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Corticosterone mediates stress-related increased intestinal permeability in a region-specific manner

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Abstract

Background *Chronic psychological stress (CPS) is associated with increased intestinal epithelial permeability and visceral hyperalgesia. It is unknown whether corticosterone (CORT) plays a role in mediating alterations of epithelial permeability in response to CPS.* ***Methods*** *Male rats were subjected to 1-h water avoidance (WA) stress or subcutaneous CORT injection daily for 10 consecutive days in the presence or absence of corticoid receptor antagonist RU-486. The visceromotor response (VMR) to colorectal distension (CRD) was measured. The in situ single-pass intestinal perfusion was used to measure intestinal permeability in jejunum and colon simultaneously.* ***Key Results*** *We observed significant decreases in the levels of glucocorticoid receptor (GR) and tight junction proteins in the colon, but not the jejunum in stressed rats. These changes were largely reproduced by serial CORT injections in control rats and were significantly reversed by RU-486. Stressed and CORT-injected rats demonstrated a threefold increase in permeability for PEG-400 (MW) in colon, but not jejunum and significant increase in VMR to CRD, which was significantly reversed by RU-486. In addition, no differences in permeability to PEG-4000 and PEG-35 000 were detected between control and WA groups.* ***Conclusions & Inferences*** *Our findings indicate that CPS was associated with region-specific decrease in epithelial tight junction protein levels in the colon, increased colon epithelial permeability to low molecular weight macromolecules which were largely reproduced by CORT treatment in control rats and prevented by RU-486. These observations implicate a novel, region-specific role for CORT as a mediator of CPS-induced increased permeability to macromolecules across the colon epithelium.*

Keywords *chronic stress, corticosterone, epithelial permeability, region specificity, tight junction proteins.*

Abbreviations: CPS, Chronic psychological stress; CORT, corticosterone; CRF, corticotropin releasing factor; GR, glucocorticoid receptor; HPA, hypothalamic-pituitary-adrenal; IBS, Irritable Bowel Syndrome; PEG, polyethylene glycol; WA, water avoidance; ZO-1, zona occludens-1.

INTRODUCTION

The intestinal epithelial barrier regulates transport and host defense mechanisms at the mucosal interface with the outside environment to maintain gut homeostasis. Increased gut permeability and defects in intestinal barrier function are associated with several functional gastrointestinal disorders such as irritable bowel syndrome (IBS).[1] Studies using urine excretion showed increased intestinal permeability to ^{50}Cr-EDTA and low molecule polyethylene glycol (PEG-400) in patients with IBS.[2,3] More recently, Rao *et al.*[4] reported that both small bowel and colon permeability were increased in IBS patients with diarrhea by measuring saccharides lactulose and mannitol secretion in urine collections. Another study using intestinal biopsies in Ussing chambers demonstrated that colonic paracellular permeability to sulfonic acid was significantly increased in IBS patients regardless of IBS subtype.[5]

Chronic psychological stress (CPS) activates the hypothalamic-pituitary-adrenal (HPA) axis that affects various physiologic functions of the gastrointestinal tract including visceral sensitivity, gut permeability,

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and barrier function.[6–8] Stress-based animal models, reproducing several important pathological features of IBS such as visceral pain and epithelial barrier dysfunction, have been widely used to study the mechanistic aspects of IBS physiopathology. In an animal model of chronic stress, neonatal maternal deprivation increased total gut and colonic paracellular permeability in the adult rats.[9] Consistent with this, it was reported that chronic crowding stress caused significant increases in jejunal and colonic permeability in the rat that was associated with visceral hyperalgesia.[10] However, the mechanism(s) underlying chronic stress-induced alterations of intestinal permeability is largely unknown. Recently, the corticotropin releasing factor (CRF) that mediates the gastrointestinal responses to stress has been suggested to play a role in stress-related pathophysiology of IBS.[11] For example, peripheral administration of CRF in control animals mimicked the effect of stress, and treatment with the CRF agonists cortagine and stressin-1 produced similar effects,[12,13] suggesting that the CRF pathway may represent a potential therapeutic target in management of IBS.[14] The results of therapeutic trials have been mixed. Peripheral administration of the non-selective CRF antagonist alpha-helical CRF $^{9–41}$ showed a beneficial effect on symptoms including altered permeability in IBS patients,[15] but the selective oral CRF1 subtype receptor antagonist Pexacerfont (BMS-562086) demonstrated no improvement in symptoms in female IBS patients.[16]

An important component of the intestinal barrier is the intercellular tight junction complexes that restrict paracellular diffusion of hydrophilic macromolecules in a charge- and size-selective manner[8,17] and are crucial for the maintenance of barrier integrity. The transmembrane tight junction proteins include claudin-1, occludin, and zona occludens-1 (ZO-1) which are embedded in the lateral domains of both plasma membranes. Increasing evidence suggests that claudins are important in establishing the tight junction pore, and ZO-1 and occludin are important in leaky pathway.[18,19] Several studies showed that the barrier dysfunction in IBS was associated with down-regulation of ZO-1 and degradation of occludin by the proteasome in the intestinal tissues.[5,20,21] Consistently, it was reported that stress is associated with changes in tight junction protein levels and epithelial permeability.[22,23] However, little is known about how chronic stress regulates tight junction protein expression and function.

In a recent study, we reported that chronic intermittent stress-induced visceral hyperalgesia was reproduced in control rats treated with serial injections of glucocorticoid corticosterone (CORT) *in vivo* that mimicked the enhanced levels of CORT observed in the chronically stressed rat and was prevented by co-administration with corticoid receptor antagonist RU-486.[24] Relevant to this observation, it was reported that RU-486 attenuated acute restraint stress-induced decrease in ZO-1 proteins in colonic epithelial cells in mice,[25] suggesting that CORT may be involved in regulation of tight junction protein expression and intestinal barrier function.

The goal of the present study was to examine the novel hypothesis that the elevated levels of CORT under chronic intermittent stress conditions are linked to down-regulation of colon epithelial tight junction protein and enhanced permeability to macromolecules. Furthermore, we applied a new strategy using *in situ* single-pass intestinal perfusion methodology for precise measurement of the epithelial permeability for specific molecular weights in small bowel (jejunum) and large bowel (colon) simultaneously.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (200–220 g) were obtained from Charles River Laboratories (Wilmington, MA). Female rats were excluded in this study to avoid the influence of hormonal changes associated with the estrus cycle. Animals were housed in an animal facility that was maintained at 22 °C with an automatic 12 h light/dark cycle. All experiments were in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals. All experiments were approved by the University of Michigan Committee on Use and Care of Animals (animal protocol number 10137).

