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ORIGINAL ARTICLE

The 5-HT4 Receptor Agonist Prucalopride Stimulates Mucosal Growth and Enhances Carbohydrate Absorption in the Ileum of the Mouse

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Abstract

Background Enteric serotonin may function as a mucosal growth factor. Previous work demonstrated increased crypt cell proliferation and intestinal mucosal surface area with potentiation of serotonin. While an indirect mechanism was postulated to explain these effects, the presence of 5-HT4 receptors on enterocytes raises the possibility of a direct action of serotonin. We hypothesized that a 5-HT4 specific agonist, prucalopride, would stimulate intestinal mucosal growth and enhance absorptive function in the murine small intestine.

Methods Adult wild-type mice were treated parenterally with prucalopride for 14 days via surgically implanted osmotic pumps. In vivo D-xylose absorption was assessed by oral gavage and serum D-xylose measurements. On day 14, glucose absorption was assessed by instilling a glucose solution into isolated segments of small intestine. The bowel was harvested and examined for morphologic parameters and crypt cell proliferation.

Results Villus height, crypt depth, and crypt proliferation were significantly increased in the distal small bowel of prucalopride-treated mice compared with control animals. Crypt depth was also increased in the proximal and middle small intestine in treated mice. There was no difference in D-xylose absorption throughout the study period; however, glucose absorption was significantly increased in the distal small intestine of prucalopride-treated mice.

Conclusion Parenteral administration of the 5-HT4 receptor specific agonist, prucalopride, results in morphologic and functional changes in the murine small intestine that are most prominent in the distal small bowel. While further studies are necessary to delineate the mechanism, it is plausible that the effects are mediated by 5-HT4 receptors on enterocytes.

Keywords Serotonin · Small intestine · Mucosa · Growth factor · 5-HT4 receptor

Introduction

Serotonin (5-HT) is a monoamine neurotransmitter with diverse actions that affects virtually all organ systems. Ninety-five percent of the body's 5-HT, however, is found in the gastrointestinal (GI) tract, where the majority of 5-HT is produced by enterochromaffin (EC) cells and to a lesser extent by enteric neurons of the submucosal and myenteric plexus.[1]

5-HT was first implicated as a mitogen by Hedinger and Langmann, who found an increase in number of circulating platelets in rats treated with serotonin.[2] Subsequent studies have shown that 5-HT can stimulate cell division in both plants and animals including root organogenesis in the thale cress,[3] liver regeneration in mice and rats,[4,5] mitogenic effects on vascular endothelial cells of dogs and cows,[6] and the development of neurons in cultures of isolated enteric crest-derived cells.[7]

Consistent with these findings, in the gut, 5-HT produced by enteric neurons promotes growth of the intestinal mucosa as seen in mice with potentiated 5-HT signaling.[8,9] This is evident both in mice with targeted deletion or pharmacologic blockade of the serotonin reuptake transporter (SERT), which is the primary method for inactivating the actions of 5-HT.

At least 7 types and as many as 14 subtypes of 5-HT receptors have been identified, underscoring the complexity of the 5-HT signaling system. In the GI tract, the main subtypes

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of 5-HT receptors include 5-HT1A, 5-HT1B/D, 5-HT2A, 5-HT2B, 5-HT3, 5-HT4, and 5-HT7.[10] 5-HT2A receptors have been localized to submucosal cholinergic neurons, which provide muscarinic innervation to epithelial effectors and act as the final driving force for promoting proliferation in the crypt base stem cell compartment.[8] This indirect pathway by which the cholinergic system mediates serotonergic effects is further evidenced by the mitigation of increased mucosal proliferation when SERT knock-out (SERTKO) mice are treated with scopolamine, a nonselective cholinergic antagonist.[8]

However, among the many 5-HT receptor types, 5-HT4 receptors are uniquely present directly on enterocytes themselves,[11] raising the possibility of a direct pathway of action where 5-HT would stimulate receptors directly on mucosal cells. The idea that 5-HT4 agonism may have trophic effects is supported by the robust evidence for its role in neuroprotection and neurogenesis in the enteric nervous system (ENS). In knock-out mice lacking 5-HT4 receptors, the normal increase in enteric neurons seen during the postnatal period is absent, and autophagy is increased compared to wild-type (WT) littermates.[12] Additionally, neurogenesis in the adult ENS was first demonstrated in mice and was found to be mediated by 5-HT4 receptors.[12] In an in vivo model of enteric neural circuit injury, the 5-HT4 agonist mosapride citrate promoted regeneration of the impaired neural circuit, possibly involving neural stem cells.[13]

Based on evidence that 5-HT is an intestinal mucosal growth factor and since it is clear that enterocytes express 5-HT4 receptors, we developed the hypothesis that 5-HT may directly interact with 5-HT4 mucosal receptors to induce intestinal mucosal growth and increase absorptive capacity.

