RESEARCH

Altered colonic sensory and barrier functions by CRF: roles of TLR4 and IL-1

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Abstract

Visceral allodynia and increased colonic permeability are considered to be crucial pathophysiology of irritable bowel syndrome (IBS). Corticotropin-releasing factor (CRF) and immune-mediated mechanisms have been proposed to contribute to these changes in IBS, but the precise roles have not been determined. We explored these issues in rats *in vivo*. The threshold of visceromotor response, i.e., abdominal muscle contractions induced by colonic balloon distention was electrophysiologically measured. Colonic permeability was estimated by quantifying the absorbed Evans blue in colonic tissue. Intraperitoneal injection of CRF increased the permeability, which was blocked by astressin, a non-selective CRF receptor antagonist, but astressin₂-B, a selective CRF receptor subtype 2 (CRF₂) antagonist did not modify it. Urocortin 2, a selective CRF₂ agonist inhibited the increased permeability by CRF. Eritoran, a toll-like receptor 4 (TLR4) antagonist or anakinra, an interleukin-1 receptor antagonist blocked the visceral allodynia and the increased gut permeability induced by CRF. Subcutaneous injection of lipopolysaccharide (immune stress) or repeated water avoidance stress (WAS, psychological stress), 1 h daily for 3 days induced visceral allodynia and increased gut permeability (animal IBS models), which were also blocked by astressin, eritoran or anakinra. In conclusion, stress-induced visceral allodynia and increased colonic permeability were mediated via peripheral CRF receptors. CRF induced these visceral changes via TLR4 and cytokine system, which were CRF₁ dependent, and activation of CRF₂ inhibited these CRF₁-triggered responses. CRF may modulate immune system to alter visceral changes, which are considered to be pivotal pathophysiology of IBS.

Key Words

- ▶ corticotropin-releasing factor
- ▶ cytokine
- ▶ gut permeability
- ▶ toll-like receptor 4
- ▶ visceral pain

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Introduction

Irritable bowel syndrome (IBS) is a functional gastrointestinal (GI) disorder characterized by the presence of chronic abdominal pain with altered bowel habits without any organic cause (Mearin *et al.* 2016). Stress alters colonic sensorimotor function and has a significant impact on the development and exacerbation

of IBS symptoms (Taché *et al.* 2009). Since exogenous administration of corticotropin-releasing factor (CRF) mimics these colonic functional changes, and CRF antagonist abolishes these responses to stress (Taché *et al.* 2009, Nozu & Okumura 2015), CRF is considered to be a key molecule in the pathophysiology of IBS.

The actions of CRF are mediated through the activation of two receptors, CRF receptor subtypes 1 (CRF₁) and 2 (CRF₂) (Perrin & Vale 1999, Hillhouse & Grammatopoulos 2006). Classically, increased colonic contractility and visceral hypersensitivity induced by CRF were considered to be exclusively mediated through the activation of CRF₁ (Taché *et al.* 2009). However, we previously demonstrated that these functional changes were CRF₁ dependent indeed, but activating CRF₂ suppressed these CRF₁-triggered responses, and the signaling balance of CRF₁ and CRF₂ may determine these colonic functional changes (Nozu *et al.* 2014). According to these results, we advocated the balance theory of peripheral CRF₁ and CRF₂ signaling (Nozu *et al.* 2014, Nozu & Okumura 2015).

There is ample evidence that compromised gut barrier function manifested by increased gut permeability is observed in the patients with IBS (Taché *et al.* 2009). Stress is also known to increase gut permeability, which is mediated via CRF (Santos *et al.* 1999, 2008, Overman *et al.* 2012, Yu *et al.* 2013). However, the precise role of CRF receptor subtypes on gut permeability has not been determined, and both CRF₁ and CRF₂ have been reported to increase gut permeability (Barreau *et al.* 2007, Gareau *et al.* 2007, Teitelbaum *et al.* 2008, Ayyadurai *et al.* 2017). Moreover, it is not known whether this change follows the balance theory of CRF signaling.

Impaired gut barrier induces bacterial translocation resulting in increased lipopolysaccharide (LPS) and proinflammatory cytokine, which is also thought to be an important aspect of IBS (Barbara *et al.* 2012, Dlugosz *et al.* 2015, Nozu *et al.* 2017b). Actually, plasma proinflammatory cytokine and serum LPS are increased in IBS (Dinan *et al.* 2006, Ortiz-Lucas *et al.* 2010, Scully *et al.* 2010, Dlugosz *et al.* 2015). Moreover, LPS-induced stimulation of cytokine release from peripheral blood mononuclear cells is enhanced in this disease, and higher symptoms severity such as urgency, diarrhea, etc., are associated with higher cytokine response induced by LPS (Liebrechts *et al.* 2007). Since LPS is a ligand of toll-like receptor 4 (TLR4), these results suggest that activating TLR4-cytokine signaling may contribute to the visceral functional changes in IBS.