Water avoidance stress paradigm and in situ CORT treatment

Repeated WA stress and CORT treatment were performed in adult rats as described previously.[24] Briefly, the rats were subjected to WA stress for 1 h daily in between 9 and 11 AM for 10 consecutive days corresponding to the chronic stress protocol. In a separate study, several groups of rats were injected subcutaneously with 2 mg kg^{-1} RU-486 (Cayman Chemical, Ann Arbor, MI, USA) in 5% DMSO, 5% Cremophor EL (Sigma-Aldrich Corp, St. Louis, MO, USA), and 90% H_2O or vehicle only 10 min before the WA stress procedure consecutively for 10 days. To reproduce the serum CORT level observed in the stressed rats, 5% ethanol and 95% saline was used as vehicle, CORT (Sigma-Aldrich) was resolved in ethanol at 80 °C and then mixed with saline to a final concentration of 3 mg mL^{-1}, and injected subcutaneously at the dose of 3 mg kg^{-1} in between 9 and 11 AM daily for 10 days in a group of healthy control rats. For intervention studies, several groups of control rats were injected simultaneously with optimal doses of CORT (3 mg kg^{-1}) or vehicle in the presence and absence of RU-486 (2 mg kg^{-1}) during the serial injection period as described previously.[24] The optimal doses of CORT and RU-486 were determined by dose-response studies.[24] The animals were housed singly during the stress and injection procedures.

Immunofluorescence and western blotting

Immunofluorescence study was performed following the protocol as described in previous study.[26] Sections of jejunum and colon tissue (2–5 cm from the anal opening) from several groups of rats were dissected out and stored at −80 °C. The frozen tissues were sliced and postfixed with 1% paraformaldehyde (4 °C). Sections were then blocked with 5% normal goat serum and incubated with monoclonal anti-occludin with anti-ZO-1 or anti-claudin-1 antibody (1 : 100; Invitrogen, Carlsbad, CA, USA) and Alexa 488-conjugated or Alexa 594-conjugated secondary antibody, respectively (1 : 400; Molecular Probes, Carlsbad, CA, USA) and Hoechst 33342 (Molecular Probes). Slides were imaged using an Olympus BX 60 (Olympus Optical Co. Ltd., Tokyo, Japan) upright fluorescence microscope. The pixel density of immunofluorescence staining was measured *via* Image J software (National Institutes of Health) by randomly selecting six regions from each picture along the outer surface of epithelia cells that were labeled by tight junction antibodies.

For immunoblot analysis, colon and jejunum mucosa were scraped off and lysed with NP40 lysis buffer (50 mmol L^{-1} Tris-HCl, 150 mmol L^{-1} NaCl, 1% NP40, pH 8.0) supplied with protease inhibitor cocktail (Roche, Indianapolis, IN, USA).[27] Samples were sonicated and centrifuged at 11 750 ***g*** at 4 °C for 10 min. Supernatants were collected for SDS-PAGE analysis. The following primary antibodies were used: anti-claudin-1 antibody (1 : 5000; Invitrogen), anti-occludin antibody (1 : 2000; Invitrogen), anti-ZO-1 antibody (1 : 500; Invitrogen) that detects both α^+ and α^- isoforms of ZO-1,[28] anti-glucocorticoid receptor (GR; 1 : 1000; Santa Cruz Biotech, Santa Cruz, CA, USA), anti-E-cadherin (1 : 5000; Abcam, Cambridge, MA, USA), or anti-β-actin antibody (1 : 5000; Sigma-Aldrich) overnight at 4 °C. The corresponding immunoblot bands were scanned at 1200 dots per inch and semi-quantified with Image J software from the National Institutes of Health.

Regional *in situ* single-pass intestinal perfusion

Intestinal perfusion experiments were performed on rats *in situ* from different treatment groups according to the methods described previously.[27] After anesthesia with ketamine (40–60 mg kg^{-1} i.p.), surgery was performed on each animal while lying on top of a heating pad to maintain the body temperature. Isopropyl alcohol was used to sterilize the abdominal area, and a 1.5 cm midline incision was made longitudinally to expose the small intestine. Before cannulations of different intestinal segments, the common bile duct was ligated by silk suture. Eight centimeter long segment of jejunum (i.e., 2 cm distal to the ligament of Treitz) and 3 cm segment of colon (i.e., 0.5 cm distal to the cecum) were used for simultaneous perfusion, and incisions were then made at the proximal and distal ends. Glass cannulas (2.0 mm outer diameter), attached to Tygon tubing, were inserted at each end of the jejunal segment and secured in place with silk sutures. After cannulations, the isolated intestinal segment was covered with saline-wetted gauze and parafilm to prevent dehydration of the tissue. The animals were then transferred to a temperature-controlled Plexiglas perfusion chamber (31 °C) to maintain body temperature during the perfusion experiment. The inlet tubing was connected to a 10 mL syringe placed on a perfusion pump (model 22; Harvard Apparatus, South Natick, MA), and the outlet tubing was placed in a collection vial. The perfusate (pH 6.5) contained 10 mmol L^{-1} MES, 135 mmol L^{-1} NaCl, 5 mmol L^{-1} KCl, [^{14}C]-polyethylene glycol (PEG)-200, and [^{3}H]-PEG-400 0.01% (w/v) to examine the intestinal permeability to intermediate molecules, which flowed through the proximal jejuna and colonic segment at a rate of 0.14 mL min^{-1}. A 100 μL aliquot of perfusate was added to 5.5 mL of scintillation cocktail (Ecolite MP Biomedicals, Solon, OH), and the samples were analyzed with a dual-channel liquid scintillation counter (Beckman LS 6000 SC; Beckman Coulter Inc., Fullerton, CA, USA). In some experiments, [^{14}C]-PEG-4000 and [^{3}H]-PEG-35 000 (final concentration = 0.01%) were perfused in the jejunum and colon simultaneously to examine the permeability to large molecules. The perfusion last for 120 min, samples were collected every 10 min, and the effective permeability (P_{eff}) was calculated after reaching steady state (30 min) using the following formula. The water flux was corrected by using gravimetric method.

$$P_{eff} = \frac{-Q \times \ln(C_{out}/C_{in})}{2\pi RL}$$

In the above equation, Q is the perfusate flow rate, R is the intestinal radius (0.1 cm), L is the length of intestine, C_{in} is the inlet drug concentration, and C_{out} is the outlet drug concentration (corrected for water flux).