Materials and Methods

Animals

C57Bl/6 mice were bred and housed under pathogen-free conditions with 12-h light/dark cycle and food/water ad libitum. Male and female mice ages 8–18 weeks were used for experiments. Animal protocols were approved by Yale University's Institutional Animal Care and Use Committee.

Prucalopride Administration

Eight to 18-week-old C57Bl/6 mice were treated with prucalopride (Sigma-Aldrich, St. Louis, MO; dissolved in DMSO), a highly selective and specific 5-HT4 receptor agonist,[14] and compared with control mice that were given vehicle alone (Fig. 1). Prucalopride (5 mg/kg/day, $n = 6$) and the vehicle ($n = 5$) were administered continuously for 14 days via a micro-osmotic pump (0.25 μl/h; Durect, Cupertino, CA) surgically implanted into a subcutaneous pocket created between the scapulae in isoflurane-anesthetized mice.

D-xylose Gavage

On days 0, 7, and 14 of the treatment period, mice were fasted for 4 h then received 100 μl of 2 mg/g body weight D-xylose (Sigma-Aldrich; dissolved in deionized water) by oral gavage. Blood was collected by retroorbital draw after 1 h, and serum D-xylose levels were quantified spectrophotometrically utilizing a D-xylose assay kit (Chondrex, Redmond, WA).

Extracorporeal Glucose Absorption Studies

At the end of the 14-day treatment period, animals were fasted for 4–6 h prior to induction of isoflurane anesthesia (3% induction; 1.5% maintenance) and laparotomy. The small bowel was identified from the ligament of Treitz to the ileocecal valve. From here, 2-cm segments of proximal, middle, and distal small intestine were isolated by application of a silk suture tie with special caution to preserve adjacent mesenteric vessels. Two hundred microliter of a 1:1 mixture of glucose (2 mg/ml) and phenol red (50 mg/L; G-Biosciences, St. Louis, MO), a non-absorbable substrate, was injected into each isolated segment. Contents were aspirated after 15 min and centrifuged. Glucose concentration within the aspirate was quantified with a Glucose (HK) Assay kit (Sigma-Aldrich, St. Louis, MO), and samples were analyzed spectrophotometrically against standard glucose curves. Phenol red concentration was assessed spectrophotometrically by measuring absorption at 560 nm against standard curves to determine fluid shifts. Final concentration of glucose was adjusted according to calculated fluid shifts and expressed as a rate of glucose absorbed per unit length (μg/ml/min/cm).

Bowel Harvest

After completion of the extracorporeal glucose absorption studies, mice were euthanized by cervical dislocation. Two-centimeter segments of unmanipulated intestine were harvested by sharp dissection from proximal, middle, and distal regions and flushed with 10% neutral buffered formalin (NBF). The sections were fixed in 10% NBF for at least 12 h before paraffin embedding and mounting on slides for staining.

Anatomic Measurement of Mucosal Parameters

Paraffin sections underwent standard hematoxylin and eosin staining. The slides were examined at ×200–×400 using standard brightfield microscopy (Axio Imager M1, Zeiss, OberKochen, Germany) and analyzed using ImageJ software (NIH, Bethesda, MD). Villi were measured only when intact from crypt-villus junction to crypt-villus junction with a

