Gene plasticity in colonic circular smooth muscle cells underlies motility dysfunction in a model of postinfective IBS

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Choudhury BK, Shi X-Z, Sarna SK. Gene plasticity in colonic circular smooth muscle cells underlies motility dysfunction in a model of postinfective IBS. Am J Physiol Gastrointest Liver Physiol 296: G632–G642, 2009. First published January 8, 2009; doi:10.1152/ajpgi.90673.2008.—The cellular mechanisms of motility dysfunction in postinfectious irritable bowel syndrome (PI-IBS) are not known. We used a rat model of neonatal inflammation to test the hypothesis that gene plasticity in colonic circular smooth muscle cells underlies motility dysfunction in PI-IBS. Mild/moderate or severe inflammation was induced in neonatal and adult rats. Experiments were performed in tissues obtained at 7 days (short term) and 6–8 wk (long term) after the induction of inflammation. Severe inflammation in neonatal rats induced persistent long-term smooth muscle hyperreactivity to acetylcholine (ACh), whereas in adult rat caused smooth muscle hyporeactivity that showed partial recovery in the long term. Mild/moderate inflammation had no effect in neonatal rats, but it induced smooth muscle hyporeactivity to ACh in adult rats, which recovered fully in the long term. Smooth muscle hyperreactivity to ACh resulted in accelerated colonic transit and increase in defecation rate, whereas hyporeactivity had opposite effects. Smooth muscle hyperreactivity to ACh was associated with increase in transcription rate of key cell-signaling proteins of the excitation-contraction coupling in colonic circular smooth muscle cells to enhance their contractility and accelerate colonic transit and defecation rate.

irritable bowel syndrome; enteric nervous system; gastrointestinal motility; neurotransmitters; gene expression

MOtILITY DYSFUNCTION RESULTING in diarrhea, constipation, or alternating diarrhea and constipation and visceral hypersensitivity are the defining symptoms of irritable bowel syndrome (IBS). Motility dysfunction in IBS is thought to be attributable to defects in smooth muscle and/or enteric neuronal regulatory systems. However, the cellular and molecular mechanisms of these defects in the absence of an organic disease, infection, or structural abnormality are not known.

Clinical observations indicate that a subset (about 10–25%) of the subjects exposed to enteric infections in an individual or a community setting go on to develop predominantly the symptoms of diarrhea-predominant IBS (IBS-D) (8, 15–17, 23, 28, 30). Why only less than a quarter of the subjects exposed to enteric infections develop the chronic symptoms of IBS has remained an enigma. However, two major risk factors that predispose the individuals to develop postinfectious IBS (PI-IBS) symptoms following an enteric infection have emerged. 1) The duration of enteritis lasting greater than 3 wk significantly increases the risk for developing PI-IBS over a duration lasting less than 1 wk. 2) The presence of comorbid psychiatric disorders or a lifetime history of anxiety and depression at the time of infection increases the risk of developing PI-IBS. The longer duration of enteritis reflects its severity of inflammation (15, 28). The psychosomatic disorders represent dysregulation/impairment of the central nervous system (16, 24).

We investigated whether a combination of the above two risk factors in an animal model would result in colonic motility dysfunction similar to that seen in patients with PI-IBS in the absence of a structural abnormality, inflammation, or organic disease. Stress resulting from psychological trauma or physical insult in early stages of life is known since the works of Sigmund Freud (14) to result in persistent neural disorders in adulthood. Therefore, early life stress has been used extensively to investigate major depressive disorders and posttraumatic stress disorders (20). More recently, neonatal maternal separation (2, 10, 37) and colonic irritation (1) have been used to model visceral hypersensitivity in response to colorectal distension. The immaturity of the stress response system in neonates is, therefore, a risk factor for the development of persistent adverse effects later in life. However, the molecular mechanisms of persistent cellular dysfunction long after the neonatal insult remain unknown. We also do not know whether neonatal insult has persistent adverse effects on other cell types, such as the gut smooth muscle cells. Therefore, we induced colonic inflammation in neonates and in adults to investigate whether the long-term persistent effects in the two groups differ from each other. The second risk factor of severity of inflammation was incorporated into the model by inducing mild/moderate and severe inflammations in neonates and in adults by using low and high doses of 2,4,6-trinitrobenzenesulfonic acid (TNBS).