Assessment of visceral pain: visceromotor response to colorectal distention

Visceral motor response (VMR) to colorectal distension (CRD) is frequently used to measure enhanced visceral pain (hyperalgesia).[24,29,30] Assessment of VMR to CRD was conducted in rats on day 11 of the chronic WA stress procedure as described previously.[24,26] Briefly, rats were deeply anesthetized with subcutaneous injection of a mixture of ketamine (60 mg kg^{-1}) and xylazine (5 mg kg^{-1}), and teflon-coated, 32-gauge stainless steel wires were inserted into the external oblique pelvic muscles superior to the inguinal ligament. A minimum of 5 days were allowed for recovery from surgery before the distension experiments. Before test, rats were habituated in the testing room and placed in the testing Plexiglas cylinders for 30 min per day for three consecutive days prior to experiments. During the test, a series of CRD was conducted to constant pressures of 10, 20, 40, 60 mmHg by a custom-made distension control device. Each distention consisted three segments: a 20-s predistention baseline period, a 20-s distention period, and a 20-s period after termination of CRD with a 4-min inter-stimulus interval. The electromyographic (EMG) activity was amplified and digitized using a SPIKE2/CED 1401 data acquisition interface. The EMG activity was rectified, and the increase in the area under the curve (AUC) of EMG amplitude during CRD over the baseline period before CRD was recorded as the response.

Statistical analysis

The electromyographic amplitudes, represented by calculating AUC, were normalized as percentage of baseline response for the highest pressure (60 mmHg) for each rat. The effects of stress and/or pharmacologic treatment on the VMR to CRD was analyzed by comparing the poststress or posttreatment measurements with the baseline values at each distention pressure using a repeated-measures, two-way ANOVA followed by Bonferroni posttest comparisons. For comparisons of intestinal permeability, one-way analysis of variance followed by Tukey's analysis was performed to examine the statistical significance. Unpaired Student's t-test was used to examine the data between two groups for other studies. Results were expressed as means ± SE. $P < 0.05$ was considered statistically significant.

RESULTS

I. Water avoidance stress and CORT treatment in control rats were associated with decreased immunofluorescence signal for colon epithelial tight junction proteins that were largely reversed by treatment with the corticoid receptor antagonist RU-486.

As depicted in Fig. 1A, significant decreases in the immunofluorescence signals for tight junction proteins including claudin-1, occludin, and ZO-1 were observed in the tight junction region in colon tissue samples from WA stress rats compared with controls which were largely blocked by treatment with RU-486. Colon samples from the WA stressed rat demonstrated a 58.9 ± 5.1% decrease in claudin-1, 61.9 ± 2.3% decrease in occludin, and 51.6 ± 2.6% decrease in ZO-1 ($P < 0.01$; $n = 4$), respectively, in the fluorescence signal intensity in the tight junction region compared with the control samples (Fig. 1B). Treatment with RU-486 significantly blocked this effect. The intensities of claudin-1, occludin, and ZO-1 in RU-486-treated WA samples were reversed to 71.8 ± 7.5%, 65.9 ± 3.8%, 84.8 ± 6.4% of their corresponding control levels, respectively ($P < 0.05$; $n = 4$).

To examine whether corticosterone (CORT) plays a role in modulation of epithelial tight junctions, a groups of rats were serially injected with CORT (3 mg kg^{-1}) to mimic the levels of CORT observed during WA stress. The serum CORT levels were significantly increased in WA stressed and CORT-injected control rats after 10 days of treatment, similar to what we previously reported.[24] As shown in Fig. 1B, treatment of control rats with serial CORT injections reproduced the effect of WA stress on the reduction of