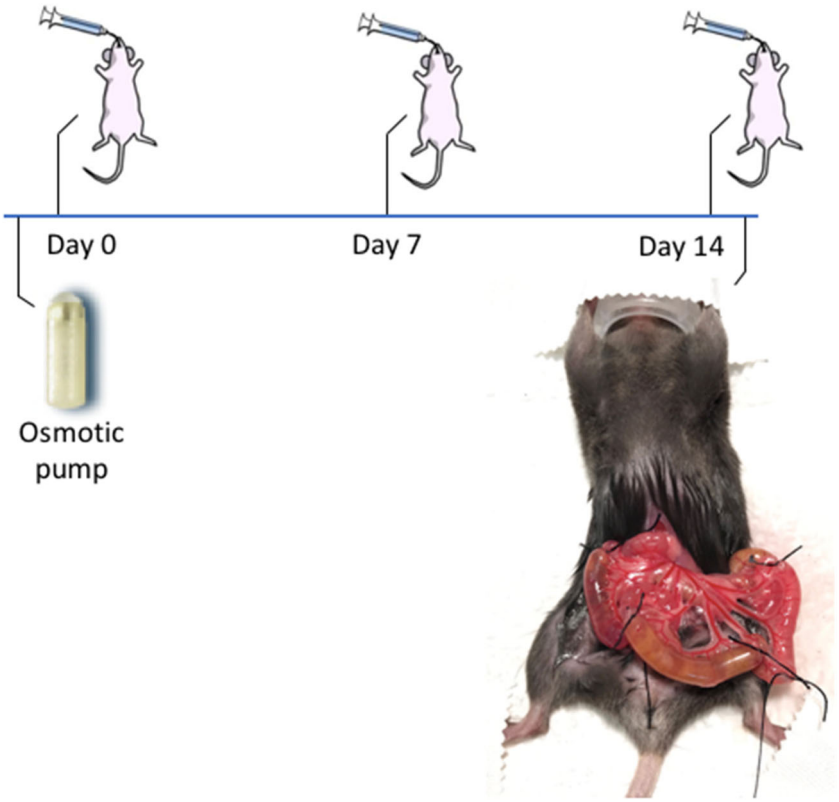

Fig. 1 Schematic drawing of experimental design. Day 0: micro-osmotic pump containing prucalopride or vehicle is surgically implanted followed by in vivo gavage of D-xylose and serum measurement. Day 7: in vivo gavage of D-xylose and serum measurement. Day 14: in vivo gavage of D-xylose and serum measurement followed by extracorporeal glucose absorption study

visible central lacteal. Crypts were measured when intact from crypt-villus junction to crypt villus junction and with at least partial visualization of adjacent villi. At least 30 villi and 15 crypts were measured per animal. Mucosal surface area (MSA) was calculated using methods described previously.[15]

Immunohistochemistry

For Ki-67 staining, rabbit polyclonal primary antibody against Ki-67 (Thermo Fisher Scientific, Waltham, MA) at a dilution of 1:1000 was used with an HRP-conjugated anti-rabbit secondary at 1:200 dilution (Biotium, Fremont, CA). Chromogen was developed using DAB substrate (Thermo Fisher Scientific, Waltham, MA) according to manufacturer protocols and counterstained with hematoxylin.

Crypt Proliferation Index (CPI)

Ki-67-stained slides were used to calculate the CPI. Slides were examined at ×400 using brightfield microscopy (Leica DM 1000 LED, Leica Microsystems, Buffalo Grove, IL), and images were captured using Leica MC120 HD digital microscope camera (Leica Microsystems). Color and exposure parameters were set manually for consistency. Images were analyzed utilizing the ImageJ software (NIH, Bethesda, MD) "color threshold" function with the following filter settings optimized for immunohistochemical DAB stain as described previously[16]: Hue 44/255 (stop); Saturation 37/255 (pass); Brightness 0/255 (pass). The number of Ki67-positive cells (cells filtered with color thresholding, Fig. 3) were divided by the total number of crypt cells and converted to a percentage to express CPI. Crypts were counted if a single epithelial cell layer was present from crypt-villus junction to crypt-villus junction and at least a portion of the adjacent villi were visible. At least 20 crypts were counted per group and used to calculate the mean.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 7.0a for MAC OS X (Graphpad Software, La Jolla, CA). Group means were compared using Student's t test and proportions were compared using χ^2 test with significance assumed for $p < 0.05$.

Results

5-HT4 Agonist-Mediated Intestinal Mucosal Growth

Prucalopride treatment caused no recognizable adverse events, and prucalopride-treated mice exhibited similar weight gain when compared to mice treated with vehicle. VH and CD in the distal small intestine was significantly greater ($p <$

0.0001) in mice treated with prucalopride compared to vehicle (Fig. 2a–d). In the proximal and middle small intestine, no significant change was seen in VH but CD was significantly greater ($p < 0.0001$) in both regions in prucalopride-treated animals compared to those given vehicle. For prucalopride-treated mice, the mean ± SEM values were as follows: proximal VH = 410.2 ± 4.8 μm; proximal CD = 86.4 ± 2 μm; middle VH = 246.9 ± 2 μm; middle CD = 91.8 ± 1.4 μm; distal VH = 210.7 ± 1.2 μm; distal CD = 85.8 ± 1 μm. For mice treated with vehicle, the mean values were as follows: proximal VH = 413.6 ± 6.4 μm; proximal CD = 64.3 ± 1.6 μm; middle VH = 254.2 ± 4 μm; middle CD = 67 ± 1.3 μm; distal VH = 180.8 ± 2.5 μm; distal CD = 61.5 ± 1.2 μm.

