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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 astressin$_2$-B, a selective CRF receptor subtype 2 (CRF_2) antagonist did not modify it. Urocortin 2, a selective CRF_2 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 CRF_1 dependent, and activation of CRF_2 inhibited these CRF_1-triggered

responses. CRF may modulate immune system to alter visceral changes, which are considered to be pivotal pathophysiology of IBS.

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 (Nozu and Okumura 2015; Taché et al. 2009), 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 (CRF_1) and 2 (CRF_2) (Hillhouse and Grammatopoulos 2006; Perrin and Vale 1999). Classically, increased colonic contractility and visceral hypersensitivity induced by CRF were considered to be exclusively mediated through the activation of CRF_1 (Taché et al. 2009). However, we previously demonstrated that these functional changes were CRF_1 dependent indeed, but activating CRF_2 suppressed these CRF_1-triggered responses, and the signaling balance of CRF_1 and CRF_2 may determine these colonic functional changes (Nozu, et al. 2014). According to these results, we advocated the balance theory of peripheral CRF_1 and CRF_2 signaling (Nozu and Okumura 2015; Nozu et al. 2014).

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

(Overman, et al. 2012; Santos, et al. 1999; Santos, et al. 2008; Yu, et al. 2013). However, the precise role of CRF receptor subtypes on gut permeability has not been determined, and both CRF_1 and CRF_2 have been reported to increase gut permeability (Ayyadurai, et al. 2017; Barreau, et al. 2007; Gareau, et al. 2007; Teitelbaum, et al. 2008). 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; Dlugosz et al. 2015; Ortiz-Lucas, et al. 2010; Scully, et al. 2010). 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 (Liebregts, 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 through modulating the cytokine release in rats (Nozu et al. 2017b; Nozu, et al. 2017c). 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; Nozu et al. 2017b, c).

Materials and methods

Animals

Adult male Sprague-Dawley rats (Charles River Laboratory, Atsugi, Japan) weighing about 300 g were used. The animals were housed grouply (3–4 rats/cage) under controlled conditions of illumination (12 h light/dark cycle starting at 7 a.m.) with temperature regulated at 23–25 ºC. 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 CRF_2 agonist (Bachem AG, Bubendorf, Switzerland), LPS obtained from Escherichia coli with the serotype 055:B5 (Sigma-Aldrich, St. Louis, MO, USA) and anakinra, an IL-1 receptor antagonist (Swedish Orphan Biovitrum, Stockholm, Sweden) were dissolved in normal saline. Astressin, a non-selective CRF receptor antagonist, astressin$_2$-B, a selective CRF_2 antagonist (Sigma-Aldrich) and cortagine, a selective CRF_1 agonist (PolyPeptide Laboratories, Torrance, CA, USA) were dissolved in double-distilled water. The doses of the chemicals were determined according to the previous reports (Nozu et al. 2017b, c; Santos et al. 1999). 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 phosphate-buffered saline (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 the small hole was made at the 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 ºC) 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 and 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 electrodes 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 sec 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 min 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 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 3 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 ± standard error. Multiple comparisons were performed by two-way analysis of variance 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 μg/kg) significantly increased colonic permeability (Fig. 2A). Cortagine (50 μg/kg), a selective CRF_1 agonist also increased (Fig. 2B), but urocortin 2 (50 μg/kg), a selective CRF_2 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 astressin$_2$-B (200 μg/kg), a selective CRF_2 antagonist did not alter it (effect of CRF: $F = 219.5$, $p < 0.05$, effect of astressin$_2$-B: $F = 2.7$, $p > 0.05$, interaction between CRF and astressin$_2$-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 CRF_1 dependent and activating CRF_2 suppressed this response. We already have shown that visceral hypersensitivity by CRF followed the 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).

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

LPS (1 mg/kg) increased gut permeability, and astressin (200 μg/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 (50 μg/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 (50 μg/kg) injected 10 min before each stress session abolished the response (effect of WAS: $F = 25.3$, $p < 0.05$, effect of 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 (Taché, et al. 2004). As described before, classically visceral hypersensitivity was considered to be induced exclusively via CRF_1. However, recently inhibitory effects of CRF_2 signaling on CRF_1-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 (Gourcerol, et al. 2011; Million, et al. 2006; Nozu et al. 2014). Then, we previously have shown that both CRF receptor subtypes were involved, and the signaling balance of CRF_1 and CRF_2 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 (Lewis, et al. 2001; Reyes, et al. 2001; Vaughan, et al. 1995). 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. 2016; Yuan, et al. 2010). 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 (Larauche, et al. 2009; Overman et al. 2012; Santos et al. 1999; Santos et al. 2008; 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 (Overman et al. 2012; Santos et al. 1999; Santos et al. 2008). On the other hand, Larauche et al. showed that peripheral injection of selective CRF_1 agonist,

331 cortagine increased colonic permeability in vivo in rats (Larauche et al. 2009), but
332 the precise roles of CRF receptor subtypes have not been determined. Both CRF_1
333 and CRF_2 have been reported to increase gut permeability (Ayyadurai et al. 2017;
334 Barreau et al. 2007; Gareau et al. 2007; Teitelbaum et al. 2008).

335 Current study clearly showed that exogenous peripheral CRF increased
336 colonic permeability in vivo, which was CRF_1 dependent. Moreover, activating CRF_2
337 per se did not alter the permeability but suppressed this CRF_1-triggered change.
338 Additionally, endogenous CRF also mediated this visceral change induced by LPS or
339 repeated WAS (animal IBS model), which was suppressed by the activation of CRF_2.
340 In this context, increased colonic permeability by both exogenous and endogenous
341 CRF may also follow the balance theory of CRF signaling. Since we previously have
342 shown that the quite similar roles of CRF_1 and CRF_2 signaling on gastric
343 contractility (Nozu, et al. 2013), the balance theory might be a fundamental rule in
344 the GI functional changes induced by peripheral CRF.

345 Whereas, we demonstrated inconsistent result with the balance theory, i.e.,
346 astressin$_2$-B did not enhance the increased colonic permeability by CRF. The
347 blocking CRF_2 would further enhance CRF_1 signaling activated by CRF and
348 increase the permeability. There is the fact of the predominant expression of
349 functional CRF_1 relative to CRF_2 in colonic myenteric neurons in guinea-pig
350 suggesting that CRF_1 is the dominant signaling in colon (Liu et al. 2010), which
351 may lead to induce strong activation of CRF_1, and consequently, inhibition of CRF_2
352 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 CRF_1 signaling may not permit further enhancement by blocking CRF_2.

The mechanisms of CRF_1 and CRF_2 interaction has not been determined. Gourcerol et al. (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 CRF_2. 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 CRF_1 dependent, and CRF_2 suppressed the changes. These results suggest that activation of CRF_2 inhibits the increased concentration of second messenger and the phosphorylation state of protein kinases leading to inhibiting activation of target cells, thereby suppressing the CRF_1-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 CRF_1, 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 CRF_2 signaling could inhibit maladaptation to stress. Moreover, the balance theory suggests that CRF signaling might be shifted toward CRF_1 resulting in altered visceral functions in IBS, and resetting abnormal CRF signaling balance by blocking

375 CRF_1 or stimulating CRF_2, may become a promising therapeutic approach to the
376 disease (Nozu and Okumura 2015).

377 As described before, TLR4 (LPS)-proinflammatory cytokine system is thought
378 to be involved in the pathophysiology of some portion of IBS patients (Dinan et al.
379 2006; Dlugosz et al. 2015; Ortiz-Lucas et al. 2010; Scully et al. 2010). TLR4 in
380 colonic tissue of IBS patients is elevated (Kocak, et al. 2016), and TLR4 messenger
381 RNA expression in colonic mucosa correlates significantly with duration of
382 symptoms in the IBS patients (Belmonte, et al. 2012). In animal studies, WAS
383 significantly increased colonic TLR4 expression (Nebot-Vivinus, et al. 2014).
384 Additionally, He et al. showed that chronic stress induced diarrhea with increased
385 colonic expression of TLR4 and NF-κB in rats, which was inhibited by TLR4/NF-κB
386 inhibitor (He, et al. 2017). Since our current and previous studies showed that the
387 visceral changes observed in our tested animal models were mediated via peripheral
388 CRF, TLR4 and cytokine (Nozu et al. 2017b, c), the existence of the link between
389 CRF and TLR4-cytokine system was expected.

