Gut Microbial Products Regulate Murine Gastrointestinal Motility via Toll-Like Receptor 4 Signaling

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BACKGROUND & AIMS: Altered gastrointestinal motility is associated with significant morbidity and health care costs. Toll-like receptors (TLR) regulate intestinal homeostasis. We examined the roles of TLR4 signaling in survival of enteric neurons and gastrointestinal motility. **METHODS:** We assessed changes in intestinal motility by assessing stool frequency, bead expulsion, and isometric muscle recordings of colonic longitudinal muscle strips from mice that do not express TLR4 (*Tlr4*$^{Lps\text{-}d}$ or *TLR4*$^{-/-}$) or Myd88 (*Myd88*$^{-/-}$), in wild-type germ-free mice or wild-type mice depleted of the microbiota, and in mice with neural crest-specific deletion of Myd88 (*Wnt1Cre*$^{+/-}$/*Myd88*$^{fl/fl}$). We studied the effects of the TLR4 agonist lipopolysaccharide (LPS) on survival of cultured, immortalized fetal enteric neurons and enteric neuronal cells isolated from wild-type and *Tlr4*$^{Lps\text{-}d}$ mice at embryonic day 13.5. **RESULTS:** There was a significant delay in gastrointestinal motility and reduced numbers of nitrergic neurons in *TLR4*$^{Lps\text{-}d}$, *TLR4*$^{-/-}$, and *Myd88*$^{-/-}$ mice compared with wild-type mice. A similar phenotype was observed in germ-free mice, mice depleted of intestinal microbiota, and *Wnt1Cre*$^{+/-}$/*Myd88*$^{fl/fl}$ mice. Incubation of enteric neuronal cells with LPS led to activation of the transcription factor nuclear factor (NF)-κB and increased cell survival. **CONCLUSIONS: Interactions between enteric neurons and microbes increases neuron survival and gastrointestinal motility in mice. LPS activation of TLR4 and NF-κB appears to promote survival of enteric neurons. Factors that regulate TLR4 signaling in neurons might be developed to alter gastrointestinal motility.**

Keywords: Intestinal Transit; Microbiota; Apoptosis; Mouse Model.

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Toll-like receptors (TLRs) are pattern recognition receptors involved in innate immune responses and are highly conserved in species such as Drosophila and humans.[1] TLRs recognize molecules of microbial origin, called microbial-associated molecular patterns (MAMPs). Recently, TLRs have been shown to have a novel function in controlling intestinal epithelial homeostasis and protecting epithelial injury.[2] Inflammation and altered intestinal homeostasis underlie several diseases affecting the gastrointestinal tract including diabetes and inflammatory bowel disease. During a study performed to understand the role of TLRs in the pathophysiology of gastrointestinal motility disorders in diabetes, we noticed a distinct phenotype in the TLR4 mutant mice (*Tlr4*$^{Lps\text{-}d}$) with a significant reduction in stool pellet frequency compared with controls. This led us to study the role of TLR4 in regulating intestinal enteric neurons and motility. Gastrointestinal motility disorders are associated with significant morbidity and are a key reason for outpatient clinic visits.

TLRs are expressed in the cells of the immune system; however, recently several studies have demonstrated the presence of TLRs in neurons of the central nervous system,[3] and they have been implicated in neurodegeneration.[4] TLR 3, 4, and 7 expression has been demonstrated in the human enteric nervous system.[5] TLR4 detects lipopolysaccharide (LPS), a major outer membrane component of gram-negative bacteria, and can signal through the NF-κB pathways resulting in the production of proinflammatory cytokines.