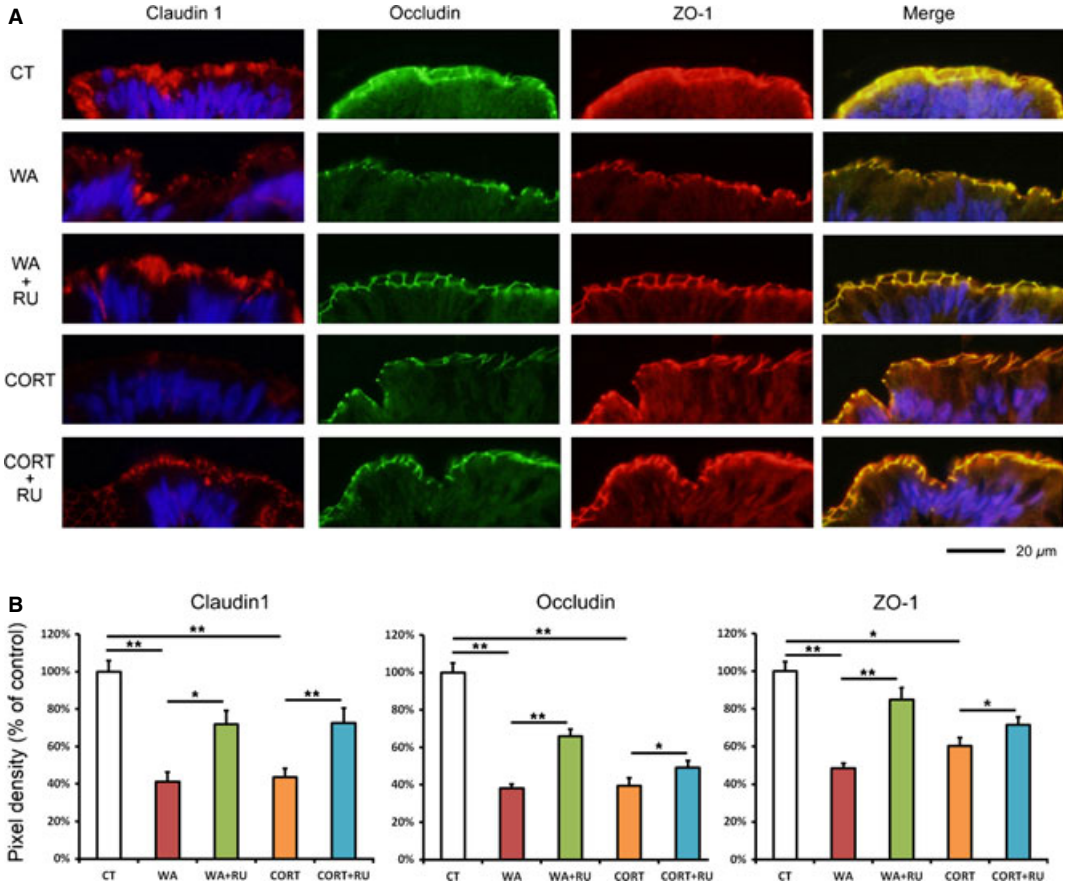

Figure 1 Effects of water avoidance (WA) stress and corticosterone treatment (CORT) in control rats on colon epithelial tight junction protein immunofluorescence levels ± co-administration of the corticoid receptor antagonist RU-486. (A) Immunofluorescence images of colon were obtained from the following groups: Control (CT), WA stress, WA stress together with RU-486 (WA+RU), CORT, and CORT together with RU-486 treatment (CORT+RU). Representative images are shown. (B) The immunofluorescence signals of colon epithelial tight junction proteins measured included: claudin-1, occludin, and ZO-1. WA stressed rats demonstrated a significant reduction in tight junction immunofluorescence signals which was significantly reversed by treatment with RU-486. Treatment of control rats with serial injections of CORT largely reproduced the results observed in the colon in WA stressed rats which were prevented by co-administration of RU-486. Data are expressed as mean ± SE, $n = 4$ in each group. *, $P < 0.05$; **, $P < 0.01$.

claudin-1 (decreased 56.3 ± 4.6%) and occludin (decreased 60.5 ± 4.1%), and a somewhat lower effect on ZO-1 (decreased 39.8 ± 4.4%) compared with non-treated controls ($P < 0.05$; $n = 4$). The CORT-induced reduction in immunofluorescence signals of the tight junction proteins including claudin-1, occludin, and ZO-1 were significantly reversed by RU-486 treatment ($P < 0.05$; $n = 4$), similar to those of WA samples treated with RU-486. No significant changes in staining intensities of these tight junction proteins were observed between RU-486-treated controls and non-treated controls (data not shown). To examine whether the changes observed in colon were region-specific, we performed parallel studies using samples from the jejunum prepared from the same rats. As shown in Fig. 2, no significant differences were observed in the tight junction protein immunofluorescence signals in the jejunum from WA stress and CORT-injected rats compared with controls ($P > 0.05$, $n = 4$).

II. Water avoidance stress and CORT treatment in control rats demonstrated region-specific reduction in epithelial tight junction protein levels in the colon that were significantly blocked by treatment with RU-486

In order to confirm that the decrease in epithelial tight junction immunofluorescence signals correlated with decreased tight junction protein levels, the mucosa from colon and jejunum were removed for Western immunoblot analysis. As shown in representative western blotting images and histogram analysis (Fig. 3), colon epithelia cells from the WA stressed rats demonstrated a 86.9 ± 1.0% decrease in claudin-1, 71.3 ± 0.7% decrease in occludin, and 80.4 ± 11.9% decrease in ZO-1 ($P < 0.01$; $n = 5$), respectively, in the band intensity compared with the control samples

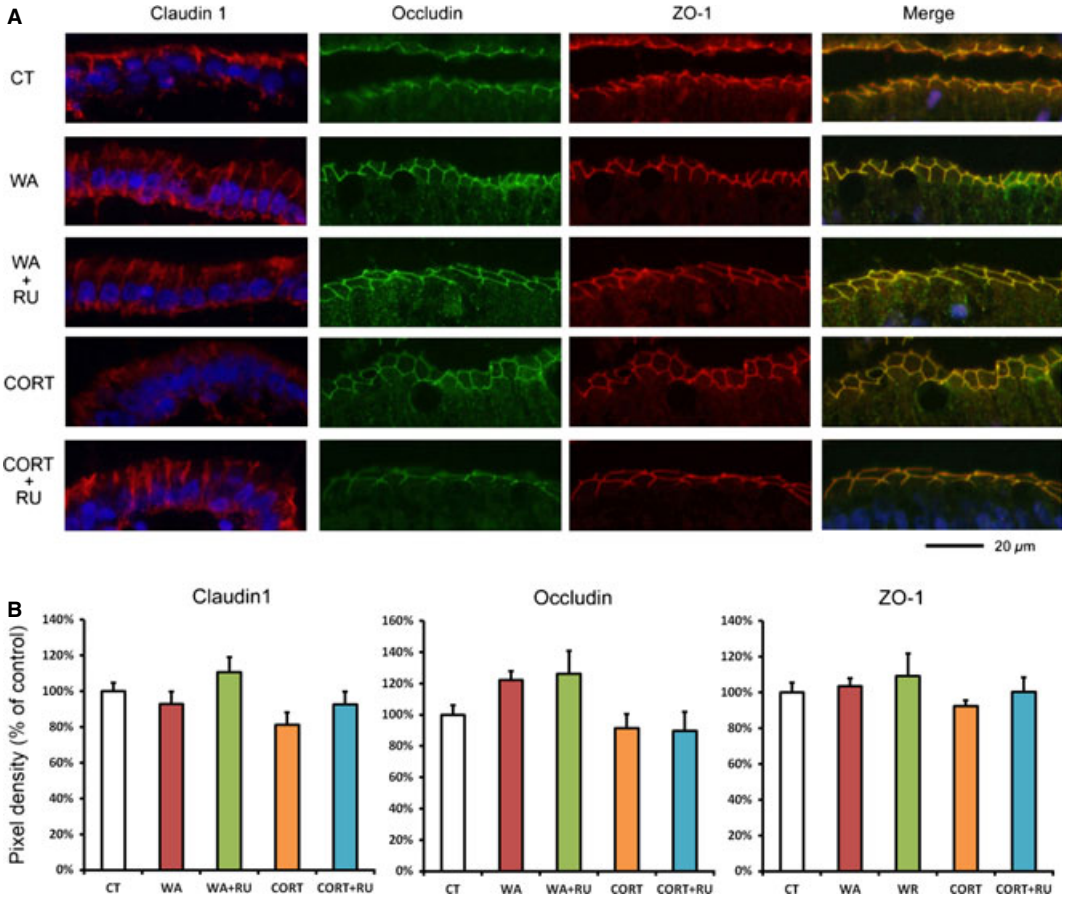

Figure 2 Effect of water avoidance (WA) stress and CORT treatment in control rats ± RU-486 on jejunum epithelial tight junction protein immunofluorescence levels. (A) Immunofluorescence images of jejunum were obtained from the following groups: Control (CT), WA stress, WA stress + corticoid receptor antagonist RU-486 (WA+RU), corticosterone injection (CORT), CORT + RU-486 (CORT+RU). Representative images are shown. No significant changes were observed in the immunofluorescence signals for claudin-1, occludin, and ZO-1 in the jejunum ($n = 4$). (B) This panel presents the summary data in histogram format (pixel densities) for the jejunum epithelial tight junction proteins measured. No significant differences were observed in jejunum epithelial tight junction protein immunofluorescence signals ($n = 4$).