390 In the current study, we clearly demonstrated for the first time that
391 peripheral exogenous or endogenous CRF induced visceral allodynia and increased
392 colonic permeability, which were mediated via TLR4 and cytokine signaling in vivo.
393 The mechanisms of these visceral changes induced by peripheral CRF have not been
394 determined definitely so far. Since CRF receptors are proved to be expressed in
395 dorsal root ganglia (Million et al. 2006), CRF may act directly to the receptors to
396 alter visceral sensation. Additionally, enterochromaffin or mast cells have CRF

receptors and release chemical mediators, such as serotonin, cytokines etc. (Overman et al. 2012; Wu, et al. 2011), which may also contribute to the changes through activating visceral afferents (Barbara, et al. 2007; Mawe, et al. 2006) and altering tight junction proteins which regulate gut epithelial barrier (Piche 2014). Our results indicated that CRF may modulate TLR4 and cytokine signaling to alter visceral function, which is 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 (Kidd, et al. 2009; McCurdy, et al. 2001). 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 and Okumura 2015). CRF-CRF_1 signaling are

419 thought to be a key factor in IBS (Taché et al. 2009), and CRF signaling balance is
420 abnormally shifted toward CRF_1 according to the balance theory (Nozu and
421 Okumura 2015). CRF activates TLR4 signaling, which triggers to produce
422 proinflammatory cytokines, thereby increasing the colonic permeability via
423 modifying tight junction proteins (Suzuki, et al. 2011) and inducing visceral
424 hypersensitivity by activating sensory neurons (Obreja, et al. 2002).

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

433 The current study had several limitations. Our method required minor
434 surgery, which is inevitable for assessing visceral sensation by EMG. However, it
435 might have some influence on the immune system, which could modify the results.
436 The cellular mechanisms of CRF were not evaluated. Since the targets of peripheral
437 CRF have not been determined definitely so far, we have to explore this issue in the
438 first place. CRF or CRF antagonists used in this study have poor penetrance into
439 brain (Taché and Brunnhuber 2008), but LPS is known to increase the permeability
440 of blood-brain barrier (Ghosh, et al. 2014). Thus, the possibility that peripheral

441 administration of CRF agonists or antagonists act to brain inducing the visceral
442 changes was not completely denied. Further studies were needed to evaluate these
443 issues.

444 In summary, stress-induced visceral allodynia and increased colonic
445 permeability were mediated via peripheral CRF pathway. CRF induced these
446 visceral changes possibly via TLR4-cytokine system, which were CRF_1 dependent,
447 and activation of CRF_2 inhibited these CRF_1-triggered responses. CRF may
448 modulate immune system to alter visceral changes, which are considered to be
449 pivotal pathophysiology of IBS.

450

451 **Declaration of interest**

452 The authors declare that they have no conflict of interest.

453

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459

460 **Author contribution statement**

TN designed and performed the experiment, analyzed the data and wrote the paper; SM and RN performed the experiment; KT contributed to establishing the experimental system monitoring visceral sensation; TO designed the experiment, analyzed the data and was involved in critical revision of the manuscript.

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Figure legends

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 3 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.

Figure 2

The effects of CRF receptor agonists and antagonists on colonic permeability. CRF (50 μg/kg) increased the permeability (**A**), and a selective CRF_1 agonist, cortagine (50 μg/kg) also increased it (**B**). Selective CRF_2 activation by urocortin 2 (50 μg/kg) did not alter the permeability (**C**). This change by CRF was abolished by astressin (200 μg/kg), a non-selective CRF receptor antagonist (**D**), while $astressin_2$-B (200 μg/kg), a selective CRF_2 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.

Figure 3

The roles of toll-like receptor 4 (TLR4) and cytokine on CRF-induced visceral allodynia and increased colonic permeability. Eritoran (10 mg/kg) fully reversed CRF-induced visceral allodynia (**A**) and increased colonic permeability (**B**). In addition, anakinra (20 mg/kg) also blocked these changes (**C**, **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 ± standard error. The number of rats examined is shown in parentheses.

Figure 4

Effect of astressin or urocortin 2 on increased colonic permeability induced by LPS (1 mg/kg) injection or repeated water avoidance stress (WAS). Astressin (200 μg/kg) or urocortin 2 (50 μg/kg) blocked the response by LPS (**A, B**). Besides, the repeated injections of astressin (50 μg/kg) or urocortin 2 (50 μg/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 ± standard error. The number of rats examined is shown in parentheses.

Figure 5

Roles of toll-like receptor 4 (TLR4) and cytokine 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**, **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

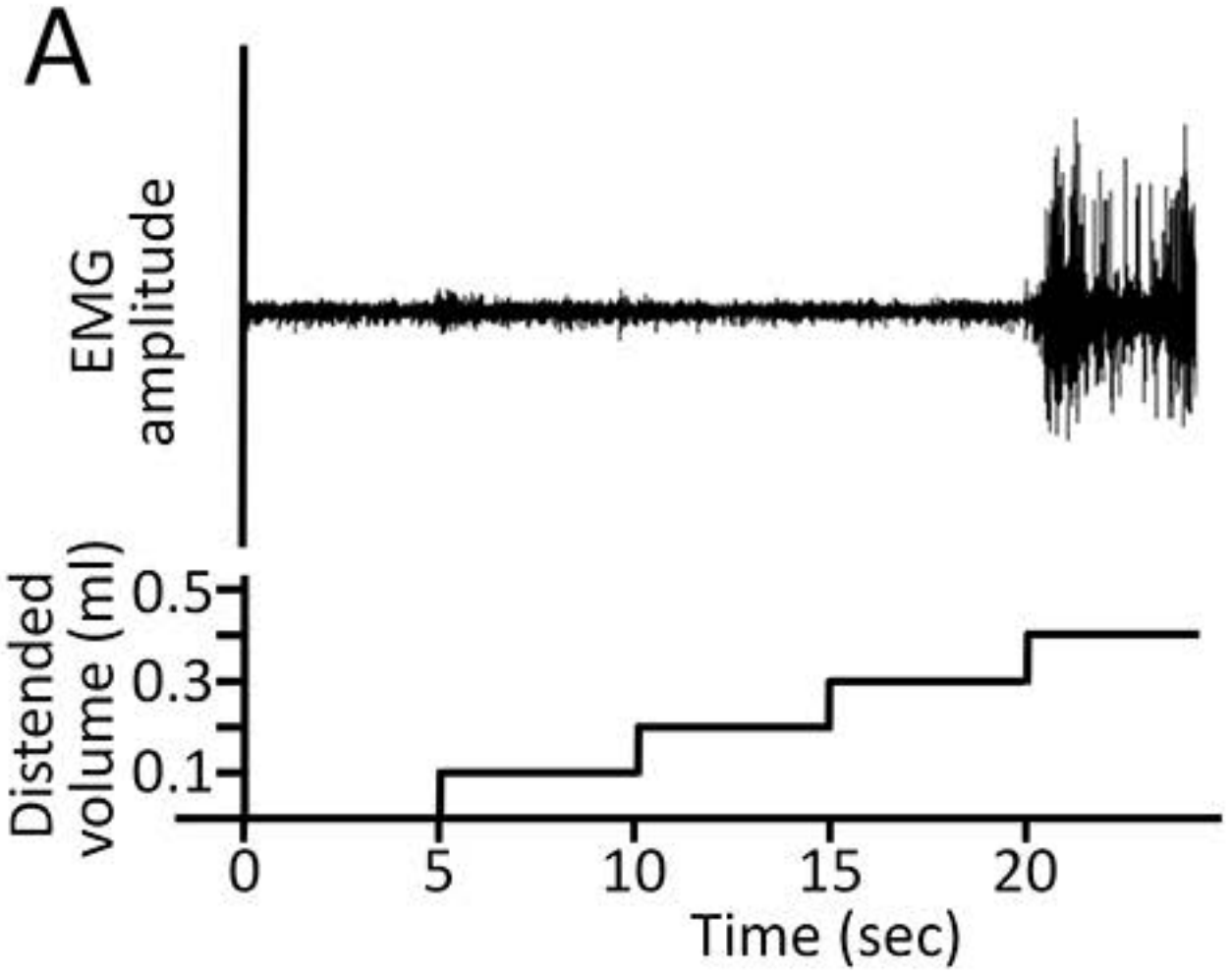
742 column represents the mean ± standard error. The number of rats examined is
743 shown in parentheses.