We have recently demonstrated that injection of LPS- or repeated water avoidance stress (WAS)-induced visceral allodynia was interleukin (IL)-1 and IL-6-dependent response, and peripheral CRF signaling also mediated this change possibly by modulating the cytokine release in rats (Nozu *et al.* 2017b,c). Additionally, peripheral administration of LPS upregulates CRF ligands in colon, and LPS-induced cytokine response is mediated via

peripheral CRF receptor (Yuan *et al.* 2016). In this context, it may be considered that peripheral CRF alters visceral sensation and gut permeability via modulating TLR4 and cytokine signaling. However, there has been no study to ascertain this notion definitely, especially *in vivo*.

In this study, we explored the roles of peripheral CRF receptor subtypes and immune system such as TLR4 and cytokine on visceral sensation and gut permeability *in vivo* and tried to confirm the link between CRF and TLR4, cytokine signaling. In addition, the roles were also evaluated on these altered visceral changes induced by LPS (immune stress) or repeated WAS (psychological stress), which are considered to be experimental animal models of IBS (Larauche *et al.* 2012, Nozu *et al.* 2017a,b,c).

Materials and methods

Animals

Adult male Sprague–Dawley rats (Charles River Laboratories) weighing about 300g were used. The animals were housed groupwise (3–4 rats/cage) under controlled conditions of illumination (12-h light/darkness cycle starting at 07:00h) with temperature regulated at 23–25°C. Rats were allowed free access to standard food (Solid rat chow, Oriental Yeast, Tokyo, Japan) and tap water.

Chemicals

A rat/human CRF (Peptide Institute Inc., Asagi, Japan), human urocortin 2, a selective CRF₂ agonist (Bachem AG, Bubendorf, Switzerland), LPS obtained from *Escherichia coli* with the serotype 055:B5 (Sigma-Aldrich) and anakinra, an IL-1 receptor antagonist (Swedish Orphan Biovitrum, Stockholm, Sweden) were dissolved in normal saline. Astressin, a non-selective CRF receptor antagonist, astressin₂-B, a selective CRF₂ antagonist (Sigma-Aldrich) and cortagine, a selective CRF₁ agonist (PolyPeptide Laboratories, Torrance, CA, USA) were dissolved in double-distilled water. The doses of the chemicals were determined according to the previous reports (Santos *et al.* 1999, Nozu *et al.* 2017b,c). The volume of injection was 0.2 mL/rat. Additionally, eritoran tetrasodium, a TLR4 antagonist (a kind gift from Eisai Inc., Andover, MA, USA) was dissolved in PBS with the concentration of 3.5 mg/mL. LPS was subcutaneously injected. Other drugs were administered via intraperitoneal route.

Measuring colonic permeability

Colonic permeability measurement was performed as previously described (Nozu *et al.* 2017a). The rats anesthetized by administration of the mixture of medetomidine hydrochloride (Orion Pharma Ltd., Dhaka, Bangladesh, 0.15 mg/kg), midazolam (Sandoz, Tokyo, Japan, 2 mg/kg) and butorphanol tartrate (Meiji Seika Pharma, Tokyo, Japan, 2.5 mg/kg) intraperitoneally were placed in a supine position on a heating pad, and laparotomy was performed. The colon was ligated at the junction with the cecum, and a small hole was made at 1 cm from the ileocecal junction by a puncture using 18 G needle. Then, an open-tipped catheter (3-Fr, Atom, Tokyo, Japan) was inserted into the proximal colon through the hole and fixed by purse-string sutures. The colon was gently flushed with PBS (37°C) using the catheter until all stools were washed out. Normally, the required volume of PBS was less than 10 mL and the perfusion rate was 5 mL/min. Later, another ligation was added on the colon at approximately 4 cm from the proximal one, and 1 mL of 1.5% Evans blue in PBS was instilled into the colon through the catheter. The animals were killed after 15 min, and the colons were excised. Later, they were washed with PBS and 1 mL of 6 mM N-acetyl-cysteine and were opened and placed in 2 mL of N,N-dimethylformamide for 12 h. The permeability was calculated by measuring the Evans blue concentration in the supernatant using a spectrophotometer at 610 nm.

