly, naturally occurring *MDR-1* deletion mutations are common in certain dog herding breeds, making them vulnerable to severe side effects (which can be fatal) from various therapeutics such as parasiticides. Intestinal enteroids are a promising research tool which can be used *in vitro* to predict therapeutic drug effects *in vivo*.

Methods: In this study, we utilize a novel method for mimicking a wild-type (WT) mutation found in the *MDR-1* gene of herding breed dogs in duodenal enteroids. We sought the most efficient methodology possible as this type of editing can be used in future contexts to rescue diseased phenotypes. The usage of a ribonucleoprotein (RNP) complex and delivery via electroporation were used to increase the efficiency of the genetic editing components.

Results: Previous studies had created P-gp knockout variants in mammalian cell lines; however, these mutations simply led to frameshift mutations. Here, we present a novel usage of two gRNAs in canine enteroids which remove a ~300 base pair (bp) area and results in the ends of the gene being spliced together via a Homology Directed Repair (HDR) template. This is done to precisely mimic the WT four bp deletion mutation. This more difficult method is required as the WT mutation is not proximal to a PAM site which the CRISPR/Cas9 system relies on.

Conclusions: This study allows for future assessment of the effect of *MDR-1* mutation on intestinal drug transport within the same individual dog. Additionally, by creating the exact WT deletion, we laid the framework to rescue this genotype by simply replacing the HDR repair template with a template containing the four missing bp in the *MDR-1* gene. Finally, with optimization of gene editing in canine organoids, a variety of additional organoid tissue models can be used to study other mutation/drug combinations. This optimization paired with new tissue types being grown in the organoid system allows for rapid and novel advances to be made in the field.

Su125

STANDARD PURIFICATION METHODS REDUCE BACTERIOPHAGE NUMBER AND ACTIVITY

Amanda Carroll-Portillo, Cristina Coffman, Matthew G. Varga, Sudha Singh, Henry C. Lin

Background: Bacteriophage (phage), bacterial viruses, are a major component of the gastrointestinal (GI) microbiome occurring in ~10-fold higher numbers than bacteria as a heterogeneous population varying considerably in size, shape and density. However, methods for purifying samples containing a mixed, GI phage population including filtration (for removal of bacterial component), PEG precipitation, and CsCl gradient purification are techniques originally optimized for purifying a single phage type from large cultures of their paired bacterial hosts. In this study, we tested the hypothesis that these standard purification methods may variably decrease phage numbers or activity depending on their type. **Methods:** Three coliphagic phages from different families- T4, fX 174, and M13- varying in size, shape, and density (see Table 1)- were processed individually or as mixed samples by PEG precipitation followed with a CsCl gradient. Quantitative (qPCR and fluorescent microscopy) and qualitative (phage overlay and TEM) measurements were performed to determine the effects of phage concentration and purification methods on phage numbers and activity. Data for numbers (microscopy) and activity (phage overlay) are presented as mean % change from the expected value (not graphed, set to zero) based on the unprocessed, starting phage concentration. **Results:** Processing of phage, for both individual and mixed samples, in almost all instances resulted in a loss in the number and activity of phage tested (exception for M13 numbers which were enhanced by PEG precipitation)(Figure 1). Additionally, CsCl gradient purification was almost always significantly more detrimental to both phage number and activity as compared to PEG precipitation alone (exception for M13 activity which was not significantly affected beyond loss from PEG precipitation with further CsCl purification; $p<0.0001$). Activity for each phage within the mixed population was unmeasurable as individual phage are indistinguishable in the phage overlay assay (all are coliphage). Of all phage tested, fX demonstrated the largest loss (almost 100%) in both activity and number following CsCl gradient purification. Of note, this degree of loss correlates to a log-fold decrease in the overall measure as compared to the expected value (e.g. activity fX expected = 6×10^{10} PFU/mL, fX CsCl purified = 1.2×10^{9}) rather than a complete loss of phage number or activity. **Conclusion:** Our data demonstrate that processing of samples containing phage by the standard purification methods of PEG precipitation or CsCl gradient significantly decreases phage number and activity across different size, shape and density. These changes may bias results such that true population dynamics cannot be properly interpreted.

Table 1. ***Phage characteristics***

Phage family	Dimensions	Density
M13 Inoviridae	 Length = 880 nm, Width = 6 nm	1.28 g/cm^3
T4 Myoviridae	 Body length = 190 nm, Head Width = 65 nm	1.5 g/cm^3
Phi X174 Microviridae	 Diameter = 32 nm	1.3 – 1.41 g/cm^3
Mixed (to scale)		

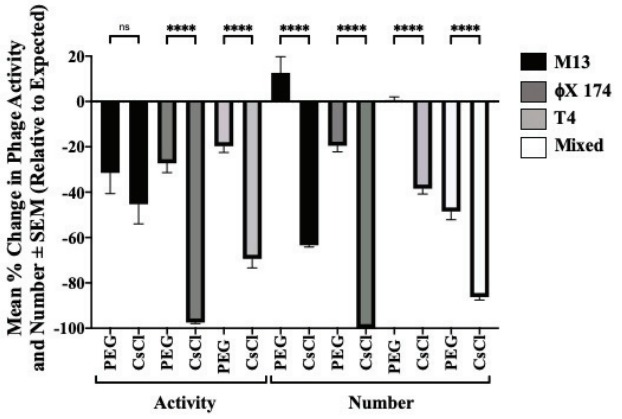


Figure 1. *CsCl gradient purification results in loss of phage number and activity.* Graphical representation of mean % change in phage activity and number as compared to expected values (set as zero baseline) based on starting phage concentrations. Samples processed by PEG precipitation followed by CsCl gradient centrifugation were tested after each step (PEG and CsCl bars respectively). **** = $p<0.0001$, ns = no significance

Su126

PRECLINICAL STUDIES OF PATHOPHYSIOLOGICAL ROLE AND DIAGNOSTIC POTENTIAL OF OSTEOPONTIN IN CROHN'S DISEASE

You-Min Lin, Xuan-Zheng P. Shi

Background and Aims: Multifunctional glycoprotein osteopontin (OPN) plays an important role in Th 1 immune response, and was found increased in the blood of Crohn's disease (CD) patients. However, what leads to increased OPN in CD is not known. CD is characterized by transmural inflammation, stenosis, and distention, which represent mechanical stress (MS) in the gut. We hypothesize that MS induces up-regulation of OPN expression in stenotic CD, and that OPN may contribute to neuromuscular dysfunction. **Methods:** Rat model of CD was induced by intracolonic instillation of TNBS (65 mg/kg in 250 microliter of 40% ethanol) to the distal colon. TNBS treatment induced localized transmural inflammation (~ 2 cm length) in the distal colon (site I), with a distended colon segment (site P) prior to, and a non-distended segment (site D) distal to the inflammation site. We studied the site-specific changes of OPN expression in sites I, P, and D, and in additional experimental conditions with or without MS. The role of OPN in motility function was tested in wild-type (WT) and OPN$^{-/-}$ mice. **Results:** 1) OPN mRNA expression was increased 77.8(± 20.6)-fold in site I of CD rats (7 day), compared to controls (saline instillation). Interestingly, OPN expression was also increased in the distended site P [69(±11.4)-fold], but not in the non-distended site D. This data indicate a mechanosensitive mechanism in OPN expression.

