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Stress-Induced Corticotropin-Releasing Hormone-Mediated NLRP6 Inflammasome Inhibition and Transmissible Enteritis in Mice

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Abstract

BACKGROUND & AIMS—Stress alters brain–gut interactions and could exacerbate intestinal disorders, including irritable bowel syndrome. Alterations in the intestinal microbiota have been associated with irritable bowel syndrome. Maintenance of healthy microbiota requires nucleotide-binding oligomerization domain protein-like receptors, pyrin- domain containing (NLRP)-6 inflammasomes. We investigated the involvement of NLRP6 in water-avoidance stress (WAS)-induced intestinal disorders in mice.

METHODS—B57BL6 mice were subjected to WAS for 1 hour each day for 10 days; body weights and intestinal inflammation and permeability were analyzed. We investigated signaling via the NLRP3 and NLRP6 inflammasomes, and the role of corticotropin-releasing hormone (CRH) in WAS-associated inflammation and NLRP6 inhibition. Mice that were not exposed to stress were co-housed with mice subjected to WAS to determine the effects of WAS-induced

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Supplementary Material

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Conflicts of interest

The authors disclose no conflicts.

dysbiosis, measured by sequencing bacterial 16S ribosomal RNA. We also assessed the effects of a peroxisome proliferator-activated receptor- agonist and probiotics.

RESULTS—WAS-induced small-bowel inflammation (enteritis) was associated with inhibition of NLRP6, but not NLRP3, and was prevented by a peroxisome proliferator-activated receptor-agonist, which induced epithelial expression of NLRP6. CRH was released during WAS and inhibited NLRP6 expression. WAS induced alterations in the gut microbiota of mice; co-housed nonstressed mice developed enteritis associated with increased CRH and decreased levels of NLRP6. Probiotic therapy reduced intestinal inflammation in mice with WAS-induced enteritis.

CONCLUSIONS—Exposure of mice to stress inhibits NLRP6 and alters the composition of the gut microbiota, leading to intestinal inflammation. These findings might explain the benefits of probiotics for patients with stress-associated gastrointestinal disorders.

Keywords

Treatment; GI Flora; Mouse Model; Inflammatory Response

Gastrointestinal responses to stress include alterations in motility, visceral hypersensitivity, and intestinal permeability.¹ Psychosocial stress alters brain– gut interactions and may exacerbate a wide range of disorders, including irritable bowel syndrome (IBS).¹ There is growing evidence that IBS patients harbor altered gut microbiota.^{2,3} In fact, probiotics and antibiotics have been shown to be beneficial in patients with IBS, suggesting that the gut microbiota may play an important role in the pathogenesis of IBS.^{4,5}

Elinav et al⁶ recently shed light on the maintenance of the healthy microbiota, reporting that the nucleotide-binding oligomerization domain protein-like receptors, pyrin-domain containing (NLRP)-6 inflammasome regulates intestinal microbial ecology. They observed that a deficiency of NLRP6 inflammasome in mouse colonic epithelial cells resulted in reduced interleukin (IL)-18 levels and altered fecal microbiota. NLRP6 inflammasome–deficient mice were characterized by an exacerbation of chemical colitis induced by exposure to dextran sulfate sodium. Thus, they concluded that NLRP6 is important in maintaining a homeostatic gut microbiota and NLRP6-deficient animals have an altered gut microbiota that predisposes them to intestinal inflammation. Normand et al⁷ and Chen et al⁸ reported that NLRP6 also plays a role in colonic carcinogenesis. Anand et al⁹ further showed that NLRP6 negatively regulates innate immunity and host defense against bacterial pathogens. It is unclear, however, whether inhibition of NLRP6 is involved in normal physiological responses.

We studied the involvement of NLRP6 in stress-related intestinal pathology using a murine water-avoidance stress (WAS) model. We found that WAS-induced smallbowel inflammation (enteritis) is associated with inhibition of NLRP6, but not NLRP3, and is prevented by a peroxisome proliferator–activated receptor (PPAR-) agonist, which induces epithelial NLRP6 expression. Corticotropin- releasing hormone (CRH), an important stress signal in the brain– gut axis,¹⁰ is released during WAS and inhibits NLRP6 expression. WAS induced an altered gut microbiota and co-housed nonstressed mice developed enteritis associated with increased CRH and decreased NLRP6 levels. The role of dysbiosis was supported further by improving WAS intestinal pathology with probiotic therapy. Our study proposes a mechanism of stress-induced dysbiosis that involves the CRH–NLRP6–microbiota axis to explain the benefits of probiotic therapy for stress-associated gastrointestinal disorders.

