# Effects of norepinephrine on colonic tight junction protein expression during heat stress

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**Abstract.** Stress induced by changes in the internal or external environment in humans and animals leads to intestinal epithelial damage, in a manner that is associated with impaired intestinal barrier function. However, the role of the stress hormone norepinephrine (NE) in impairments in barrier function remains poorly understood. In the present study, a rat heat-exposed model was used to observe changes in the tight junction proteins Occludin and zonula occludens-1 (ZO-1), in addition to those in protease-activated receptor 2 (PAR-2) and transient receptor potential ankyrin 1 channel (TRPA1) in colon. The levels of plasma NE were detected using an ELISA kit. Different concentrations of NE were used to culture the human colon cell line Caco-2 for 6 and 24 h to investigate the cell viability using Cell Counting Kit-8 assay, whilst the expression levels of Occludin, ZO-1, PAR-2 and TRPA1 were examined using western blotting and immunofluorescence in Caco-2 cells and immunohistrochemistry in rat colon tissues. Although there was no clear histological damage to the rat colonic mucosa, there were decreased expression levels of tight junction proteins Occludin and ZO-1 after heat exposure. In addition, PAR-2 expression was increased by heat exposure. It was found that TRPA1 expression was concentrated to the luminal surface of the colon in the heat exposed group compared with that in the control group. After the administration of increasing concentrations of NE for 6 h, treatment did not affect cell viability. Furthermore, after application of NE for 24 h, cell viability gradually increased as the NE concentration was elevated from 10 to 100 $\mu$M. However, no significant increase in viability was observed when the cells were treated with 120 and 160 $\mu$M NE. Occludin expression was decreased when 10 $\mu$M NE was applied for 6 or 24 h. By contrast, 60 $\mu$M NE significantly downregulated Occludin expression in the 6 h group, but caused an insignificant decrease in the 24 h group. It was found that ZO-1 expression was upregulated after treatment with 10 $\mu$M NE for 6 h, whilst downregulation was observed after treatment with 10 $\mu$M NE for 24 h. PAR-2 protein expression was increased after application of NE for both 6 and 24 h, but not after treatment with 60 $\mu$M NE. In addition, TRPA1 expression was not affected by the treatment of NE, but increased positive staining was observed on the luminal side of the mucosa, which appeared to be concentrated in the cells of the luminal side in the rat colon after heat exposure. Collectively, the present results suggested that expression of tight junction proteins Occludin and ZO-1, in addition to that of PAR-2, can be regulated by NE, which may contribute to impairments in barrier function observed during heat stress.

## Introduction

Stress is a pivotal factor for inflammation and reactive oxygen species (ROS) production, which can in turn induce damage to the epithelial barrier (1,2). Heat stress induced by continuous high ambient temperatures or strenuous exercise in humans and animals can also lead to epithelial damage in the digestive tract due to the induction of cellular stress responses (3-5). Damage to the intestinal barrier is an important cause of bacterial translocation, inflammation, sepsis and multiple organ dysfunction (3). In particular, tight junction proteins are important for the maintenance of intestinal barrier integrity. For instance, zonula occludens-1 (ZO-1) and Occludin can both enhance steady-state barrier function in primary cultured Sertoli cells (4). Occludin function appear to require the cytoplasmic C terminus, which is highly phosphorylated in tight junction-associated Occludin, which binds to ZO-1, ZO-2 and ZO-3 (5). Occludin or ZO-1 knockdown increases the leak pathway permeability in cultured epithelial monolayers (6). In addition, the expression pattern and intracellular localization of Occludin and ZO-1 can change under stress, ischemic or inflammation conditions (1,7).

Protease-activated receptor 2 (PAR-2) is highly expressed in the gastrointestinal tract, which has dual effects on inflammation and serves a key role in visceral hypersensitivity (8).

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Expression levels of intestinal tight junction proteins are reduced following water-avoidance stress (9). Furthermore, PAR-2 expression and mast cells are elevated under acute or chronic restraint stress, which contributes to the impaired epithelial barrier function in the colon and esophagus (10,11). It has also been reported previously that increased mast cell numbers and mucosal PAR2 expression in the colon is mediated by the release of corticotrophin-releasing factor (10). Subsequently, the activation of PAR2 disrupts tight junctions and increases barrier permeability through the activation of p38 MAPK (12). PAR2 activation can also compromise vascular endothelial barrier function by suppressing the expression of vascular endothelial (Ve)-cadherin (13).