Measuring visceral sensation

Visceral sensation was assessed by abdominal muscle contractions induced by colonic distention (visceromotor response (VMR)) using electromyogram (EMG) in conscious rats, which was validated as quantitative measure of visceral nociception (Ness & Gebhart 1988).

Implantation of electrodes and placement of colonic distention balloon

Under brief ether anesthesia, a small skin incision was made in non-fasted rats and electrodes (Teflon coated stainless steel, 0.05 mm diameter, MT Giken, Tokyo, Japan) for EMG were inserted approximately 2 mm into left side external oblique musculature through the incision. They were fixed to musculature by cyanoacrylate instant adhesive together with the incised skin. The electrode leads were externalized directly through this closed incision without a subcutaneous tunnel and threaded through a urethane tube. Neither analgesics nor antibiotics were administered

after the surgery. Distension balloon (6-Fr disposable silicon balloon-urethral catheter, JU-SB0601, Terumo Corporation, Tokyo, Japan) was inserted intra-anally with the distal end positioned 2 cm proximal to the anus. The volume and length of maximally inflated balloon were 1.5 mL and 1.2 cm.

Colonic distention and measuring abdominal muscle contractions

After completing electrode implantation and balloon placement, the rats were placed in Bollmann cages and acclimated to the experimental condition for 30 min before testing. Later, the electrode leads were connected to an EMG amplifier, and EMG signals were amplified, filtered (3000 Hz), digitized by a PowerLab system (AD Instruments, Colorado Springs, CO, USA) and recorded using computer software (LabChart 7, AD Instruments). Colonic distension was performed according to a previous publication (Nozu *et al.* 2017a), namely, ascending method of limits phasic distension was applied in increments of 0.1 mL for 5 s by inflating the balloon by water using a syringe manually until significant abdominal muscle contractions, i.e., VMR were detected (Fig. 1A). The VMR threshold was defined as the distended balloon volume (mL) inducing VMR. The threshold was measured twice (2-min interval), and the threshold mean was calculated as the data of the animals. The percentage change threshold, i.e., the threshold value after treatment divided by the basal threshold value and multiplied by 100, was calculated.

Experimental protocols

The basal VMR threshold was measured, and then the electrodes and distention balloon were removed followed by administration of the vehicle or drug ('Injection', Fig. 1B). Ten minutes later, the vehicle or CRF was injected, and the rats were returned to their home cages. At 3.5 h later, the rats underwent surgery for electrode implantation and balloon placement again, and the second measurement of threshold was performed at 4 h after the injection followed by the measurement of colonic permeability (Fig. 1B). This protocol was decided according to the previous study demonstrating that intraperitoneal CRF (50 µg/kg) increased gut permeability with maximal response at 4 h after the injection in rats (Santos *et al.* 1999).

For evaluating the effect of LPS, the second measurement of threshold was performed at 3 h after the injection (Fig. 1C). We previously confirmed that

Figure 1

(A) The threshold of visceromotor response (VMR) was determined by the distended balloon volume (mL) inducing apparent sustained abdominal muscle contractions detected by electromyogram (EMG). The threshold was 0.4 mL in this animal. (B) Schematic representation of the experimental protocol. The basal VMR threshold was measured at 30 min after the surgery for implanting EMG electrodes and placing the balloon, then the vehicle or drug was injected (Injection). Ten min later, the vehicle or CRF was injected, and the second measurement of threshold was performed at 4 h after the injection followed by measuring colonic permeability. (C) The protocol evaluating the mechanism of LPS-induced visceral changes. The basal VMR threshold was measured, and vehicle or drug was injected (Injection). Ten min later, the vehicle or LPS was injected and the visceral changes were evaluated at 3 h after the injection. (D) The protocol regarding repeated water avoidance stress. The basal threshold was measured, and the rats were subjected to either water avoidance or sham stress for 1 h daily for three consecutive days. The measurement of second threshold and colonic permeability were performed at 24 h after undergoing the last stress session. The vehicle, astressin or urocortin 2 (Injection A) was injected 3 times at 10 min prior to each stress session. The vehicle, eritoran or anakinra (Injection B) was administered twice at 15 h and 30 min before the measurement of second threshold of VMR.

LPS (1 mg/kg) injected subcutaneously induced visceral allodynia at 3 h after the injection (Nozu *et al.* 2017b).