Materials and Methods

Animals

Female C57BL/6 mice (age, 5–6 wk) were purchased from Charles River Laboratories (Wilmington, MA) and housed in the animal maintenance facility at the University of Michigan under specific pathogen-free conditions. All animal experiments were reviewed and approved by the University Committee on Use and Care of Animals at the University of Michigan.

WAS

Mice were put on a small platform surrounded by shallow water (25°C) for 1 hour daily for 10 days.¹¹ Mice avoided the water by remaining on the platform during each of the daily 1-hour periods of water exposure. The number of stool pellets produced during WAS was recorded and the mice were analyzed at specified time points. The intestines of the mice were gently washed with phosphate-buffered saline (PBS) to remove the fecal contents, then processed for RNA or protein analysis, and stained with H&E for inflammation scoring.¹² Lamina propria (LP) CD11c+ cells were obtained after collagenase digestion and sorted by EasySep (Stemcell Technologies, Tukwila, WA). For the co-housing experiments, age- and sex-matched mice were co-housed at 1:1 ratios for the duration of the WAS exposure.

Isolation of LP CD11c+ Cells From Mouse Small Intestine

Mouse small intestine was washed in PBS and Peyer's patches were removed. The gut was opened longitudinally, cut into small pieces, then incubated in RPMI-1640 containing 2 mmol/L EDTA and 1 mmol/L dithiothreitol for 15 minutes to remove epithelial and intraepithelial cells. This was followed by incubation in RPMI-1640 containing 3.7 U Liberase TM Research Grade (Roche Diagnostics, Indianapolis, IN) and 1% DNase I for 15 minutes for digestion of the basement membrane to obtain lamina propria mononuclear cells (LPMCs). The LPMCs were passed through 100- and 40- μ m cell strainers. After centrifugation, 6×10^7 LPMCs on average were isolated from the small intestine of a normal mouse. Three percent of these cells were collected as CD11c+ cells using the EasySep Mouse CD11c Positive Selection Kit (Stemcell Technologies).

Intestinal Permeability Measurement

This intestinal permeability measurement is based on the permeability of the intestine to 4000 daltons of fluorescent dextran (Sigma-Aldrich, St. Louis, MO), as described previously.¹³ Briefly, 6-hour-fasted mice were treated with fluorescein isothiocyanate (FITC)-dextran by oral gavage (600 mg/kg body weight, 125 mg/mL). After 1 hour, 120 μ L of blood was collected from a vein at the tip of the tail. The blood was centrifuged at 4°C, 12,000 *g*, for 3 minutes. Plasma was diluted in an equal volume of PBS (pH 7.4). The FITC-dextran concentration was analyzed with a fluorescence spectrophotometer (HTS-7000 Plus-plate-reader; PerkinElmer, Wellesley, MA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Standard curves for calculating the FITC-dextran concentration in the samples were obtained by diluting FITC-dextran in nontreated plasma diluted with PBS (1:2 vol/vol).

The Role of CRH

To determine if endogenous CRH contributed to the colonic and intestinal stress responses, mice were injected with 50 μ g/kg CRH (Sigma-Aldrich) daily for 10 days.¹² Control mice were given an intraperitoneal injection of either PBS or 5 μ g -helical CRH_{9–41} (Sigma-Aldrich), a CRH antagonist, 30 minutes before WAS exposure.

Cell Culture

Murine (CT26) and human (HCT116) colonic epithelial cells were grown for 48 hours in 12-well plates at 1×10^6 cells per mL^{-1} per well. The cells then were incubated for 16 hours with CRH (10 $\mu\text{g}/\text{well}$), Biofermin (1×10^7 CFU/mL; Biofermin Pharmaceutical Co, Kobe, Japan), or rosiglitazone (2 $\mu\text{mol}/\text{L}$ per well).

Results

WAS-Induced Enteritis in Mice

WAS for 1 hour each day for 10 days induced acute small intestinal inflammation characterized by erythema along with increased histologic scores and CCL5 chemokine expression (Figure 1A and B), leukocytic infiltration (Supplementary Figure 1B), failure to thrive and increased stool pellet counts during stress (Figure 1C), increased serum levels of tumor necrosis factor- α (Figure 1D), increased intestinal permeability (Figure 1E), and increased mucosal expression of small intestine proinflammatory cytokines IL-17 and IL-6 (Figure 1F). In the colon, inflammation was insignificant (data not shown) and there was no appreciable increase in colonic tissue IL-17 and IL-6 expression (Figure 1F).