Transient receptor potential ankyrin 1 channel (TRPA1) serves as a key sensor for temperature and is permeable to $Ca^{2+}$ (14). In addition, TRPA1 can be activated by mustard oil, cinnamic acid, garlicin, oxidative stress products and inflammatory mediators, such as prostanoids (15,16). TRPA1 is mainly expressed on sensory neurons, afferent nerve endings and some non-neuronal cells, including immune cells, where it is involved in the process of nociception and inflammatory responses (17). Although TRPA1 is sensitive to temperature, this differs among species. For instance, mouse TRPA1 has been implicated in noxious cold detection but was also identified as one of the prime noxious heat sensors (18). Moreover, human TRPA1, which was originally considered to be temperature-insensitive, is also capable of sensing both hot and cold, where it is suggested that an allosteric mechanism could account for the variability in TRPA1 temperature responsiveness (18). TRPA1 can also serve as a sensor of cellular stress and tissue damage (19). This channel has been found to be upregulated in various tumors and is associated with tumor proliferation and metastasis, as well as promoting ROS and chemotherapy tolerance through the $Ca^{2+}$-dependent anti-apoptotic pathway (20). Hypoxia and ischemia are associated with oxidative stress, which can activate TRPA1 in cerebral artery endothelial cells, leading to vasodilation, thereby reducing ischemic damage (21). These previous findings suggest a protective role of TRPA1. However, TRPA1 can also induce stress-induced duodenal lesions in a water immersion restraint stress rat model by promoting the release of substance P (22).

Norepinephrine (NE) is a stress hormone that is elevated due to activation of the hypothalamic-pituitary-adrenal axis, the locus coeruleus (LC) and involves noradrenergic neurons, the sympathetic adrenal medulla and the renin-angiotensin-aldosterone system during stress (23). NE constricts the blood vessels to change blood distribution, which is important during heat stress (24). In addition, previous studies have reported the effect of NE in the regulation of barrier function. Degeneration of noradrenergic fibers from the LC causes tight-junction disorganization in the rat brain (25). Moreover, another previous study revealed that in the presence of NE, some *Campylobacter* strains show increased invasion into T84 epithelial cells and induced a greater breakdown of tight junctions (26). However, whether NE can directly contribute to the regulation of barrier function remains poorly understood.

Therefore, the present study aimed to investigate the expression changes in tight junction proteins Occludin and ZO-1, in addition to PAR-2 and TRPA1, in the rat colon after heat stress. Additionally, the present study aimed to evaluate the effects of NE on the expression levels of these proteins in cultured Caco-2 cells.

## Materials and methods

*Animals*. A total of 14 male Sprague-Dawley rats (weight, 220±20 g, age, 8 weeks) were randomly divided into control and heat exposure groups (n=7 per group). Rats were provided with standard laboratory diet and tap water *ad libitum*. The experimental procedures were approved by the Animal Ethics Committee of the Ningxia Medical University and Use Committee (Yinchuan, China). After an adaptation period of 3 days, rats were acclimatized to heat exposure with increasing durations from 2 h (day 1), 6 h (day 2), 14 h (day 3) and 18 h (day 4) at a temperature of 32±0.1˚C in a closed, temperature-controlled chamber with a relative humidity of 54±5%, 12-h light/dark cycle. After a rest on day 5, the rats received continuous 24-h heat exposure (32˚C) for 9 days. The rats in the control group were raised in normal conditions (24±0.1˚C, relative humidity of 54±5%, 12-h light/dark cycle).

*Tissue preparation*. All animals were anesthetized with intraperitoneal injections of 2% sodium pentobarbital (40 mg/kg) and sacrificed by exsanguination immediately after the 9-day 32˚C heat exposure procedure. After anesthesia, the rats exhibited no signs of peritonitis, pain or discomfort. The abdominal cavity was rapidly opened and the distal colon (1-cm from the rectum; length, 2-3 cm) was carefully excised, which was then placed into modified cold Krebs' solution (120.6 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgCl_2$, 15.4 mM $NaHCO_3$ and 11.5 mM glucose) for rinsing. Part of the distal colon was fixed with 4% paraformaldehyde for 24 h at room temperature. Blood was collected from the inferior vena cava into a heparinized tube and centrifuged at 1,449 x g for 10 min at 4˚C to obtain plasma samples, which was then frozen at -80˚C to measure NE levels.

