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Abstract

Stress-induced altered visceral sensation and impaired gut barrier play an important role in the pathophysiology of irritable bowel syndrome (IBS). These responses were demonstrated to be peripheral corticotropin-releasing factor (CRF) dependent and also mediated via proinflammatory cytokine in animal IBS model. Dehydroepiandrosterone sulfate (DHEA-S) is known to have anti-inflammatory properties by suppressing proinflammatory cytokine release. We hypothesized that DHEA-S improves stress-induced visceral changes and is beneficial for IBS treatment. We explored the effects of DHEA-S on lipopolysaccharide (LPS)- or repeated water avoidance stress (WAS)-induced visceral allodynia and increased colonic permeability (rat IBS models). The threshold of visceromotor response, i.e. abdominal muscle contractions induced by colonic balloon distention was electrophysiologically measured. Colonic permeability was estimated in vivo by quantifying the absorbed Evans blue in colonic tissue. DHEA-S abolished visceral allodynia and colonic hyperpermeability induced by LPS in a dose-dependent manner. It also blocked repeated WAS- or peripheral injection of CRF-induced visceral changes. These effects by DHEA-S in LPS model were reversed by bicuculline, a γ-aminobutyric acid $(GABA)_A$ receptor antagonist, N^G-nitro-L-arginine methyl ester, a nitric oxide (NO) synthesis inhibitor, naloxone, an opioid receptor antagonist, or sulpiride, a dopamine D_2 receptor antagonist. However, domperidone, a peripheral dopamine D_2 receptor antagonist did not modify the effects. Peripheral injection of $astressin_2$-B, a selective CRF receptor subtype 2 (CRF_2) antagonist also reversed these effects. In conclusion, DHEA-S blocked stress-induced visceral changes via $GABA_A$, NO, opioid, central dopamine D_2 and peripheral CRF_2 signaling. DHEA-S may be useful for IBS treating.

48 Key words: dehydroepiandrosterone sulfate; visceral pain; gut barrier; irritable bowel syndrome

1. 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 substantial impact on the development and exacerbation of IBS symptoms (Taché et al., 2009). Since stress-induced colonic functional changes are abolished by corticotropin-releasing factor (CRF) antagonist (Nozu and Okumura, 2015; Taché et al., 2009), CRF may be a key molecule in the pathophysiology of IBS.

Incidentally, there is ample evidence that compromised gut barrier function manifested by increased gut permeability is observed in some patients with IBS (Taché et al., 2009). Impaired gut barrier induces bacterial translocation leading to increased lipopolysaccharide (LPS) and proinflammatory cytokines, which is also an important aspect of IBS (Barbara et al., 2012; Dlugosz et al., 2015; Nozu et al., 2017b, 2018).

We have recently shown that LPS injection or repeated water avoidance stress (WAS) induced visceral allodynia and increased colonic permeability in rats (animal IBS models), and these changes were mediated via peripheral CRF, toll-like receptor 4 (TLR4) and proinflammatory cytokine system (Nozu et al., 2017b, c, 2018). Furthermore, we also demonstrated that peripheral injection of CRF mimicked these visceral changes, which were mediated via TLR4 and proinflammatory cytokine (Nozu et al., 2018). These results suggest that peripherally released CRF triggered by stress may evoke the visceral changes by modulating TLR4-cytokine pathway, which seems to be one of the possible pathophysiology of IBS.

Dehydroepiandrosterone (DHEA) and its sulfate ester (DHEA-S) are weak androgen and the most abundant circulating steroid hormones in humans (Baulieu et al., 1965). Like cortisol, DHEA(-S) is released by hypothalamic-pituitary-adrenocortical axis triggered by CRF in response to stress. Although the precise physiological roles have yet to be fully determined, several studies have reported that it modulates vascular endothelial function and improves insulin sensitivity, body composition, cognitive and sexual function (Woda et al., 2016). Additionally, DHEA(-S) also displays anti-inflammatory effects by inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and proinflammatory cytokine release (Ben-Nathan et al., 1999; Danenberg et al., 1992; Du et al., 2001; Poynter and Daynes, 1998). At the same time, DHEA(-S) plays a significant role in nociception, and it exerts antinociceptive action on somatic pain (Kibaly et al., 2008; Patte-Mensah et al., 2010). However, the information regarding the effects of DHEA(-S) on GI function has been very scarce. Incidentally, although several drug candidates such as cannabinoids (Capasso et al., 2014; Pagano et al., 2016), lovastatin (Nozu et al., 2017a) or metformin (Nozu et al., 2019), etc., have been recently proposed for IBS treatment, the therapeutic options are still limited.

In this context, we hypothesized that DHEA-S improves the visceral function by suppressing proinflammatory cytokine or modulating CRF signaling, and it may be beneficial for IBS treatment. In this study, in order to examine the hypothesis, we attempted to determine the effects of DHEA-S on visceral allodynia and increased gut permeability induced by LPS or repeated WAS in rats.

2. Materials and Methods

92 *2.1. Animals*

93 Adult male Sprague-Dawley rats (Charles River Laboratory, Atsugi, Japan) weighing
94 about 300 g were used. The animals were housed in groups (3−4 rats/cage) in metallic cages. The
95 animal room was maintained at a controlled condition of illumination (12 h light/dark cycle
96 starting at 0700 h) with temperature regulated at 23−25 ºC. Rats were allowed free access to
97 standard food (Solid rat chow, Oriental Yeast, Tokyo, Japan) and tap water.

98

99 *2.2. Chemicals*

100 DHEA-S sodium hydrate (Tokyo Chemical Industry, Tokyo, Japan), LPS obtained from
101 Escherichia coli with the serotype 055:B5 (Sigma-Aldrich, St. Louis, MO, USA), rat/human
102 CRF (Peptide Institute Inc., Asagi, Japan), N^G-nitro-L-arginine methyl ester (L-NAME), a nitric
103 oxide (NO) synthesis inhibitor, naloxone hydrochloride, an opioid receptor antagonist and
104 domperidone (Wako Pure Chemical Industries, Osaka, Japan), a peripheral dopamine D_2
105 receptor antagonist were dissolved in normal saline. Sulpiride (Wako Pure Chemical Industries),
106 a dopamine D_2 receptor antagonist and bicuculline (Sigma-Aldrich), a γ-aminobutyric acid
107 $(GABA)_A$ receptor antagonist was dissolved in saline containing 10 % dimethyl sulfoxide
108 (DMSO). Astressin$_2$-B, a selective CRF receptor subtype 2 (CRF_2) antagonist (Sigma-Aldrich)
109 was dissolved in double-distilled water. The doses of the chemicals were determined according
110 to the previous reports (Nozu et al., 2017a, 2019; Nozu et al., 2017b; Samardzic et al., 2017).
111 The volume of injection was 0.2 ml/rat. DHEA-S, L-NAME, CRF or astressin$_2$-B was
112 intraperitoneally injected. Other chemicals were administered via subcutaneous route.

113

2.3. Measuring visceral sensation

Visceral sensation was evaluated 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).

2.3.1. Implantation of electrodes and placement of colonic distention balloon

Under brief ether anesthesia, a small abdominal skin incision approximately 3 mm in length was made in non-fasted rats, and four electrodes, i.e. for positive, negative, ground and spare (Teflon coated stainless steel, 0.05 mm diameter, MT Giken, Tokyo, Japan) for EMG were inserted approximately 2 mm into left side external oblique muscle through the incision. They were fixed to the muscle 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. Distension balloon (6-Fr disposable silicon balloon-urethral catheter, JU-SB0601, Terumo Corporation, Tokyo, Japan) was inserted intra-anally into the colon 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.

2.3.2. 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., 2018), 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. The VMR threshold was defined as the distended balloon volume (ml) inducing VMR (Fig. 1A). Tang et al. (Tang et al., 2013) previously demonstrated using the balloon quite similar to ours that the pain threshold induced by colonic distention assessed by the observation of abdominal withdrawal reflex could be determined as distended balloon volume in rats, and also showed that intracolonic pressure was linearly associated with intraballoon volume. 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.

2.4. Measuring colonic permeability

Colonic permeability measurement was performed as previously described (Nozu et al., 2018). The rats anesthetized by intraperitoneal 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) 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 by a puncture using 18 G needle at the 1 cm from the ileocecal junction. 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.