For repeated WAS (Fig. 1D), the basal threshold of VMR was measured, and 10 min later, either WAS or sham stress was applied for 1 h. This daily stress session was implemented for three consecutive days. The threshold was again measured at 24 h after undergoing the last stress session followed by the measurement of colonic permeability. This protocol was demonstrated successfully to induce visceral allodynia in rats (Nozu *et al.* 2017c). In this model, astressin or urocortin 2 (Injection A) was injected at 10 min prior to each stress session, i.e., injected 3 times. Anakinra or eritoran (Injection B) was administered at 30 min and 15 h before the second measurement of the threshold.

Stress procedure

Exposure to WAS was performed as previously described (Martínez *et al.* 1997). Rats were individually placed on a plastic platform (height, 8 cm; length, 6 cm; width, 6 cm) positioned in the middle of a plastic cage filled with water up to 7 cm of the platform height. Control animals were individually placed in the same plastic cage, which was not filled with water (sham stress).

Statistical analysis

Data are expressed as means $\pm$ standard error. Multiple comparisons were performed by two-way ANOVA followed by Tukey's honestly significant difference test. Comparisons between two groups were performed using Student's *t*-test. The SYSTAT 13 software (Systat Software, Chicago, IL, USA) was used for the study.

Ethical considerations

Approval by the Research and Development and Animal Care Committees at the Asahikawa Medical University (#15132, approved on April 1, 2015) was obtained for all studies.

Results

The roles of CRF receptor subtypes on colonic permeability

Intraperitoneal CRF (50 μ g/kg) significantly increased colonic permeability (Fig. 2A). Cortagine (50 μ g/kg), a selective CRF₁ agonist also increased (Fig. 2B), but urocortin 2

Figure 2

The effects of CRF receptor agonists and antagonists on colonic permeability. CRF (50 µg/kg) increased the permeability (A), and a selective CRF₁ agonist, cortagine (50 µg/kg) also increased it (B). Selective CRF₂ activation by urocortin 2 (50 µg/kg) did not alter the permeability (C). This change by CRF was abolished by stressin (200 µg/kg), a non-selective CRF receptor antagonist (D), while stressin₂-B (200 µg/kg), a selective CRF₂ antagonist did not modify it (E). Urocortin 2 attenuated this change by CRF (F). **P* < 0.05 vs vehicle, or vehicle + vehicle, #*P* < 0.05 vs vehicle + CRF by two-way analysis of variance followed by Tukey's honestly significant difference test. Each column represents the mean ± standard error. The number of rats examined is shown in parentheses.

(50 µg/kg), a selective CRF₂ agonist did not alter it (Fig. 2C). Astressin (200 µg/kg), a non-selective CRF receptor antagonist blocked the CRF-induced increased permeability (effect of CRF: *F* = 61.6, *P* < 0.05, effect of astressin: *F* = 120.6, *P* < 0.05, interaction between CRF and astressin: *F* = 121.9, *P* < 0.05; Fig. 2D), while astressin₂-B (200 µg/kg), a selective CRF₂ antagonist did not alter it (effect of CRF: *F* = 219.5, *P* < 0.05, effect of astressin₂-B: *F* = 2.7, *P* > 0.05, interaction between CRF and astressin₂-B: *F* = 1.58, *P* > 0.05; Fig. 2E). Urocortin 2 per se did not modify the basal permeability but inhibited the increased permeability by CRF (effect of CRF: *F* = 24.9, *P* < 0.05, effect of urocortin 2: *F* = 8.91, *P* < 0.05, interaction between CRF and urocortin 2: *F* = 8.44, *P* < 0.05; Fig. 2F). These results indicate that CRF increased colonic permeability, which was CRF₁ dependent and activating CRF₂ suppressed this response. We already have shown that visceral hypersensitivity by CRF followed similar rules to these changes of colonic permeability (Nozu *et al.* 2014).

The roles of TLR4 and cytokine signaling on CRF-induced visceral changes

CRF induced visceral allodynia, which was blocked by eritoran (10 mg/kg), a TLR4 antagonist (effect of CRF: *F* = 14.5, *P* < 0.05, effect of eritoran: *F* = 18.3, *P* < 0.05, interaction between CRF and eritoran: *F* = 17.8, *P* < 0.05; Fig. 3A). Moreover, the drug also reversed increased colonic permeability by CRF (effect of CRF: *F* = 16.6, *P* < 0.05, effect of eritoran: *F* = 29.4, *P* < 0.05, interaction between CRF and eritoran: *F* = 29.7, *P* < 0.05; Fig. 3B).