744

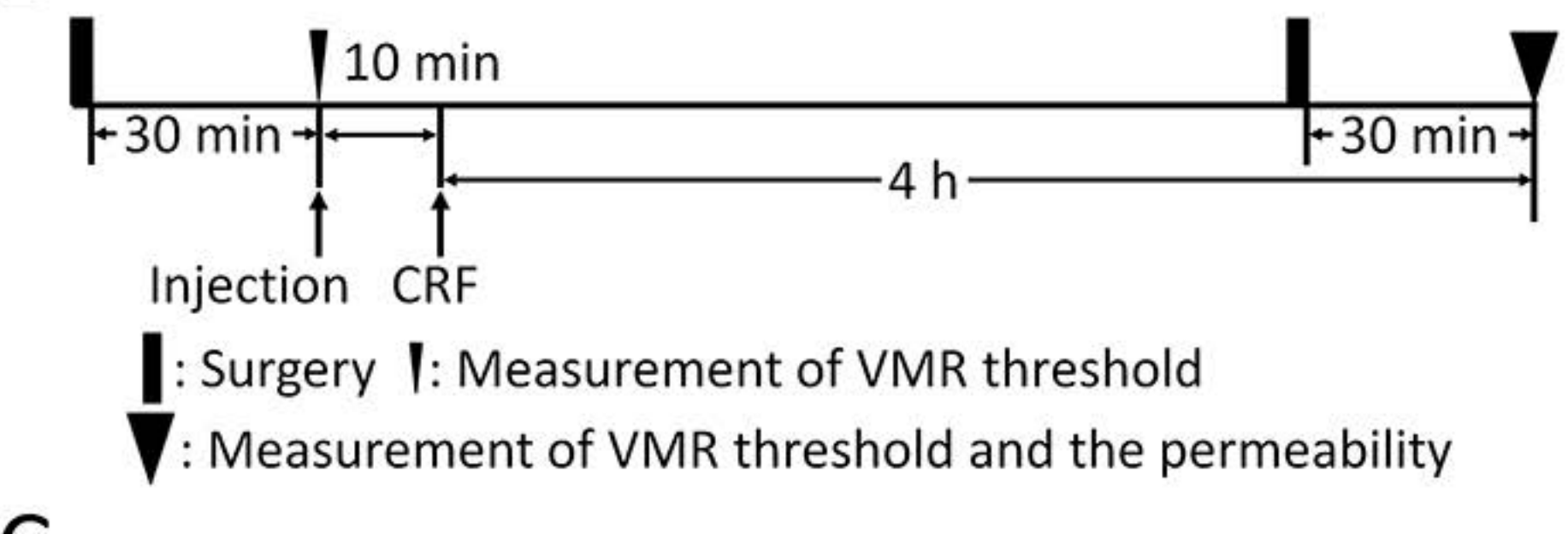
745 Figure 6

746 Schematic illustration of our hypothesis in terms of peripheral CRF, toll-like
747 receptor 4 (TLR4) and proinflammatory cytokine on visceral hypersensitivity and
748 increased gut permeability in IBS (peripheral mechanisms). CRF signaling are
749 activated (Taché et al. 2009) and its signaling balance is abnormally shifted toward
750 CRF_1 (Nozu and Okumura 2015). CRF activates TLR4 to trigger producing
751 proinflammatory cytokines, which increases the permeability (Suzuki et al. 2011)
752 and induces visceral hypersensitivity (Obreja et al. 2002). Incidentally, increased
753 gut permeability activates immune system to release LPS. LPS not only stimulates
754 TLR4 but also activates peripheral CRF signaling (Nozu et al. 2017b). In this
755 context, CRF and TLR4-cytokine signaling are considered to form a vicious cycle to
756 activate each other to induce these visceral changes in IBS.

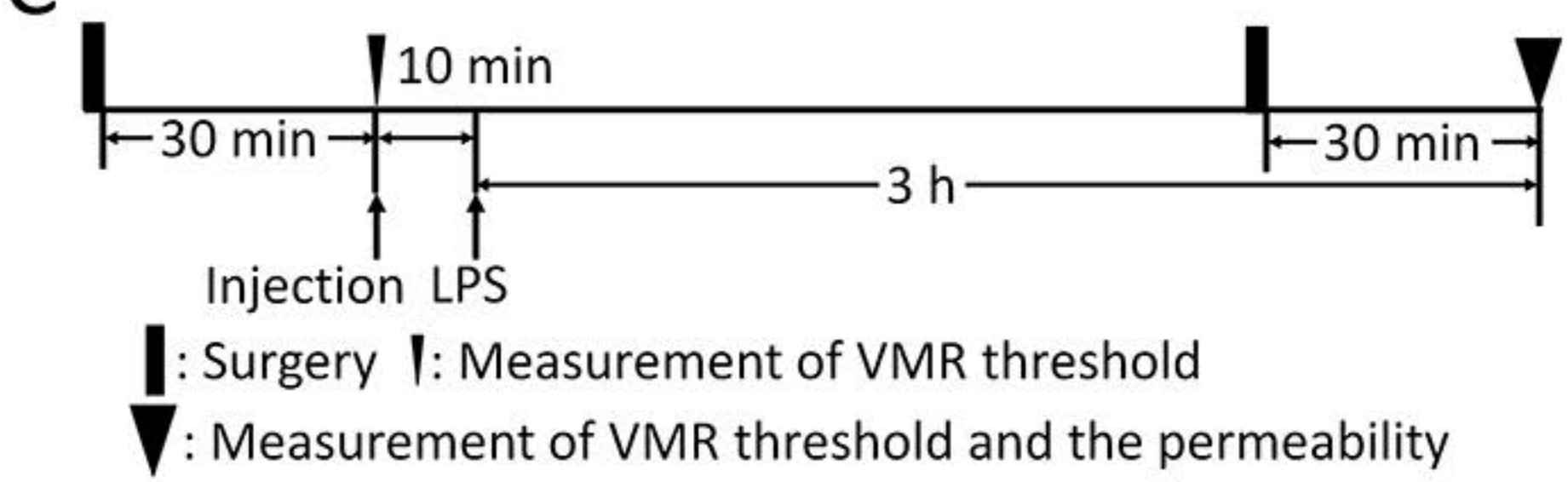
A


EMG amplitude
Distended volume (ml)
0.5
0.3
0.1
0
5
10
15
20
Time (sec)

B


10 min
30 min
30 min
4 h
Injection
CRF
: Surgery
: Measurement of VMR threshold
: Measurement of VMR threshold and the permeability

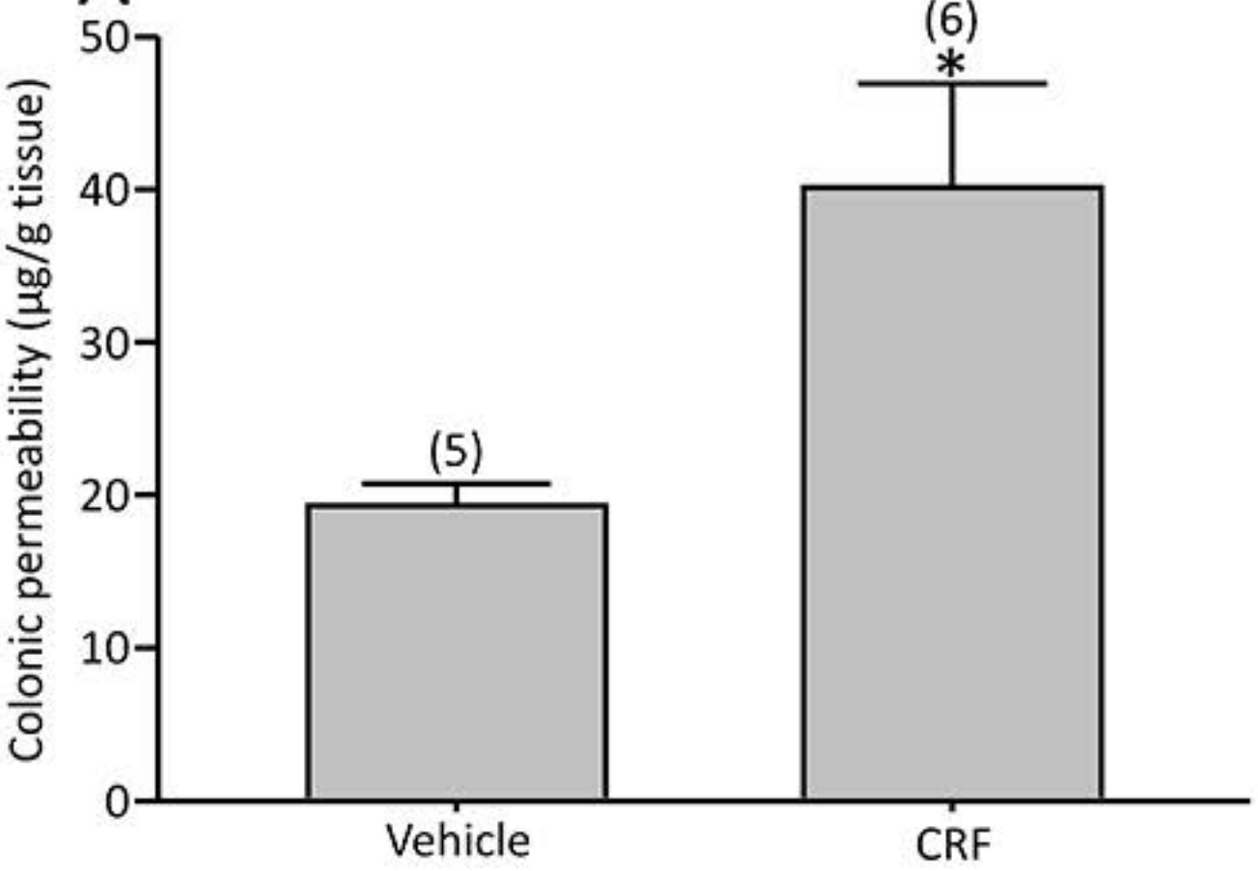
C


10 min
30 min
30 min
3 h
Injection
LPS
: Surgery
: Measurement of VMR threshold
: Measurement of VMR threshold and the permeability