158 The colon was gently flushed with phosphate buffered saline (PBS, 37 ºC) using the catheter
159 until all stools were washed out. Generally, the required volume of PBS was approximately 10
160 ml and the perfusion rate was 5 ml/min. Then another ligation was added on the colon at
161 approximately 4 cm from the proximal ligation, and 1 ml of 1.5 % Evans blue in PBS was
162 instilled into the colon segment between ligations through the catheter. Fifteen min later, the
163 animals were killed and the colons were excised. Later they were washed with PBS and 1 ml of 6
164 mM N-acetyl-cysteine, and were opened and placed in 2 ml of N,N-dimethylformamide for 12 h.
165 The permeability was calculated by measuring the Evans blue concentration in the supernatant
166 using a spectrophotometer at 610 nm.

167

168 *2.5. Experimental protocols*

169 First, the basal VMR threshold was measured. Then the electrodes and distention balloon
170 were removed, and either LPS (1 mg/kg) or the vehicle was injected (Fig. 1B). The rats were
171 returned to their home cages, and after 2.5 h, they underwent surgery for electrode implantation
172 and balloon placement again. The second measurement of threshold was performed 3 h after the
173 injection followed by the measurement of colonic permeability (Nozu et al., 2017b). The vehicle
174 or DHEA-S (5, 15 or 40 mg/kg) was injected thrice at 48 h, 24 h and 30 min before injecting
175 LPS or the vehicle.

176 Next, in a separate experiment, the effects of DHEA-S on repeated WAS-induced
177 visceral changes were explored (Fig. 1C). The basal threshold was measured, and 10 min later,
178 either WAS or sham stress was applied for 1 h daily for 3 consecutive days. The threshold was
179 again measured at 24 h after undergoing the last stress session followed by the measurement of

colonic permeability (Nozu et al., 2017c, 2018). DHEA-S or the vehicle was administered at 10 min prior to each stress session and 30 min before the second measurement of threshold.

The effects were also tested in CRF model. The vehicle or DHEA-S was injected thrice at 48 h, 24 h and 30 min before injecting CRF (50 μg/kg) or the vehicle. The second measurement of threshold was performed at 4 h after injecting CRF or the vehicle (Fig. 1D) (Nozu et al., 2018).

Next, to explore the mechanisms of actions of DHEA-S, the effects of bicuculline (2 mg/kg), sulpiride (200 mg/kg), domperidone (10 mg/kg), L-NAME (10 mg/kg), naloxone (1 mg/kg) or astressin$_2$-B (100 μg/kg) was examined. These drugs were administered together with DHEA-S. In the current study, the rats were not reused in a separate series of experiments.

2.6. Stress procedure

Water avoidance stress consisted of placing rat individually 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 as previously described (Martínez et al., 1997). Control rats were individually placed in the same plastic cage, which was not filled with water (sham stress).

2.7. Statistical analysis

Data are expressed as means ± S.E.M. Multiple comparisons were performed by one-way or 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.

2.8. Ethical considerations

For all studies, approval was obtained by the Research and Development and Animal Care Committees at the Asahikawa Medical University (#16191, approved on April 1, 2016).

3. Results

3.1. DHEA-S eliminated LPS-induced visceral allodynia and increased colonic permeability

DHEA-S inhibited LPS-induced visceral allodynia in a dose-responsive manner ($F = 5.9$, $P < 0.05$; Fig. 2A). DHEA-S at 40 mg/kg fully reversed the response by LPS, and this dose of DHEA-S per se did not alter the basal threshold of VMR (ml, 0.59 ± 0.03 for vehicle, $n = 10$ vs. 0.60 ± 0.02 for DHEA-S, $n = 11$, $P > 0.05$). Similarly, DHEA-S abolished the increased colonic permeability induced by LPS in a dose-responsive manner ($F = 8.4$, $P < 0.05$; Fig. 2B), and a 40 mg/kg-dose fully reversed the response. However, DHEA-S per se did not alter the permeability. According to the results above, 40 mg/kg of DHEA-S was employed for the following experiments.

3.2. DHEA-S blocked repeated WAS- or peripheral CRF-induced visceral changes

DHEA-S also abolished repeated WAS induced visceral allodynia (effect of WAS: $F = 29.0$, $P < 0.05$; effect of DHEA-S: $F = 18.5$, $P < 0.05$; interaction between WAS and DHEA-S: F

221 = 27.4, P < 0.05; Fig. 3A) and increased colonic permeability (effect of WAS: F = 13.3, P <
222 0.05; effect of DHEA-S: F = 12.5, P < 0.05; interaction between WAS and DHEA-S: F = 18.4, P
223 < 0.05; Fig. 3B).

224 Since LPS- or repeated WAS-induced visceral allodynia and increased colonic
225 permeability were mediated via peripheral CRF receptors as described before (Nozu et al.,
226 2017b, c, 2018), we also determined the effects of DHEA-S in CRF model. Peripheral injection
227 of CRF reduced the threshold of VMR and increased colonic permeability, which were blocked
228 by DHEA-S (% change threshold, effect of CRF: F = 14.0, P < 0.05; effect of DHEA-S: F =
229 21.5, P < 0.05; interaction between CRF and DHEA-S: F = 17.1 P < 0.05; Fig. 3C, colonic
230 permeability, effect of CRF: F = 25.9, P < 0.05; effect of DHEA-S: F = 24.6, P < 0.05;
231 interaction between CRF and DHEA-S: F = 23.5, P < 0.05; Fig. 3D).

232

233 *3.3. Bicuculline reversed the inhibitory effects of DHEA-S on LPS-induced visceral changes*

234 DHEA(-S) is a potent allosteric modulator of $GABA_A$ receptor (Perez-Neri et al., 2008).
235 Therefore, we tested the effects of $GABA_A$ receptor antagonist, bicuculline on the actions of
236 DHEA-S. Bicuculline did not alter the basal threshold (ml, 0.59 ± 0.02 for vehicle, n = 10 vs.
237 0.59 ± 0.01 for bicuculline, n = 10, P > 0.05). Moreover, the drug did not modify the sensory
238 response (effect of bicuculline: F = 0.01, P > 0.05; effect of LPS: F = 27.6, P < 0.05; interaction
239 between bicuculline and LPS: F = 0.07, P > 0.05) or increased colonic permeability (effect of
240 bicuculline: F = 1.3, P > 0.05; effect of LPS: F = 61.2, P < 0.05; interaction between bicuculline
241 and LPS: F = 0.75, P > 0.05) by LPS.

Later the effects of bicuculline on the inhibitory effects of DHEA-S on LPS-induced visceral changes were determined. The drug blocked the antinociceptive effect by DHEA-S (effect of bicuculline: $F = 9.7$, $P < 0.05$; effect of DHEA-S: $F = 10.6$, $P < 0.05$; interaction between bicuculline and DHEA-S: $F = 6.0$, $P < 0.05$; Fig. 4A). Additionally, it also abolished the improvement of colonic permeability by DHEA-S (effect of bicuculline: $F = 10.0$, $P < 0.05$; effect of DHEA-S: $F = 12.9$, $P < 0.05$; interaction between bicuculline and DHEA-S: $F = 11.8$, $P < 0.05$; Fig. 4B). We also confirmed that DMSO used as a solvent for bicuculline per se neither modified the basal threshold nor the permeability as compared with saline (data were not shown).

3.4. Sulpiride reversed but domperidone did not alter the effects of DHEA-S on LPS-induced visceral changes

Since dopamine signaling is an important modulator of visceral pain (Okumura et al., 2015), we explored its role on the actions of DHEA-S. Sulpiride did not alter the basal threshold (ml, 0.60 ± 0.02 for vehicle, $n = 11$ vs. 0.60 ± 0.03 for sulpiride, $n = 10$, $P > 0.05$). In addition, the drug did not alter the changes by LPS (% change threshold, effect of sulpiride: $F = 0.02$, $P > 0.05$; effect of LPS: $F = 23.4$, $P < 0.05$; interaction between sulpiride and LPS: $F = 0.05$, $P > 0.05$, colonic permeability, effect of sulpiride: $F = 1.7$, $P > 0.05$; effect of LPS: $F = 83.2$, $P < 0.05$; interaction between sulpiride and LPS: $F = 1.72$, $P > 0.05$).