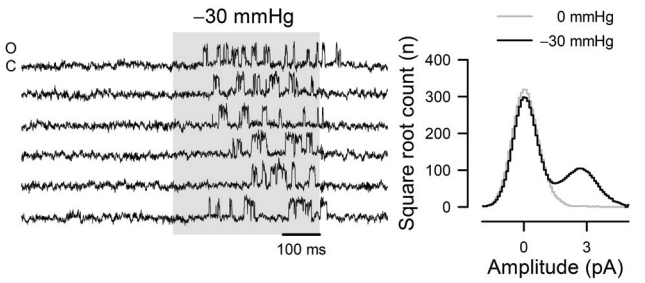
2) If mechanical distention in CD was prevented by feeding rats exclusively with clear liquid diet (Ensure), the increase of OPN mRNA expression was significantly attenuated to 13.2(±5.8)-fold in site I, and completely blocked in site P. 3) In a separate model, where mechanical distention, but no inflammation, was induced by an obstruction band in the distal colon, OPN mRNA expression was increased significantly in the distended segment prior to obstruction, but not in the segment distal to obstruction. 4) Plasma OPN level was increased significantly in rats with mechanical obstruction (608±116 vs. 222±39 ng/mL in sham, 7 day), and in TNBS induced colitis when rats were fed with regular pellet diet (424±118 ng/mL), but not in colitis rats when colon distention was prevented with clear liquid diet. 5) Our studies showed that OPN mRNA was increased mainly in the muscularis externae in the inflamed or distended colons. We then compared smooth muscle contractile function in wild-type and $OPN^{-/-}$ mice, and found that circular muscle contractility was significantly lower in the $OPN^{-/-}$ mice comparing to WT. Colon inflammation led to a decreased muscle contractility in WT mice, but not in $OPN^{-/-}$ mice. **Conclusions:** Our preclinical studies suggest that mechanical stress plays a critical role in up-regulation of OPN in CD. Increased plasma OPN in gut inflammation may indicate stenosis and obstruction. OPN may contribute to motility dysfunction in CD.

Su127

SMALL INTESTINE SMOOTH MUSCLE MECHANO-GATED CHANNELS AT SINGLE CHANNEL RESOLUTION

Peter R. Strege, Vikram Joshi, Kaitlyn R. Knutson, Andrew J. Wegner, Arthur Beyder, Gianrico Farrugia

BACKGROUND: Smooth muscle cells (SMCs) are indispensable for gastrointestinal (GI) motility. SMCs can be dysfunctional in diseases like intestinal pseudo-obstruction and slow transit constipation. Mechanical sensitivity of SMCs is epitomized by the myogenic reflex (MR), a rapid contraction in response to stretch independent of neighboring cell types like neurons. Mechano-gated ion channels (MGICs) are critical regulators of rapid cellular mechano-sensing. Select MGICs are involved in MR in vascular SMCs while the MGICs required for the MR in GI remain unknown. **AIM:** Determine whether mouse jejunum SMCs have functional mechano-gated single channels. **METHODS:** Myh11 codes for smooth muscle myosin and lineage traces SMCs. We generated a Myh11-creER^{T2}::tdTomato/GCaMP5 (Myh11-tomato) mouse model and dissociated jejunum smooth muscle strips into single SMC primary cultures. Single tdTomato+ (Myh11) SMCs were patch clamped in cell-attached mode. High-K^+ bath solution, along with high-Na^+ pipette solution containing TEA to block K^+ channels was used to isolate non-selective cation mechano-gated SC activity elicited by high-speed pressure clamp. To screen for pressure-dependent SC activity, cells were held at -80 mV and stepped for 400 ms to 0-50 mmHg patch suction. To determine SC conductance, cells were held at -120 mV, and 200 ms of suction was applied to each 400-ms voltage step from -100 to -20 mV. SC amplitudes and dwell times were calculated by event search, and dwell time histograms were fit with Gaussian curves. **RESULTS:** Myh11-tomato primary cultures contained tdTomato+ spindle-shaped cells consistent with SMCs. Pressure clamp elicited single channel openings. Pressure-dependent SC openings at -80 mV were 2.8±0.1 pA in amplitude (n = 6 cells from 4 cultures). Voltage dependence was linear and reversed ~0 mV; SC conductance was 13.4±2.2 pS. The EC_{50} of pressure was -32±5 mmHg. The mean dwell time for the open state was 2.6 ms, and the mean dwell times for 2 closed states were 0.7 and 32.0 ms. Averaged SC activity showed activation kinetics accelerated with pressure (τ = 540 ms at -30 mmHg, 120 ms at -50 mmHg), while deactivation slowed with pressure (τ = 31 ms at -30 mmHg, 140 ms at -50 mmHg). The net result of pressure-induced effects on kinetics would substantially increase inward currents with increasing SMC stretch. **CONCLUSION:** Lineage-traced mouse small bowel SMCs express functional MGICs with biophysical properties required to contribute to the MR. The identity of MGICs and downstream mechanisms are the focus of ongoing and future investigations. Support: NIH DK52766, DK106456.



Left, representative single channel traces recorded from Myh11-tomato SMC at -80 mV with 0 (unshaded) or -30 mmHg (shaded region) applied to the patch. O=open, C=closed. Right, all-points histograms constructed from 185 traces at 0 (gray) or -30 mmHg pressure (black).