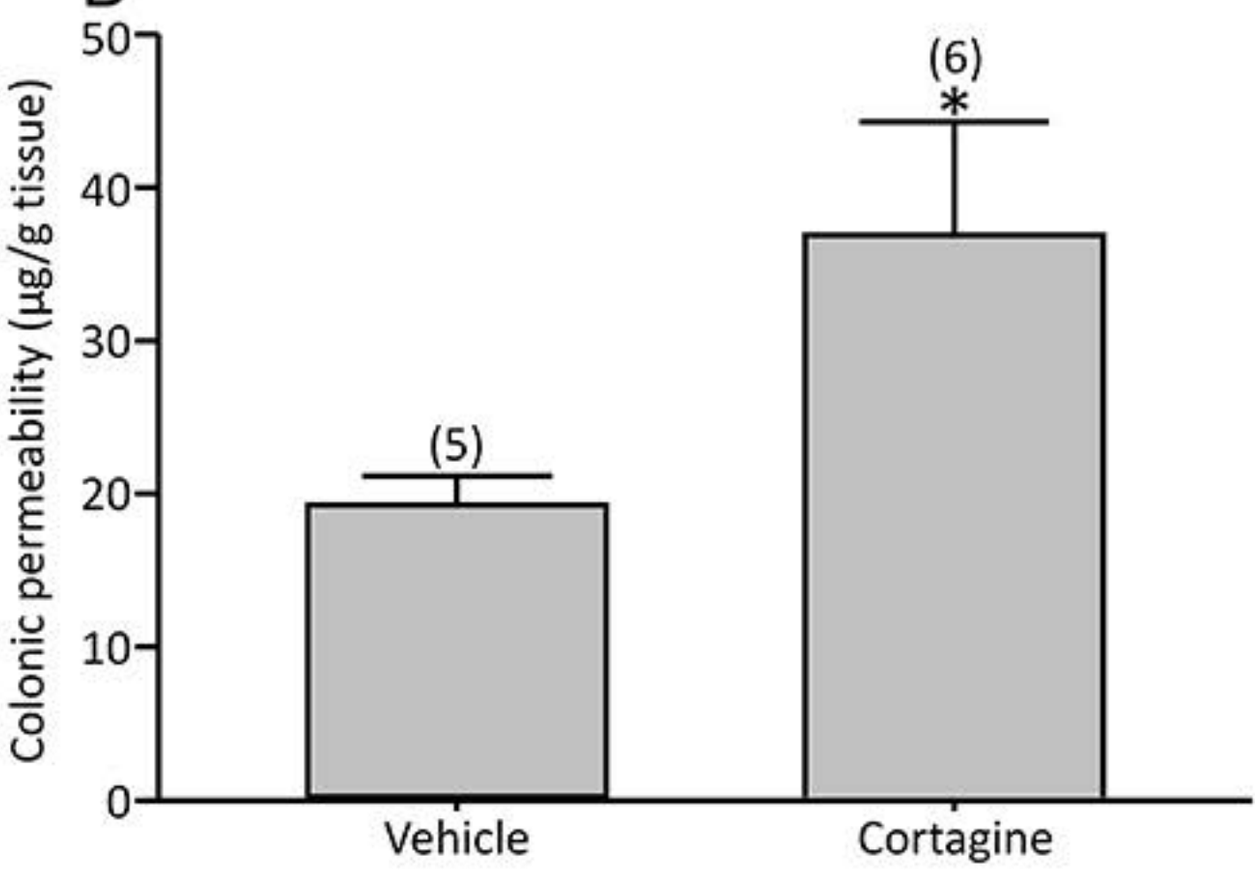
D

30 min
24 h
24 h
24 h
Injection B
10 min
10 min
10 min
Injection A
Injection A
Injection A
30 min
15 h
Injection B
: Surgery
: Measurement of VMR threshold
: Water avoidance or sham stress
: Measurement of VMR threshold and the permeability