MSA of prucalopride-treated mice was significantly greater in the distal small intestine (Fig. 2e). For prucalopride-treated mice, the mean ± SEM values were as follows: proximal MSA = 904.7 ± 146.2 cm^2; middle MSA = 570.9 ± 218.3 cm^2; distal MSA = 447.9 ± 14.9 cm^2. For vehicle-treated mice, the mean values were as follows: proximal MSA = 1027 ± 138.2 cm^2; middle MSA = 452.1 ± 75.2 cm^2; distal MSA = 346 ± 34.2 cm^2.

Crypt Proliferation Index

Prucalopride-treated mice had significantly increased CPI in all three regions of the small intestine compared to control mice (Fig. 3). The mean ± SEM CPI of the proximal segments were 13.19 ± 1.1% and 50.51 ± 3.2% in control and treated animals respectively. Middle segments had a CPI of 17.6 ± 1.3% and 73.4 ± 1.0% in control and treated animals respectively, and distal segments had a CPI of 31.4 ± 2% and 60.1 ± 1.7% in control and treated animals respectively. All pairwise comparisons had $p < 0.0001$.

In Vivo Carbohydrate Absorption

To evaluate if the anatomic changes to the mucosa translate into increased absorptive functional capacity of the total intestine, we studied absorption of D-xylose, a simple carbohydrate in whole animals by oral gavage. There were no significant differences in D-xylose absorption between prucalopride-treated and control mice throughout the treatment period (Fig. 4a).