Su128

HUNGER CONTRACTIONS DRIVING THE GASTRIC EMPTYING AS MEASURED BY WIRELESS MOTILITY CAPSULE (SMARTPILL®): HIGH CORRELATION WITH GHRELIN BUT NOT MOTILIN

moeen ud din, Hetzel O. Diaz Tartera, Dominic-Luc Webb, Per M. Hellström

Introduction: Gastric emptying can be quantified by several methods (e.g., gastric scintigraphy or paracetamol absorption). The SmartPill® wireless motility capsule (WMC) is increasingly used to quantify gastric emptying time (GET). Ghrelin receptor agonists are of interest as prokinetic drugs. How ghrelin relates to GET and hunger contractions obtained by WMC is poorly understood. Correlations between GET, hunger contractions and the metabolic response to a meal in terms of plasma glucose and peptide hormones were studied. **Methods:** Fasted subjects (N=41) ingested a 260-kcal mixed meal and the WMC. Plasma was obtained -10, 0 ,10, 20, 30, 40, 50, 60, 90, 120, 180 and 240 min into the meal. Peptide hormones (ghrelin, glucagon like peptide-1 (GLP-1), gastric inhibitory peptide (GIP), peptide YY(PYY), insulin) were measured by ELISA; glucose and triglycerides by clinical chemistry multianalyzer and motilin by radioimmunoassay. WMC recordings were analysed by MotiliGI 3.0 software. GET was found by pH drop in stomach followed by neutralization upon reaching duodenum. Last hunger contraction (LHC, >100 mmHg) was identified from pressure recordings within 5 min before GET. Correlations of GET to LHC, plasma glucose, insulin, ghrelin, GIP, GLP-1, motilin and PYY as evaluated by plasma concentrations as time to peak (Tmax), maximum concentration (Cmax) and exposure (AUC) were quantified by Pearson correlation coefficients. **Results:** Hunger contractions preceded GET in 31 subjects. Of all peptide hormones, as well as glucose and triglycerides, ghrelin showed strongest correlations to LHC (R 0.69; p<0.001) (fig.1a) and further related to GET (R 0.56, p <0.001) (fig.1b). The timing of the GLP-1 Tmax in response to meal approached significance (R 0.24, p<0.07), but not Cmax or AUC. Neither did Cmax, Tmax or AUC of glucose, insulin, GIP, motilin or PYY reveal any correlations to GET. However, a correlation between motilin and ghrelin was seen (R 0.50, p <0.01). This means that high ghrelin levels correlated with the induction of hunger contractions leading to gastric emptying. **Conclusions:** GET as measured by SmartPill WMC displays a robust correlation to hunger contractions occurring at high ghrelin levels, but does not correlate either with motilin or metabolic parameters, glucose absorption or insulin release. Hence, hunger contractions seem to be a strong physiological force behind gastric emptying time of the WMC. Since a relationship was found between ghrelin and hunger contractions, this speaks in favour of ghrelin as a major physiological driver for induction of hunger contractions in the terminal phase of the gastric emptying process.

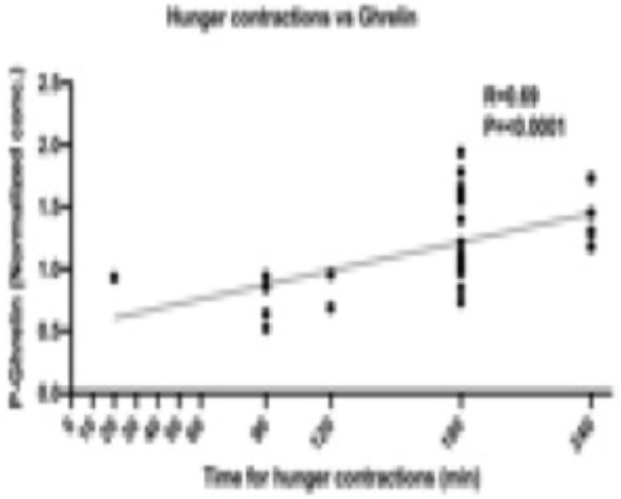


Fig. 1a. Relationship between the time for hunger contractions after food intake versus normalized plasma ghrelin concentrations.

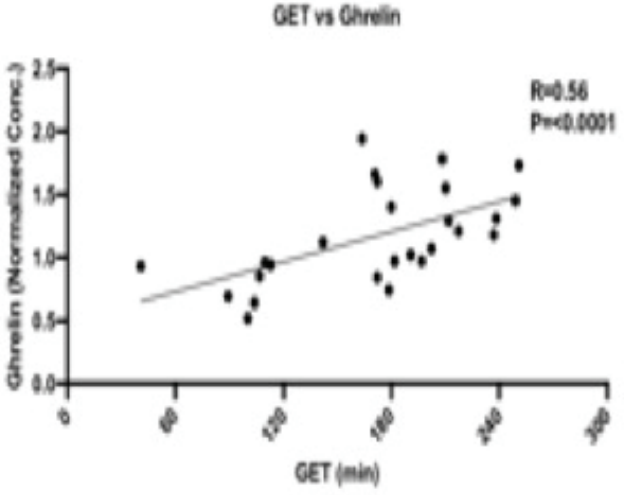


Fig. 1b. Relationship between the gastric emptying time as measured by WMC (SmartPill) versus the normalized plasma concentrations of ghrelin.

Sa129

LOSS OF GLUTATHIONE PEROXIDASE 1 ATTENUATES COLITIS AND ACTIVATES EPITHELIAL REGENERATIVE RESPONSES

Jared R. Hendren, Koral M. Kasnyik, Mary K. Washington, Christopher S. Williams, Sarah P. Short