Then we determined the effects of sulpiride on the actions of DHEA-S in LPS model. The drug reversed the inhibitory actions by DHEA-S on LPS-induced visceral changes (% change threshold, effect of sulpiride: $F = 8.2$, $P < 0.05$; effect of DHEA-S: $F = 6.5$, $P < 0.05$;

interaction between sulpiride and DHEA-S: $F = 10.8$, $P < 0.05$; Fig. 5A, colonic permeability, effect of sulpiride: $F = 20.2$, $P < 0.05$; effect of DHEA-S: $F = 5.0$, $P < 0.05$; interaction between sulpiride and DHEA-S: $F = 18.7$, $P < 0.05$; Fig. 5B).

Domperidone, a peripherally acting dopamine D_2 receptor antagonist neither modified the basal threshold (ml, 0.61 ± 0.03 for vehicle, n = 10 vs. 0.60 ± 0.03 for domperidone, n = 10, $P > 0.05$) nor the visceral changes induced by LPS (% change threshold, effect of domperidone: $F = 0.12$, $P > 0.05$; effect of LPS: $F = 76.7$, $P < 0.05$; interaction between domperidone and LPS: $F = 1.33$, $P > 0.05$, colonic permeability, effect of domperidone: $F = 0.007$, $P > 0.05$; effect of LPS: $F = 211.6$, $P < 0.05$; interaction between domperidone and LPS: $F = 0.003$, $P > 0.05$). Additionally, it did not alter the effects of DHEA-S in LPS model (% change threshold, effect of domperidone: $F = 0.15$, $P > 0.05$; effect of DHEA-S: $F = 38.8$, $P < 0.05$; interaction between domperidone and DHEA-S: $F = 2.27$, $P > 0.05$; Fig. 5C, colonic permeability, effect of domperidone: $F = 0.21$, $P > 0.05$; effect of DHEA-S: $F = 318.8$, $P < 0.05$; interaction between domperidone and DHEA-S: $F = 2.27$, $P > 0.05$; Fig. 5D). These results suggested that central dopamine D_2 signaling mediated the effects by DHEA-S.

3.5. L-NAME reversed the effects of DHEA-S in LPS model

Since it is known that DHEA-S increases NO synthesis (Reddy and Kulkarni, 1998), its role on the actions of DHEA-S was explored. L-NAME did not change either the basal threshold (ml, 0.61 ± 0.01 for vehicle, n = 10 vs. 0.61 ± 0.02 for L-NAME, n = 10, $p > 0.05$) or the changes induced by LPS (% change threshold, effect of L-NAME: $F = 0.42$, $P > 0.05$; effect of LPS: $F = 71.9$, $P < 0.05$; interaction between L-NAME and LPS: $F = 0.02$, $P > 0.05$, colonic

permeability, effect of L-NAME: $F = 0.04$, $P > 0.05$; effect of LPS: $F = 60.1$, $P < 0.05$; interaction between L-NAME and LPS: $F = 0.17$, $P > 0.05$).

Meanwhile, the drug blocked the antinociceptive effect (effect of L-NAME: $F = 8.84$, $P < 0.05$; effect of DHEA-S: $F = 11.4$, $P < 0.05$; interaction between L-NAME and DHEA-S: $F = 9.07$, $P < 0.05$; Fig. 6A) and the improvement of increased colonic permeability (effect of L-NAME: $F = 7.53$, $P < 0.05$; effect of DHEA-S: $F = 6.90$, $P < 0.05$; interaction between L-NAME and DHEA-S: $F = 5.29$, $P < 0.05$; Fig. 6B) of DHEA-S.

3.6. Naloxone abolished the effects of DHEA-S

Endogenous opioid signaling is well known to modulate visceral pain (Reiss et al., 2017), and its role was also determined. The basal threshold was not changed by naloxone (ml, 0.58 ± 0.02 for vehicle, n = 10 vs. 0.59 ± 0.02 for naloxone, n = 10, $P > 0.05$). Moreover, naloxone did not alter the changes by LPS (% change threshold, effect of naloxone: $F = 0.079$, $P > 0.05$; effect of LPS: $F = 39.9$, $P < 0.05$; interaction between naloxone and LPS: $F = 0.045$, $P > 0.05$, colonic permeability, effect of naloxone: $F = 0.002$, $P > 0.05$; effect of LPS: $F = 157.2$, $P < 0.05$; interaction between naloxone and LPS: $F = 0.15$, $P > 0.05$).

The drug fully reversed the effects of DHEA-S (% change threshold, effect of naloxone: $F = 6.96$, $P < 0.05$; effect of DHEA-S: $F = 8.56$, $P < 0.05$; interaction between naloxone and DHEA-S: $F = 5.53$, $P < 0.05$; Fig. 7A, colonic permeability, effect of naloxone: $F = 11.2$, $P < 0.05$; effect of DHEA-S: $F = 9.43$, $P < 0.05$; interaction between naloxone and DHEA-S: $F = 7.77$, $P < 0.05$; Fig. 7B) in LPS model.

3.7. Astressin$_2$-B blocked the effects of DHEA-S

As described before, LPS- or repeated WAS-induced visceral changes were mediated by peripheral CRF receptors (Nozu et al., 2017b, c, 2018), and the role of CRF signaling was explored. Astressin$_2$-B did not alter the basal threshold of VMR (ml, 0.58 ± 0.02 for vehicle, n = 10 vs. 0.58 ± 0.02 for astressin$_2$-B, n = 10, $P > 0.05$), and did not modify the changes by LPS (% change threshold, effect of astressin$_2$-B: $F = 0.12$, $P > 0.05$; effect of LPS: $F = 20.3$, $P < 0.05$; interaction between astressin$_2$-B and LPS: $F = 0.008$, $P > 0.05$, colonic permeability, effect of astressin$_2$-B: $F = 1.84$, $P > 0.05$; effect of LPS: $F = 44.6$, $P < 0.05$; interaction between astressin$_2$-B and LPS: $F = 1.95$, $P > 0.05$).

The antagonist reversed the effects by DHEA-S in LPS model (% change threshold, effect of astressin$_2$-B: $F = 14.9$, $P < 0.05$; effect of DHEA-S: $F = 23.5$, $P < 0.05$; interaction between CRF and DHEA-S: $F = 13.7$ $P < 0.05$; Fig. 8A, colonic permeability, effect of astressin$_2$-B: $F = 11.6$, $P < 0.05$; effect of DHEA-S: $F = 15.3$, $P < 0.05$; interaction between CRF and DHEA-S: $F = 5.8$, $P < 0.05$; Fig. 8B).

4. Discussion

The current study clearly demonstrated for the first time that DHEA-S blocked visceral allodynia and increased colonic permeability induced by LPS or repeated WAS. Moreover, it also abolished the CRF-induced visceral changes.

As described before, LPS-, repeated WAS- or CRF-induced visceral changes were mediated via TLR4-proinflammatory cytokine signaling (Nozu et al., 2017b, c, 2018). Incidentally, peripheral injection of interleukin (IL)-1β or IL-6 induces visceral allodynia (Nozu

et al., 2017b), which is considered to be mediated through the activation of the cytokine receptors located in the visceral afferent neurons (Obreja et al., 2002; von Banchet et al., 2005). Furthermore, cytokine also increases gut permeability via modifying tight junction proteins (Suzuki et al., 2011). Meanwhile, DHEA(-S) is known to inhibit NF-κB and cytokine production (Ben-Nathan et al., 1999; Danenberg et al., 1992; Du et al., 2001; Poynter and Daynes, 1998). These lines of evidence suggest that DHEA-S may exert the action by suppression of proinflammatory cytokine production.

We found that $GABA_A$ receptor antagonist, bicuculline blocked the actions of DHEA-S in LPS model. GABA receptors exist not only within brain but also have been identified in GI tract (Gladkevich et al., 2006). DHEA(-S) is a potent allosteric modulator of $GABA_A$ receptor. DHEA(-S) binds to the picrotoxin site of the $GABA_A$ receptor (Perez-Neri et al., 2008), and may modulate GABA neurotransmission (Majewska et al., 1986). Although DHEA(-S) is generally considered to display an inhibitory effect on $GABA_A$ receptor function (Perez-Neri et al., 2008), but the evidence that it stimulated $GABA_A$ receptor was also reported (Lapchak et al., 2000). Additionally, macrophages express a functional $GABA_A$ receptor, and activating the receptor reduces the production of IL-6 triggered by LPS (Reyes-Garcia et al., 2007). These findings suggest that DHEA-S may inhibit cytokine signaling via activating $GABA_A$ receptor, thereby improving the visceral changes. At the same time, GABA is the principal inhibitory neurotransmitter within the spinal dorsal horn, and activating $GABA_A$ receptor is known to exert anti-nociceptive actions (Rode et al., 2005).