Our hypothesis is that the coexistence of the above two clinical risk factors alters gene expression of key cell-signaling proteins of the excitation-contraction coupling in colonic circular smooth muscle cells. The altered gene expression results in colonic circular smooth muscle hyperreactivity to acetylcholine.

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line (ACh), faster colonic transit, and increase in defection rate of fecal pellets. The immature development of the stress and immune response systems at the time of inflammation predisposes the neonatal pups to motility dysfunction in adulthood, which mimics the clinical symptoms of patients with PI-IBS. Similar inflammatory insult in adults does not mimic the diarrhea-like symptoms of PI-IBS after the same duration of postinflammatory lapse. We also found that only a severe, but not mild/moderate, inflammatory insult alters the gene expression in the long term, which may explain why the PI-IBS develops predominantly in subjects with long durations of enteritis (15, 28).

MATERIALS AND METHODS

Animals. Sprague Dawley rats were housed in a controlled environment (22°C, 12-h light/dark cycle) and allowed food and water ad libitum. The Institutional Animal Care and Use Committee at the University of Texas Medical Branch approved all procedures performed on animals.

Induction of colonic inflammation in neonatal and adult rats. Six- to eight-week-old male adult rats were fasted for 24 h before the induction of inflammation. During this period, regular water was replaced with Colyte to cleanse the colon for consistent exposure of TNBS to the mucosal surface. Colonic inflammation in 10-day-old male neonatal pups and in 6–8 wk-old male adult Sprague-Dawley rats was induced by intraluminal administration of two doses of(TNBS, 50 mg/kg and 130 mg/kg. The adult animals were anesthetized mildly with 2% isoflurane. TNBS was dissolved in 10% ethanol (vol/vol) for neonates and in 40% ethanol (vol/vol) in adults. Two hundred microliters of TNBS solution was injected via a catheter advanced to 2 cm oral to the anal verge in neonatal pups, and 250 μl were injected via a catheter advanced to 8 cm in adult rats. Age-matched rats treated with vehicle only served as controls. Each group of rats was euthanized either 7 days (short term) or 6–8 wk (long term) after the induction of inflammation.

Tissue procurement, protein and total RNA extraction, and myeloperoxidase assay. Three- to four-centimeter-long segments of the distal colon (starting from ~1 cm oral to the pelvic flexure) were obtained, opened along the mesenteric border, cleaned, and pinned flat in a Petri dish with Sylgard base. The mucosal/submucosal layers were separated by microdissection. The muscularis propria were quick frozen in liquid nitrogen and broken into small particles with a chilled pestle for protein and RNA extractions. The tissue particles were homogenized on ice in phosphate-buffered saline supplemented with protease inhibitors for protein extraction. Total RNA was extracted from tissues by using the Qiagen RNeasy kit (Qiagen, Valencia, CA).

Western blotting. The proteins in the samples were resolved by a standard immunoblotting method. Equal quantities (25 μg) of total protein were loaded and run on premaid 8–16% Tris-glycine SDS-PAGE (Invitrogen). They were transferred to nitrocellulose membranes (Invitrogen) for incubation with primary and secondary antibodies. The following antibodies were used: for α1C subunit of Ca3.1, 2 channels (1:400; Alomone Labs, Jerusalem, Israel), G protein αq (Gαq, 1:1000; Calbiochem, Gibbstown, NJ), 20-kDa myosin light chain (MLC20, 1:2000, Sigma, St. Louis, MO), myosin light chain kinase (MLCK, 1:10,000; Covance, Berkeley, CA), secondary antibody IRDye 800-conjugated anti-mouse IgG (Rockland, Gilbertsville, PA), or Alexa Fluor 680 goat anti-rabbit IgG (Invitrogen). B-Actin (1:5,000, Sigma) was used as loading control. The detection was done by ODYSSEY Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Real-time PCR. Real-time PCR was performed at the real-time PCR core facility, Sealy Center for Cancer Cell Biology, UTMB. We used Applied Biosystems 7000 real-time PCR system (Foster City, CA). The assay IDs for Gαq (Gnaq), MLC20 (mlcb) and MLCK (mlck) are Rn00578978_m1, Rn00820921_g1, and Rn 00679008_m1, respectively. The probe and primer sequences used for rat Ca3.1 intermediate form (Ca3.1b2) are as follows: probe spanning exon 1b and exon 2, CACCAAGGTCCACTAT; forward primer, CCATGGTCATGAGATACGGAGT; reverse primer, GCCGCAATGGCATCATTAGTT. For relative quantization of gene transcription, real-time PCR was performed with 40 ng cDNA for the target genes and the endogenous control (18S rRNA). We used universal PCR master mix reagent kit (P/N 4304437). The cycling parameters for real-time PCR were as follows: uracil N-glycosylase activation at 50°C for 2 min, AmpliTaq activation at 95°C for 10 min, denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min (repeat times 40) on ABI7900. Duplicate Cq values were analyzed in Microsatin Excel using the comparative Ct (2^-ΔΔCt) method, as described by the manufacturer (Applied Biosystems). The amount of target (2^-ΔΔCt) was obtained by normalization to endogenous reference (18S rRNA).