Many selenium-containing "selenoproteins" function as antioxidants, and work by our group and others has demonstrated that selenoproteins often protect against intestinal inflammatory diseases, including colitis and colitis-associated cancer (CAC). Glutathione peroxidase 1 (GPx1) is a ubiquitous antioxidant selenoprotein which catalyzes the reduction of hydrogen peroxide by glutathione. Previously, we determined that despite its antioxidant role, loss of GPx1 greatly reduced disease severity in the dextran sodium sulfate (DSS) colitis model. Furthermore, GPx1 loss increased baseline intestinal cell proliferation, enhanced enteroid plating efficiency, and induced expression of stem cell-associated genes, such as *Lgr5*. Here, we next aimed to determine the mechanism by which GPx1 modifies response to DSS. First, we investigated whether GPx1 loss also affects stem cell function in the setting of colitis, as observed at baseline. We determined that both proliferation (Fig. 1A, $p<0.01$) and *Lgr5* expression (Fig. 2B $p<0.05$) were increased in the crypts of *Gpx1*$^{-/-}$ DSS-treated mice as compared to those from WT controls. Similarly, organoids established from ulcerative colitis tissue displayed increased proliferation (Fig. 1C, $p<0.05$), expression of stem cell and expression of Wnt target genes, such as *AXIN2* and *LGR5* (Fig. 1D, $p<0.01$), following *GPX1* knockdown. Interestingly, RNA-sequencing and gene set enrichment analysis identified a positive association with oxidative phosphorylation-associated genes in DSS-treated *Gpx1*$^{-/-}$ mice (NES: 1.78; FDR q-val: 0.01), and further studies in *GPX1* knockdown colorectal cancer cells observed higher basal respiration ($p<0.0001$) and ATP generation ($p<0.0001$). Together, these results suggest that GPx1 loss protects from colitis via augmenting epithelial proliferation and regenerative responses. However, while intestinal proliferation and regenerative responses can mitigate colitis and promote wound healing, these pathways may also drive cancer development and growth. To next determine whether GPx1 increases susceptibility to inflammatory tumorigenesis, we treated cohorts of WT and *Gpx1*$^{-/-}$ mice with azoxymethane followed by 3 cycles of DSS (AOM/DSS). Again, *Gpx1*$^{-/-}$ mice had preserved weights, lower mortality rates, reduced and endoscopic colitis scores. However, there was no change in tumor incidence, tumor number, tumor size, or degree of dysplasia between *Gpx1*$^{-/-}$ and WT AOM/DSS-treated mice. Taken together, these results indicate that unlike other intestinal selenoproteins studied to date, loss of GPx1 augments stem cell injury responses to protect against intestinal inflammation and injury. Furthermore, because GPx1 loss can confer protection from colitis without inducing pro-tumorigenic changes, GPx1 may serve as a novel therapeutic target.

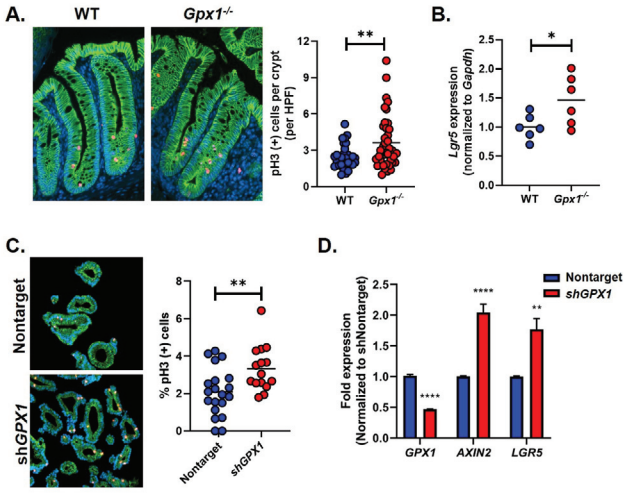


Figure 1. GPx1 loss activates proliferation in colitis. (A) Mice were treated with 2.5% DSS for 5 days and allowed to recover for 3 days prior to sacrifice. Proliferation was assessed by IHC for phospho-histone H3 (red), E-cadherin (green), and DAPI (blue). **(B)** Samples of colon were collected from DSS treated mice and *Lgr5* expression was assessed by qPCR. Values were normalized to *Gapdh* and represented as fold change over WT. **(C)** Organoids were established from an ulcerative colitis patient and *GPX1* was knocked down by shRNA while control cells received a nontargeted shRNA. Organoids were fixed and stained for phospho-histone H3 (red), E-cadherin (green), and DAPI (blue). **(D)** Gene expression of *GPX1*, *AXIN2*, and *LGR5* was assessed in GPx1 knockdown and control organoids. All statistics by Student's t test.

Sa130

MECHANICAL STRESS PLAYS A CRITICAL ROLE IN INTESTINAL FIBROSIS AND SMOOTH MUSCLE HYPERPLASIA IN A RODENT MODEL OF CROHN'S DISEASE

You-Min Lin, Suimmin Qiu, Amosy E. M'Koma, Don W. Powell, Steven Cohn, Xuan-Zheng P. Shi

Background and Aims: Intestinal fibrosis and smooth muscle hyperplasia are two main pathological changes in stenotic Crohn's disease (CD). Even if stenosis is resected, the previously distended pre-stenotic region may become new sites of recurrent inflammation and fibrosis, of which mechanisms are not well understood. Transmural inflammation in CD is associated with deformation, stenosis, and distention, which represent mechanical stress (MS) to the gut. We hypothesize that MS induces gene expression of pro-fibrotic and proliferative mediators, i.e. connective tissue growth factor (CTGF) and brain-derived neurotrophic factor (BDNF), and contribute to fibrosis and muscle hyperplasia. Methods: Rat model of CD was induced by intracolonic instillation of TNBS (65 mg/kg in 250 µL of 40% ethanol) to the distal colon. Control rats were treated with saline. TNBS treatment induced localized transmural inflammation (~ 2 cm length) in the distal colon (site I), with a distended colon segment (site P) prior to, and a non-distended segment (site D) distal to the site of inflammation. Micro- and macroscopic studies found that site I encompasses inflammation and MS, site P has MS but no inflammation, and site D has neither inflammation nor MS. We determined the site-specific changes of gene expression, inflammation, fibrosis and muscle hyperplasia in sites I, P, and D, and in additional experimental conditions with or without MS. Results: (1) Significant fibrosis and muscle hyperplasia were detected 7 to 21 days after TNBS treatment, not only in site I (inflammation site), but also in site P (distended segment prior to inflammation). RT-PCR and Western blot studies found that CTGF and BDNF expression was markedly up-regulated also in sites P and I. The increased CTGF and BDNF were mainly from muscularis externa. Interestingly, there was no up-regulation of CTGF and BDNF in the non-distended site D, suggesting a mechanosensitive mechanism in CTGF and BDNF expression. (2) In the CD model, if mechanical distention was prevented by feeding rats exclusively with clear liquid diet (Ensure), expression of CTGF and BDNF was completely blocked in site P, and dramatically attenuated in site I. Inflammation, fibrosis, and hyperplasia were significantly improved with liquid diet treatment. (3) In a separate model, where only mechanical distention, but no inflammation, was induced with an obstruction band in the distal colon, we found that collagen deposition, muscle hyperplasia, and expression of CTGF and BDNF were present only in the distended segment prior to obstruction, but not in the segment distal to obstruction. Conclusions: Transmural inflammation in CD causes MS in the inflammation site and distended segment prior to inflammation, and induces expression of pro-fibrotic and proliferative mediators, which may play a critical role in fibrosis and muscle hyperplasia.