Garrido-Gil et al. (Garrido-Gil et al., 2018) showed that central dopaminergic depletion, i.e. Parkinson's disease model, increased the level of IL-1β in colon, suggesting that brain dopamine reduces the vulnerability of gut inflammation. Incidentally, it was reported that

DHEA(-S) increased dopamine release in hypothalamic cell cultures or PC12 cells (Perez-Neri et al., 2008). These findings may support our results that sulpiride but not domperidone reversed the effect of DHEA-S, suggesting that central dopamine D_2 signaling mediated the effects by DHEA-S possibly through suppressing cytokine production. Moreover, we previously demonstrated that central dopamine signaling is an important modulator of visceral pain. Intracisternal injection of dopamine agonist displayed antinociceptive action against colonic distention (Okumura et al., 2015).

It was demonstrated that NO inhibited expression of proinflammatory cytokine genes in various immune cells (Kroncke et al., 2001), and suppressed IL-1β release from macrophage (Kim et al., 1998). In addition, the chemical mediators including cytokines released by mast cells induce stress-induced visceral hypersensitivity and increased gut permeability (Nozu et al., 2017c; Nozu and Okumura, 2015; Taché et al., 2009), and NO inhibits mast cell degranulation resulting in improved gut permeability (Kanwar et al., 1994). Meanwhile, DHEA-S increases NO synthesis (Reddy and Kulkarni, 1998), and it was reported that anxiety-like behavior induced by restraint stress in elevated plus maze test was abolished by DHEA-S, which was reversed by L-NAME (Chakraborti et al., 2011). These findings are consistent with our results that the effects of DHEA-S were prevented by L-NAME, suggesting that DHEA-S exerted the action via NO pathway.

Opioid receptors are expressed in immune cells and modulate cytokine response (Ninkovic and Roy, 2013). Chronic morphine treatment was reported to decrease the production of IL-1β and TNF-α from mouse splenocyte cultures (Pacifici et al., 2000). Meanwhile, DHEA-S blocks stress-induced elevation of plasma aldosterone concentration in rats via opioid receptor, suggesting that DHEA-S activates opioid signaling (Obut et al., 2012). These findings are

consistent with our results that the effects of DHEA-S were reversed by naloxone. At the same time, several studies showed that NO facilitated neuronal release of endogenous opioids to stimulate opioid receptors in brain and spinal cord (Branda et al., 2000; Chung et al., 2006). In this context, DHEA-S may activate opioid receptors via stimulating NO pathway.

Peripheral CRF modulates the visceral changes through the activation of two receptors, CRF receptor subtype 1 (CRF_1) and CRF_2 (Hillhouse and Grammatopoulos, 2006; Perrin and Vale, 1999). Additionally, we have recently demonstrated that the visceral changes induced by exogenous or endogenous CRF activated by stress, i.e. LPS or WAS, are CRF_1 dependent, and CRF_2 signaling inhibits the CRF_1-triggered changes (Nozu et al., 2017b, 2018; Nozu et al., 2014). Therefore, we hypothesized that DHEA-S activates CRF_2 signaling to suppress the responses, and it actually happened, i.e. astressin$_2$-B reversed the effects of DHEA-S.

Stress induces integrated responses to maintain homeostasis, which is thought to be favorable for survival of organisms. However, in the absence of appropriate counter regulatory system, the stress response may run in an overdrive state, that can become maladaptive and fatal (Chrousos, 2009). Thus, CRF_2 signaling is considered to be the system preventing maladaptation to stress and can be beneficial for survival.

Cortisol is also one of the major players in modulating stress response (Kamin and Kertes, 2017; Wiley et al., 2016). Meanwhile, DHEA(-S) is known to largely antagonize the effects of cortisol (Kamin and Kertes, 2017), and upregulation of DHEA(-S) accompanied with that of cortisol may be important in adaptation to stress (Maninger et al., 2010). In this context, DHEA(-S) is considered to have counter regulatory action to stress response.

It has been recently demonstrated that exogenous cortisol mimicked the visceral response induced by repeated WAS, i.e. increased gut permeability with altered tight junction proteins in rat colon (Zong et al., 2018). Moreover, repeated WAS-induced visceral hyperalgesia was prevented by corticoid-receptor antagonist (Hong et al., 2011). These findings indicate that the visceral changes induced by repeated WAS are mediated via cortisol signaling in addition to CRF, and we found that DHEA-S displayed counter actions to these changes, which may support the notion above. Therefore, CRF_2 and DHEA-S might have similar role on the visceral stress response, and our results indicated that the regulatory actions by DHEA-S were mediated via CRF_2. There is no evidence indicating that DHEA-S modulates CRF_2 signaling, and the mechanisms of the regulatory action of CRF_2 have not been demonstrated either yet. Further studies are needed to clarify these issues, which possibly lead to further understanding the mechanisms of the action of DHEA-S on GI function.

Although the molecular or cellular mechanism was not shown, we clearly showed that visceral hypersensitivity and impaired colonic barrier in animal IBS models were improved by DHEA-S. Since these visceral changes are considered to be significant contributors to the pathophysiology of IBS (Taché et al., 2009), our results suggest that DHEA-S is effective for IBS treating. Incidentally, psychological problems such as anxiety and depression are common in IBS, and treatment directly towards the psychiatric conditions can reduce the symptom severity of IBS (Lee et al., 2017). DHEA(-S) exhibits anxiolytic activity in mice (Melchior and Ritzmann, 1994), and several clinical studies showed the beneficial effect of DHEA(-S) in the patients with depression (Eser et al., 2006). These pharmacological properties may also beneficial for IBS. Large scale clinical trials to evaluate the effectiveness of DHEA(-S) in the patients with IBS should be conducted in future.

5. Conclusions

DHEA-S blocked visceral allodynia and colonic hyperpermeability in animal IBS models via $GABA_A$, central dopamine D_2, NO, opioid and peripheral CRF_2 signaling. DHEA-S may be useful for IBS treating.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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604

Figure legends

Figure 1

A The threshold of visceromotor response (VMR) was determined by the distended balloon volume (ml) inserted into the colon inducing apparent sustained abdominal muscle contractions. Demonstrable EMG recording is represented. The threshold of VMR was 0.4 ml in this animal. **B** Schematic representation of the experimental protocol to explore the effects of DHEA-S on LPS-induced visceral allodynia and increased colonic permeability. The basal VMR threshold was measured at 30 min after the surgery for implanting EMG electrodes and placing the balloon. Then LPS (1 mg/kg, subcutaneously) or the vehicle was administered, and the second measurement of threshold was performed at 3 h after the injection followed by the measurement of colonic permeability. DHEA-S or the vehicle was intraperitoneally injected thrice before injection of LPS or the vehicle. **C** The protocol determining the effects of DHEA-S on repeated water avoidance stress (WAS)-induced visceral changes. The basal threshold was measured, and then the rats were subjected to either WAS or sham stress for 1 h daily for 3 consecutive days. The measurements of second VMR threshold and colonic permeability were performed at 24 h after the last stress session. DHEA-S or the vehicle was injected 4 times before the second measurement. **D** The protocol examining the effects of DHEA-S on CRF-induced visceral changes. DHEA-S or the vehicle was injected thrice. The changes were assessed at 4 h after the injection of CRF (50 μg/kg, intraperitoneally) or the vehicle.

Figure 2

The effects of DHEA-S on LPS-induced visceral changes. LPS induced visceral allodynia, and DHEA-S dose-dependently blocked the change (**A**). DHEA-S also reversed LPS-induced increased colonic permeability (**B**). * $P < 0.05$ vs. vehicle (DHEA-S 0) + vehicle, # $P < 0.05$ vs. vehicle (DHEA-S 0) + LPS by one-way analysis of variance followed by Tukey's honestly significant difference test. Each column represents the mean ± S.E.M. The number of rats examined is shown in parentheses.