Immunoregulatory IL-10 expression was increased in the colon, perhaps explaining the lack of colonic inflammation. Interestingly, WAS inhibited IL-1 β messenger RNA expression in both the small intestine and colon (Figure 1F).

WAS-Induced NLRP6 Inhibition

Because IL-1 β generation requires inflammasome signaling¹⁴ we measured intestinal expression of NLRP3 and NLRP6. WAS for 1 hour inhibited NLRP6 expression and this inhibition persisted through day 10 (Figure 2A); NLRP3 down-regulation was insignificant. Inflammasome adaptor proteins, apoptosis-associated speck-like protein containing a caspase recruitment domain and caspase 1, and downstream effector proteins, IL-1 β and IL-18, similarly were inhibited (Figure 2B). These findings suggest that WAS-induced disruption of NLRP6 inflammasome signaling may lead to small intestinal pathology. To investigate further if NLRP6 is a critical target in the treatment of stress-induced intestinal pathology, we administered the PPAR- γ agonist rosiglitazone, which has been shown to up-regulate NLRP6 in vitro.¹⁵ We found that rosiglitazone increased mouse and human epithelial NLRP6 expression (Figure 2C). During WAS, rosiglitazone normalized NLRP6 expression and function in the small intestine (Figure 2D and Supplementary Figure 2B) and reversed WAS-induced intestinal pathology (Figure 2E and Supplementary Figure 2E). Because NLRP6 expression was found in the intestinal epithelium,⁶ we performed immunofluorescent confocal microscopy to assess the effect of WAS on epithelial NLRP6 expression. E-cadherin was used to mark the epithelial compartment. We measured significant inhibition of epithelial NLRP6 and IL-1 β in the small intestine but not in the colon after WAS (Figure 2F and Supplementary Figure 3A–C).

WAS-Induced Intestinal Pathology Is Mediated by CRH

To elucidate the mechanism of WAS-induced NLRP6 inhibition in mice, we examined the role of CRH, a known mediator of the effects of stress on the intestinal epithelium.^{12,16} The serum level of CRH during WAS increased over time and NLRP6 expression in the small intestine correlated inversely with serum levels of CRH (Figure 3A). To show that CRH is sufficient to inhibit intestinal NLRP6 expression, normal mice were given a daily intraperitoneal injection of CRH. Intestinal pathology was recapitulated; enteritis and decreased NLRP6 messenger RNA expression and protein level (Figure 3B–E). In fact, CRH neutralization during WAS improved intestinal pathology (Figure 3B–E and Supplementary Figure 2A). Direct treatment of mouse and human colonic epithelial cell

lines with CRH inhibited NLRP6 expression and function (Figure 3F), and treatment with a CRH antagonist reversed mucosal NLRP6 inhibition by CRH (Supplementary Figure 4). These findings indicate that CRH down-regulates NLRP6 expression in the small intestine and brings to light a previously unrecognized role of CRH.

WAS-Induced Gut Dysbiosis and Transmissible Enteritis

Elinav et al⁶ showed that the NLRP6 inflammasome regulates intestinal microbial ecology. Furthermore, disturbance of the microbiota in NLRP6-deficient mice is transmissible to co-housed NLRP6-sufficient mice.⁶ To determine if WAS induces gut dysbiosis, we performed cohousing experiments and examined if WAS-induced intestinal pathology is transmissible to co-housed mice. As shown in Figure 4A, co-housed mice developed enteritis and increased intestinal permeability. Evidence of disruption of the healthy microbiota (dysbiosis) in the WAS and co-housed mice was shown by rank abundance distributions using terminal restriction fragment length polymorphism analysis (Figure 4B). WAS-induced or cohoused dysbiosis was not the result of a loss of diversity (eg, similar richness, evenness, or α -diversity; Supplementary Figure 5A–C) but rather a change in community membership and structure (eg, low Jaccard's coefficient α -diversity; Supplementary Figure 5D), indicating that WAS or co-housed fecal microbiota are significantly different from nonstress fecal microbiota. Further molecular characterization revealed a 2-fold decrease in Bacteroidetes, a 2-fold increase in Firmicutes, and a 3-fold increase in Gammaproteobacteria after WAS (Supplementary Figure 5E and F). Further evidence supporting the role of dysbiosis in WAS enteritis was shown by broadspectrum antibiotic treatment, which ameliorated WAS-induced enteritis (data not shown).