Sa131

DEVELOPMENT OF A PERSONALIZED MODEL OF INTESTINAL FIBROSIS USING HUMAN INTESTINAL ORGANOIDS DERIVED FROM INDUCED PLURIPOTENT STEM CELLS

Hannah Q. Estrada, Shachi Patel, Shervin Rabizadeh, Stephan R. Targan, Robert J. Barrett

Background: Intestinal fibrosis is a serious complication of inflammatory bowel disease (IBD) with > 20% of Crohn's disease patients developing this complication within 10 years of diagnosis. Despite improvements in anti-inflammatory medication, its incidence remains stubbornly high and thus far surgical intervention remains the only treatment option. Numerous cell types including intestinal epithelial and mesenchymal cells are implicated in this process, yet studies are hampered by the lack of personalized in vitro models. One potential avenue that would permit a personalized approach is to utilize human intestinal organoids (HIOs) derived from induced pluripotent stem cells (iPSCs). iPSCs can be generated from any individual, faithfully recapitulate the genetics of the host and can be directed to form HIOs that contain both epithelial and mesenchymal cells. Our goal was to determine the feasibility of utilizing iPSC-HIO technology to model intestinal fibrotic responses in vitro.
Methods: iPSCs from two control individuals and two very early onset-IBD (VEOIBD) patients with stricturing complications were obtained and directed to form HIOs. Given HIOs are heterogeneous in terms of size, shape and ratio of mesenchymal to epithelial cells, they were firstly dissociated to a single cell suspension and EpCAM was used to positively select for epithelial cells using magnetic activated cellular sorting. These EpCAM+ cells were then seeded onto transwells and EpCAM- cells were seeded as monolayers in 10% serum containing media. Both cell types were treated with the profibrotic cytokine TGFβ, and changes in the expression of selected profibrogenic genes were analyzed.
Results: iPSCs from all 4 individuals could be directed to form HIOs containing both epithelial (E-cadherin+) and mesenchymal (vimentin+) cells (see Fig. 1). In the TGFβ-treated mesenchymal cell population, expression of Col1a1 and Col5a1, was increased in all four lines after 48hrs (see Table 1). In the TGFβ-treated epithelial cell population, Col1a1 and fibronectin expression were also increased in all lines after 96hrs.
Conclusion: We demonstrate the feasibility of utilizing iPSC-HIO technology to model intestinal fibrotic responses in vitro. We show that iPSCs generated from all selected individuals could be directed to form HIOs and that responses to the profibrotic cytokine TGFβ can be examined in both intestinal epithelial and mesenchymal cells. This now permits the generation of near unlimited quantities of patient specific cells that could be used to reveal cell and environmental specific mechanisms underpinning intestinal fibrosis which may ultimately lead to personalized treatments.

Table 1

	Mesenchymal cells		Epithelial cells	
	Fold change in Col1a1 after 48 hrs	Fold change in Col5a1 after 48 hrs	Fold change in Col1a1 after 96 hrs	Fold change in Fibronectin after 96 hrs
Control 1	2.19 ± 0.19 *	2.25 ± 0.27*	5.00 ± 2.60	2.95 ± 0.95
Control 2	2.34 ± 0.21 *	2.99 ± 0.41 *	4.84 ± 1.93	3.18 ± 1.69
VEOIBD patient 1	1.81 ± 0.38	1.65 ± 0.40	4.92 ± 2.42	3.67 ± 0.95
VEOIBD patient 2	2.65 ± 0.44	3.39 ± 0.16 **	6.09 ± 1.34 *	2.35± 0.95

Fold changes in gene expression of iPSC-derived epithelial and mesenchymal cells in response to 1ng/ml of TGFβ. A minimum of three independent experiments were carried out for each group. Results expressed as mean ± S.E.M. *P<0.05, **P<0.01 as compared to each line's respective control at time 0.

Preclinical studies of pathophysiological role and diagnostic potential of osteopontin in Crohn's disease

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Abstract

Background and Aims: Multifunctional glycoprotein osteopontin (OPN) plays an important role in Th 1 immune response, and was found increased in the blood of Crohn's disease (CD) patients. However, what leads to increased OPN in CD is not known. CD is characterized by transmural inflammation, stenosis, and distention, which represent mechanical stress (MS) in the gut. We hypothesize that MS induces up-regulation of OPN expression in stenotic CD, and that OPN may contribute to neuromuscular dysfunction. **Methods:** Rat model of CD was induced by intracolonic instillation of TNBS (65 mg/kg in 250 microliter of 40% ethanol) to the distal colon. TNBS treatment induced localized transmural inflammation (~ 2 cm length) in the distal colon (site I), with a distended colon segment (site P) prior to, and a non-distended segment (site D) distal to the inflammation site. We studied the site-specific changes of OPN expression in sites I, P, and D, and in additional experimental conditions with or without MS. The role of OPN in motility function was tested in wild-type (WT) and OPN$^{-/-}$ mice. **Results:** 1) OPN mRNA expression was increased 77.8(± 20.6)-fold in site I of CD rats (7 day), compared to controls (saline instillation). Interestingly, OPN expression was also increased in the distended site P [69(±11.4)-fold], but not in the non-distended site D. This data indicate a mechanosensitive mechanism in OPN expression. 2) If mechanical distention in CD was prevented by feeding rats exclusively with clear liquid diet (Ensure), the increase of OPN mRNA expression was significantly attenuated to 13.2(±5.8)-fold in site I, and completely blocked in site P. 3) In a separate model, where mechanical distention, but no inflammation, was induced by an obstruction band in the distal colon, OPN mRNA expression was increased significantly in the distended segment prior to obstruction, but not in the segment distal to obstruction. 4) Plasma OPN level was increased significantly in rats with mechanical obstruction (608±116 vs. 222±39 ng/mL in sham, 7 day), and in TNBS induced colitis when rats were fed with regular pellet diet (424±118 ng/mL), but not in colitis rats when colon distention was prevented with clear liquid diet. 5) Our studies showed that OPN mRNA was increased mainly in the muscularis externae in the inflamed or distended colons. We then compared smooth muscle contractile function in wild-type and OPN$^{-/-}$ mice, and found that circular muscle contractility was significantly lower in the OPN$^{-/-}$ mice comparing to WT. Colon inflammation led to a decreased muscle contractility in WT mice, but not in OPN$^{-/-}$ mice. **Conclusions:** Our preclinical studies suggest that mechanical stress plays a critical role in up-regulation of OPN in CD. Increased plasma OPN in gut inflammation may indicate stenosis and obstruction. OPN may contribute to motility dysfunction in CD.