Multiplex immunoassay of cytokines and chemokines. Rat colonic muscularis propria was homogenized in cold PBS supplemented with protease inhibitors for protein extraction. LINCO rat cytokine/chemokine multiplex immunoassay kit (LINCO, St. Charles, Missouri) was used to quantify cytokine/chemokine levels in the homogenates following the manufacturer’s protocols. The assay results were read and analyzed by a Bio-Rad BioPlex System powered by Luminex xMAP Technology (Bio-Rad Laboratories, Hercules, CA).

Measurement of vasoactive intestinal polypeptide content in muscularis propria. Vasoactive intestinal polypeptide (VIP) content in the muscularis propria was measured by a competitive enzyme immunoassay (EIA) kit (Peninsula Laboratories, San Carlos, CA). The tissue was homogenized in PBS with protease inhibitors cocktail (Sigma). The absorbance was read at 450 nm with an Emax precision microplate reader (Molecular Devices, Sunnyvale, CA).

Measurement of norepinephrine, corticosterone, and CRH in plasma. Blood samples were collected at the time of animal euthanasia. Plasma levels of norepinephrine and corticosterone were measured using respective radioimmunoassay kits from MP Biomedicals (Solon, OH). Corticotrophin-releasing hormone (CRH) plasma levels were measured with EIA kit (Peninsula Laboratories).

Muscle bath experiments. Freshly obtained 3–4 cm-long segments of the distal colon (starting from ~1 cm oral to the pelvic flexure) were obtained, opened along the mesenteric border, cleaned, and pinned flat in a Petri dish with Sylgard base in carbogenated Krebs solution (in mmol/L: 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1 Na2HPO4, 1.2 MgCl2, 11 d-glucose, and 25 NaHCO3). The mucosal/submucosal layers were separated and discarded by microdissection. Circular muscle strips (4 mm X 10 mm) were mounted in individual muscle baths (Radnoti Glass, Monrovia, CA) filled with 5 ml carbogenated Krebs solution at 37°C. The contractile activity was recorded with Grass isometric force transducers and amplifiers connected to Biopac data-acquisition system (Biopac Systems, Goleta, CA). The effects of inflammation were tested by obtaining concentration-response curves to ACh in the muscle bath. The bathing solution was replaced every 15 min and 5 min after the addition of each concentration of ACh. The strips were left to equilibrate for at least 15 min before addition of the next concentration of ACh. The contractile response of circular muscle strips was quantified as the increase in area under contractions during 4 min after addition of ACh to the bath, over the baseline area under contractions for 4 min before the addition of ACh.