Several studies have demonstrated the role of TLRs in mediating neuronal injury. Human neurons express TLR3 and have a role in the neurodegeneration that occurs with inflammation.[6,7] Cerebral cortical neurons express TLR2 and TLR4 and are activated in response to energy deprivation and ischemia.[8] Mouse hippocampal neurons are protected from ischemia-induced damage in *TLR4*$^{-/-}$ mice.[9] Neurons lacking functional TLR2 or TLR4 exhibit increased resistance to death by energy deprivation and stroke.[10] TLR4 expression has been identified on sensory neurons and involved in pain modulation.[11]

TLR4 expression has been demonstrated in the myenteric plexus of the murine jejunum. However, its function

Abbreviations used in this paper: ACh, Acetylcholinesterase; ChAT, choline acetyltransferase; EFS, electrical field stimulation; ENS, enteric nervous system; IM-FEN, immorto fetal enteric neurons; LAL, Limulus amebocyte lysate; LPS, lipopolysaccharide; MAMPs, microbial-associated molecular patterns; NADPH, Nicotinamide adenine dinucleotide phosphate; nNOS, neuronal nitric oxide synthase; TLR, Toll-like receptor; WT, wild type.

0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2012.06.034

in these neurons has not been examined.[12] Enteric neurons form a complex network of neurons within the gastrointestinal tract, populating the submucosa and muscularis externa. The enteric neurons release neurotransmitters such as acetylcholine, nitric oxide, adenosine aminotransferase, vasoactive intestinal peptide (VIP), and serotonin. The enteric nervous system can also be exposed to bacterial products during times of compromised barrier function. Intestinal bacterial products can translocate, circulate, and affect distant organs such as the bone marrow.[13] In Parkinson's disease, there is evidence for increased intestinal permeability with enhanced bacterial translocation into the intestinal mucosa and circulation.[14] In the colon, bacterial products have been shown to activate nociceptive dorsal root ganglion neurons.[15]

The role of TLR4 in the enteric nervous system and how it can modulate intestinal motility has not been studied. In rodent models of sepsis, damage to enteric neurons due to LPS has been proposed. Rat myenteric neurons exposed to high doses of LPS in culture undergo cell death.[16] We sought to examine the role of TLR4 signaling in enteric neuronal survival and in the modulation of gastrointestinal motility disturbances. To do this, we used both in vitro and in vivo models; *Tlr4*$^{Lps\text{-}d}$, *TLR4*$^{-/-}$, and *Myd88*$^{-/-}$ mice; germ-free mice; and mice with enteric neuronal-specific deletion of Myd88 that we generated. In these models, we assessed TLR4 signaling, enteric neuronal apoptosis, and associated changes in intestinal motility.

Materials and Methods

Animals

C3H/HeJ (spontaneous mutation in TLR4 gene, *Tlr4*$^{lps\text{-}d}$), *C3H/HeOuJ* mice (controls for TLR4 mutant mice), and *TLR4*$^{-/-}$ mice on BL/10 background were obtained from Jackson Laboratories (Bar Harbor, ME). *Myd88*$^{-/-}$ mice on BL/6 background and their controls were bred in the Emory animal facility. Swiss Webster germ-free mice and conventional mice were obtained from Taconic (Hudson, NY). Hemizygous for transgenic (Tg) (*Wnt1-cre*) male mice were bred with noncarrier (wild type) female mice to obtain *Wnt1-Cre* transgenic mice that express Cre recombinase. Female or male heterozygous for *Myd88*^{tm1Defr} were bred to obtain heterozygous or homozygous mice that possess *loxP* sites on either side of exon 3 of the targeted gene. These mutant mice were bred to mice that express Cre recombinase to obtain offspring with exon 3 deleted in the *cre*-expressing tissue (*Wnt1Cre*$^{+/-}$/*Myd88*$^{fl/fl}$). *Wnt1Cre*$^{-/-}$/*Myd88*$^{fl/fl}$ *or Wnt1Cre*$^{-/-}$/*Myd88*$^{fl/-}$ mice were used as controls. The original breeding pairs were obtained from Jackson Laboratories. All experimental mice used were 8- to 12-week-old male mice. All animal studies were approved by the Emory University Institutional Review Board.