Background

Multifunctional glycoprotein osteopontin (OPN) plays an important role in Th 1 immune response, and was found increased in the blood of Crohn's disease (CD) patients. However, what leads to increased OPN in CD is not known.

CD is characterized by transmural inflammation, stenosis, and distention, which represent mechanical stress (MS) in the gut.

We hypothesize that MS induces up-regulation of OPN expression in stenotic CD, and that OPN may contribute to neuromuscular dysfunction.

Specific Aims

We tested the hypothesis in a rodent model of Crohn's like colitis that MS induces up-regulation of OPN expression in stenotic CD, and that OPN may contribute to neuromuscular dysfunction.

Methods

1. Rat model of CD was induced by intracolonic instillation of TNBS (65 mg/kg in 250 µL of 40% ethanol) to the distal colon. Control rats were treated with saline.

2. TNBS treatment induced localized transmural inflammation (~ 2 cm length) in the distal colon (site I), with a distended colon segment (site P) prior to, and a non-distended segment (site D) distal to the site of inflammation. Micro- and macroscopic studies found that site I encompasses inflammation and MS, site P has MS but no inflammation, and site D has neither inflammation nor MS (Fig. 1).

3. We studied the site-specific changes of OPN expression in sites I, P, and D, and in additional experimental conditions with or without MS.

4. The role of OPN in colonic smooth muscle contractile function was studied in wild-type (WT) and OPN$^{-/-}$ mice.

Results

1. OPN mRNA expression was increased 77.8(± 20.6)-fold in site I of CD rats (7 day), compared to controls (saline instillation). Interestingly, OPN expression was also increased in the distended site P [69(±11.4)-fold], but not in the non-distended site D. This data indicate a mechanosensitive mechanism in OPN expression (Fig. 2a).
2. If mechanical distention in CD was prevented by feeding rats exclusively with clear liquid diet (Ensure) (Fig. 1), the increase of OPN mRNA expression was significantly attenuated to 13.2(±5.8)-fold in site I, and completely blocked in site P (Fig. 2b).
3. Our studies showed that OPN mRNA was increased mainly in the muscularis externae in the inflamed or distended colons (Fig. 3a).
4. In a separate model, where mechanical distention, but no inflammation, was induced by an obstruction band in the distal colon, OPN mRNA expression was increased significantly in the distended segment prior to obstruction, but not in the segment distal to obstruction (Fig. 4).
5. Plasma OPN level was increased significantly in rats with mechanical obstruction (608±116 vs. 222±39 ng/mL in sham, 7 day), and in TNBS induced colitis when rats were fed with regular pellet diet (424±118 ng/mL), but not in colitis rats when colon distention was prevented with clear liquid diet (Figs. 3,4).
6. We compared smooth muscle contractile function in wild-type and OPN$^{-/-}$ mice, and found that circular muscle contractility was significantly lower in the OPN$^{-/-}$ mice comparing to WT. Colon inflammation led to a decreased muscle contractility in WT mice, but not in OPN$^{-/-}$ mice (Fig. 5)

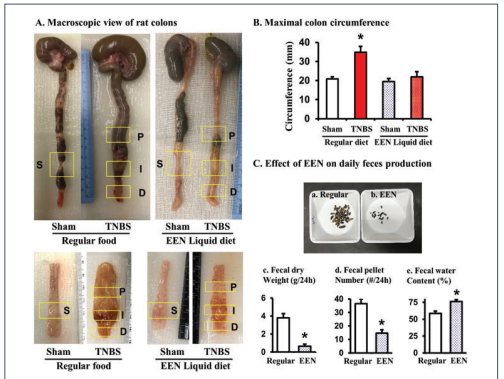

Fig. 1 Rat model of Crohn's-like colitis and effect of liquid diet treatment. (A) Outlook view of colons (top) and macroscopic view of distal colons (bottom). (B) Exclusive enteral nutrition (EEN) prevents colon circumference increase in TNBS colitis rats. (C) EEN treatment in control rats significantly decreased fecal production. Daily fecal production in one rat taking regular diet (a) and EEN for 3 days (b) is shown. Average fecal weight (c), number (d), and % water content (e) are shown (N=4 or 5). *p < 0.05 vs control rats fed with regular diet. The yellow boxes indicate different sites of colon tissue for the study. S, sham control; I, localized inflammation site; P, distended colon site proximal to inflammation site; D, no-distended site distal to inflammation.

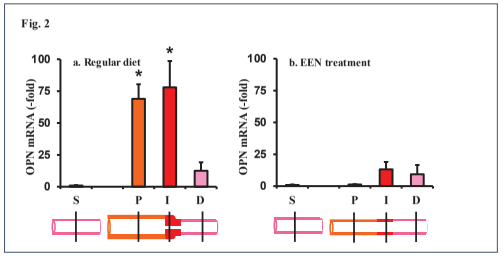

Fig. 2 Site-specific expression of OPN in colitis and effect of liquid diet. OPN mRNA expression was markedly induced in sites I and P, but not D in TNBS rats (7 day) when fed with regular diet (a). However, EEN treatment almost completely blocked up-regulation of OPN (b). N=5 in a, N=3 or 4 in b. *p < 0.05 vs S (sham).

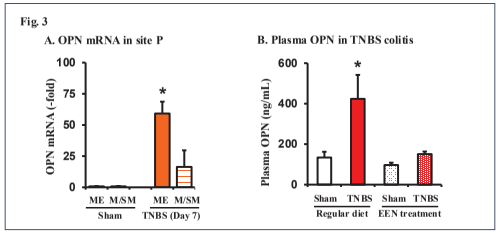

Fig. 3 OPN mRNA was increased mainly in the muscularis externae, not in the mucosa/submucosa of the TNBS colitis model (A). Plasma OPN level was significantly increased in TNBS colitis rats when fed with regular pellet diet (B), but not when treated with EEN. N= 4 or 5. *p < 0.05 vs S (sham).