Colonic transit. Colonic transit was measured by the geometric center method as described previously (34, 40). A Silastic catheter (1 mm inside diameter and 2.1 mm outside diameter) was implanted
under general anesthesia (2% isoflurane inhalation) into the proximal colon, with its tip resting ~2 cm distal to the cecum. The rats were allowed to recover from surgery for at least 5 days. A bolus of 1.5 ml of 1.5% methylcellulose (Fisher Scientific, Fair Lawn, NJ) containing 0.75 mg nonabsorbable phenol red was injected into the colon via the catheter, and the catheter was flushed with 0.5 ml saline. Ninety minutes later, the rats were euthanized by CO2 inhalation. The entire colon distal to the tip of the catheter was removed immediately and divided into six segments of equal length. The contents expelled from the anus during this period were collected and referred to as segment 7 for the measurement of center of gravity. Each segment, along with its contents, was placed in 25 ml of 0.1 N NaOH and homogenized. The homogenate was kept at room temperature for 1 h. One milliliter of the supernatant was mixed with 0.1 ml of 20% trichloroacetic acid solution to precipitate the protein. After centrifugation at 10,000 g for 5 min, 0.5 ml of 0.5 N NaOH was added to 0.5 ml supernatant. Phenol red was determined by measuring the absorption at 560 nm by use of a spectrophotometer (Beckman Instruments, Palo Alto, CA). Colonic transit was calculated as the geometric center of distribution of phenol red described as follows: Σ (optical density of phenol red per segment × segment number)/total optical density.

Infusion of VIP antagonist by an osmotic pump. Adult male rats were anesthetized with 2% isoflurane inhalation (E-Z. Anesthesia vaporizer, Palmer, PA). A 7-day micro-osmotic pump (Alzet, Cupertino, CA) filled with 90 µl of VIP and pituitary adenylate cyclase-activating peptide receptors (VPAC)1,2 receptor antagonist (p-chloro-4-[2-hydroxyethyl]piperazine-1-ethanesulfonic acid (TNBS). Experiments were performed 7 days (short term) or 6–8 wk (long term) after the induction of inflammation.

Enzymatic dispersion of rat colonic circular smooth muscle cells and measurement of cell contraction. Single smooth muscle cells were isolated by enzymatic digestion with collagenase, as described previously with minor modification (39). The colonic circular muscle sheet was cut into pieces (0.5 mm length), and the digestion was performed using 0.5 ml collagenase (type II, 175 U/ml; Sigma) for 40 min. The digested tissue was washed with enzyme-free fresh HEPES medium (Invitrogen), and the muscle cells were allowed to disperse spontaneously under gentle to-and-fro motion. Dispersed cells were relaxed at rest, and they responded with cell length shortening in the presence of ACh (10−3−10−5 M). To quantitate muscle cell contraction, an aliquot (0.45 ml) of cells was exposed to 50 µl of ACh or vehicle control for 40 s at 31°C and fixed with 1% acrolein. The lengths of 30 consecutive intact healthy cells were measured by scanning micrometry, as described previously (39).

NIH Image 1.61 was used to measure the cell length.

Statistical analysis. ANOVA with repeated measures was used for statistical analysis. All pairwise multiple comparisons were done by the Student-Newman-Keuls or Fisher method. A P value of <0.05 was considered statistically significant.

RESULTS

Short-term and long-term inflammatory responses induced by low (50 mg/kg) and high (130 mg/kg) doses of TNBS. One of our aims in this study was to investigate whether the severity of the inflammatory response relates to persistent motility dysfunction, such as that seen in patients with PI-IBS (28). Therefore, we used two doses (50 mg/kg and 130 mg/kg) of TNBS to induce colonic inflammation (Fig. 1). We also wished to investigate whether immature development of the immune and stress response systems in neonates is a prerequisite for the development of persistent motility dysfunction. Therefore, we induced inflammation in 10-day old neonatal pups and in 6–8-wk-old adult rats (Fig. 1).

Both doses of TNBS significantly raised the levels of MPO in the muscularis propria of the adult rats (Fig. 2C). The increase in MPO by the larger dose was significantly greater than that by the smaller dose. The 130-mg/kg TNBS dose also increased MPO in the muscularis propria of neonatal rats, but the increase was significantly less than that with the same dose in adult rats (Fig. 2A). The 50-mg/kg dose of TNBS in neonatal rats had no significant effect on MPO in the muscularis propria (Fig. 2A). There were no residual effects of the higher or the lower dose of TNBS on MPO, 6–8 wk after the induction of inflammation in adult or in neonatal rats (Figs. 2, B and D).