Interestingly, serum CRH also increased in co-housed mice with the associated decrease in intestinal NLRP6. These changes, however, lagged behind those observed in WAS mice (Figure 4C), suggesting that the CRH increase in co-housed mice may come from the intestine, as previously reported.¹⁷ Indeed, we observed increased intestinal CRH expression in both WAS and co-housed mice (Figure 4D). These results suggest that WAS-induced dysbiosis is transmissible and induces inflammation in the small intestine. The subsequent increase in the intestinal CRH and down-regulation of NLRP6 may perpetuate intestinal inflammation by a failure to restore healthy microbiota. To investigate the role of dysbiosis in WAS enteritis, we examined if the administration of probiotics would prevent WAS enteritis. Pretreatment of mice with a commercial probiotic supplement containing 3 lactic acid bacteria—*Bifidobacterium bifidum*, *Lactobacillus acidophilus*, and *Streptococcus faecalis*—significantly reduced the severity of WAS-induced intestinal pathology and reversed the WAS-induced fecal microbiota alterations (Figure 4E and F and Supplementary Figure 2E). This effect was not observed when probiotics were administered after the initiation of WAS (Supplementary Figure 6). Taken together, our findings indicate that stress induces a cycle of events that begins with increased circulating CRH, and subsequent intestinal NLRP6 inhibition and gut microbiota alterations leading to intestinal inflammation.

454 Pyrosequencing of 16S Ribosomal RNA Revealed WAS-Induced Dysbiosis of the Small Intestinal Luminal Content

To further assess the alterations in the gut microbial community induced by WAS, we performed 454 pyrosequencing of amplicon libraries targeting the V3V5 regions of the 16S ribosomal RNA gene of small intestinal and colonic tissues and contents and compared microbial community structures at the family level between nonstress and WAS mice. There were minor differences in the relative abundance of a few phylotypes observed between nonstress and WAS mice for colonic mucosa, colonic content, and small intestinal mucosa (Figure 5A). The small intestinal content, however, was found to have a significant

difference in Lactobacillaceae, unclassified Clostridiales, Lachnospiraceae, Ruminococcaceae, Streptococcaceae, unclassified Bacteroidetes, unclassified Firmicutes, unclassified Lactobacillales, and Clostridiaceae (Figure 5A). We also calculated the pairwise distances between community structures of the small intestinal content using the β -diversity metric γ_C (Jaccard and the Yu and Clayton similarity coefficients) and found a significant difference between nonstress and WAS (nonmetric multidimensional scaling plots stress = 0.07 and $r^2 = 0.99$, and $P = .015$ per analysis of molecular variation) (Figure 5B). There is intersubject variability of mouse response to WAS as shown on nonmetric multidimensional scaling plots and family level histograms with greater alterations seen in WAS mice 3 and 5 (Figure 5B and C). This intersubject variability may be attributable to a variation in the development of habituation to WAS between individual mice. Factors that influenced this variation were age of mice and noisy WAS environment (data not shown). The total bacterial load in the small intestine and colon were increased by 1.5- and 1.2-fold, respectively (Figure 5D). These findings indicate that the small intestine is targeted during WAS and that the alteration in the composition of bacterial communities may play a role in the pathogenesis of WAS-associated intestinal pathologies.

Discussion

Our key observation was that 1 hour of WAS daily for 10 days produced significant small intestinal pathology in mice. Previous studies in rats have shown that WAS induces intestinal inflammation characterized by ultrastructural damage and inflammatory infiltration in the lamina propria.^{12,18} One laboratory conducting experiments in rats reported more pronounced inflammation in the small intestine than in the colon,¹⁸ similar to our observations in mice. In fact, Larsson et al¹⁹ reported that WAS had no effect on dextran sulfate sodium (DSS) colitis, further supporting the predominance of small intestinal pathology during stress.

Elinav et al⁶ showed that NLRP6 null mice did not show spontaneous inflammation but they had increased susceptibility to colonic inflammation after DSS administration. The discrepancy between our findings and those reported by Elinav et al⁶ may be attributed to global NLRP6 deficiency in their model whereas WAS predominantly inhibited NLRP6 expression in the small intestine. This may explain the lack of colonic phenotype in the WAS mice. As for the absence of small intestinal inflammation in NLRP6 null mice reported by Elinav et al,⁶ we believe a second hit is required to trigger intestinal inflammation because colonic inflammation was seen only after DSS administration. Thus, WAS-induced intestinal pathology is associated with the inhibition of NLRP6 and additional insults (eg, CRH-induced intestinal permeability²⁰) are required to induce inflammation development.