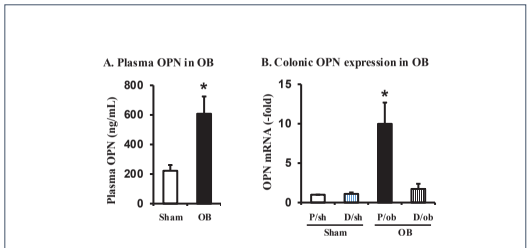

Fig. 4 Plasma and colon OPN levels in mechanical obstruction. Partial colon obstruction was induced with an obstruction band in the distal colon of rats. Tissue was taken from the sites proximal (P, with mechanical distention) and distal (D, without distention) to obstruction band 7 days after obstruction. Plasma OPN level was increased significantly in rats with mechanical obstruction (A). This is associated with increased OPN expression only in the distended proximal segment of the colon, not in the un-distended distal segment. N= 5. *p < 0.05 vs S (sham).

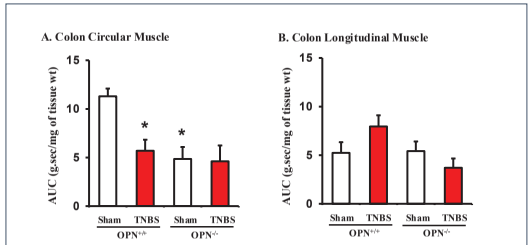

Fig. 5 Colonic smooth muscle contractility in TNBS-induced colitis in wild-type (OPN$^{+/+}$) and OPN$^{-/-}$mice. Mice were treated intra-colonically with saline or TNBS (1 mg/kg in 40% ethanol). Contractile response of colon circular (A) and longitudinal smooth muscle (B) to acetylcholine (10^{-7} to 10^{-3} M), cumulative) was determined in muscle bath recordings 7 days after induction of inflammation. N= 4 or 5. *p < 0.05 vs sham wild-type mice group.

Summary and Conclusions

Our preclinical studies suggest that mechanical stress plays a critical role in up-regulation of OPN in CD. Increased plasma OPN in gut inflammation may indicate stenosis and obstruction. OPN plays a role in regulating colonic circular muscle contractile function, and may contribute to motility dysfunction in CD.

Acknowledgements

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Mechanical stress plays a critical role in intestinal fibrosis and smooth muscle hyperplasia in a rodent model of Crohn's disease

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Abstract

Background and Aims: Intestinal fibrosis and smooth muscle hyperplasia are two main pathological changes in stenotic Crohn's disease (CD). Even if stenosis is resected, the previously distended pre-stenotic region may become new sites of recurrent inflammation and fibrosis, of which mechanisms are not well understood. Transmural inflammation in CD is associated with deformation, stenosis, and distention, which represent mechanical stress (MS) to the gut. We hypothesize that MS induces gene expression of pro-fibrotic and proliferative mediators, i.e. connective tissue growth factor (CTGF) and brain-derived neurotrophic factor (BDNF), and contribute to fibrosis and muscle hyperplasia. **Methods:** Rat model of CD was induced by intracolonic instillation of TNBS (65 mg/kg in 250 μL of 40% ethanol) to the distal colon. Control rats were treated with saline. TNBS treatment induced localized transmural inflammation (~ 2 cm length) in the distal colon (site I), with a distended colon segment (site P) prior to, and a non-distended segment (site D) distal to the site of inflammation. Micro- and macroscopic studies found that site I encompasses inflammation and MS, site P has MS but no inflammation, and site D has neither inflammation nor MS. We determined the site-specific changes of gene expression, inflammation, fibrosis and muscle hyperplasia in sites I, P, and D, and in additional experimental conditions with or without MS. **Results:** (1) Significant fibrosis and muscle hyperplasia were detected 7 to 21 days after TNBS treatment, not only in site I (inflammation site), but also in site P (distended segment prior to inflammation). RT-PCR and Western blot studies found that CTGF and BDNF expression was markedly up-regulated also in sites P and I. The increased CTGF and BDNF were mainly from muscularis externa. Interestingly, there was no up-regulation of CTGF and BDNF in the non-distended site D, suggesting a mechanosensitive mechanism in CTGF and BDNF expression. (2) In the CD model, if mechanical distention was prevented by feeding rats exclusively with clear liquid diet (Ensure), expression of CTGF and BDNF was completely blocked in site P, and dramatically attenuated in site I. Inflammation, fibrosis, and hyperplasia were significantly improved with liquid diet treatment. (3) In a separate model, where only mechanical distention, but no inflammation, was induced with an obstruction band in the distal colon, we found that collagen deposition, muscle hyperplasia, and expression of CTGF and BDNF were present only in the distended segment prior to obstruction, but not in the segment distal to obstruction. **Conclusions:** Transmural inflammation in CD causes MS in the inflammation site and distended segment prior to inflammation, and induces expression of pro-fibrotic and proliferative mediators, which may play a critical role in fibrosis and muscle hyperplasia.

Background

Intestinal fibrosis and smooth muscle hyperplasia are two main pathological changes in stenotic Crohn's disease (CD). Even if stenosis is resected, the previously distended pre-stenotic region may become new sites of recurrent inflammation and fibrosis, of which mechanisms are not well understood. Transmural inflammation in CD is associated with deformation, stenosis, and distention, which represent mechanical stress (MS) to the gut. We hypothesize that MS induces gene expression of pro-fibrotic and proliferative mediators, i.e. connective tissue growth factor (CTGF) and brain-derived neurotrophic factor (BDNF), and contribute to fibrosis and muscle hyperplasia.

Specific Aims

We tested the hypothesis in a rodent model of Crohn's like colitis that MS induces gene expression of pro-fibrotic and proliferative mediators, i.e. connective tissue growth factor (CTGF) and brain-derived neurotrophic factor (BDNF), and contribute to fibrosis and muscle hyperplasia.

Methods

1. Rat model of CD was induced by intracolonic instillation of TNBS (65 mg/kg in 250 μL of 40% ethanol) to the distal colon. Control rats were treated with saline.

2. TNBS treatment induced localized transmural inflammation (~ 2 cm length) in the distal colon (site I), with a distended colon segment (site P) prior to, and a non-distended segment (site D) distal to the site of inflammation. Micro- and macroscopic studies found that site I encompasses inflammation and MS, site P has MS but no inflammation, and site D has neither inflammation nor MS (Fig. 1).

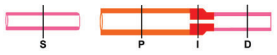

Fig. 1 Rat model of Crohn's like colitis

3. We determined the site-specific changes of gene expression, inflammation, fibrosis and muscle hyperplasia in sites I, P, and D, and in additional experimental conditions with or without MS.