In addition, anakinra (20 mg/kg), an IL-1 receptor antagonist abolished these visceral changes induced by CRF (% change threshold, effect of CRF: *F* = 17.1, *P* < 0.05, effect of anakinra: *F* = 29.7, *P* < 0.05, interaction between CRF and anakinra: *F* = 25.5, *P* < 0.05; Fig. 3C, colonic permeability, effect of CRF: *F* = 9.85, *P* < 0.05, effect of anakinra: *F* = 12.2, *P* < 0.05, interaction between CRF and anakinra: *F* = 10.5, *P* < 0.05; Fig. 3D).

Figure 3

The roles of toll-like receptor 4 (TLR4) and cytokine on CRF-induced visceral allodynia and increased colonic permeability. Eritoran (10mg/kg) fully reversed CRF-induced visceral allodynia (A) and increased colonic permeability (B). In addition, anakinra (20mg/kg) also blocked these changes (C and D). * $P < 0.05$ vs vehicle + vehicle, # $P < 0.05$ vs vehicle + CRF by two-way analysis of variance followed by Tukey's honestly significant difference test. Each column represents the mean $\pm$ standard error. The number of rats examined is shown in parentheses.

The roles of CRF receptor subtypes on stress-induced increased colonic permeability

LPS (1mg/kg) increased gut permeability, and astressin (200μg/kg) inhibited this response without affecting the basal gut permeability (effect of LPS: $F = 12.9$, $P < 0.05$, effect of astressin: $F = 10.2$, $P < 0.05$, interaction between LPS and astressin: $F = 13.2$, $P < 0.05$; Fig. 4A). Additionally,

urocortin 2 (50μg/kg) abolished the LPS-induced response (effect of LPS: $F = 57.1$, $P < 0.05$, effect of urocortin 2: $F = 26.5$, $P < 0.05$, interaction between LPS and urocortin 2: $F = 27.9$, $P < 0.05$; Fig. 4B).

Similar results were also obtained in repeated WAS-induced increased permeability, i.e., astressin (50μg/kg) injected 10min before each stress session abolished the response (effect of WAS: $F = 25.3$, $P < 0.05$, effect of

Figure 4

Effect of astressin or urocortin 2 on increased colonic permeability induced by LPS (1mg/kg) injection or repeated water avoidance stress (WAS). Astressin (200μg/kg) or urocortin 2 (50μg/kg) blocked the response by LPS (A, B). Besides, the repeated injections of astressin (50μg/kg) or urocortin 2 (50μg/kg) before each stress session also abolished the increased permeability by repeated WAS (C, D). Sham; sham stress. * $P < 0.05$ vs. vehicle + vehicle or vehicle + sham, # $P < 0.05$ vs. vehicle + LPS or vehicle + WAS by two-way analysis of variance followed by Tukey's honestly significant difference test. Each column represents the mean $\pm$ standard error. The number of rats examined is shown in parentheses.

astressin: $F=17.2$, $P<0.05$, interaction between WAS and astressin: $F=21.8$, $P<0.05$; Fig. 4C), and urocortin 2 (50 µg/kg) also blocked it (effect of WAS: $F=6.18$, $P<0.05$, effect of urocortin 2: $F=8.29$, $P<0.05$, interaction between WAS and urocortin 2: $F=11.5$, $P<0.05$; Fig. 4D).

The roles of TLR4 and cytokine signaling on stress-induced visceral changes

LPS (1 mg/kg) reduced the threshold of VMR, and eritoran (10 mg/kg) reversed this response (effect of LPS: $F=7.64$, $P<0.05$, effect of eritoran: $F=15.9$, $P<0.05$, interaction between LPS and eritoran: $F=17.3$, $P<0.05$; Fig. 5A). The increased permeability by LPS was also blocked by eritoran (effect of LPS: $F=27.2$, $P<0.05$, effect of eritoran: $F=23.6$, $P<0.05$, interaction between LPS and eritoran: $F=24.1$, $P<0.05$; Fig. 5B). Similar effects of eritoran were also found in repeated WAS model. The antagonist fully reversed the reduced threshold (effect of WAS: $F=5.48$, $P<0.05$, effect of eritoran: $F=8.62$, $P<0.05$, interaction between WAS

and eritoran: $F=6.89$, $P<0.05$; Fig. 5C) and the increased permeability (effect of WAS: $F=7.43$, $P<0.05$, effect of eritoran: $F=11.0$, $P<0.05$, interaction between WAS and eritoran: $F=10.8$, $P<0.05$; Fig. 5D) by WAS.