Figure 3

The effects of DHEA-S (40 mg/kg) on repeated water avoidance stress (WAS)- or CRF-induced visceral changes. Repeated WAS-induced visceral allodynia (**A**) and increased colonic permeability (**B**), which were abolished by DHEA-S. Similar results were also obtained on CRF (50 μg/kg, intraperitoneally)-induced visceral changes (**C**, **D**). * $P < 0.05$ vs. vehicle + sham or vehicle + vehicle, # $P < 0.05$ vs. vehicle + WAS or vehicle + CRF by two-way analysis of variance followed by Tukey's honestly significant difference test. Each column represents the mean ± S.E.M. The number of rats examined is shown in parentheses.

Figure 4

Bicuculline (2 mg/kg, subcutaneously) blocked the antinociceptive action by DHEA-S (40 mg/kg) on LPS-induced visceral allodynia (**A**). Additionally, it also reversed the suppressive effect of DHEA-S on LPS-induced increased colonic permeability (**B**). * $P < 0.05$ vs. vehicle + vehicle + LPS, # $P < 0.05$ vs. vehicle + DHEA-S + LPS by two-way analysis of variance

followed by Tukey's honestly significant difference test. Each column represents the mean ± S.E.M. The number of rats examined is shown in parentheses.

Figure 5

Sulpiride (200 mg/kg, subcutaneously) prevented the effects of DHEA-S (40 mg/kg) on LPS-induced visceral changes (**A**, **B**). However, domperidone (10 mg/kg, subcutaneously) did not modify the effects of DHEA-S on LPS-induced visceral changes (**C, D**). * $P < 0.05$ vs. vehicle + vehicle + LPS, # $P < 0.05$ vs. vehicle + DHEA-S + LPS by two-way analysis of variance followed by Tukey's honestly significant difference test. Each column represents the mean ± S.E.M. The number of rats examined is shown in parentheses.

Figure 6

L-NAME (10 mg/kg, intraperitoneally) abolished the effects of DHEA-S (40 mg/kg) on LPS-induced visceral changes (**A**, **B**). * $P < 0.05$ vs. vehicle + vehicle + LPS, # $P < 0.05$ vs. vehicle + DHEA-S + LPS by two-way analysis of variance followed by Tukey's honestly significant difference test. Each column represents the mean ± S.E.M. The number of rats examined is shown in parentheses.

Figure 7

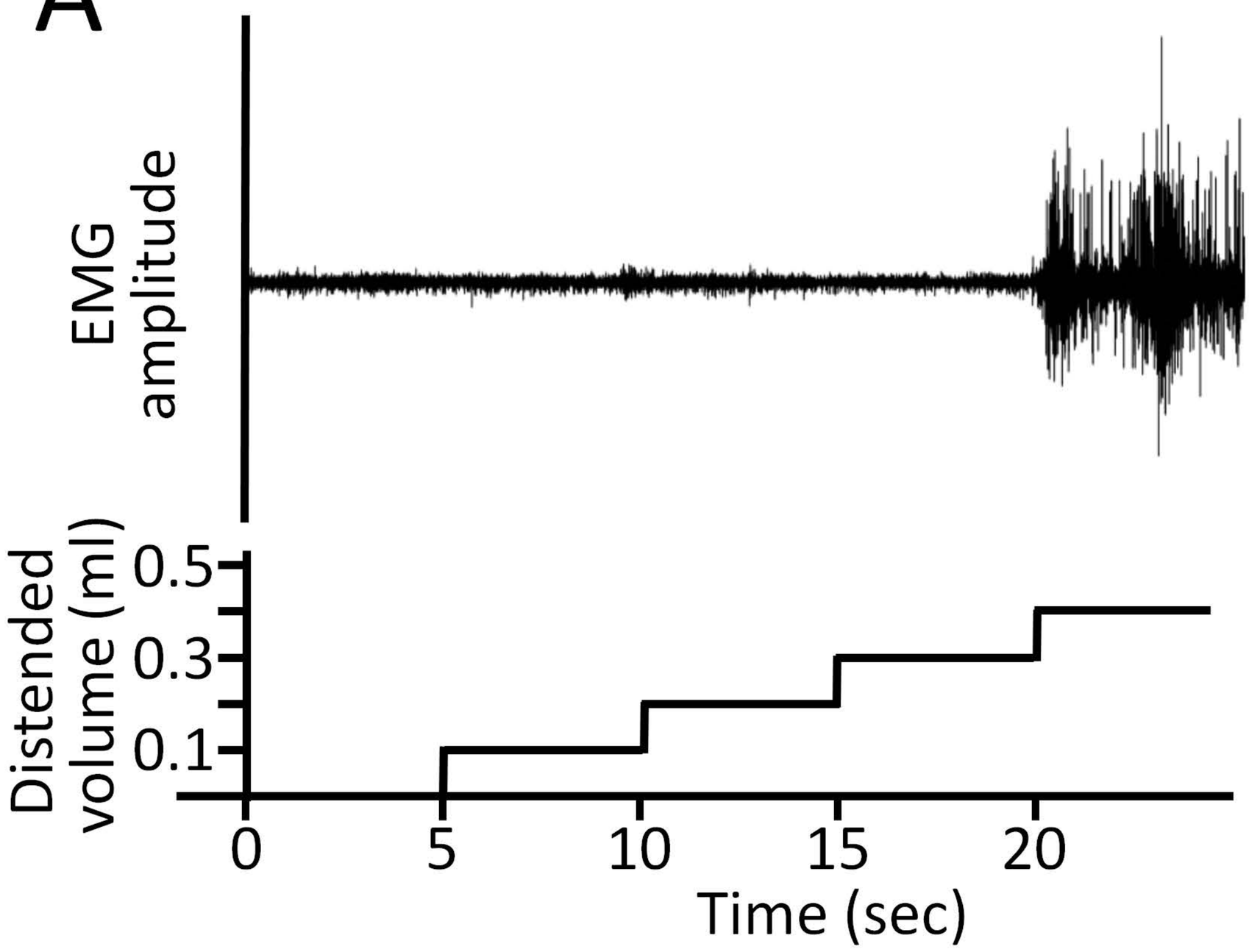
Naloxone (1 mg/kg, subcutaneously) fully reversed the effects of DHEA-S (40 mg/kg) on LPS-induced visceral changes (**A**, **B**). * $P < 0.05$ vs. vehicle + vehicle + LPS, # $P < 0.05$ vs. vehicle +

DHEA-S + LPS by two-way analysis of variance followed by Tukey's honestly significant difference test. Each column represents the mean ± S.E.M. The number of rats examined is shown in parentheses.

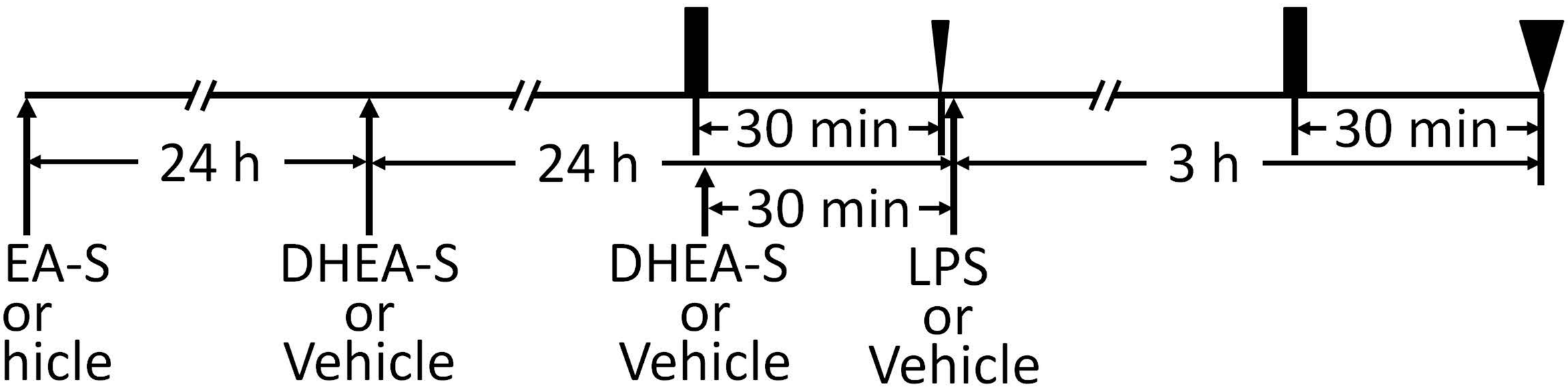
Figure 8

Astressin$_2$-B (100 μg/kg, intraperitoneally) suppressed the effects of DHEA-S (40 mg/kg) on LPS-induced visceral changes (**A**, **B**). * $P < 0.05$ vs. vehicle + vehicle + LPS, # $P < 0.05$ vs. vehicle + DHEA-S + LPS by two-way analysis of variance followed by Tukey's honestly significant difference test. Each column represents the mean ± S.E.M. The number of rats examined is shown in parentheses.