Results

1. Significant fibrosis and muscle hyperplasia were detected 7 to 21 days after TNBS treatment, not only in site I (inflammation site), but also in site P (distended segment prior to inflammation) (Fig. 2, Fig. 3). RT-PCR and Western blot studies found that CTGF, collagen, and BDNF expression was markedly up-regulated also in sites P and I. The increased CTGF and BDNF were mainly from muscularis externa. Interestingly, there was no up-regulation of CTGF and BDNF in the non-distended site D, suggesting a mechanosensitive mechanism in CTGF and BDNF expression (Fig. 4, Fig. 5).
2. In the CD model, if mechanical distention was prevented by feeding rats exclusively with clear liquid diet (Ensure), expression of CTGF and BDNF was completely blocked in site P, and dramatically attenuated in site I. Inflammation, fibrosis, and hyperplasia were significantly improved with liquid diet treatment (Fig. 3).
3. In a separate model, where only mechanical distention, but no inflammation, was induced with an obstruction band in the distal colon, we found that collagen deposition, muscle hyperplasia, and expression of CTGF and BDNF were present only in the distended segment prior to obstruction, but not in the segment distal to obstruction (Fig. 6).

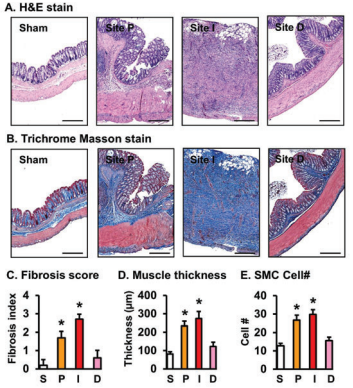

Fig. 2 Microscopic views in H&E (A) and trichrome Masson stain showing collagen deposition (B) in sham and different sites of CD rats. Quantitative analysis show increased fibrosis (C) and muscular hypertrophy (D) and hyperplasia (E) in sites I and P, but not D, in TNBS treated rats (day 21).

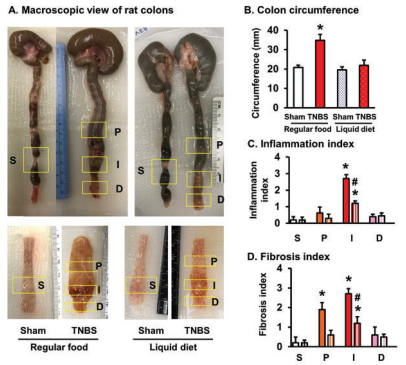

Fig. 3 Exclusive liquid diet treatment relieves lumen distention and improves inflammation and fibrosis in TNBS treated rats. (A) Outlook view of colons (top) and macroscopic view of mucosal surface of distal colons (bottom). The yellow boxes indicate different sites of colon tissue. S, sham control; I, inflammation site; P, distended colon site proximal to inflammation site; D, no-distended site distal to inflammation. Liquid diet treatment prevents increase of colon circumference in TNBS treated rats (B) and significantly improved inflammation index (C, day 7) and fibrosis index (D, day 21) in TNBS rats in both sites P and I. N=4 or 5. *p < 0.05 vs sham rats of the group. #p < 0.05 vs. same site in regular diet group. In C and D, bars in solid colors are data with regular diet, and bars in streaks are data with liquid diet.

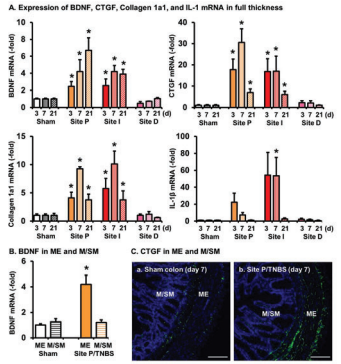

Fig. 4 (A) Time dependent expression of mechanosensitive and non-mechanosensitive inflammatory genes in different sites of the colon in rodent CD. S, sham colon; I, inflammation site; P, distended colon site proximal to inflammation; D, no-distended site distal to inflammation. Mechanosensitive expression of BDNF (B) and CTGF (C) in site P is mainly from smooth muscle tissue. ME, muscularis externae; M/SM, mucosa/submucosa. Scale bars = 100 μm. N=5 in A, N=4 or 5 in B and C. *p < 0.05 vs S (sham).

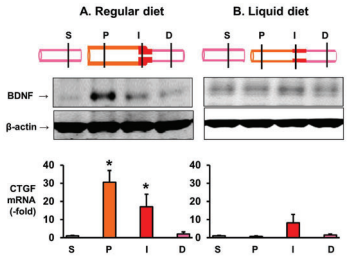

Fig. 5 Effect of exclusive liquid diet on mechanosensitive gene expression. Liquid diet treatment released mechanical stress in inflammation, and significantly attenuated expression of BDNF (Top. Shown is representative of 4 Western blot experiments) and CTGF (Bottom, Shown is qPCR result of mRNA levels) in sites P and I. Inflammation or liquid diet does not affect gene expression in site D. N= 4 or 5. *$p < 0.05$ vs S (sham).

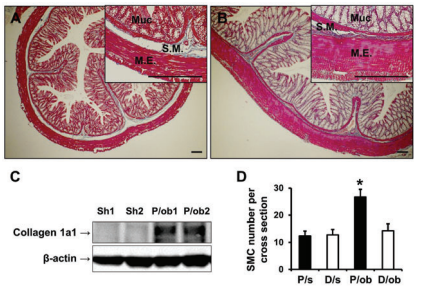

Fig. 6 ECM expression and hyperplasia in mechanical obstruction (OB) in rats. Trichrome staining of the colon specimens of the sham (A) and OB 7 day (B) (Site P, proximal to obstruction band). Scale bars = 100 μm. C, Western blot detection of collagen 1a1 expression in sham controls (Sh) and site P of obstructed colon for 7 days (P/ob). Shown are representative images of 5 independent experiments. D, Smooth muscle cell number per cross section in sites P and D in sham and obstruction (7 day) rats. N=5. *p<0.05 vs. sham.

Summary and Conclusions

Transmural inflammation in a rodent model of Crohn's colitis causes mechanical stress in the inflammation site and distended segment prior to inflammation, and induces expression of pro-fibrotic and proliferative mediators, which may play a critical role in fibrosis and muscle hyperplasia.

Acknowledgements

This study was supported in part by NIH (R01 DK124611 and R01 DK 102811) and the US DoD (W81XWH2010681) to XZS.