Anakinra (20 mg/kg) inhibited the increased permeability both induced by LPS (effect of LPS: $F=26.9$, $P<0.05$, effect of anakinra: $F=6.05$, $P<0.05$, interaction between LPS and anakinra: $F=6.36$, $P<0.05$; Fig. 5E) and repeated WAS (effect of WAS: $F=20.5$, $P<0.05$, effect of anakinra: $F=11.1$, $P<0.05$, interaction between WAS and anakinra: $F=8.85$, $P<0.05$; Fig. 5F). We have already shown that visceral allodynia observed in these animal models was abolished by anakinra (Nozu *et al.* 2017b,c).

Discussion

Visceral hypersensitivity is considered to be the most important mechanism and a hallmark of IBS (Kanazawa *et al.* 2011), which may be mediated by CRF receptors

Figure 5

Roles of toll-like receptor 4 (TLR4) and cytokine signaling on stress-induced visceral changes. Eritoran (10 mg/kg) blocked the allodynia (A) and the increased permeability (B) by LPS. The drug also abolished water avoidance stress (WAS)-induced these visceral changes (C and D). Anakinra (20 mg/kg) attenuated the increased permeability induced by LPS (E) or repeated WAS (F). Sham; sham stress. * $P<0.05$ vs vehicle+vehicle or sham+vehicle, # $P<0.05$ vs vehicle+LPS or WAS+vehicle by two-way analysis of variance followed by Tukey's honestly significant difference test. Each column represents the mean $\pm$ standard error. The number of rats examined is shown in parentheses.

(Taché *et al.* 2004). As described before, classically visceral hypersensitivity was considered to be induced exclusively via CRF₁. However, recently, inhibitory effects of CRF₂ signaling on CRF₁-triggered colonic functional changes including visceral hypersensitivity have been shown. Colorectal distention-induced visceral hyperalgesia or intraperitoneal CRF-induced defecation was blocked by peripheral administration of urocortin 2 (Million *et al.* 2006, Gourcerol *et al.* 2011, Nozu *et al.* 2014). Then, we previously have shown that both CRF receptor subtypes were involved, and the signaling balance of CRF₁ and CRF₂ determined the changes of visceral sensation and colonic contractility, i.e., balance theory of peripheral CRF signaling (Nozu *et al.* 2014).

The activity balance of each CRF receptor subtype signaling during stress may depend on the released peptides such as CRF and CRF-related peptides, urocortins (urocortin 1, 2 and 3), and their relative affinity for each CRF receptor subtype (Vaughan *et al.* 1995, Lewis *et al.* 2001, Reyes *et al.* 2001). Additionally, expression profile of CRF receptor subtypes of GI tract may also determine the balance. CRF receptors were up or downregulated by stress, and the expression profile of CRF receptor subtypes was changed dynamically (O'malley *et al.* 2010, Yuan *et al.* 2010, 2016). Moreover, dominant expression of CRF receptor subtypes varies along the GI tract (Liu *et al.* 2010, Yuan *et al.* 2012).

The role of impaired gut permeability has been recently postulated in IBS pathophysiology (Taché *et al.* 2009). Several studies showed that CRF ligands increased gut permeability and endogenous CRF-mediated stress-induced impaired gut barrier function (Santos *et al.* 1999, 2008, Larauche *et al.* 2009, Overman *et al.* 2012, Yu *et al.* 2013). Most of these studies were performed *in vitro*, using colonic cell line (Yu *et al.* 2013, Yue *et al.* 2017) or gut segment by Ussing chamber (Santos *et al.* 1999, 2008, Overman *et al.* 2012). On the other hand, Larauche *et al.* showed that peripheral injection of selective CRF₁ agonist, cortagine increased colonic permeability *in vivo* in rats (Larauche *et al.* 2009), but the precise roles of CRF receptor subtypes have not been determined. Both CRF₁ and CRF₂ have been reported to increase gut permeability (Barreau *et al.* 2007, Gareau *et al.* 2007, Teitelbaum *et al.* 2008, Ayyadurai *et al.* 2017).