A


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

B


24 h
24 h
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30 min
DHEA-S or Vehicle
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DHEA-S or Vehicle
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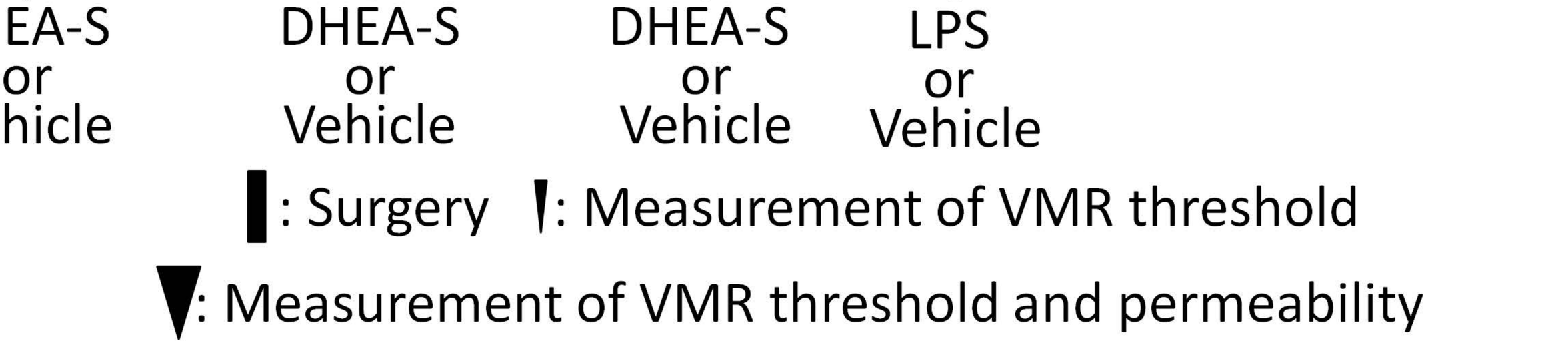
: Surgery
: Measurement of VMR threshold
: Measurement of VMR threshold and permeability

C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DHEA-S or Vehicle
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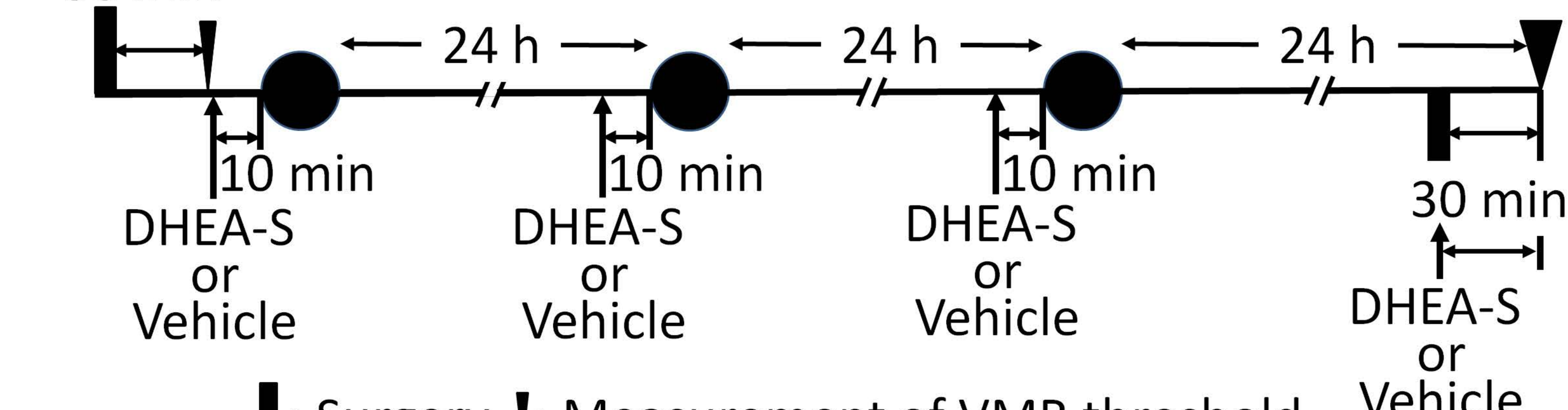
DHEA-S or Vehicle
: Surgery
: Measurement of VMR threshold
: Water avoidance or sham stress
: Measurement of VMR threshold and permeability

D


24 h
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DHEA-S or Vehicle
DHEA-S or Vehicle
DHEA-S or Vehicle
CRF or Vehicle
: Surgery
: Measurement of VMR threshold
: Measurement of VMR threshold and permeability

A

% change threshold

120
100
80
60
40
20
0

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

DHEA-S (mg/kg)

0 + Vehicle
40 + Vehicle
0 + LPS
5 + LPS
15 + LPS
40 + LPS
(1 mg/kg)

B

Colonic permeability (µg/g tissue)

70
60
50
40
30
20
10
0

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

DHEA-S (mg/kg)

0 + Vehicle
40 + Vehicle
0 + LPS
5 + LPS
15 + LPS
40 + LPS
(1 mg/kg)

A

% change threshold

120
100
80
60
40
20
0

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

Vehicle + Sham
DHEA-S + Sham
Vehicle + WAS
DHEA-S + WAS

B

Colonic permeability (µg/g tissue)

80
60
40
20
0

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

Vehicle + Sham
DHEA-S + Sham
Vehicle + WAS
DHEA-S + WAS

C

% change threshold

120
100
80
60
40
20
0

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

Vehicle + Vehicle
DHEA-S + Vehicle
Vehicle + CRF
DHEA-S + CRF

D

Colonic permeability (µg/g tissue)

80
60
40
20
0

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

Vehicle + Vehicle
DHEA-S + Vehicle
Vehicle + CRF
DHEA-S + CRF

A

Vehicle

Bicuculline

% change threshold

120

100

80

60

40

20

0

(6)

(5)

(6)
*

(5)
#

Vehicle + LPS

Vehicle + LPS

DHEA-S + LPS

DHEA-S + LPS

B

Vehicle

Bicuculline

Colonic permeability (µg/g tissue)

80

60

40

20

0

(6)

(5)