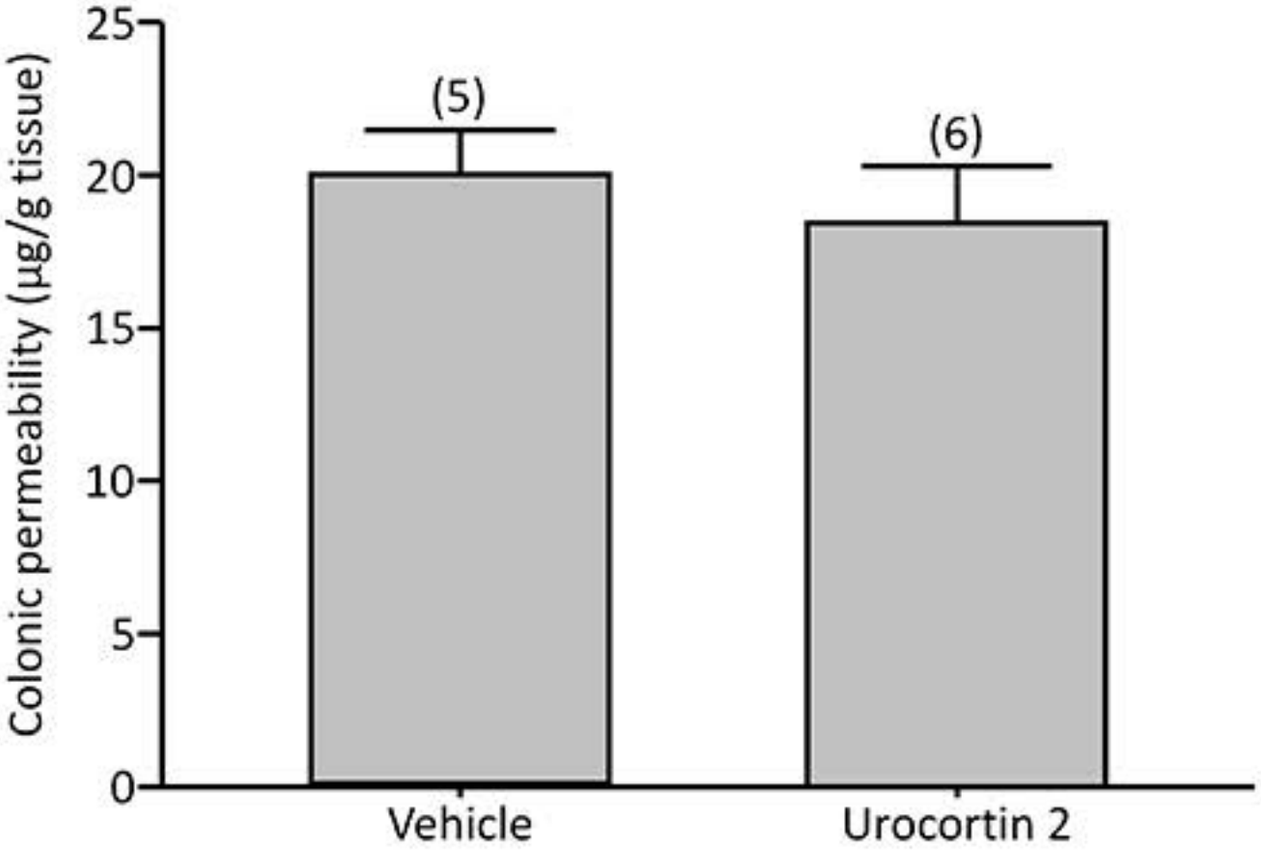
A


Colonic permeability (µg/g tissue)
50
40
30
20
10
0
(5)
(6)
*
Vehicle
CRF

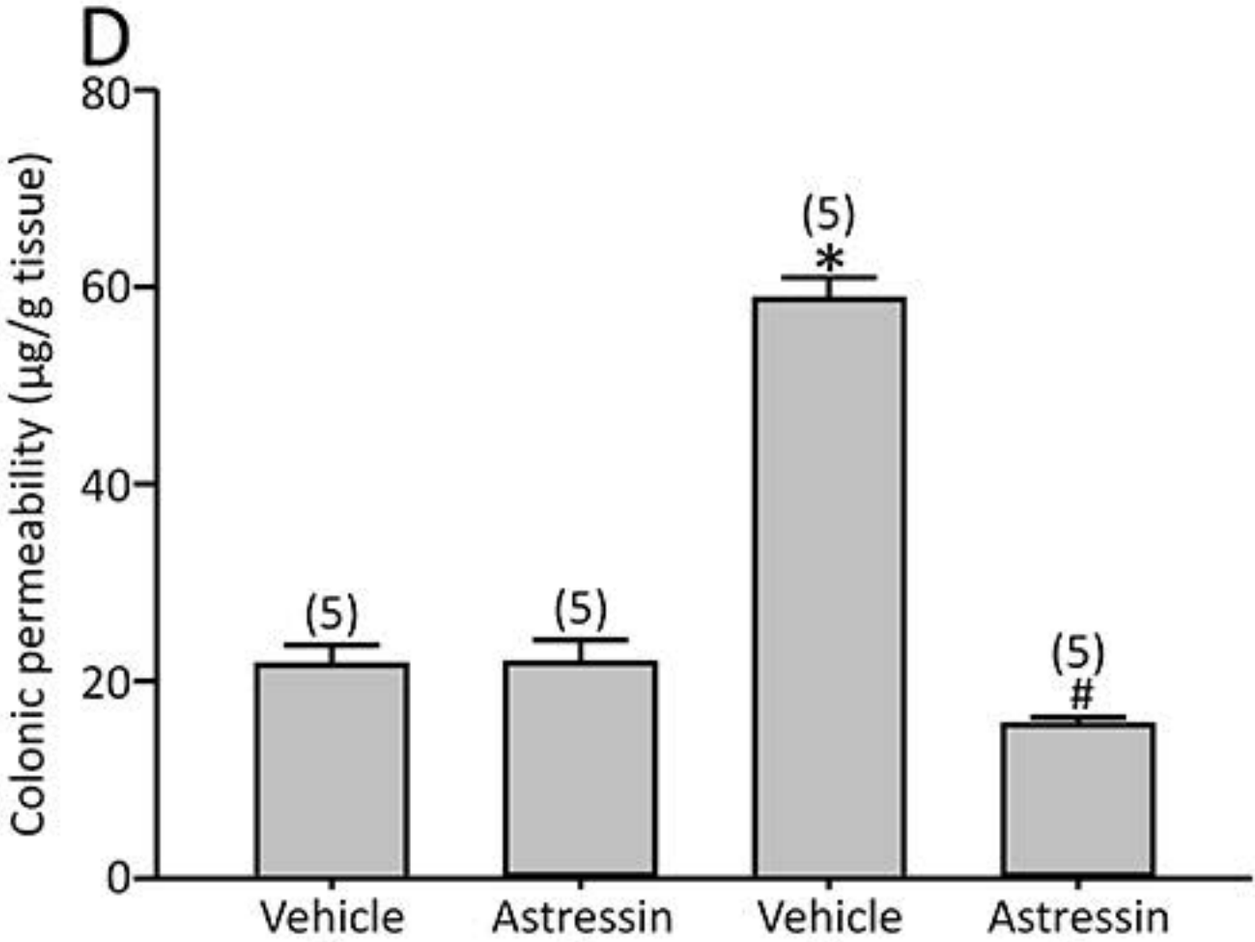
B


Colonic permeability (µg/g tissue)
50
40
30
20
10
0
(5)
(6)
*
Vehicle
Cortagine

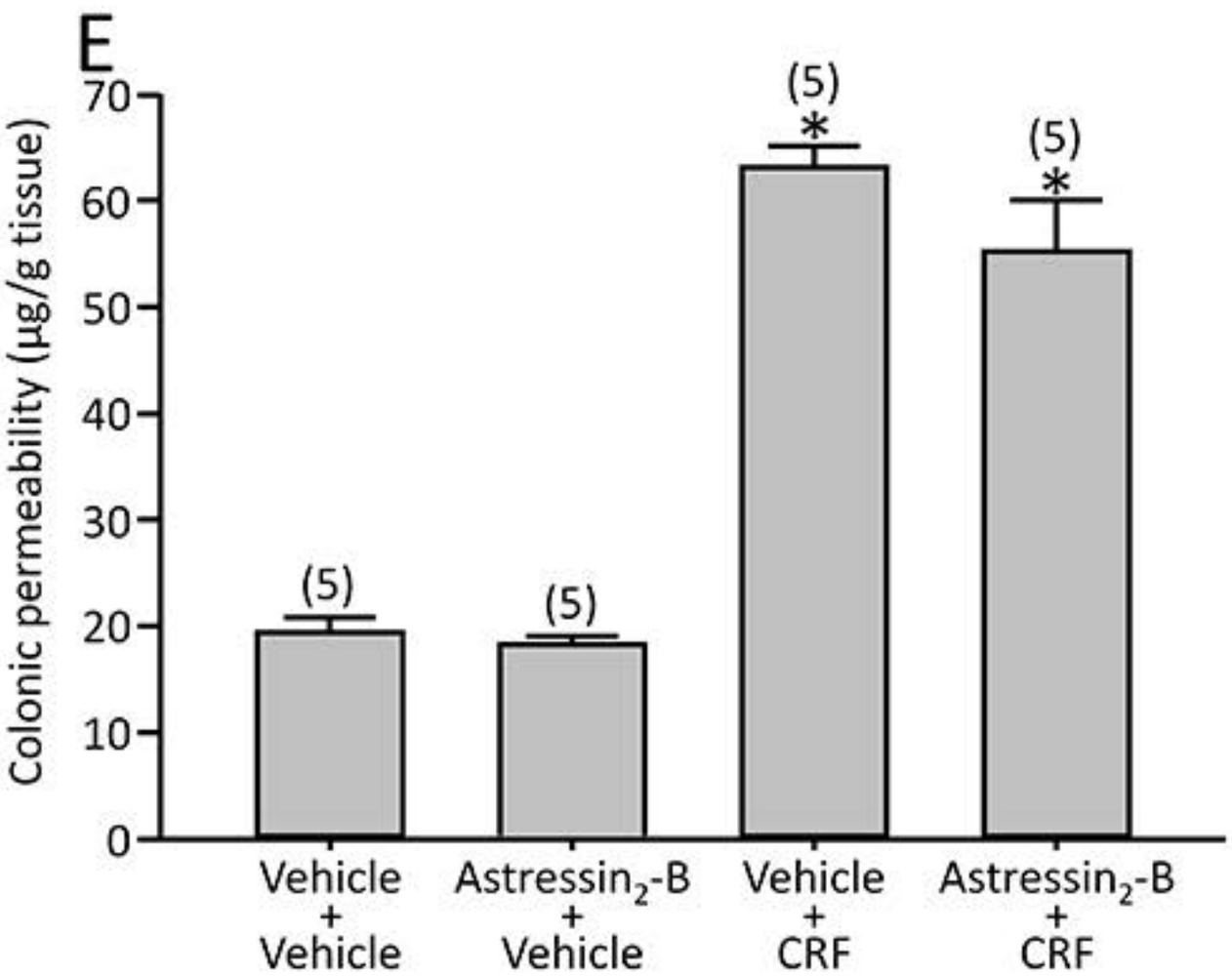
C


Colonic permeability (µg/g tissue)
25
20
15
10
5
0
(5)
(6)
Vehicle
Urocortin 2

D

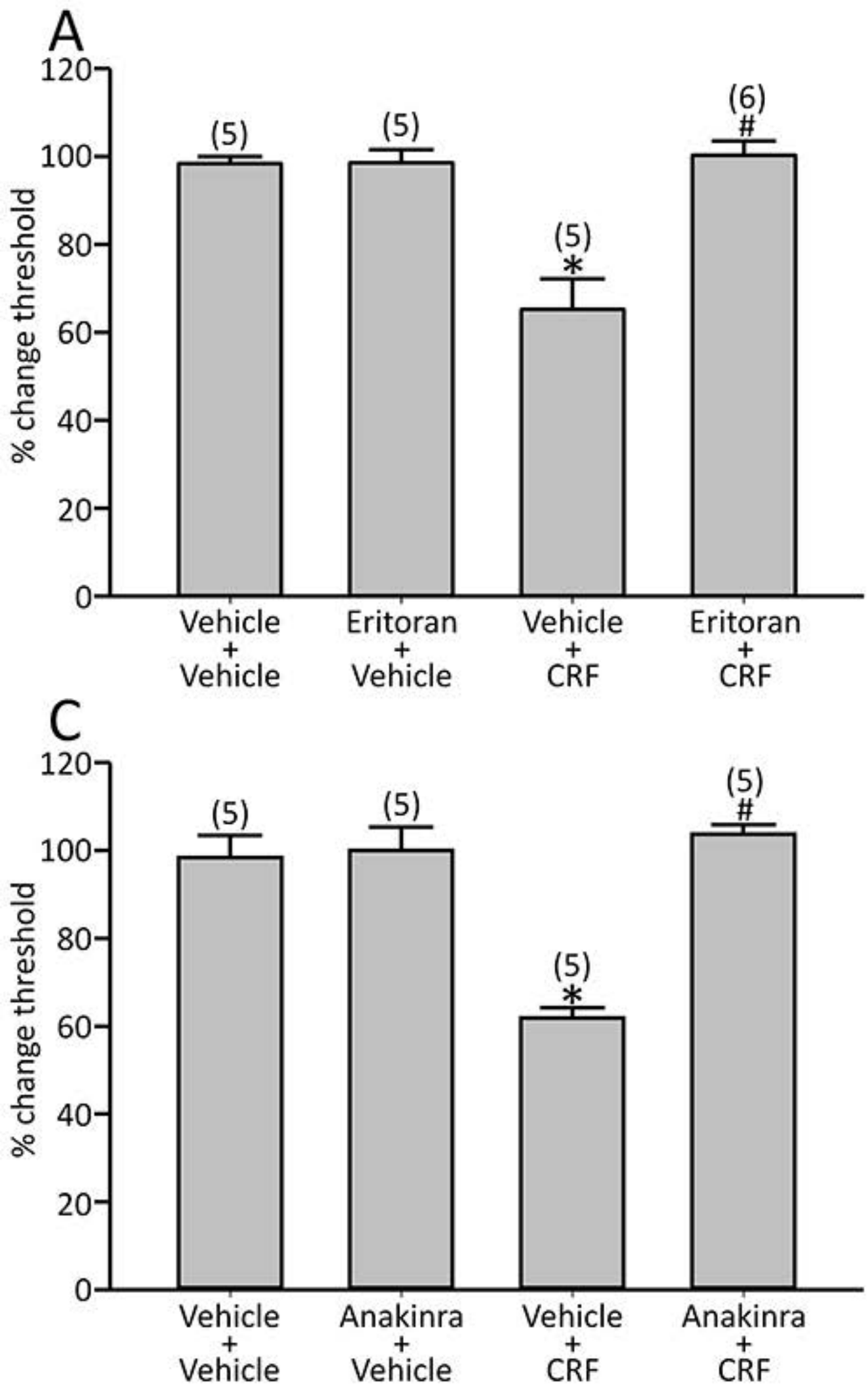

Colonic permeability (µg/g tissue)
80
60
40
20
0
(5)
(5)
(5)
*
(5)
#
Vehicle + Vehicle
Astressin + Vehicle
Vehicle + CRF
Astressin + CRF

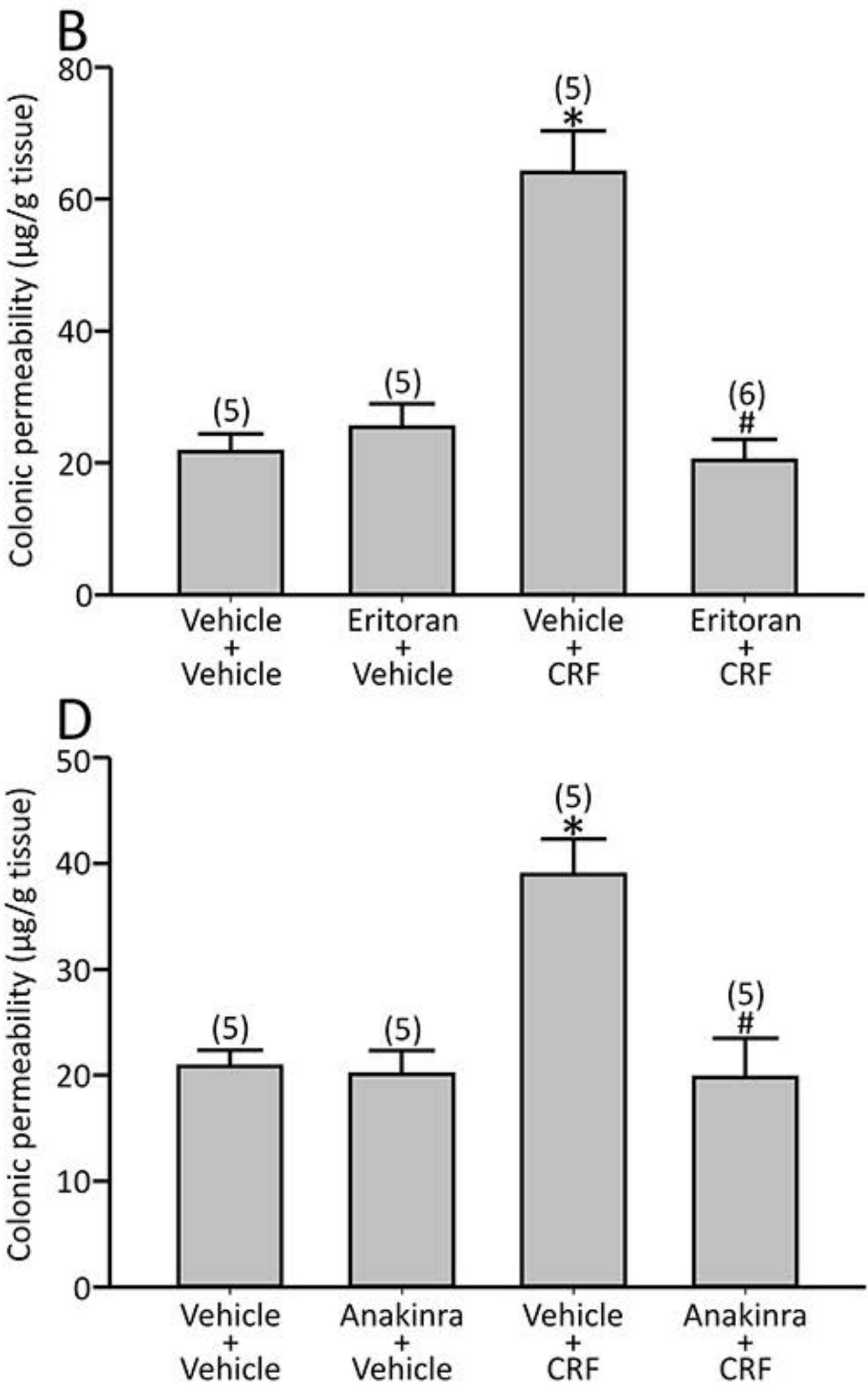
E


Colonic permeability (µg/g tissue)
70
60
50
40
30
20
10
0
(5)
(5)
(5)
*
(5)
*
Vehicle + Vehicle
Astressin2-B + Vehicle
Vehicle + CRF
Astressin2-B + CRF

F

Colonic permeability (µg/g tissue)
50
40
30
20
10
0
(5)
(5)
(5)
*
(5)
#
Vehicle + Vehicle
Urocortin 2 + Vehicle
Vehicle + CRF