Current study clearly showed that exogenous peripheral CRF increased colonic permeability *in vivo*, which was CRF₁ dependent. Moreover, activating CRF₂ *per se* did not alter the permeability but suppressed this CRF₁-triggered change. Additionally, endogenous CRF also mediated this visceral change induced by LPS or

repeated WAS (animal IBS model), which was suppressed by the activation of CRF₂. In this context, increased colonic permeability by both exogenous and endogenous CRF may also follow the balance theory of CRF signaling. Since we previously have shown that the quite similar roles of CRF₁ and CRF₂ signaling on gastric contractility (Nozu *et al.* 2013), the balance theory might be a fundamental rule in the GI functional changes induced by peripheral CRF.

Whereas we demonstrated inconsistent result with the balance theory, i.e., astressin₂-B did not enhance the increased colonic permeability by CRF. The blocking CRF₂ would further enhance CRF₁ signaling activated by CRF and increase the permeability. There is the fact of the predominant expression of functional CRF₁ relative to CRF₂ in colonic myenteric neurons in guinea pig suggesting that CRF₁ is the dominant signaling in colon (Liu *et al.* 2010), which may lead to induce strong activation of CRF₁, and consequently, inhibition of CRF₂ signaling could not enhance it. Additionally, the dose of CRF (50 µg/kg) used in the study was known to induce maximal response on colonic permeability (Santos *et al.* 1999), and such strong activation of CRF₁ signaling may not permit further enhancement by blocking CRF₂.

The mechanisms of CRF₁ and CRF₂ interaction have not been determined. Gourcerol *et al.* (2011) showed that peripheral injection of CRF increased defecation and activated colonic myenteric neurons, and these responses were inhibited by activation of peripheral CRF₂. Furthermore, the authors also demonstrated that CRF-induced phosphorylation of extracellular signal-regulated kinase in primary cultures of the neurons and cyclic adenosine monophosphate production in human embryonic kidney-293 cells transfected with CRF receptors were CRF₁ dependent, and CRF₂ suppressed the changes. These results suggest that activation of CRF₂ inhibits the increased concentration of second messenger and the phosphorylation state of protein kinases leading to inhibiting activation of target cells, thereby suppressing the CRF₁-triggered colonic functional changes.

This system of peripheral CRF signaling may be suitable for the survival of organisms under stressful condition. Acute stress induces integrated responses to maintain homeostasis via CRF₁, which may be favorable for survival of organisms. However, if the stress response is led into an overdrive state, it can become fatal (Chrousos 2009). Therefore, existence of counter regulatory action by CRF₂ signaling could inhibit maladaptation to stress. Moreover, the balance theory suggests that CRF signaling might be shifted toward CRF₁ resulting in altered visceral

functions in IBS, and resetting abnormal CRF signaling balance by blocking CRF₁ or stimulating CRF₂, may become a promising therapeutic approach to the disease (Nozu & Okumura 2015).

As described before, TLR4 (LPS)-proinflammatory cytokine system is thought to be involved in the pathophysiology of some portion of IBS patients (Dinan *et al.* 2006, Ortiz-Lucas *et al.* 2010, Scully *et al.* 2010, Dlugosz *et al.* 2015). TLR4 in colonic tissue of IBS patients is elevated (Kocak *et al.* 2016), and TLR4 messenger RNA expression in colonic mucosa correlates significantly with duration of symptoms in the IBS patients (Belmonte *et al.* 2012). In animal studies, WAS significantly increased colonic TLR4 expression (Nebot-Vivinus *et al.* 2014). Additionally, He *et al.* showed that chronic stress-induced diarrhea with increased colonic expression of TLR4 and NF- κ B in rats, which was inhibited by TLR4/NF- κ B inhibitor (He *et al.* 2017). Since our current and previous studies showed that the visceral changes observed in our tested animal models were mediated via peripheral CRF, TLR4 and cytokine (Nozu *et al.* 2017b,c), the existence of the link between CRF and TLR4-cytokine system was expected.

In the current study, we clearly demonstrated for the first time that peripheral exogenous or endogenous CRF-induced visceral allodynia and increased colonic permeability, which were mediated via TLR4 and cytokine signaling *in vivo*. The mechanisms of these visceral changes induced by peripheral CRF have not been determined definitely so far. Since CRF receptors are proved to be expressed in dorsal root ganglia (Million *et al.* 2006), CRF may act directly to the receptors to alter visceral sensation. Additionally, enterochromaffin or mast cells have CRF receptors and release chemical mediators, such as serotonin, cytokines etc. (Wu *et al.* 2011, Overman *et al.* 2012), which may also contribute to the changes through activating visceral afferents (Mawe *et al.* 2006, Barbara *et al.* 2007) and altering tight junction proteins that regulate gut–epithelial barrier (Piche 2014). Our results indicated that CRF may modulate TLR4 and cytokine signaling to alter visceral function, which is a novel mechanism of visceral changes induced by peripheral CRF.