We also showed that WAS-induced CRH production inhibited intestinal NLRP6 expression and led to the development of intestinal pathology. Several studies have shown the proinflammatory effect of CRH on mouse intestine. CRH antagonists and CRH deficiency reduces *Clostridium difficile* toxin A-mediated ileal inflammation and trinitrobenzene sulfonic acid-induced experimental colitis.^{17,21,22} Interestingly, CRH deficiency exacerbates DSS colitis by down-regulating Toll-like receptor 4 expression.²³ We did not ascertain whether CRH acts in a corticosteroid-dependent manner; however, in vitro, the addition of CRH or corticosteroid to epithelial cells was sufficient to down-regulate NLRP6 expression (Supplementary Figure 4). Hong et al²⁴ showed that chronic WAS in rats is mediated by corticosterone. Therefore, we speculate that CRH may act both directly and indirectly in a corticosteroid-dependent manner. Overall, these observations indicate that CRH exerts proinflammatory pressure on intestinal homeostasis.

Data from our co-housing experiments suggest that WAS-associated fecal dysbiosis is transmissible to nonstress co-housed mice, although we cannot exclude the possible contribution of behavioral transferability. We measured an increase in the serum CRH level on day 10 with an associated decrease in NLRP6 expression in the nonstress co-housed mice. The source of the increased serum CRH level likely was intestinal²⁵ because an increase in the mucosal CRH level was measured in WAS and co-housed mice (Figure 4D). We speculate that the early increase in CRH during WAS down-regulates NLRP6 and its downstream effectors IL-18 and IL-1, leading to intestinal dysbiosis. This CRH surge also increases intestinal permeability, leading to an increase in bacterial translocation. The ensuing intestinal inflammation is dominated by an increase in CD11c+ myeloid cells. In co-housed mice, the transmission of fecal material is sufficient to induce intestinal injury, leading to a secondary increase in CRH and a decrease in NLRP6. Cessation of WAS for 4 days led to a resolution of enteritis, indicating the recovery of intestinal pathology with cessation of daily stress.

Our study also may shed light on the pathogenesis of IBS in a subset of patients because WAS mice show visceral hypersensitivity to colonic distension,¹⁹ which is a pathologic feature of IBS. Stress also has been described as a risk factor for IBS in human beings.²⁶ Although IBS is a diagnosis of exclusion in the absence of pathology, there is a subset of patients, especially those with postinfectious IBS, who have mild inflammation in the colon.²⁷ Children with IBS also have evidence of increased intestinal permeability and low-grade intestinal inflammation, with the latter relating to the degree to which pain interferes with activities.²⁸ We also found that WAS-induced microbial alterations (Supplementary Figure 5E and F) are consistent with microbiota signatures in fecal samples from adult and pediatric patients with IBS.^{2,3}

Our study further suggests that stress-induced CRH inhibition of NLRP6 inflammasome signaling may play a role in WAS-induced intestinal pathology by inducing fecal dysbiosis. Although a tissue-specific inducible NLRP6 transgenic model could address the protective role of NLRP6 further in stress-induced intestinal pathology, such an animal model is not currently available. The observation that the PPAR- agonist induced epithelial NLRP6 and reversed stress-induced NLRP6 down-regulation and intestinal inflammation provides indirect evidence of the protective role of NLRP6 in the intestine. Rosiglitazone also has been shown to reduce DSS colitis in mice²⁹ and to benefit patients with active distal ulcerative colitis,³⁰ but it may have other epithelial protective effects. We do not advocate the use of rosiglitazone to treat IBS given its association with increased cardiovascular risk.³¹

In summary, our findings offer evidence that stress alters the gut microbiota, resulting in intestinal pathology. Confirmation of a causal relationship between gut dysbiosis and IBS in human beings would further validate the clinical use of probiotics and antibiotics to treat IBS. Moreover, we believe our results implicate NLRP6 as an important target in the development of a novel therapy for stressed-induced intestinal disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

CRH	corticotropin-releasing hormone
DSS	dextran sulfate sodium
FITC	fluorescein isothiocyanate
IBS	irritable bowel syndrome
IL	interleukin
LP	lamina propria
LPMC	lamina propria mononuclear cell
NLRP	nucleotide-binding oligomerization domain protein-like receptors, pyrin domain-containing
PBS	phosphate-buffered saline
PPAR-	peroxisome proliferator-activated receptor
WAS	water-avoidance stress