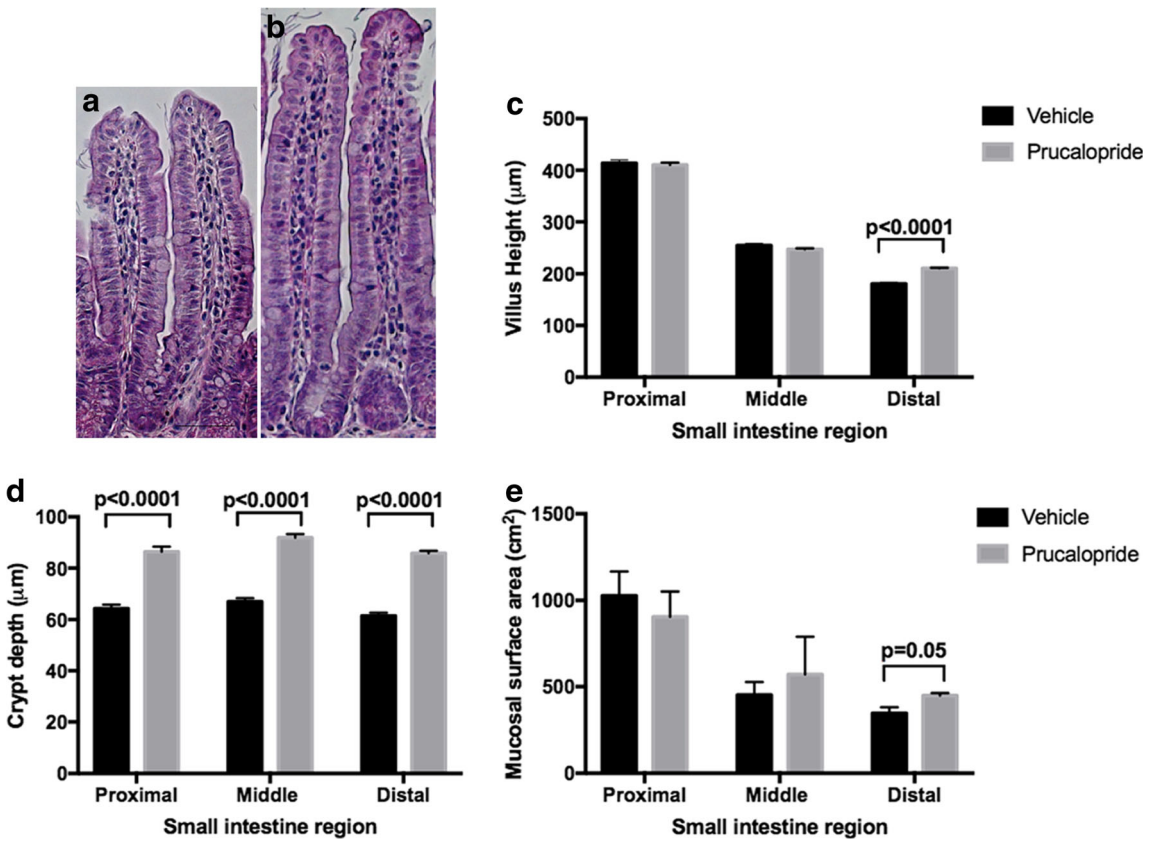

Fig. 2 Prucalopride-treated mice have taller villi, deeper crypts, and larger mucosal surface area. **a** Mucosal architecture in a section of control animal ileum stained with H&E. **b** Mucosal architecture of prucalopride-treated animal. **c** VH of proximal, middle, and distal small intestine comparing control vs. treated animals. **d** CD of proximal, middle, and distal small intestine comparing control vs. treated animals. **e** MSA of proximal, middle, and distal small intestine comparing control vs. treated animals

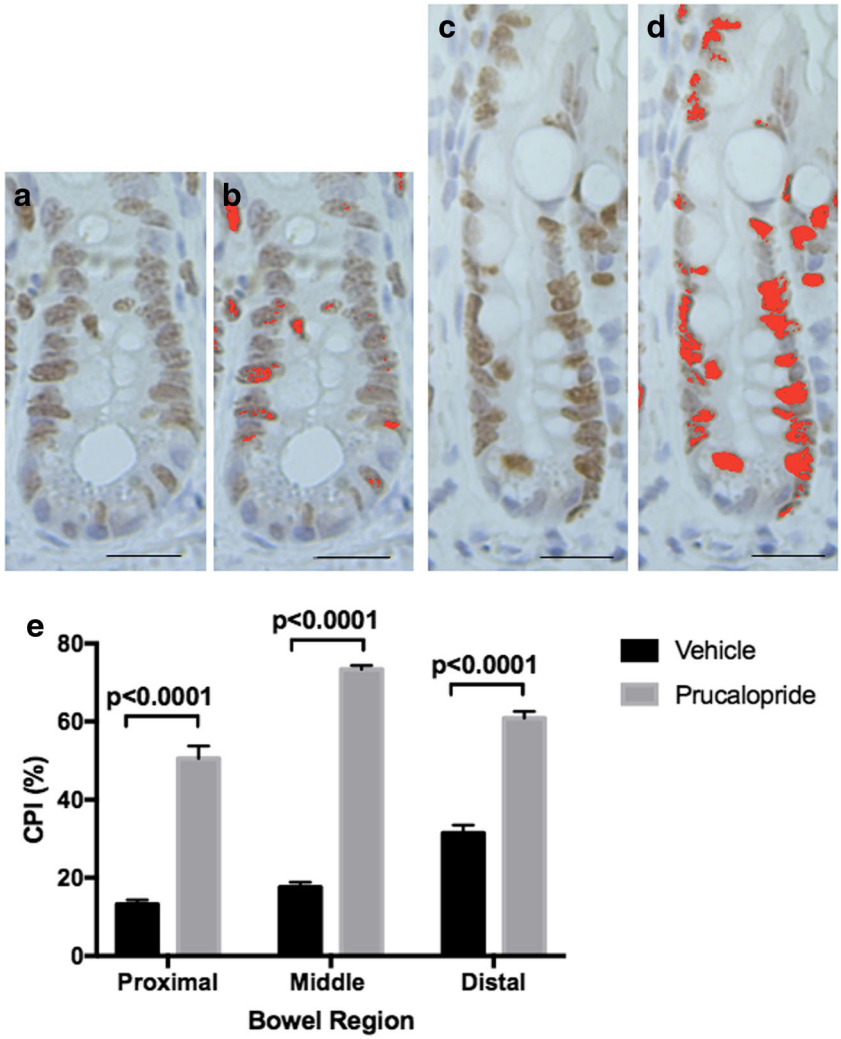

Fig. 3 Prucalopride-treated mice have deeper crypts and increased crypt proliferation in the proximal, middle, and distal small intestine compared to control mice as demonstrated by anti-Ki67 immunohistochemical DAB staining and image processing. **a** Representative crypt of control animal after anti-Ki67 staining. **b** Image "a" after color thresholding. **c** Representative crypt of prucalopride-treated animal after anti-Ki67 staining. **d** Image "c" after color thresholding. a–d scale bars 50 μm. **e** Prucalopride-treated mice have increased crypt proliferation index compared to control mice