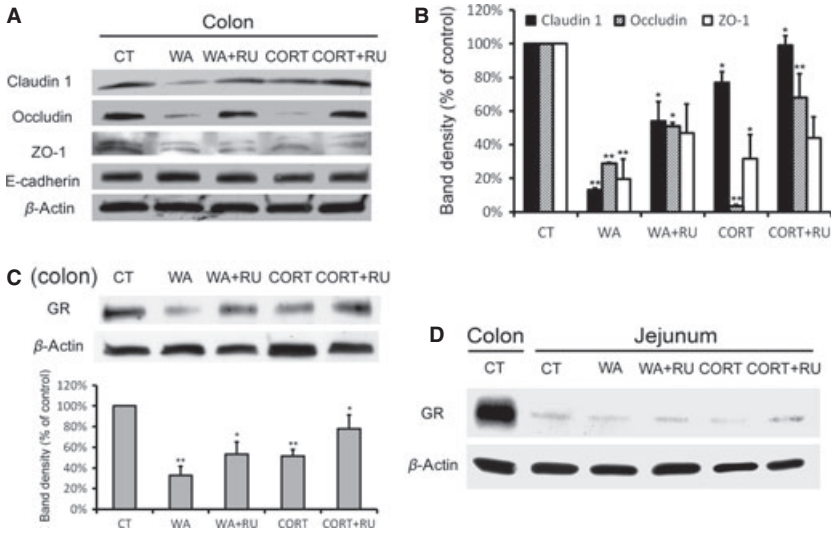

Figure 3 Effect of WA stress and corticosterone treatment in control rats ± RU-486 on colon epithelial tight junction protein levels. (A) Significant decreases in tight junction proteins claudin-1, occludin, and ZO-1 levels were detected in colon samples from WA stress rats compared with controls which were prevented by treatment with the corticoid receptor antagonist RU-486. CORT injections in control rats reproduced the effect of WA stress which was prevented by treatment with RU-486. (B) Histogram showing the immunoblot band density of claudin-1, occludin, and ZO-1 protein. ZO-1 band intensity reflected both α^+ and α^- isoforms. Statistical analysis was performed either between control group and WA stress or CORT injection group, or between WA stress group, CORT injection group, and their corresponding RU-486 intervention group. (C) Immunoblot results of GR demonstrated decreased levels of GR protein in colon epithelial cells in WA stressed and CORT-injected control rats, which were significantly reversed by RU-486 treatment. (D) No significant changes were observed in GR levels in epithelial cells in the jejunum in WA stressed and CORT-injected rats. Data are normalized to β-actin and expressed as mean ± SE, $n = 5$ in each group. *, $P < 0.05$; **, $P < 0.01$.

(Fig. 3B). Treatment with RU-486 significantly prevented the decreases in claudin-1 and occludin. The levels of claudin-1 and occludin in RU-486-treated WA samples were reversed to 54.0 ± 11.7% and 51.0 ± 2.0% of their corresponding control levels, respectively ($P < 0.05$; $n = 5$). The level of ZO-1 showed a trend of reversal after RU-486 treatment (recovery to 47.0 ± 17.2%) compared with the controls ($P = 0.20$; $n = 5$). Furthermore, treatment of control rats with serial CORT injections demonstrated similar effects compared with WA stress on the reduction of claudin-1 (decreased 23.1 ± 6.5%) and occludin (decreased 96.5 ± 0.9%), and ZO-1 (decreased 68.3 ± 14.2%) as shown in Fig. 3B ($P < 0.05$; $n = 5$). Similar to the results observed in WA stress rats treated with RU-486, the CORT-induced reduction in band intensity of the tight junction proteins including claudin-1 and occludin were significantly reversed by RU-486 treatment ($P < 0.05$; $n = 5$). Zona occludens-1 also demonstrated a slight trend of reversal (to 44.1 ± 12.4% of the controls) with RU-486 treatment ($P = 0.16$; $n = 5$). In addition, no significant changes in the expression level of E-cadherin were observed in the colon epithelial cells between these groups of rats. This data validates that non-treated rat control group is a proper control for the rat groups with different treatments that used different vehicles.

To examine the potential linkage of changes in epithelial tight junction proteins to the HPA axis and corticoid receptors, we measured the protein level of glucocorticoid receptor (GR) in WA stressed rats and CORT-treated control rats. As shown in Fig. 3C, GR levels were significantly decreased 67.2 ± 8.9% and 48.4 ± 6.2% in colon epithelial cells in stressed rats and CORT-injected control rats, respectively ($P < 0.01$; $n = 5$). Treatment with RU-486 largely prevented the changes in GR levels in both populations; specifically, the protein level of GR was reversed to 53.3 ± 11.9% and 77.9 ± 13.4% of their corresponding control levels in stressed rats and CORT-injected control rats, respectively, after treatment with RU-486 ($P < 0.05$; $n = 5$). Interestingly, the protein level of GR receptors was approximately 10-fold less in epithelial cells in jejunum compared with colon in healthy control rats. No significant changes in the GR expression level were observed in WA stressed and CORT-injected rats in the jejunum compared with controls as shown in Fig. 3D ($P > 0.05$; $n = 5$).

III. Water avoidance stress and CORT treatment in control rats were associated with region-specific increased permeability to PEG-400 in the colon that was prevented by RU-486.