References

1. Konturek PC, Brzozowski T, Konturek SJ. Stress and the gut: pathophysiology, clinical consequences, diagnostic approach and treatment options. *J Physiol Pharmacol*. 2011; 62:591–599. [PubMed: 22314561]
2. Saulnier DM, Riehle K, Mistretta TA, et al. Gastrointestinal microbiome signatures of pediatric patients with irritable bowel syndrome. *Gastroenterology*. 2011; 141:1782–1791. [PubMed: 21741921]
3. Rajilic-Stojanovic M, Biagi E, Heilig HG, et al. Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. *Gastroenterology*. 2011; 141:1792–1801. [PubMed: 21820992]
4. Pimentel M, Lembo A, Chey WD, et al. Rifaximin therapy for patients with irritable bowel syndrome without constipation. *N Engl J Med*. 2011; 364:22–32. [PubMed: 21208106]
5. O'Mahony L, McCarthy J, Kelly P, et al. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. *Gastroenterology*. 2005; 128:541–551. [PubMed: 15765388]
6. Elinav E, Strowig T, Kau AL, et al. NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. *Cell*. 2011; 145:745–757. [PubMed: 21565393]
7. Normand S, Delanoye-Crespin A, Bressenot A, et al. Nod-like receptor pyrin domain-containing protein 6 (NLRP6) controls epithelial self-renewal and colorectal carcinogenesis upon injury. *Proc Natl Acad Sci U S A*. 2011; 108:9601–9606. [PubMed: 21593405]
8. Chen GY, Liu M, Wang F, et al. A functional role for Nlrp6 in intestinal inflammation and tumorigenesis. *J Immunol*. 2011; 186:7187–7194. [PubMed: 21543645]
9. Anand PK, Malireddi RK, Lukens JR, et al. NLRP6 negatively regulates innate immunity and host defence against bacterial pathogens. *Nature*. 2012; 488:389–393. [PubMed: 22763455]
10. Elenkov IJ, Webster EL, Torpy DJ, et al. Stress, corticotropin-releasing hormone, glucocorticoids, and the immune/inflammatory response: acute and chronic effects. *Ann N Y Acad Sci*. 1999; 876:1–13. [PubMed: 10415589]

11. Cameron HL, Perdue MH. Stress impairs murine intestinal barrier function: improvement by glucagon-like peptide-2. *J Pharmacol Exp Ther.* 2005; 314:214–220. [PubMed: 15798004]
12. Saunders PR, Santos J, Hanssen NP, et al. Physical and psychological stress in rats enhances colonic epithelial permeability via peripheral CRH. *Dig Dis Sci.* 2002; 47:208–215. [PubMed: 11852879]
13. Wang Q, Fang CH, Hasselgren PO. Intestinal permeability is reduced and IL-10 levels are increased in septic IL-6 knockout mice. *Am J Physiol Regul Integr Comp Physiol.* 2001; 281:R1013–R1023.
14. van Lookeren Campagne M, Dixit VM. Immunology: in command of commensals. *Nature.* 2011; 474:42–43. [PubMed: 21637249]
15. Kempster SL, Belteki G, Forhead AJ, et al. Developmental control of the Nlrp6 inflammasome and a substrate, IL-18, in mammalian intestine. *Am J Physiol Gastrointest Liver Physiol.* 2011; 300:G253–G263. [PubMed: 21088234]
16. Soderholm JD, Yates DA, Gareau MG, et al. Neonatal maternal separation predisposes adult rats to colonic barrier dysfunction in response to mild stress. *Am J Physiol Gastrointest Liver Physiol.* 2002; 283:G1257–G1263. [PubMed: 12388189]
17. Gay J, Kokkotou E, O'Brien M, et al. Corticotropin-releasing hormone deficiency is associated with reduced local inflammation in a mouse model of experimental colitis. *Endocrinology.* 2008; 149:3403–3409. [PubMed: 18403481]
18. Soderholm JD, Yang PC, Ceponis P, et al. Chronic stress induces mast cell-dependent bacterial adherence and initiates mucosal inflammation in rat intestine. *Gastroenterology.* 2002; 123:1099–1108. [PubMed: 12360472]
19. Larsson MH, Miketa A, Martinez V. Lack of interaction between psychological stress and DSS-induced colitis affecting colonic sensitivity during colorectal distension in mice. *Stress.* 2009; 12:434–444. [PubMed: 19929445]
20. Santos J, Saunders PR, Hanssen NP, et al. Corticotropin-releasing hormone mimics stress-induced colonic epithelial pathophysiology in the rat. *Am J Physiol.* 1999; 277:G391–G399. [PubMed: 10444454]
21. Wlk M, Wang CC, Venihaki M, et al. Corticotropin-releasing hormone antagonists possess anti-inflammatory effects in the mouse ileum. *Gastroenterology.* 2002; 123:505–515. [PubMed: 12145804]
22. Anton PM, Gay J, Mykoniatis A, et al. Corticotropin-releasing hormone (CRH) requirement in *Clostridium difficile* toxin A-mediated intestinal inflammation. *Proc Natl Acad Sci U S A.* 2004; 101:8503–8508. [PubMed: 15159534]
23. Chaniotou Z, Giannogonas P, Theoharis S, et al. Corticotropin-releasing factor regulates TLR4 expression in the colon and protects mice from colitis. *Gastroenterology.* 2010; 139:2083–2092. [PubMed: 20732324]
24. Hong S, Zheng G, Wu X, Snider NT, et al. Corticosterone mediates reciprocal changes in CB1 and TRPV1 receptors in primary sensory neurons in the chronically stressed rat. *Gastroenterology.* 2011; 140:627–637. [PubMed: 21070780]
25. Kawahito Y, Sano H, Mukai S, et al. Corticotropin releasing hormone in colonic mucosa in patients with ulcerative colitis. *Gut.* 1995; 37:544–551. [PubMed: 7489943]
26. Drossman DA, Camilleri M, Mayer EA, et al. AGA technical review on irritable bowel syndrome. *Gastroenterology.* 2002; 123:2108–2131. [PubMed: 12454866]
27. Gwee KA, Collins SM, Read NW, et al. Increased rectal mucosal expression of interleukin 1 β in recently acquired post-infectious irritable bowel syndrome. *Gut.* 2003; 52:523–526. [PubMed: 12631663]
28. Shulman RJ, Eakin MN, Czyzewski DI, et al. Increased gastrointestinal permeability and gut inflammation in children with functional abdominal pain and irritable bowel syndrome. *J Pediatr.* 2008; 153:646–650. [PubMed: 18538790]
29. Adachi M, Kurotani R, Morimura K, et al. Peroxisome proliferator activated receptor γ in colonic epithelial cells protects against experimental inflammatory bowel disease. *Gut.* 2006; 55:1104–1113. [PubMed: 16547072]