*Hematoxylin and eosin (H&E) staining*. Full-thickness (4 $\mu$m) paraffin-embedded sections of the distal colon from control and heat exposed rats were stained with H&E staining for the evaluation of histological structural change. Xylene followed by a descending ethanol gradient was used for deparaffinization. Hematoxylin and eosin staining were both performed at room temperature for 60-70 min. 60-70 min refers to the total time of HE staining, from deparaffinization to the end. For just Hematoxylin and eosin staining need 10 minutes (5 min for H,and 5 min for E). Light microscopy was used for observation (Magnification, x400).

*Immunohistochemistry staining*. Expression levels of Occludin, ZO-1, TRPA1 and PAR-2 were examined in rat distal colon full-thickness paraffin-embedded sections (4 $\mu$m). Briefly, the sections were washed three times in PBS after deparaffinization using the same protocol as that used for in H&E staining aforementioned and incubated with 3% hydrogen peroxide for 10 min at room temperature to block the activity of endogenous peroxidase. EDTA buffer antigen retrieval was used for Occludin and ZO-1, whilst citrate buffer antigen retrieval was used for TRPA-1 and PAR-2. Microwave-treated antigen

retrieval was used, microwave heating in EDTA or citrate buffer was performed for 15 min under high fire, following by another 10-min heating after boiling. The sections were washed again with PBS and blocked with 10% normal goat non-immune serum (cat. no. C01-03001; BIOSS) for 30 min at room temperature, which was followed by incubation with primary antibodies against rabbit polyclonal anti-PAR-2 (1:250; cat. no. bs-1178R; BIOSS), rabbit monoclonal anti-Occludin (1:200; Abcam; cat. no. ab216327), rabbit polyclonal anti-ZO-1 (1:500; cat. no. bs-1329-R; BIOSS) and rabbit polyclonal anti-TRPA1 (1:1,000; cat. no. ab58844; Abcam) overnight at 4˚C. The slices were then stained using a two-step IHC detection reagent kit (cat. no. PV-9001; ZSGB-BIO; http://www.zsbio.com/product/PV-9001) according to manufacturer's protocol. DAB chromogenic kit (cat. no. ZLI-9018; ZSGB-BIO) was used for detection. Hematoxylin was used at room temperature for 5 min for counterstaining. Light microscopy was used to image the sections (magnification, x200).

*ELISA.* The plasma level of NE was determined using an ELISA kit (cat. no. EL-0047C; Elabscience) according to the manufacturer's protocol.

*Cell culture.* Caco-2 cells were used for detection of the cell permeability (27). Caco-2 cells were kindly gifted by Professor Jingxin Li (Cheeloo College of Medicine, Shangdong University, Jinan, China). The cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (cat. no. FB35015; Clark Bio Office; https://www.clarkbio.com/index.php?m=Content&c=Index&a=show&catid=9&id=6), 100 U/ml penicillin and 100 $\mu$g/ml streptomycin (Beijing Solarbio Science & Technology Co., Ltd.) at 37˚C under a humidified atmosphere of 5% $CO_2$. Caco-2 cells were collected when they reached 70-80% confluence and cells from passages 2-15 were used for all subsequent experiments.

*Cell viability detection using a Cell Counting Kit (CCK)-8 assay.* Relative viability of Caco-2 cells after NE treatment was detected using a CCK-8 (cat. no. BB-4202-2; BestBio Science) assay. The kit was used according to the manufacturer's protocols. Briefly, Cells ($5x10^3$ cells/well) in 100 $\mu$l medium were added into 96-well plates. After 12 h, cells were cultured with NE (cat. no. S25926; Shanghai Yuanye Bio-Technology Co., Ltd. http://www.shyuanye.com/goods-S25926.html) at different final concentrations (0, 5, 10, 20, 40, 60 and 80 $\mu$M) for 6 h and (10, 20, 40, 60, 80, 100, 120 and 160 $\mu$M) 24 h at 37˚C. In total, 10 $\mu$l CCK-8 solution was added to the 100 $\mu$l culture medium per well and incubated at 37˚C for 2 h. The optical density (OD) was measured at 450 nm using a microplate spectrophotometer (1420 Victor3; Thermo Fisher Scientific, Inc.). Relative cell viability (%)=[(OD of NE treatment group-OD of blank)/(OD of control group-OD of blank)] x100%. Viability was represented as the percentage of culture without NE that was set to 100%.