Various methods are employed to measure the intestine permeability, for example, horseradish

peroxidase, dextran, lactulose, and radiolabeled polyethylene glycols.[31] We employed *in situ* single-pass intestinal perfusion using sections of jejunum and colon to measure intestinal permeability directly in a region-specific manner. For this purpose, we used radiolabeled PEG compounds of several molecular weights. In this study, colon specimens demonstrated a threefold increase in permeability for PEG-400 (MW) in WA stress rats compared with controls ($P < 0.01$; $n = 8$). Treatment of WA stress rats with RU-486 prevented the increase in permeability for PEG-400 ($P < 0.05$; $n = 8$). Serial CORT injections in control rats mimicked this effect by increasing the permeability by threefold ($P < 0.01$; $n = 8$) and RU-486 treatment prevented this change ($P < 0.05$; $n = 8$; Fig. 4). The jejunum demonstrated no significant differences in permeability for PEG-400 (Fig. 4). No statistical significant differences in permeability for PEG-200 were detected in all groups (Fig. 5). In addition, no significant differences in permeability for PEG-4000 and PEG-35 000 were detected in both jejunum and colon between control and WA groups (Fig. 6). However, the permeability for both PEG-200 and PEG-400 are lower in the colon compared with the jejunum ($P < 0.002$ and $P < 0.001$, respectively). For PEG-4000, the difference in permeability between jejunum and colon is barely significant ($P = 0.047$; $n = 8$), while for PEG-35 000 there was no difference in permeability between these intestinal segments ($P > 0.50$). These results indicate that WA stress is associated with an increase in colon epithelial permeability via a CORT-mediated pathway and that this effect is likely limited to molecules of molecular weight less than 4000.

IV. Treatment with the corticoid receptor antagonist RU-486 decreased visceral hypersensitivity in WA stressed and CORT-treated rats.

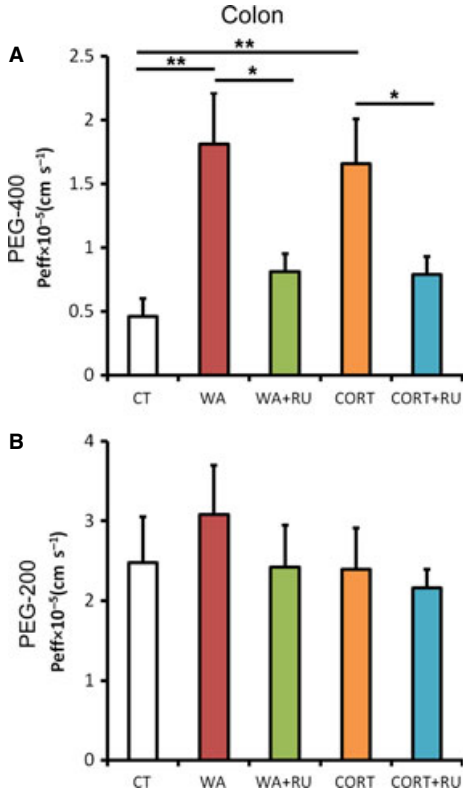

Figure 4 Effect of water avoidance stress (WA) and CORT treatment ± RU-486 in controls on colon permeability to PEG-400. Eight rats in each group were subjected to *in situ* single-pass intestinal perfusion analysis. [^{14}C]-PEG-200 and [^{3}H]-PEG-400 (final concentration = 0.01%) were perfused in the colon of rats from all groups. (A) WA stress was associated with a significant (3-fold) region-specific increase in the colon permeability to PEG-400. Co-administration of corticoid receptor antagonist RU-486 prevented the changes observed in the colon. Treatment of control rats with serial CORT injections mimicked the results observed in the WA stress rat which was prevented by co-administration of RU-486 ($P < 0.05$; $n = 8$). (B) No significant change in permeability to PEG-200 was observed in the colon from all groups ($n = 8$). Statistical significance indicated by *, $P < 0.05$; **, $P < 0.01$.

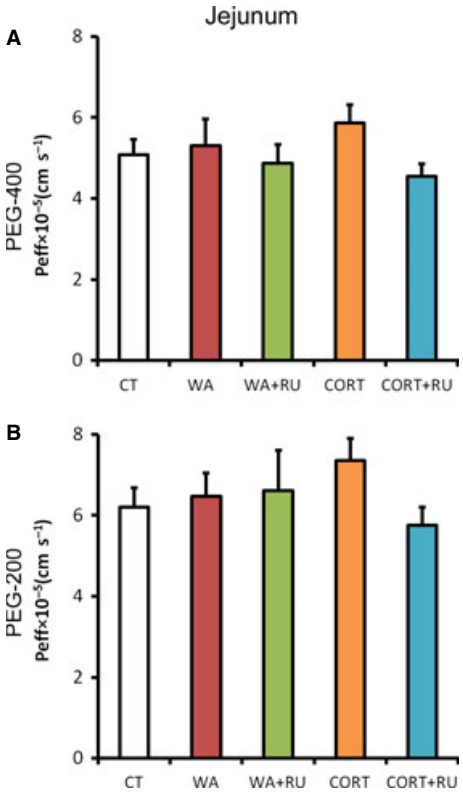

Figure 5 Effect of water avoidance (WA) stress and corticosterone (CORT) treatment in control rats on jejunum permeability to PEG-400 ± RU-486. Eight rats in each group were subjected to *in situ* single-pass intestinal perfusion analysis. [^{14}C]-PEG-200 and [^{3}H]-PEG-400 (final concentration = 0.01%) were perfused in the jejunum of rats from all groups. No significant change in permeability to PEG-400 (A) and PEG-200 (B) was observed in the jejunum from all groups ($n = 8$).

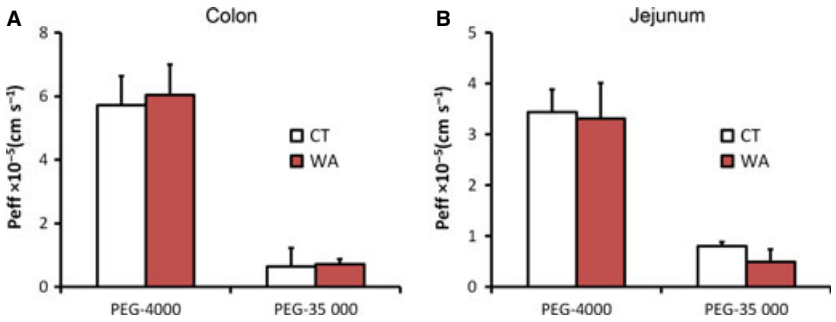

Figure 6 Effects of water avoidance (WA) stress and CORT treatment in control rats on colon and jejunum permeability to PEG-4000 and PEG-35 000. [^{14}C]-PEG-4000 and [^{3}H]-PEG-35 000 (final concentration = 0.01%) were perfused in the jejunum and colon in both control and WA stressed rats. Neither colon (A) nor jejunum (B) showed significant differences in permeability to PEG-4000 and PEG-35 000 between the control and WA groups (n = 6).