(5)
#

(6)
*

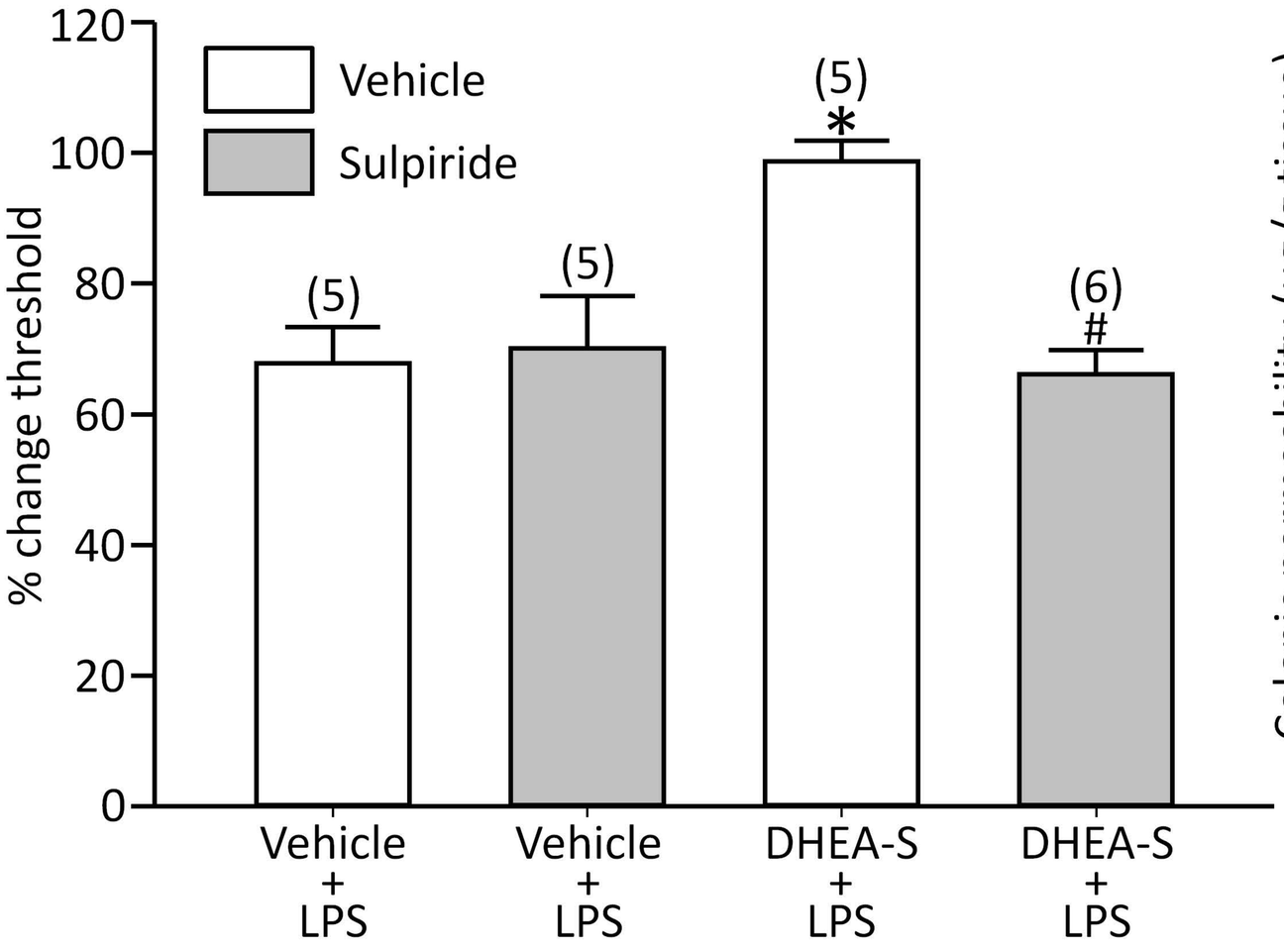
Vehicle + LPS

Vehicle + LPS

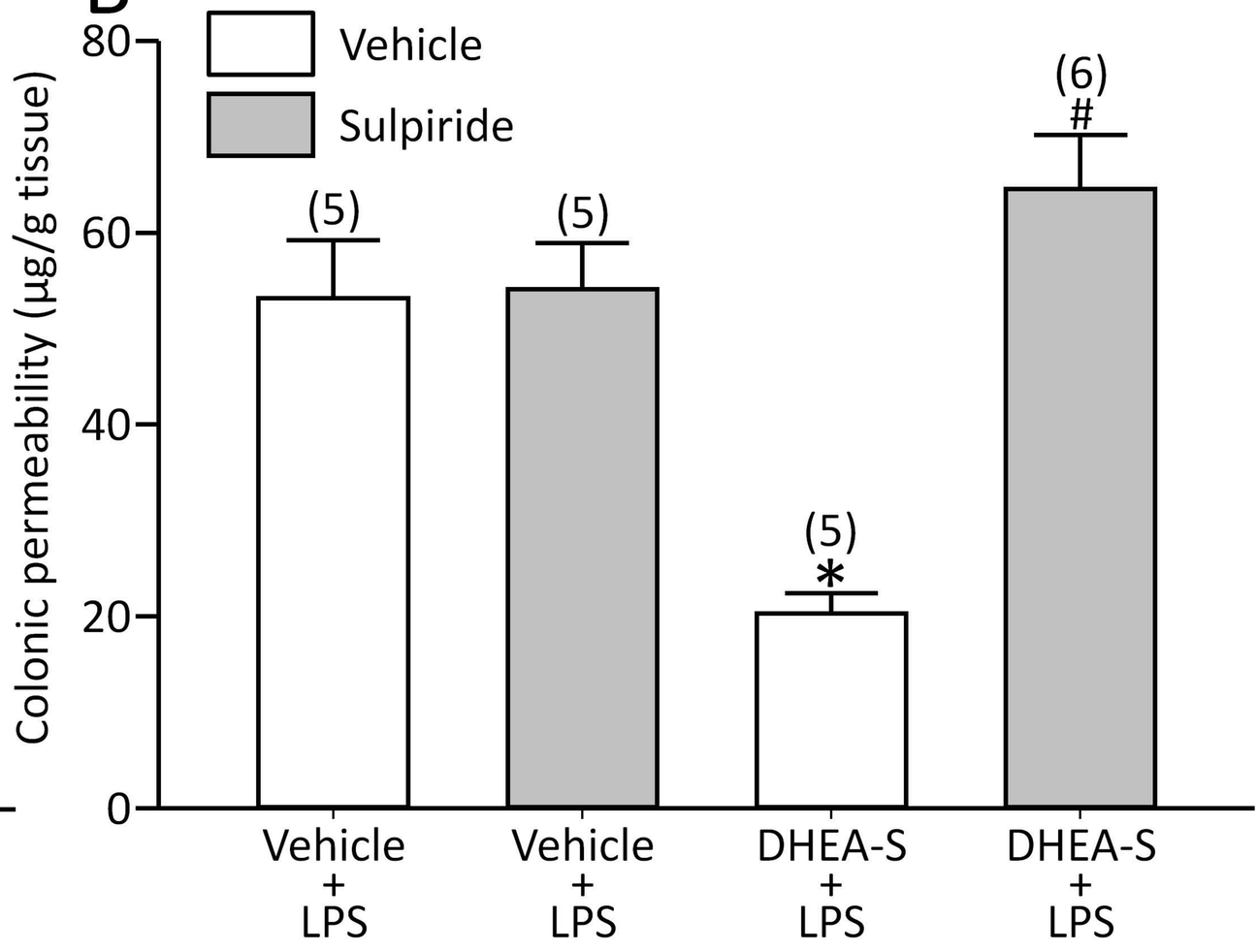
DHEA-S + LPS

DHEA-S + LPS

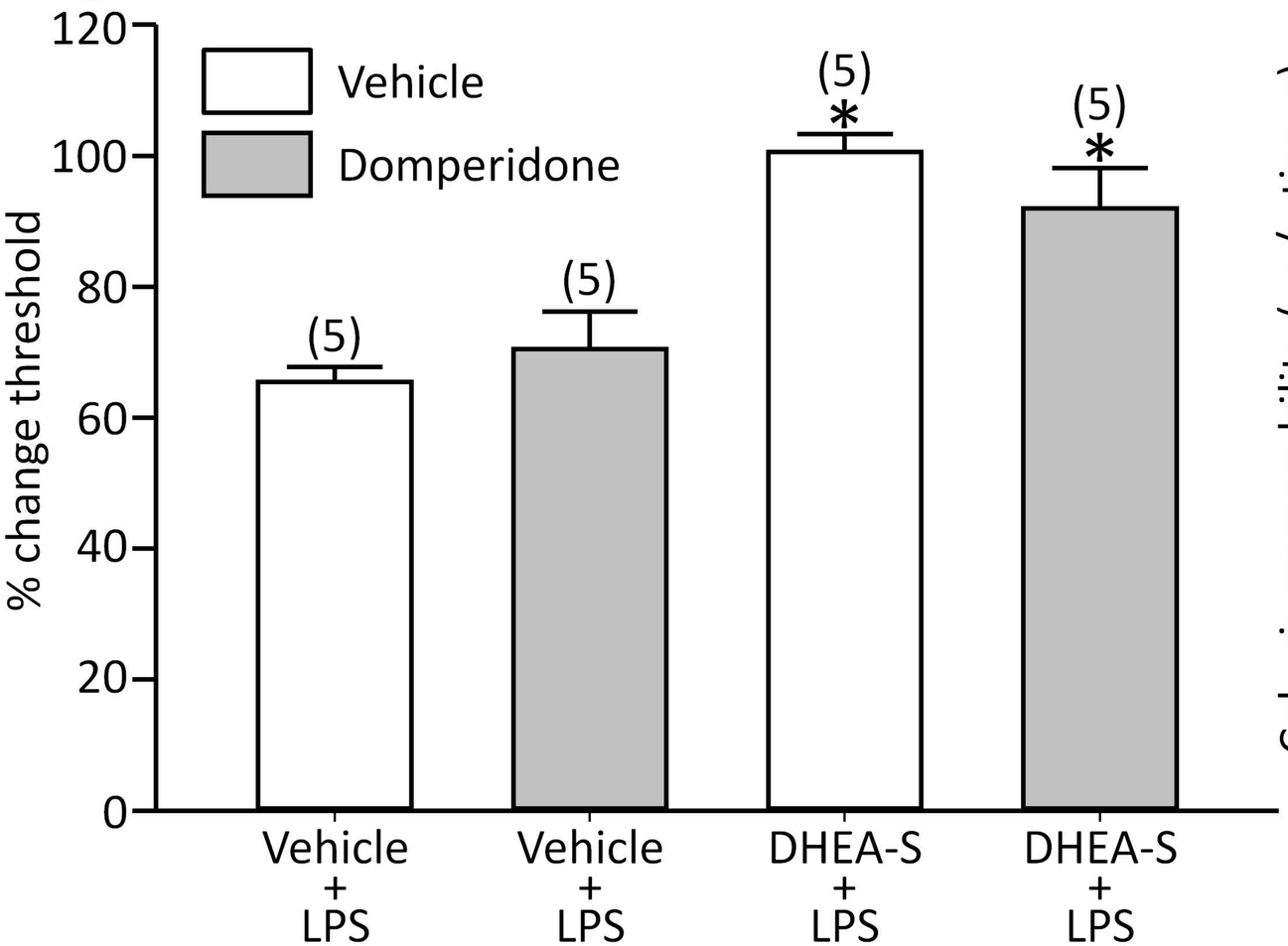
A


120
100
80
60
40
20
0
% change threshold
Vehicle
Sulpiride
(5)
(5)
(5)
*
(6)
#
Vehicle + LPS
Vehicle + LPS
DHEA-S + LPS
DHEA-S + LPS

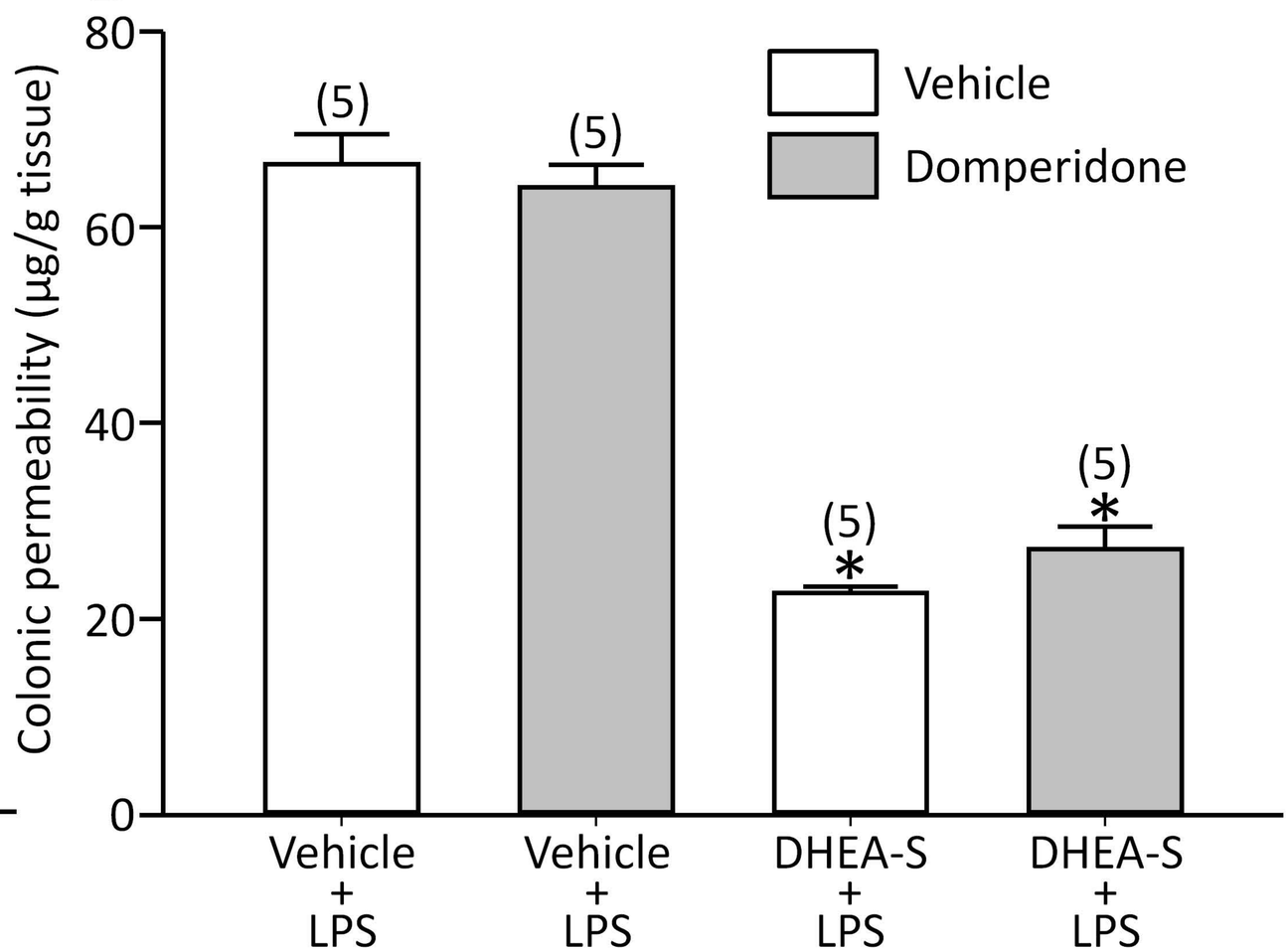
B


80
60
40
20
0
Colonic permeability (µg/g tissue)
Vehicle
Sulpiride
(5)
(5)
(5)
*
(6)
#
Vehicle + LPS
Vehicle + LPS
DHEA-S + LPS
DHEA-S + LPS

C


120
100
80
60
40
20
0
% change threshold
Vehicle
Domperidone
(5)
(5)
(5)
*
(5)
*
Vehicle + LPS
Vehicle + LPS
DHEA-S + LPS
DHEA-S + LPS

D


80
60
40
20
0
Colonic permeability (µg/g tissue)
Vehicle
Domperidone
(5)
(5)
(5)
*
(5)
*
Vehicle + LPS
Vehicle + LPS
DHEA-S + LPS
DHEA-S + LPS

A

Vehicle

L-NAME

% change threshold

120
100
80
60
40
20
0

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

Vehicle + LPS
Vehicle + LPS
DHEA-S + LPS
DHEA-S + LPS

B

Vehicle

L-NAME

Colonic permeability (µg/g tissue)

60
50
40
30
20
10
0

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

Vehicle + LPS
Vehicle + LPS
DHEA-S + LPS
DHEA-S + LPS

A

Vehicle

Naloxone

% change threshold

120
100
80
60
40
20
0

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

Vehicle + LPS
Vehicle + LPS
DHEA-S + LPS
DHEA-S + LPS

B

Vehicle

Naloxone

Colonic permeability (µg/g tissue)

60
50
40
30
20
10
0

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

Vehicle + LPS
Vehicle + LPS
DHEA-S + LPS
DHEA-S + LPS

A

Vehicle

Astressin$_2$-B

% change threshold

120
100
80
60
40
20
0

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

Vehicle + LPS
Vehicle + LPS
DHEA-S + LPS
DHEA-S + LPS

B

Vehicle

Astressin$_2$-B

Colonic permeability (µg/g tissue)

70
60
50
40
30
20
10
0

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

Vehicle + LPS
Vehicle + LPS
DHEA-S + LPS
DHEA-S + LPS