Several studies showed that CRF altered TLR4-cytokine signaling. CRF increases the expression of TLR4 on macrophage and enhances the cytokines production by LPS (Tsatsanis *et al.* 2006). Additionally, colonic TLR4 expression is reduced in CRF-deficient mice (Chaniotou *et al.* 2010). Incidentally, enterochromaffin and mast cells were reported to have functional TLR4 to secrete chemical mediators including cytokine (McCurdy *et al.* 2001, Kidd

et al. 2009). Since these cells having CRF receptors possibly contribute to visceral changes induced by CRF as described before, the link between CRF and TLR4-cytokine signaling might also occur in these cells. Further studies are needed to explore the precise mechanisms of link between these signaling on visceral changes.

Figure 6 depicted the schematic illustration of the speculated peripheral mechanisms of increased gut permeability and visceral hypersensitivity in IBS regarding peripheral CRF, TLR4 and proinflammatory cytokines. CRF is released from various cells such as neuronal, enterochromaffin and immune cells (mast cells, lymphocytes, etc.) in the colon (Nozu & Okumura 2015). CRF-CRF₁ signaling is thought to be a key factor in IBS (Taché *et al.* 2009), and CRF signaling balance is abnormally shifted toward CRF₁ according to the balance theory (Nozu & Okumura 2015). CRF activates TLR4 signaling, which triggers to produce proinflammatory

Figure 6

Schematic illustration of our hypothesis in terms of peripheral CRF, toll-like receptor 4 (TLR4) and proinflammatory cytokine on visceral hypersensitivity and increased gut permeability in IBS (peripheral mechanisms). CRF signaling are activated (Taché *et al.* 2009) and its signaling balance is abnormally shifted toward CRF₁ (Nozu & Okumura 2015). CRF activates TLR4 to trigger producing proinflammatory cytokines, which increases the permeability (Suzuki *et al.* 2011) and induces visceral hypersensitivity (Obreja *et al.* 2002). Incidentally, increased gut permeability activates immune system to release LPS. LPS not only stimulates TLR4 but also activates peripheral CRF signaling (Nozu *et al.* 2017b). In this context, CRF and TLR4-cytokine signaling are considered to form a vicious cycle to activate each other to induce these visceral changes in IBS.

cytokines, thereby increasing the colonic permeability via modifying tight junction proteins (Suzuki *et al.* 2011) and inducing visceral hypersensitivity by activating sensory neurons (Obreja *et al.* 2002).

Increased gut permeability induces bacterial translocation, resulting in activation of immune system leading to inflammation. In this process, LPS is released and proinflammatory cytokines are also produced through the activation of TLR4 by LPS (Dlugosz *et al.* 2015). Incidentally, LPS is also known to increase CRF messenger RNA in the rat colon (Yuan *et al.* 2010) and to activate peripheral CRF signaling (Nozu *et al.* 2017b), which further stimulates TLR4-cytokine system. In this context, peripheral CRF and activation of TLR4-cytokine system may form a vicious circle to activate each other.

The current study had several limitations. Our method required minor surgery, which is inevitable for assessing visceral sensation by EMG. However, it might have some influence on the immune system, which could modify the results. The cellular mechanisms of CRF were not evaluated. Since the targets of peripheral CRF have not been determined definitely so far, we have to explore this issue in the first place. CRF or CRF antagonists used in this study have poor penetrance into brain (Taché & Brunnhuber 2008), but LPS is known to increase the permeability of blood–brain barrier (Ghosh *et al.* 2014). Thus, the possibility that peripheral administration of CRF agonists or antagonists act to brain inducing the visceral changes was not completely denied. Further studies were needed to evaluate these issues.

In summary, stress-induced visceral allodynia and increased colonic permeability were mediated via peripheral CRF pathway. CRF induced these visceral changes possibly via TLR4-cytokine system, which were CRF₁ dependent, and activation of CRF₂ inhibited these CRF₁-triggered responses. CRF may modulate immune system to alter visceral changes, which are considered to be pivotal pathophysiology of IBS.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

T N designed and performed the experiment, analyzed the data and wrote the paper; S M and R N performed the experiment; K T contributed to establishing the experimental system monitoring visceral sensation; T O designed the experiment, analyzed the data and was involved in critical revision of the manuscript.

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