30. Pedersen G, Brynskov J. Topical rosiglitazone treatment improves ulcerative colitis by restoring peroxisome proliferator-activated receptor-gamma activity. *Am J Gastroenterol.* 2010; 105:1595–1603. [PubMed: 20087330]
31. Friedland SN, Leong A, Filion KB, et al. The cardiovascular effects of peroxisome proliferator-activated receptor agonists. *Am J Med.* 2012; 125:126–133. [PubMed: 22269613]

Figure 1. WAS induced enteritis in mice. C57BL/6 mice (n = 5–10 mice per time point) subjected to WAS were analyzed on days 1, 3, 6, and 10. (A) Representative gross photographs and micrographs (H&E) and histologic scores of the small intestine from nonstress mice and day 10 WAS mice are shown. *Scale bar*, 100 μ m. (B) Relative CCL5 messenger RNA expression of mouse small intestine was measured using real-time polymerase chain reaction. (C) Daily body weight and stool pellet number during 1-hour WAS were recorded. (D) Serum tumor necrosis factor (TNF)- α level was measured by enzyme-linked immunosorbent assay. (E) Intestinal permeability was measured by the FITC-dextran concentration in sera after oral FITC-dextran administration 10 days after WAS. (F) Relative cytokine messenger RNA expression of mouse small intestine and colon was measured by real-time polymerase chain reaction normalized to glyceraldehyde-3-phosphate dehydrogenase messenger RNA expression. INF, interferon; TGF, transforming growth factor (* P < .05, ** P < .01, *** P < .001).

Figure 2.

WAS-induced NLRP6 inhibition. C57BL/6 mice ($n = 5$ –10 mice per time point) were subjected to WAS for 10 days. (A) Relative Nlrp3, Nlrp6, Asc, and caspase 1 messenger RNA expression in mouse small intestine was measured by real-time polymerase chain reaction. (B) NLRP6, IL-1 β , and IL-18 protein expression in intestinal tissue extracts of mice were assessed by Western blot analysis normalized to β -actin. (C) Relative Nlrp6 messenger RNA expression of vehicle-treated (control) and rosiglitazone (Rosi)-treated mouse CT26 and human HCT116 colonic epithelial cell lines and relative NLRP6 protein expression of control and rosiglitazone-treated human HCT116 cells. C57BL/6 mice ($n = 5$ per group) were subjected to WAS for 10 days with or without daily oral rosiglitazone (0.2

mg). (D) Relative Nlrp6 mRNA expression of mouse small intestine. (E) Histologic scores of mouse small intestine and intestinal permeability measured by FITC-dextran permeability assay (* $P < .05$, ** $P < .01$, *** $P < .001$). (F) NLRP6 epithelial expression (epithelium is colocalized with E-cadherin expression) in the mucosa of mouse small intestine in nonstress and WAS mice measured by immunofluorescent confocal microscopy (*left panels*: NLRP6 epithelial expression is shown in *white*; *right panels*: IL-1 epithelial expression is shown in *yellow*).