Reagents

The following reagents were obtained: guanethidine sulfate (TCI America, Portland, OR); DNAse I (Worthington Biochemical, Lakewood, NJ); Limulus amebocyte lysate (LAL) kit (Lonza, Walkersville, MD); Histogene LCM staining kit and Picopure RNA isolation kit (Arcturus; Life Technologies Corporation, Carlsbad, CA); and Sensiscript RT kit, QIAamp DNA Stool Mini Kit, QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA), and Duoset ELISA kits (R&D Systems, Minneapolis, MN). All the other reagents were obtained from Sigma (St. Louis, MO).

Antibodies

The following antibodies were obtained: beta Tubulin 3 (TUJ1) (Covance, Princeton, NJ); protein gene product 9.5 (PGP9.5) (Abcam, Cambridge, MA); myeloid differentiation protein (MyD88) (ENZO Life Sciences, Farmingdale, NY); neuronal nitric oxide synthase (nNOS) and choline acetyltransferase (ChAT) (Millipore, Billerica, MA); cleaved caspase-3, NF-κB p65, and phospho-NF-κB p65 (Ser536) (93H1) (Cell Signaling Technology, Danvers, MA); and Alexa fluor secondary antibodies (Life Technologies Corporation).

Assessment of Colonic Emptying by Stool Frequency and Bead Expulsion Test

Stool output measurement was done as described previously.[17] Distal colonic transit and emptying were assessed by bead expulsion test as described previously.[18]

Intestinal Transit Assessment

Intestinal transit was determined by assessing the distribution of 70-kilodalton fluorescein isothiocyanate conjugated dextran in the intestines of mice as described previously.[19] Cecum was collected as the eleventh segment. Transit was analyzed using the intestinal geometric center of the distribution of dextran throughout the intestine or colon and was calculated.[20]

Isometric Colonic Muscle Strip Recording

Longitudinal muscle strips (with myenteric plexus) were obtained from the proximal colon of mice. Recording parameters for longitudinal muscle strips has been established in our laboratory. Colonic relaxation was recorded by electrical field stimulation (EFS) (24 V, 4 Hz, 0.03-ms pulse for duration of 20 seconds). Strips were precontracted with 5-hydroxytryptamine (10 μmol/L) for 30 seconds before EFS. Colonic contraction was measured using EFS (24 V, 10 Hz, 0.3-ms pulse for duration of 20 seconds). Contraction or relaxation was expressed as a percentage change from baseline muscle tone.[21]

Whole Mount Tissue Staining

Proximal colon was used for nicotinamide adenine dinucleotide phosphate diaphorase and acetylcholine staining and was performed and analyzed as described previously.[21] Proximal, middle, and distal colon diameter was assessed in wild-type (WT) and *Tlr4*$^{Lps\text{-}d}$ mice (n = 3 mice in each group). Longitudinal muscle strips (with myenteric plexus) from distal ileum were fixed in 4% paraformaldehyde; blocked for 1 hour in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 and 5% normal donkey serum (NDS); and incubated with Rb nNOS (1:200), Rb TUJ1 (1:750), or Gt ChAT (1:100) antibodies in PBS containing 1.5% NDS, 0.3% Triton X-100, and 0.01% sodium azide, /72 hours at room temperature. Secondary detection was performed by incubation with anti-Goat (Gt) immunoglobulin (Ig) G (1:500) or anti-Rabbit (Rb) IgG (1:200) conjugated to Alexa Fluor 594 antibody.

Immunohistochemistry

Paraffin sections of distal ileum from *Wnt1Cre*$^{+/-}$/*Myd88*$^{fl/fl}$, *Wnt1Cre*$^{-/-}$/*Myd88*$^{fl/fl}$, *Wnt1Cre*$^{-/-}$/*Myd88*$^{fl/-}$ mice were blocked in PBS containing 3% bovine serum albumin and 0.02% Triton X-100 and then incubated with MyD88 antibody (1:200)