Extracorporeal Carbohydrate Absorption

To assess the effects of prucalopride on specific regions of the small intestine, we evaluated glucose absorption in surgically isolated segments of perfused bowel at the end of treatment. Glucose absorption was significantly greater ($p < 0.01$) in the distal small intestine in prucalopride-treated mice compared to those that received vehicle (Fig. 4b). No differences were

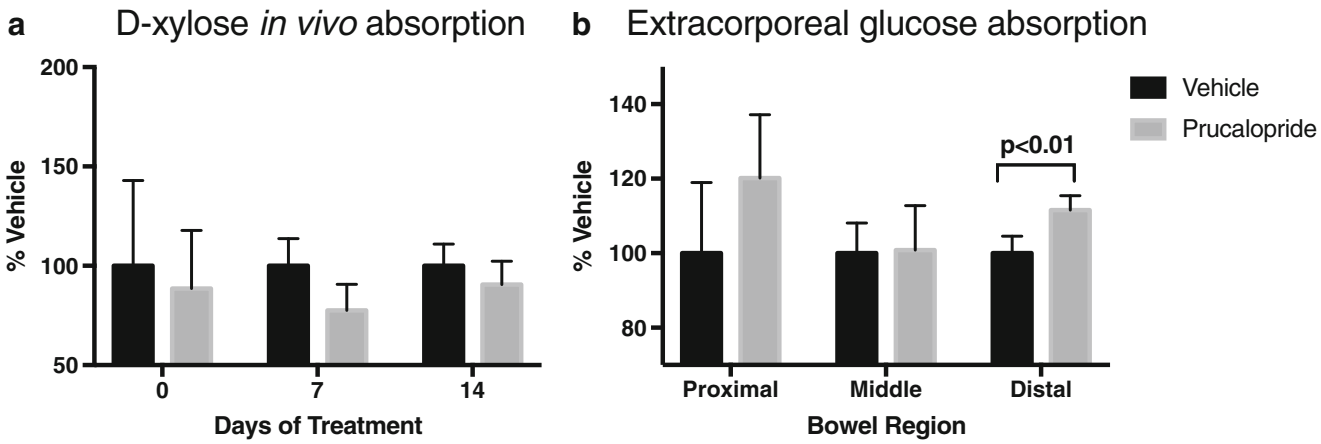

Fig. 4 Absorption studies comparing prucalopride-treated and control mice. **a** There was no difference in absorption when comparing in vivo D-xylose absorption between prucalopride-treated and control mice. **b** Prucalopride-treated mice had increased extracorporeal glucose absorption in the distal small intestine when compared to control mice

found in glucose absorption in the proximal and middle sections of the small intestine. In prucalopride-treated mice, mean values were as follows: proximal absorption 25.1 ± 3.4 μg/ml/min/cm; middle absorption 27.7 ± 2.9 μg/ml/min/cm; distal absorption 32.2 ± 0.4 μg/ml/min/cm. In control mice, mean values were as follows: proximal absorption 25.1 ± 3.4 μg/ml/min/cm; middle absorption 27.4 ± 1.6 μg/ml/min/cm; distal absorption 28.2 ± 0.9 μg/ml/min/cm.

Discussion

In this study, the hypothesis that prucalopride, a 5-HT4 receptor agonist, would lead to increase in small intestinal mucosal proliferation and absorptive capacity was tested. Our results strengthen the evidence for 5-HT as a mucosal growth factor, particularly one that produces functional intestinal mucosa. Additionally, based on evidence that enterocytes express 5-HT4 receptors, we demonstrate that a direct mechanism of 5-HT, bypassing the cholinergic system, is plausible.

We found that mucosal morphologic parameters were increased in prucalopride-treated mice compared to controls; specifically, we observed increased VH in the distal small intestine and increased CD in all regions of the small intestine. When the parameters were applied to a mathematical model, the MSA of the distal small intestine of prucalopride-treated animals was significantly greater than control animals. These changes were associated with enhanced crypt cell proliferation suggesting that prucalopride stimulated new growth.