We demonstrated previously that chronic WA stress is associated with visceral hyperalgesia in the rat as assessed by measurements of the VMR to CRD and fecal output.[26] Here, we examined whether reversal of tight junction protein expression and colon permeability by corticoid receptor antagonist RU-486 is associated with prevention of visceral hyperalgesia in WA stressed rats. As shown in Fig. 7A, chronic WA stress resulted in significant increases in VMR to CRD at the pressures of 40 and 60 mmHg compared with the baseline ($P < 0.05$), indicating visceral hyperalgesia in stressed rats. Repeated treatment with RU-486 during the stress procedure significantly decreased the stress-induced increases in the VMR to CRD at 40 and 60 mmHg pressures ($P < 0.05$), while it had no significant effect on VMR in sham control rats. Similarly, as shown in Fig. 7B, repeated CORT-injection increased VMR to CRD at pressures of 40 and 60 mm Hg ($P < 0.05$). RU-486 treatment during the injection period significantly decreased VMR to CRD at 40 and 60 mmHg pressures in CORT-injected rats ($P < 0.05$). These results are consistent with our previous report.[24]

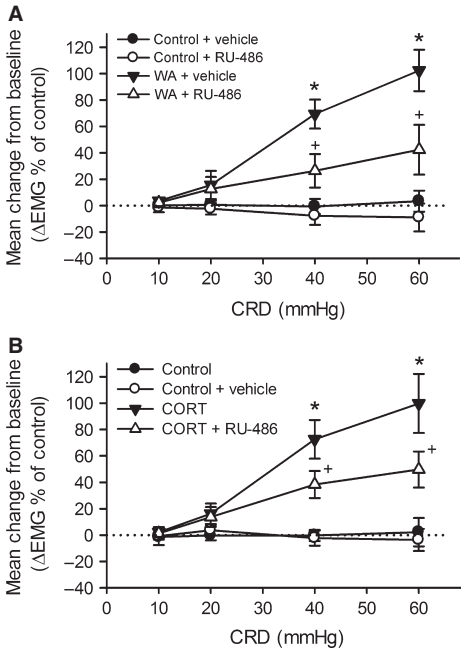

Figure 7 Effect of the corticoid receptor antagonist RU-486 on the VMR to CRD in WAS rats and CORT-treated control rats. (A) VMR to CRD in control rats, WA stress rats, and WA stress rats treated with RU-486 during the stress procedure. (B) VMR to CRD in control rats, CORT-injected rats, and CORT-injected rats treated with RU-486 during the injection period. RU-486 significantly prevented the enhanced VMR to CRD in both the WAS rats and CORT-injected control rats.EMG amplitude expressed as mean change from baseline. Data are expressed as mean ± SE, n = 8 in each group. *, $P < 0.05$ between control and WA stress or CORT-injected rats; +, $P < 0.05$ between WA stress or CORT-injected rats receiving RU-486 or vehicle.

DISCUSSION

The objective of the current study was to answer the question: Is chronic intermittent WA stress in the rodent linked to CORT-mediated effects on intestinal epithelial tight junction protein expression and function? Our results support the novel observation that chronic intermittent WA stress induces CORT-mediated region-specific reduction in epithelial tight junction proteins in the colon, but not in the jejunum. Complementary functional studies revealed the corresponding region-specific alterations of epithelial permeability because PEG-400 demonstrated increased permeability across the colonic epithelium, but not the jejunum in WA stress rats. Treatment of stressed rats with the corticoid receptor antagonist RU-486 during the WA stress period significantly reversed these changes in colon tight junction protein expression and permeability. These results were largely reproduced in control rats treated with serial injections of CORT that mimicked the serum levels of CORT

observed in the WA stressed rats, and treatment with RU-486 prevented these effects.

Chronic psychological stress is associated with activation of the HPA axis culminating in the release of the cortisol in the human and corticosterone in the rodent.[32] Chronic psychological stress affects the digestive system resulting in visceral hyperalgesia and altered bowel function in rodent models and the human,[8,22,33] which may be related to alterations in gastrointestinal epithelial permeability.[34,35] The CRF/CRF-receptor pathway has been implicated in the increased epithelial permeability to macromolecules in acute and chronic stress animal models.[13,36] Few studies have been conducted to specifically examine the potential role of corticosterone (CORT) in relation to barrier function and visceral hyperalgesia in stress. In a previous study, we observed increased CORT levels associated with visceral hyperalgesia in the chronic, intermittent WA stress rat model that was reproduced in control rats treated with CORT *in vivo* and prevented by treatment with the corticoid receptor antagonist RU-486, supporting a role for CORT in mediating these effects.[24] In this study, we demonstrated that chronic WA stress induced visceral hyperalgesia that was significantly reversed by treatment with corticoid receptor antagonist RU-486. Moreover, we showed that CORT induced down-regulation of tight junction proteins and increase in colon epithelial permeability, similar to the effects caused by chronic WA stress, further supporting the role of CORT in mediating visceral hyperalgesia and alterations of barrier function. Consistent with this, increased CORT levels and recruitment of mast cells were observed and associated with increased colon epithelial permeability and mucosal inflammation in a crowding-stress model.[35] We recognize that our results using the WA stress model do not likely explain all presentations of visceral hyperalgesia in IBS. Nevertheless, it is well accepted that chronic intermittent stress is associated with visceral hyperalgesia in animal models and a significant percentage of individuals with IBS.[37–39]