Visual observations indicated severe ulcerations (>5 mm in length), scarring, edema, and hyperemia in response to the higher dose of TNBS in adult rats in the short term. The smaller dose caused scattered small (<2 mm long) ulcerations and moderate hyperemia. The mucosal injuries in the neonates in the short term were limited predominantly to edema and occasional small ulcers. On the basis of these data, the short-term inflammatory response to 130-mg/kg TNBS was rated severe compared with that with the 50-mg/kg dose, which was rated mild to moderate.

Short- and long-term effects of severe and mild/moderate inflammation on the contractility of colonic circular smooth muscle strips in neonates and adult rats. The higher dose of TNBS in neonates induced colonic circular smooth muscle hyperreactivity to ACh in the short term (Fig. 3A). This hyperreactivity persisted in adulthood (Fig. 3B). The hyperreactivity to ACh was noted also in freshly dissociated single circular smooth muscle cells obtained from the colons of the adult rats (Fig. 4). By contrast, the smaller dose of TNBS in neonates had no significant short- or long-term effects on the contractile responses to ACh (Fig. 3, C and D). Age-matched sham-treated rats served as controls.

In contrast to the induction of circular smooth muscle hyperreactivity to ACh in neonates, severe inflammatory insult in adult rats induced colonic circular smooth muscle hyporeactivity to ACh in the short term (Fig. 5A). This hyporeactivity recovered partially in the long term (Fig. 5B). In contrast to the absence of any short-term effect of the smaller dose of TNBS...
on smooth muscle reactivity to ACh in neonates, this dose in adults induced smooth muscle hyporeactivity to ACh (Fig. 5C). This hyporeactivity to ACh recovered fully in the long term (Fig. 5D). Age-matched sham-treated rats served as controls.

Short-term and long-term effects of mild/moderate and severe inflammations on gene expression of key cell-signaling proteins for excitation-contraction coupling in colonic circular smooth muscle cells. Recent findings show that gene transcription of the cell signaling proteins for excitation-contraction coupling in smooth muscle cells is subject to regulation by inflammatory mediators (21, 39) in the muscularis propria and by the enteric neurotransmitters, such as VIP (38, 40). Therefore, we chose four key proteins of the excitation-contraction coupling, [the pore-forming α1C subunit of Cav1.2 (L-type) calcium channels, Goq, MLC20, and MLCK] to investigate whether severe and mild/moderate inflammatory insults in neonates and in adults differentially alter their transcription to explain motility dysfunction. The first two proteins are at the beginning and the last two at the end of the excitation-contraction coupling cascades (26, 35, 36).

Severe neonatal inflammatory insult significantly enhanced expression of the pore-forming α1C subunit of Cav1.2 (L-type) calcium channels in the muscularis propria in neonates and in adults differentially alter their transcription to explain motility dysfunction. The first two proteins are at the beginning and the last two at the end of the excitation-contraction coupling cascades (26, 35, 36).

Severe neonatal inflammatory insult significantly enhanced expression of the pore-forming α1C subunit of Cav1.2 (L-type) calcium channels in the muscularis propria in neonates in short-term (Fig. 6A). The increase in expression of the α1C subunit persisted in adulthood in these rats (Fig. 6B). The long-term effect of severe neonatal insult also was to enhance significantly the expressions of Goq and MLC20 proteins (Fig. 6B).

The mild/moderate inflammatory insult slightly, but significantly, enhanced the expression of Goq in the short term (Fig. 6A), but it had no significant short-term or long-term effects on the expression of any of the other cell-signaling proteins we investigated.

In contrast to the effects of inflammation in neonates, severe inflammation in adult rats significantly suppressed the expression of α1C, Goq, and MLC20 proteins in the short term; it had no significant effect on the expression of MLCK (Fig. 6C). The expression of all three proteins, which were suppressed in the short term, returned to normal levels in the long term (Fig. 6D). However, at this time the expression of MLCK was significantly less than that in age-matched sham-treated controls.

Real-time PCR showed that severe inflammatory insult in neonates also significantly increased the mRNA levels of α1C, MLC20, and Goq in the short term (Fig. 7). By contrast, the mRNA level of MLCK was not different from that in age-matched and sham-treated rats (Fig. 7). These changes in mRNA expressions mirror the changes in their respective proteins.