We then assessed the absorptive capacity of the small intestine in two ways. The in vivo approach involved oral gavage and serum analysis, thus avoiding any significant manipulation of the animal and alterations in physiology. When absorption of D-xylose was compared in this fashion, there was no differences in absorption seen between prucalopride-treated mice and control animals. The extracorporeal approach involved operative intervention under anesthesia and direct manipulation of the bowel in an effort to localize any potential changes in absorption that are only seen in a particular region of the intestine. With this approach, we found significantly greater glucose absorption in isolated segments of distal small intestine of prucalopride-treated mice compared to control animals, and this mirrored the location where an increase in villus height was noted.

The morphologic changes seen in the current study represent hallmark features of intestinal adaptation that are observed in the remnant small intestine after small bowel resection in animals and human subjects.[17] During the postresection adaptation period, structural changes include hyperplasia of enterocytes, angiogenesis within villi, bowel elongation, and bowel dilation.[17–19] Simultaneously, functional adaptations occur in the remnant bowel involving increased expression of transporter proteins and exchangers involved in nutrient, electrolyte and water absorption,[20–22] which notably, is not fully attributed to increase in enterocyte mass. Accelerated crypt cell differentiation and slower intestinal transit time also contribute to functional adaptations that increase absorptive capacity. Additionally, an accelerated maturation process of enterocytes has been suggested, whereby digestive enzymes and transporters are found to be expressed more rapidly after small bowel resection.[23,24]

However, it is important to distinguish between true intestinal adaptation and accelerated mucosal proliferation. Earlier work in our lab established a well-defined association between potentiated 5-HT signaling and accelerated mucosal proliferation.[8,9,25] In particular, taller villi, deeper crypts, and higher CPI were seen in both SERTKO animals and mice treated with SSRI's. We further determined that in the setting of 5-HT-mediated mucosal growth, the cellular composition of the villi is preserved, therefore likely producing functional mucosa.[9] Consistent with this logic, in follow-up experiments, unpublished work revealed enhanced absorption of both D-xylose and medium chain fatty acid (MCFA) analogs when administered as an oral gavage in SERTKO mice. While it cannot be concluded that our findings confer a stimulation of the natural adaptation process, the shared features accompanied by enhanced absorptive function imparts an adjunctive mechanism by which we can influence mucosal homeostasis.

It is not surprising that there was no difference in carbohydrate absorption with the in vivo approach. While significant, the anatomic and functional changes appear to be localized to the distal small intestine, which may not reach the threshold to be reflected in the whole animal. Of note, the findings of our present study are consistent with the notion that the adaptive capacity of the distal small intestine is greater than that of the jejunum as seen in both animal models[26,27] and humans.[28]

The exact molecular mechanism responsible for our findings is unclear. Aside from enterocytes, 5-HT4 receptors are also found on cholinergic neurons of the ENS and smooth muscles of the bowel wall,[29] and 5-HT4 agonists are known to facilitate acetylcholine release from myenteric neurons.[30–33] These receptors play an important role in colonic propulsion by producing a coordinated combination of relaxation of circular muscles and contraction of longitudinal muscles.[29] While 5-HT4 receptors in the small intestine have been implicated in the control of intestinal secretion,[34] the role and mechanism by which enterocyte 5-HT4 receptors influence the intestinal mucosa remains unresolved. It is possible that serotonergic neurons project into the mucosa and stimulate 5-HT4 receptors directly, thus avoiding the cholinergic system, which would provide a more selective therapeutic target. Further evidence is needed to delineate these pathways and lays the groundwork for future experiments.

Conclusion

Stimulation of the 5HT-4 receptor leads to distal small intestinal mucosal growth, increased crypt cell proliferation, and regional enhancement in carbohydrate absorption in the

mouse. It is plausible that the effects are mediated by 5-HT4 receptors on enterocytes. These findings may represent pharmacologically induced intestinal adaptation, or alternatively, an adjunctive pathway that augments the adaptation process.

Author Contribution Statement

All authors listed have made substantial contributions to the conception or design of the work, acquisition, analysis or interpretation of data, drafting the work or revising it critically for important intellectual content, final approval of the version to be published, and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Specifically, study design—all authors; data acquisition—CJP, SJA, and LZ; data analysis and interpretation—all authors; manuscript writing—CJP and RAC; manuscript editing—all authors.

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