*Western blot analysis.* The expression levels of proteins were determined by western blotting. Cells ($1x10^6$) were plated into 6-cm dishes and cultured until reaching 80% confluence. Cells were then separated into the control group, NE-treated 6 h group and NE-treated 24 h group. After 6 and 24 h culture at 37˚C,

cells were washed three times with cold PBS and collected. The cell samples were lysed in lysis buffer supplemented with 0.1% protease inhibitor, 1% phosphatase inhibitor and 1% PMSF for 30 min on the ice. All reagents were from the BCA whole protein extraction kit (cat. no. KGP250; Nanjing KeyGen Biotech Co., Ltd.). Samples were centrifuged at 13,684 x g for 5 min at 4˚C. Protein concentration in the lysate was measured using the bicinchoninic acid protein assay kit (cat. no. KGPBCA; Nanjing KeyGen Biotech Co., Ltd.). After boiling the samples with SDS sample buffer for 5 min, equal amounts of protein (40 $\mu$g) were separated by 10% SDS-PAGE (Nanjing KeyGen Biotech Co., Ltd) and were transferred onto PVDF membranes (EMD Millipore). The membranes were blocked for 2 h at room temperature with 5% non-fat dry milk diluted in PBS. The membranes were then incubated with primary antibodies against Occludin (1:1,000; cat. no. ab216327; Abcam), ZO-1 (1:500; cat. no. bs-1329-R; BIOSS), TRPA1 (1:1,000; cat. no. ab58844; Abcam) and PAR-2 (1:500; cat. no. bs-1178R; BIOSS) at 4˚C overnight. $\beta$-actin was used as an internal loading control (1:1,000; cat. no. TA09; ZSGB-BIO; OriGene Technologies, Inc.). Following three washes with TBS-T (0.2% Tween-20), membranes were then incubated with the horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (1:5,000; cat. no. ZB2301; ZSGB-BIO; OriGene Technologies, Inc.) and anti-mouse IgG secondary antibody (1:5,000; cat. no. ZB2305; ZSGB-BIO; OriGene Technologies, Inc.) respectively for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence detection reagents (Affinity Biosciences). Protein expression levels were analyzed using an ImageJ Imaging System (version 1.37; National Institutes of Health).

*Immunofluorescence.* Caco-2 cells were seeded into 24-well plates containing 15-mm slides. The complete medium was replaced by fresh complete medium (fully-supplemented DMEM) before further treatment with NE at a final concentration of 10 $\mu$M. After incubation for 6 and 24 h at 37˚C, the cells were washed three times with cold PBS and fixed in ice-cold 4% paraformaldehyde at room temperature for 20 min. After washing with cold PBS, the cells were blocked with 1% BSA (cat. no. B1010; Biotopped;; http://www.bjbiotopped.com/showinfo-20-98588-0.html) for 30 min at room temperature. The cells were then incubated with primary antibodies against PAR-2 (1:300; cat. no. bs-1178R; BIOSS), Occludin (1:100; cat. no. ab216327; Abcam), ZO-1 (1:300; cat. no. bs-1329-R; BIOSS) and TRPA1 (1:200; cat. no. ab58844; Abcam) at 4˚C overnight, followed by incubation with FITC-conjugated goat-anti-rabbit secondary antibodies (1:50; cat. no. bs-0295G-FITC; BIOSS) for 1 h at room temperature in the dark after washing in cold PBS. The slices were sealed with mounting medium containing DAPI (cat. no. DZ0125; Beijing Leagene Biotech Co., Ltd.; https://www.leagene.com/Catalogue/DZ0125-DAPIrsfpj_ID482.html). Images were captured using an Olympus fluorescence microscope (Olympus Corporation) and analyzed using Adobe Photoshop CS3 10.0.1 version (Adobe Systems, Inc.) at x400 magnification.