Inflammatory mediators in neonatal and adult inflammations. The direct exposure of the inflammatory mediators in the muscularis propria alters phenotypes of the cells, such as the smooth muscle cells and the enteric neurons (9, 39). Therefore, we examined whether differential generation of some of the prominent cytokines and chemokines in the muscularis propria in short-and long-term neonatal and adult inflammation account for the differential inflammatory responses and motility dysfunctions. Neither mild/moderate nor severe inflammatory insult significantly increased the levels of IL-1β, IL-8, RANTES, monocyte chemoattractant protein-1 (MCP-1) (Fig. 8),...
TNF-α, and eotaxin (data not shown) in the muscularis propria of the neonates in the short term. The levels of these inflammatory mediators in the long term were also not different from those in age-matched and sham-treated controls (Fig. 8).

In contrast to the above, severe inflammation in adult rats significantly increased the levels of IL-1β, IL-8, RANTES, and MCP-1 in the muscularis propria (Fig. 8), but it did not affect the levels of TNF-α and eotaxin (data not shown) in the short term. The mild/moderate inflammation increased the muscularis propria levels only of IL-1β and RANTES in the short term. However, in the long term with the induction of severe or mild/moderate inflammation, the levels of all inflammatory mediators in the muscularis propria were not different from those in age-matched and sham-treated rats (Fig. 8).

**Stress mediators in neonatal and adult inflammations.** Peripheral inflammation activates the hypothalamus-pituitary-adrenal gland axis that plays a critical role in the course of the inflammatory response (25, 33, 45). Therefore, we investigated the plasma levels of the three prominent peripheral stress mediators, CRH, corticosterone, and norepinephrine (7), in neonatal and adult inflammation. Mild/moderate inflammatory insult in neonates had no significant effect on the plasma norepinephrine levels in the short term or long term (data not shown). The severe inflammatory insult in neonates also had no significant effect on the plasma levels of norepinephrine in the short term (data not shown). However, in the long term, the plasma level of norepinephrine in these rats was significantly greater than that in age-matched control rats (Fig. 9A). By contrast, the mild/moderate or severe inflammations in adult rats had no short-term or long-term effects on the plasma levels of norepinephrine (data shown only for adult severe inflammation in Fig. 9A).

The plasma levels of corticosterone increased significantly by 94 ± 19% 7 days after the application of mild/moderate inflammatory insult in neonates. They returned to normal levels 6–8 wk later. The plasma corticosterone levels were not affected by severe inflammatory insult in the neonates or in adults (data not shown). The plasma CRH levels also were not affected by any of the insults in the neonates and in adults (data not shown).

**Differential effects of severe neonatal and adult inflammation on the expression of VIP in the muscularis propria.** We reported recently that the enteric neurotransmitter VIP regulates gene expression of Ca,1.2 (L-type) calcium channels
Therefore, we investigated the short- and long-term effects of severe neonatal and adult inflammation on VIP level in the muscularis propria. We found that severe neonatal inflammation has no short-term effect on VIP in the muscularis propria (data not shown). However, in the long term it significantly enhances it when compared with age-matched and sham-treated controls \( (P < 0.05, \text{Fig. 9B}) \). On the other hand, severe inflammation in adult rats significantly suppresses the VIP level in the short term \( (P < 0.05, \text{Fig. 9B}) \). This suppression recovers completely in the long term (data not shown).

The long-term effects of severe neonatal and adult inflammation on colonic transit and defecation rate. We investigated whether persistent alterations in the expression of cell-signaling proteins of excitation-contraction coupling and circular smooth muscle reactivity to ACh affect colonic transit and defecation rate of pellets. Severe inflammatory insult in the neonatal stage of development significantly enhanced the geometric center of a dye bolus injected into the proximal colon in the long term compared with sham-treated age-matched controls (Fig. 10A). The defecation rate in these rats was also greater than that in control rats (Fig. 10B). By contrast, severe inflammatory insult in adult rats significantly decreased the geometric center and the defecation rate in the long term when compared with age-matched and sham-treated controls (Fig. 10, C and D).