The epithelial tight junction complex includes the proteins claudin, occludin, and ZO-1. Some claudins such as claudin-1 together with occludin and ZO-1 are important in assembly and maintenance of the tight junctions, while other claudins such as claudin-2 tend to loosen the tight junctions.[40] In the chronically WA stressed and repeatedly CORT-injected rats, we observed significant reduction of claudin-1, occludin, and ZO-1 protein expression, which contributes to the increased colon epithelial permeability. Similar observations have been reported in animal models and human patients with IBS symptoms.[5,25,41] The mechanism(s) underlying down-regulation of these tight junction proteins remains largely unknown. However, it has been reported that clinically diarrhea-prone IBS patients showed significant down-regulation in occludin and claudin-1 mRNA and protein in the colonic mucosa.[5,42] In our study, we observed similar changes in expression in glucocorticoid receptor (GR) in parallel with the changes in tight junction proteins in colon epithelial cells in the WA stressed and CORT-treated rats that were largely blocked by corticoid receptor antagonist RU-486. Glucocorticoid receptors are progressively activated during acute stress and at the peaks of the diurnal rhythm for ACTH levels.[43] Several studies have shown that GR receptor itself is down-regulated in the brain following chronic stress or long-term treatment of CORT.[44–46] Consistent with this, our results demonstrated decreased protein expression of GR receptor in colon epithelial cells in the rat under chronic stress condition. It has been reported that corticoid receptors regulates the expression of tight junction proteins such as claudin-1 and occludin.[47–49] Being a positive transcription factor, down-regulation of GR may decrease the expression level of claudin-1 and occludin. In contrast, tight junction proteins in jejunum may be regulated by transcription factors other than GR because of the significantly lower protein level of GR receptor detected (compared with colon) and the relatively stable tight junction protein levels observed under WA stress conditions. Alternatively, increased tight junction protein degradation might explain the down-regulation of these proteins under stressful conditions as proteasome-mediated degradation has been reported to play an important role in regulating occludin levels in IBS patients.[20]

While the overall trends were similar, we observed some differences in the levels of epithelial tight junction proteins after chronic intermittent WA stress compared with serial CORT injections. These differences might be explained by several factors. Firstly, the experimental design (10 days of intermittent WA stress *vs* control rats serially treated with CORT injections) is inherently different. As tight junction proteins turn over rapidly, the individual variations might be larger in CORT rats than those in WA stress rats. Secondly, although we used the optimal dose of CORT to mimic the response of intermittent WA stress-induced changes, the serum CORT level and the dynamic change might not be exactly the same in the two groups of rats. Thirdly, WA stress procedure induces elevation in CORT, as well as the levels of other stress-response molecules. In addition, the rapid turnover and

trafficking of the tight junction proteins may cause the modest difference in the data obtained by immunoblot and immunofluorescence methodologies. We used RU-486 in this study because of its well-documented utility as an anti-glucocorticoid in *in vivo* studies.[50] However, it is relevant to point out that RU-486 has non-specific actions, including well-described effects as an anti-progestin[51] and a partial agonist in high doses, which may explain the partial reversal of the stress-induced changes by this corticoid receptor antagonist.

The intestinal barrier function is usually assessed by permeability assays *in vivo*, which involve oral administration of water-soluble molecules including sugars and radiolabeled EDTA.[31] Among these molecules, the saccharides lactulose and mannitol have been considered ideal for assessment of small intestinal permeability. However, because lactulose and mannitol are partially degraded by colonic bacteria,[52] the use of these two molecules for measuring colonic permeability is limited. In contrast, the artificial disaccharide sucralose and chromium-labeled EDTA are used for characterizing colonic permeability because they are not affected by colonic bacteria fermentation. Nevertheless, sucralose is not ideal for small intestinal permeability measurement as it is absorbed in small intestine.[53] Therefore, the use of excretion of sugars for assessing barrier function needs to be designed precisely and the data obtained should be interpreted carefully. Alternatively, intestinal permeability is measured using biopsies from the intestinal tissues and assessing the permeability to molecule markers of different sizes using the Ussing chamber *in vitro*. In this study, we employed the *in situ* single-pass intestinal perfusion method using two different sizes of PEGs to measure intestinal permeability *in vivo* at different intestinal segments at the same time. This allowed us to study barrier function at different segments of the gastrointestinal tract simultaneously and obtain data reflecting the permeability for specific molecular weights directly and precisely. We observed significantly increased epithelial permeability for PEG-400 in colon, but not in jejunum in WA stress and CORT-injected rats. This molecular weight (400 Da) includes a number of low molecular weight species such as fermentation products and some cytokines. Soluble mediators such as histamine or tryptase released in the intestinal mucosa of patients with IBS have been implicated in visceral hyperalgesia as a result of increased paracellular permeability.[5,54] Future experiments will be required to elucidate the upper limit of the molecular weight (less than 4000 Da) that demonstrates increased permeability in the chronic intermittent WA stressed rat model, as our results indicate that no differences in permeability were observed to PEG-4000 and PEG-35 000 in preparations from jejunum and colon in stressed rats and controls.

Compared with the jejunum, the permeability to PEG-200, PEG-400, and PEG-4000 was significantly lower in the colon. There was no difference in permeability for PEG-35 000 between these two regions. The permeability data indicate that there is more variability in the colon compared with the jejunum. The molecular basis for the regional differences observed in epithelial tight junction protein expression and epithelial permeability associated with chronic stress remains unknown. Previous studies support that the expression of epithelial tight junction proteins is region-specific along the gastrointestinal tract, which reflects the properties of permeability in different regions.[55,56] Furthermore, epithelial tight junctions are affected by luminal conditions including the presence of enteropathogenic bacteria. For example, exposure to enteropathogenic *Escherichia coli* changes distribution of occludin and ZO-1 in tight junction membrane microdomains culminating in disruption of epithelial cell tight junctions.[57,58] It has been reported that probiotics prevented bacterial translocation, improved intestinal barrier function and prevented visceral hyperalgesia in stressed rat.[59] We propose that the increased permeability observed for low molecular weight molecules in the colon in the WA stressed rats and control rats treated with CORT implicates a potential role for molecular species present in the microbiome in stress-related visceral hypersensitivity.

In summary, our results suggest that visceral hyperalgesia observed in chronic, intermittent psychological stress was associated with decreased epithelial tight junction protein levels and increased colonic permeability to low molecular weight macromolecules in the colon, but not the jejunum. These stress-induced changes were significantly reversed by treatment with the corticoid receptor antagonist RU-486. Serial injections of corticosterone in control rats largely reproduced these alterations in epithelial tight junction protein levels that were also prevented by treatment with RU-486. These observations support a novel, region-specific role for corticosterone as a mediator of chronic psychological stress-induced reduction in colon epithelial barrier function by down-regulating epithelial tight junction proteins which are responsible for regulating molecular weight-specific paracellular diffusion. These observations provide a rationale for novel treatments that

target colon epithelial tight junction protein expression and function.

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DISCLOSURE

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

ZG, JWW, and SH planned and designed the study; DES provided assistance for experiment planning and design; ZG, SPW, YH, and SH conducted experiments and analyzed data; ZG, JWW, and SH wrote and revised the article.

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