Urocortin 2 + CRF


A
% change threshold
120
100
80
60
40
20
0
(5)
(5)
(5)
*
(6)
#
Vehicle + Vehicle
Eritoran + Vehicle
Vehicle + CRF
Eritoran + CRF


B
Colonic permeability (µg/g tissue)
80
60
40
20
0
(5)
(5)
(5)
*
(6)
#
Vehicle + Vehicle
Eritoran + Vehicle
Vehicle + CRF
Eritoran + CRF

C
% change threshold
120
100
80
60
40
20
0
(5)
(5)
(5)
*
(5)
#
Vehicle + Vehicle
Anakinra + Vehicle
Vehicle + CRF
Anakinra + CRF

D
Colonic permeability (µg/g tissue)
50
40
30
20
10
0
(5)
(5)
(5)
*
(5)
#
Vehicle + Vehicle
Anakinra + Vehicle
Vehicle + CRF
Anakinra + CRF

A

Colonic permeability (µg/g tissue)

100
80
60
40
20
0

(5)
(5)
(5)
*
(6)
#

Vehicle + Vehicle
Astressin + Vehicle
Vehicle + LPS
Astressin + LPS

B

Colonic permeability (µg/g tissue)

100
80
60
40
20
0

(5)
(5)
(5)
*
(6)
#

Vehicle + Vehicle
Urocortin 2 + Vehicle
Vehicle + LPS
Urocortin 2 + LPS

C

Colonic permeability (µg/g tissue)