We then investigated whether a continuous 7-day systemic infusion (20 nmol/day) of VPAC1/2 receptor antagonist, \((p\text{-Chloro-D-Phe6, Leu17})\text{-VIP}\), by a surgically implanted osmotic pump would normalize motility dysfunction.
in adult rats that had developed faster transit attributable to severe inflammatory insult in the neonatal stage of development. We found that 7 days of infusion of VIP receptor antagonist significantly reduced the rate of transit in the colon in these rats (Fig. 10A).

DISCUSSION

Our findings show that severe inflammatory insult in stress and immune response compromised state in neonatal rats causes persistent motility dysfunction of accelerated colonic transit and increase in defecation rate in adulthood. There is no residual inflammatory response or structural abnormality associated with motility dysfunction at this time. The accelerated colonic transit results from colonic circular smooth muscle hyperreactivity to ACh, the physiological neurotransmitter of spontaneous contractions (35). The patients with PI-IBS and IBS-D also present with accelerated colonic transit and smooth muscle hyperreactivity (4–6, 11, 32, 47).

We found that severe inflammation in neonates stably enhances expression of the pore-forming α1C subunit of Ca\(_{\text{v}}\)1.2 channels in adulthood in colonic circular smooth muscle cells. Shi and Sarna et al. (38, 40) reported that an increase in the expression of these channels in colonic circular smooth muscle cells enhances influx of Ca\(_{\text{2+}}\) in response to ACh, which causes smooth muscle hyperreactivity, faster colonic transit, and increase in spontaneous defecation rate.

In addition, severe neonatal inflammation enhanced expressions of at least two other key cell-signaling proteins of the excitation-contraction coupling, G\(_{\text{o}}\)q and MLC20, in adulthood. Both proteins contribute to the amplitude of cell contraction in response to ACh (26). The activation of G\(_{\text{o}}\)q by the binding of ACh to M3 muscarinic receptors stimulates multiple cell-
signaling pathways that enhance Ca\(^{2+}\) sensitivity by inhibiting the activity of MLC phosphatase (26, 35, 41). An increase in the expression of MLC\(_{20}\) may enhance the availability of phosphorylated MLC\(_{20}\), which regulates cross-bridge cycling and, hence, the amplitude of smooth muscle contraction (27). The expression of MLCK was unaffected. Together, these data suggest that the enhanced expression of genes encoding specific cell signaling proteins of the excitation-contraction coupling is one of the underlying molecular mechanisms of colonic motility dysfunction of faster colonic transit and increase in defecation rate in the absence of a structural or organic abnormality.

Retrospective clinical analysis has shown that the subset of human subjects who develop PI-IBS had a longer duration of enteric infection (3, 24, 28). The longer duration of enteric infection suggests that the inflammatory response in

Fig. 8. Short-term and long-term effects of two doses of TNBS on the expression of inflammatory mediators in the muscularis propria of neonatal and adult rats. Short term and long term refer to 7 days and 6–8 wk after induction of inflammation, respectively. *P < 0.05 compared with age-matched sham-treated controls. MCP-1, monocyte chemoattractant protein-1.

Fig. 9. A: long-term effects of severe inflammation on the plasma levels of norepinephrine (NE) in neonatal and adult rats. B: severe inflammation in neonatal rats enhanced the muscularis propria (MP) level of vasoactive intestinal polypeptide (VIP) in the long term. By contrast, severe inflammation in adult rats suppressed the muscularis propria level of VIP in the short term. *P < 0.05 compared with age-matched sham-treated controls.
The affected subjects was more severe than in those who did not develop PI-PBS. Our findings show that the severity of inflammation is a key factor in determining whether an enteric infection would permanently alter gene transcription of cell-signaling proteins of the contraction-excitation coupling. In our experiments, mild/moderate neonatal inflammation did not induce long-term alterations in gene transcription of the cell-signaling proteins. The effects of inflammation in adult rats with mature stress and immune response systems are opposite to those in neonates. Inflammation in adult rats induces hyporeactivity to ACh, which recovers with time. The underlying cellular mechanisms for smooth muscle hyporeactivity in adult inflammation were also the opposite of those in neonates. Inflammation in adults suppressed the expression of key cell-signaling proteins of the excitation-contraction in the short term, but it recovered with time. The transit rate and the defecation rate of pellets 6 wk postinflammation in adult rats was slower than that in age-matched and sham-treated controls. Most patients with PI-IBS, however, are diarrhea predominant (13). The long-term residual effects of inflammation induced in adult rats, therefore, may not reflect the changes in motility function or their cellular mechanisms in PI-IBS.