Figure 3.

WAS-induced intestinal pathology is mediated by CRH. C57BL/6 mice ($n = 5-10$ per time point) were subjected to WAS for 10 days. (A) Serum CRH level after WAS was measured at the indicated time points using the mouse CRH enzyme-linked immunosorbent assay kit (NovaTeinBio, Cambridge, MA) and correlation between relative NLRP6 protein expression and serum CRH level. Inset corresponds to the Pearson r correlation coefficient and the corresponding P value. C57BL/6 mice ($n = 5-10$ per time point) subjected to WAS for 10 days with or without daily intraperitoneal injection of neutralizing anti-CRH antibodies. Nonstress mice treated with intraperitoneal CRH served as additional controls. (B) Histologic scores of mouse small intestine are shown. (C) Relative Nlrp6 mRNA expression

in mouse small intestine. (D) Intestinal permeability measured by FITC-dextran permeability assay after 10 days of WAS with or without daily intraperitoneal neutralizing anti-CRH antibodies, or with CRH treatment alone ($n = 5$ per group). Nonstress mice served as controls. (E) NLRP6 protein expression in intestinal tissue extracts of mice was assessed by Western blot analysis normalized to α -actin. (F) Relative Nlrp6 messenger RNA expression of PBS- or CRH-treated mouse (CT26) and human (HCT116) colonic epithelial cell lines ($n = 3$) and relative NLRP6 protein expression of PBS- (control) or CRH-treated HCT116 cells ($n = 3$). All data presented are representative of at least 2 independent experiments (1-way analysis of variance and Tukey post hoc t test; $*P < .05$, $**P < .01$, $***P < .001$).

Figure 4.

WAS-induced gut dysbiosis and transmissible enteritis. C57BL/6 mice ($n = 5$ per group) were subjected to WAS for 10 days and co-housed with unstressed mice at a 1:1 ratio during the 10-day period. (A) Representative gross photographs of mouse intestine, micrographs (H&E stain) of small intestine, histologic scores of small intestine, and intestinal permeability measured by FITC-dextran permeability assay. (B) Terminal restriction fragment length polymorphisms of 16S ribosomal RNA were ranked according to the abundance of terminal restriction fragments (T-RFs). (C) Serum CRH of WAS and co-housed mice was measured by enzyme-linked immunosorbent assay and relative Nlrp6 messenger RNA expression was measured by real-time polymerase chain reaction. (D)

Relative small intestinal tissue CRH and Nlrp6 messenger RNA expression of WAS and co-housed mice measured by real-time polymerase chain reaction. (E) C57BL/6 mice (n = 5 per group) were subjected to WAS for 10 days with or without oral probiotics (Biofermin [Biof]). Histologic scores of mouse small intestine and intestinal permeability measured by FITC-dextran permeability assay (1-way analysis of variance and the Tukey post hoc *t* test). (F) Changes in the mouse stool microbial community by phylum classification using real-time polymerase chain reaction of bacterium-specific 16S ribosomal RNA (**P* < .05, ***P* < .01, ****P* < .001).

Figure 5.

454 pyrosequencing of 16S ribosomal RNA revealed WAS-induced dysbiosis of the small intestinal luminal content. 454 pyrosequencing of 16S ribosomal RNA was used to compare the microbiome of nonstress vs WAS mice ($n = 5$ per group). (A) The family level diversity of the microbiota associated with the mucosa and in the luminal content of the small intestine and the colon (mean $\pm$ standard error of the mean, $*P < .05$ per Mann-Whitney U test compared with nonstress group). (B) Nonmetric multidimensional scaling plots using a γ_C distance matrix. The *circles* represent distinct communities identified from the luminal contents of the small intestine. *Larger circles* are closer in the depth axis and *smaller circles* are farther away in the depth axis. *Left panel:* x- and y-axes with the z-axis showing depth;

right panel: x- and z-axes with the y-axis showing depth. (C) A histogram of the family level diversity of the microbiome in the luminal contents of the small intestine. (D) Relative abundance of the total bacterial count measured by total numbers of 16S relative to tumor necrosis factor- messenger RNA expression. The fold increase comparing WAS with nonstress mice is shown. * $P < .05$.