100
80
60
40
20
0

(5)
(5)
(5)
*
(6)
#

Vehicle + Sham
Astressin + Sham
Vehicle + WAS
Astressin + WAS

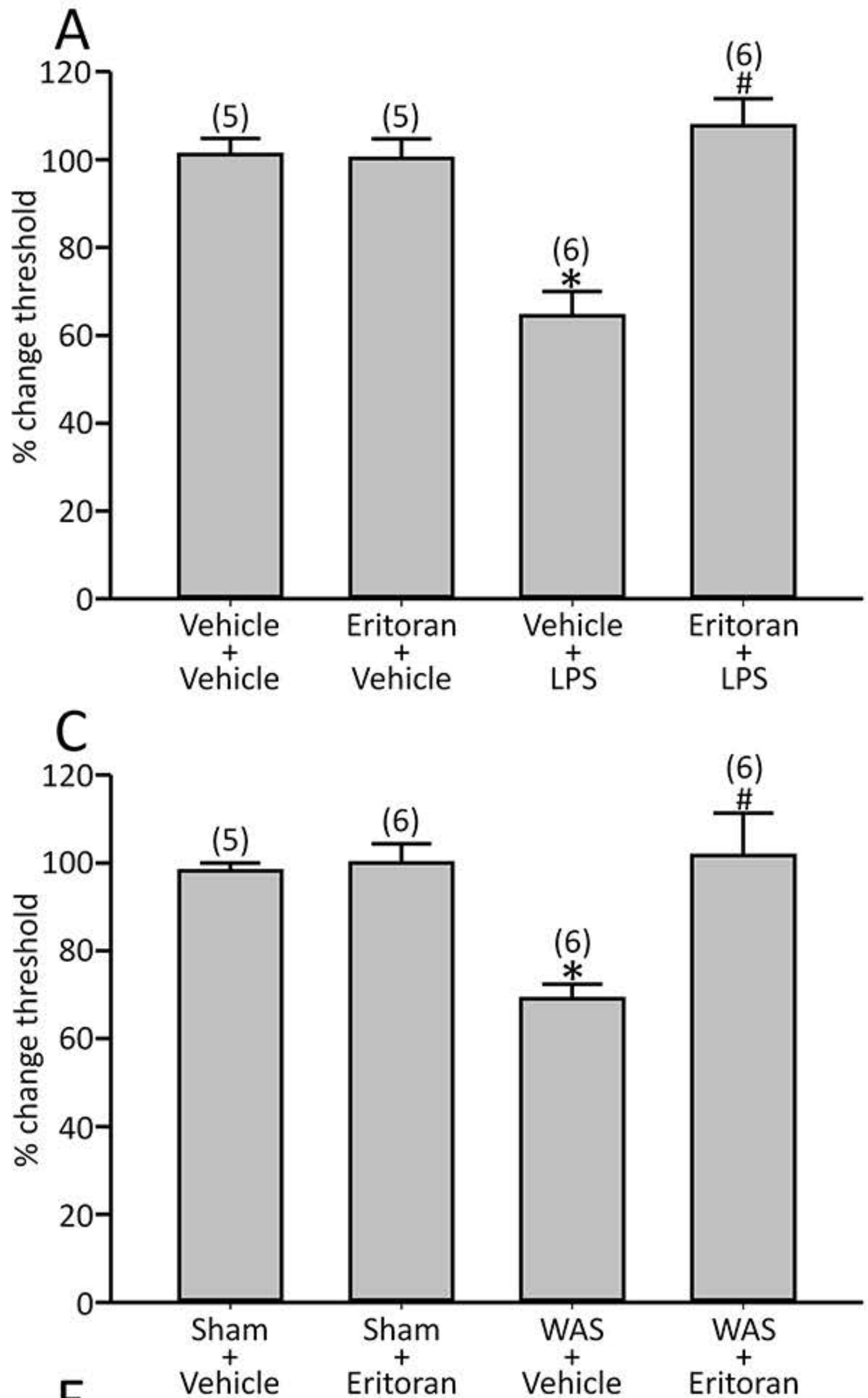
D

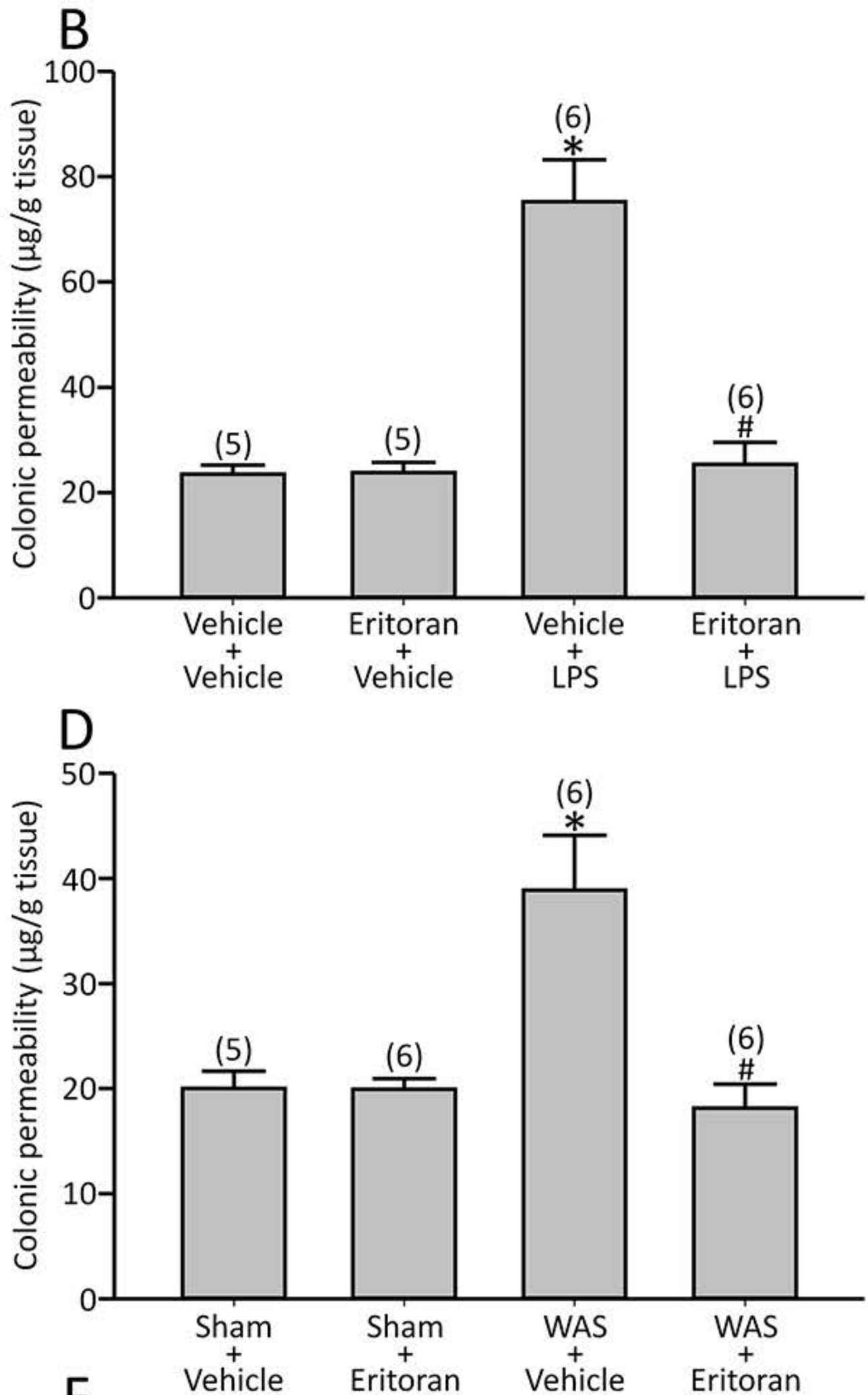
Colonic permeability (µg/g tissue)