Our findings show that the immune responses to similar inflammatory insults are dramatically different between the neonates and the adults. The classic elevation of inflammatory mediators in the muscularis propria in response to severe or mild/moderate inflammatory insult was absent in the neonates. The attenuated or total absence of the immune response is a risk factor to developing permanent long-term alterations in gene transcription (42).

The neonatal model of severe inflammation has other similarities with PI-IBS. The tissue levels of VIP significantly increased in the muscularis propria in long-term neonatal inflammation. The muscularis propria levels of VIP have not been measured in patients with IBS, but the plasma levels of this neurotransmitter/hormone are elevated in these patients (29). The plasma levels of norepinephrine, a prominent mediator of the stress response (7), increased in adult rats that had received severe inflammatory insult as neonates. The plasma and urine levels of norepinephrine are increased also in patients with IBS (18, 22, 31). On the other hand, the plasma levels of norepinephrine did not change at 7 days or 6–8 wk after the induction of severe or mild/moderate inflammation in adult rats, indicating maturity of the stress-response system. The muscularis propria level of VIP decreased at 7 days after severe inflammation in adults, but it returned to normal levels 6 wk later.

Six to eight weeks after the induction of neonatal or adult inflammation there was no evidence of an inflammatory response (increase in MPO or elevation of inflammatory mediators) in the muscularis propria. Some studies have found an increase in the presence of T lymphocytes in the rectal mucosal biopsies of patients with PI-IBS (43). An increase in T lymphocytes has been noted also in mucosal biopsies of non-PI-IBS patients (13). However, no correlation has been established between the increase in T lymphocytes in the mucosal biopsies and motility dysfunction in IBS. In part, this may be
because there is no known mechanism by which mucosal inflammatory cells could directly affect the smooth muscle cells and enteric neurons that regulate motility function. For this reason, we chose to monitor the levels of MPO and inflammatory mediators in the muscularis propria. Therefore, the persistent alterations in long-term motility dysfunctions were not due to residual inflammatory response. Instead, they were due to persistent alterations in gene transcription of specific cell-signaling proteins in circular smooth muscle cells.

VIP is an important regulator of gene expression of the α1C subunit of Ca,1.2 calcium channels (38, 40). In addition, exogenous administration of VIP enhances expression of the α1C subunit in the colonic circular smooth muscle cells, the contractile response to ACh, colonic transit, and rate of defecation (40). The persistent increase in the expression of VIP in the muscularis propria of neonates receiving severe inflammatory insult may underlie the increase in the expression of the α1C subunit resulting in hyperreactivity to ACh, faster transit, and increase in defecation rate. The blockade of VPAC1/2 receptors by the systemic administration of their antagonist retarded colonic transit.

Our findings agree with the epidemiological and clinical observations that adverse stressful life experiences, such as sexual abuse and neglect, in early stages of life predispose adult humans to developing IBS after an inflammatory episode (12, 19, 44, 46). These experiences in early life may partly impair the stress-response system, and some components of this impairment may resemble those during the immature development in neonates. Our findings demonstrate that a severe enteric infection during impairment of the stress response system increases the probability of developing PI-IBS.

We conclude that severe inflammation in stress and immune response-compromised state permanently enhance the transcription of specific genes encoding key cell-signaling proteins of excitation-contraction coupling in colonic circular smooth muscle cells. The enhanced gene transcription increases smooth muscle reactivity to ACh, accelerates colonic transit, and defecation rate. Mild/moderate inflammation does not alter the smooth muscle reactivity to ACh under these conditions. The enhanced expression of VIP in the muscularis propria is one of the factors that alter the gene transcription of the cell-signaling proteins to cause motility dysfunction. Gene plasticity in smooth muscle cells in response to environmental factors may be an underlying cause of motility dysfunction in functional bowel disorders.

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