60
50
40
30
20
10
0

(5)
(5)
(5)
*
(6)
#

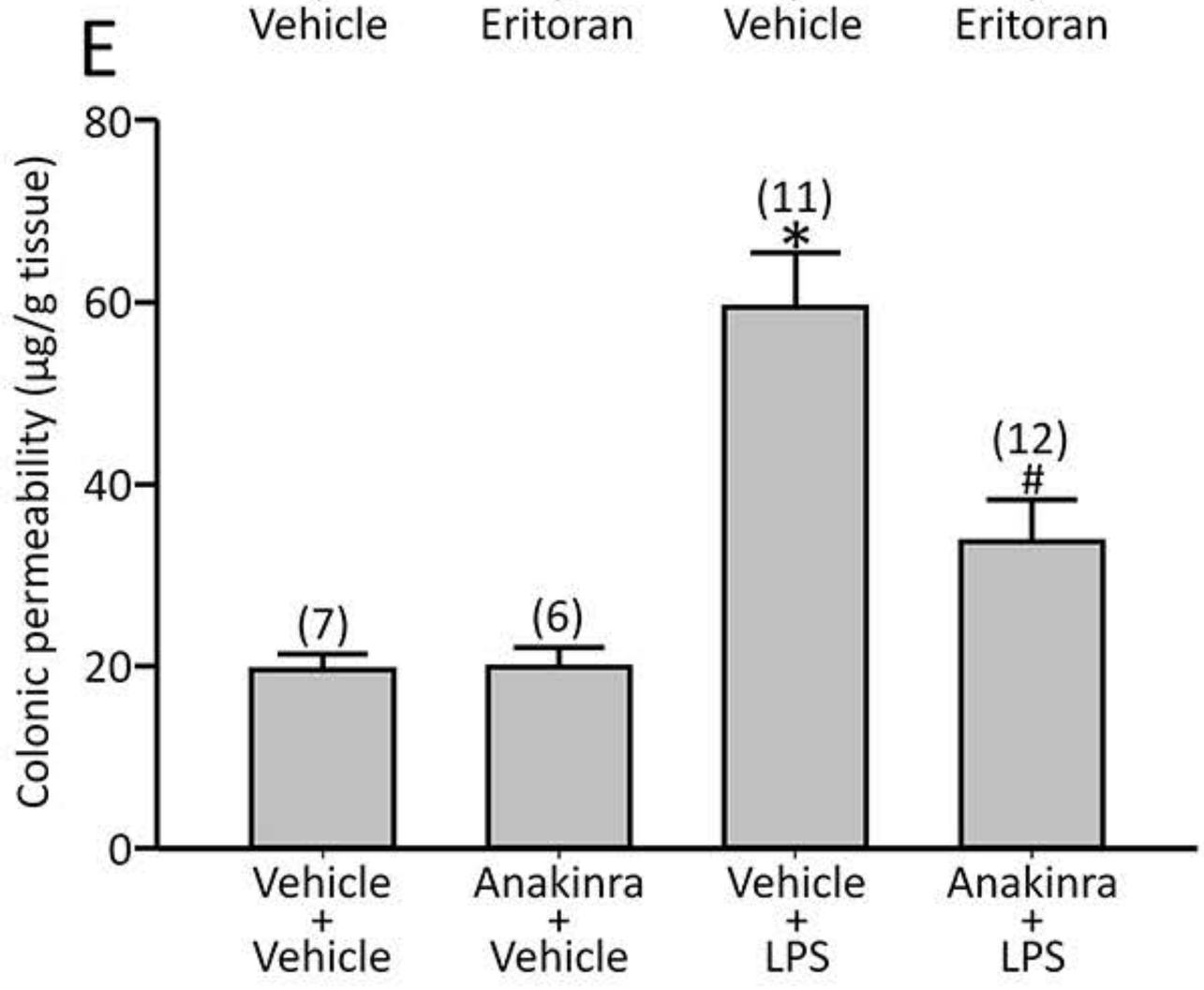
Vehicle + Sham
Urocortin 2 + Sham
Vehicle + WAS
Urocortin 2 + WAS

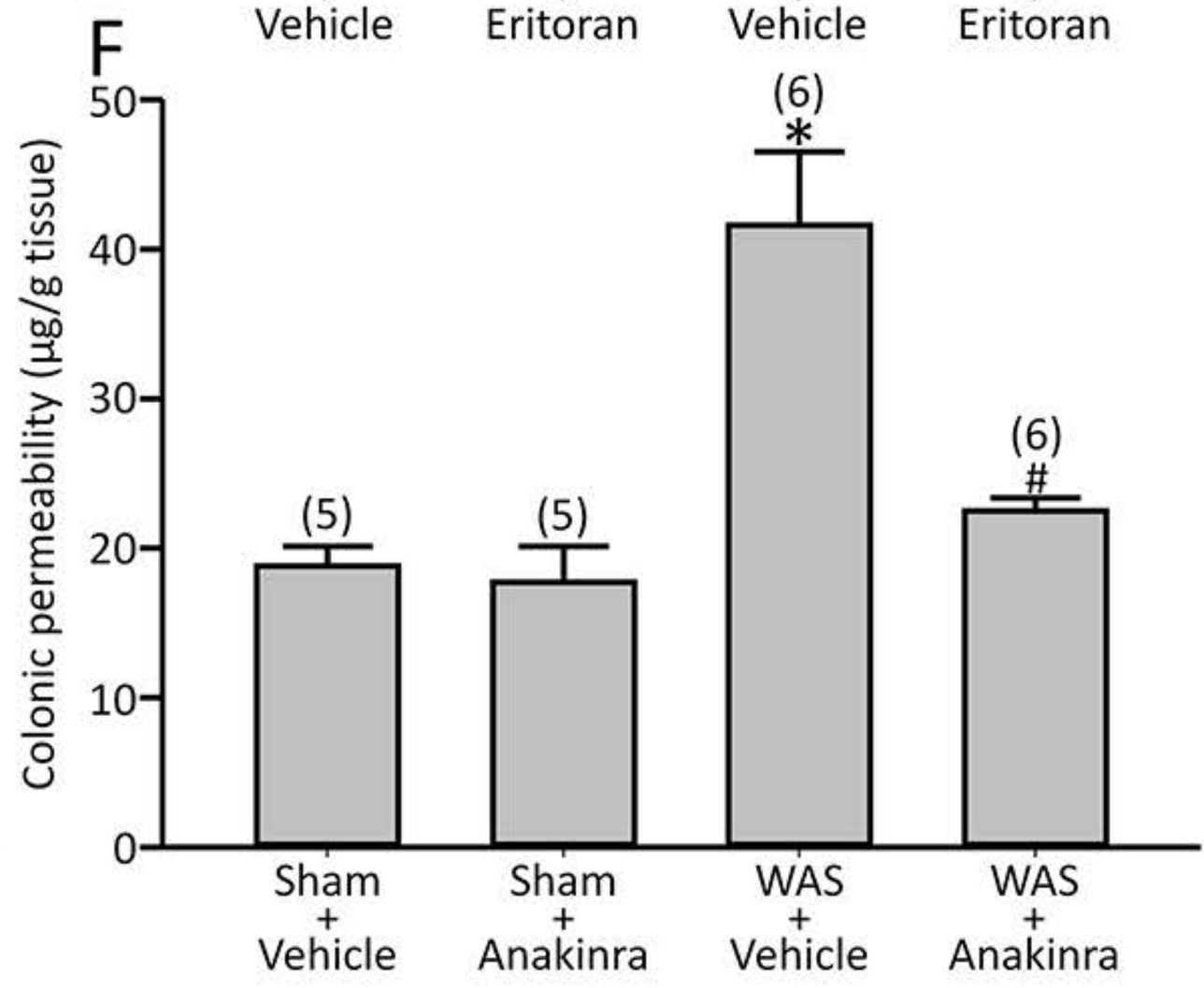

A
% change threshold
120
100
80
60
40
20
0
(5)
(5)
(6)
*
(6)
#
Vehicle + Vehicle
Eritoran + Vehicle
Vehicle + LPS
Eritoran + LPS


B
Colonic permeability (µg/g tissue)
100
80
60
40
20
0
(5)
(5)
(6)
*
(6)
#
Vehicle + Vehicle
Eritoran + Vehicle
Vehicle + LPS
Eritoran + LPS

C
% change threshold
120
100
80
60
40
20
0
(5)
(6)
(6)
*
(6)
#
Sham + Vehicle
Sham + Eritoran
WAS + Vehicle
WAS + Eritoran

D
Colonic permeability (µg/g tissue)
50
40
30
20
10
0
(5)
(6)
(6)
*
(6)
#
Sham + Vehicle
Sham + Eritoran
WAS + Vehicle
WAS + Eritoran


E
Colonic permeability (µg/g tissue)
80
60
40
20
0
(7)
(6)
(11)
*
(12)
#
Vehicle + Vehicle
Anakinra + Vehicle
Vehicle + LPS
Anakinra + LPS


F
Colonic permeability (µg/g tissue)
50
40
30
20
10
0
(5)
(5)
(6)
*
(6)
#
Sham + Vehicle
Sham + Anakinra
WAS + Vehicle
WAS + Anakinra

Shifting signal balance
toward CRF_1

CRF_1

CRF_2

Activating peripheral CRF receptors

TLR4

Cytokines

Increased gut permeability

Visceral hypersensitivity

LPS

IBS