*Statistical analysis.* Data are presented as the mean ± SD, where n refers to the number of animals or the number of duplicates.
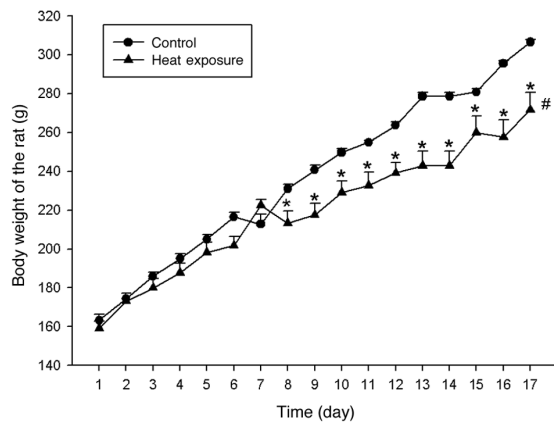
Figure 1. Changes in body weight following heat exposure. Body weight was significantly decreased in the heat exposure group. n=7. *P<0.05 vs. Control. #, the body weight increase of the rats in the heat exposure groups was lower compared with that in the control group..
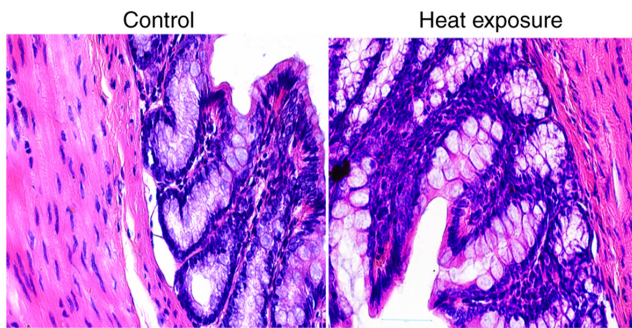


Figure 2. Representative photomicrographs of hematoxylin and eosin-stained sections (thickness, 4 $\mu$m) of the rat distal colon in control and heat exposure groups. Magnification, x400. There were no clear structural changes between control and heat exposure groups.

Unpaired Student's t-test was performed for comparisons between two groups and one-way ANOVA followed by Tukey's multiple comparisons test. Mixed two-way ANOVA followed by a Sidak corrections were used for comparisons among multiple groups with SigmaStat 3.5 software (Systat Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

## Results

*Heat exposure animal model.* All rats survived prior to sacrifice. According to the mixed two-way ANOVA (with animal treatment type as a between-subjects factor and time as the within-subjects factor) followed by post hoc testing with Sidak correction, the body weight increase of the rats in the heat exposure groups was lower compared with that in the control group (F=8.209, P<0.05), whilst time also affected the weight of the rats (F=844.208; P<0.001), with the difference more significant from day 8 onwards (F=28.15; P<0.001) and weights in rats exposed to heat lower compared with those in rats in the control group (Fig. 1). This suggests that long lasting heat exposure adversely affected the rat weight.

According to the H&E staining images, the mucosa in both control and heat-exposed rat colon tissues were found to be intact, with no notable structural changes (Fig. 2).
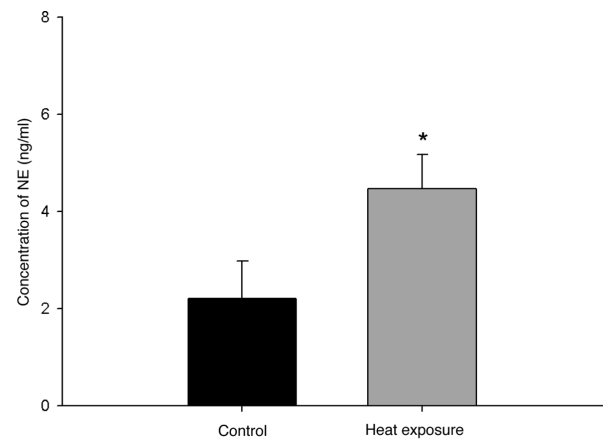


Figure 3. Plasma NE levels in control rats and rats following heat exposure. n=6. *P<0.05 vs. Control. NE, norepinephrine.

The rats were treated for 9 days continuous 24-h heat exposure (32˚C) after 5 days of acclimatization. Plasma samples were collected on day 17 and the plasma NE level was detected. In the heat exposure group, the average plasma NE level was significantly elevated compared with that in the control group (P<0.05; n=6; Fig. 3).

*Expression levels of Occludin, ZO-1, PAR-2 and TRPA1 in rat distal colon.* In the control group, strong positive staining of Occludin was observed in the mucosa, which was mainly located on the cell membranes of epithelial cells and was decreased after heat exposure (Fig. 4A). ZO-1 was observed in the mucosa and smooth muscle cells, the expression of which was found to be attenuated in the heat exposure group in comparison with that in the control group (Fig. 4A). PAR-2 staining was detected in the mucosa and the myenteric nerve plexus, in addition to within smooth muscles cells (Fig. 4B). It was found that heat exposure increased PAR-2 expression, where a weaker expression was observed in the smooth muscle layer (Fig. 4B). Additionally, TRPA1 staining was identified in the epithelial cells of the colon mucosa and myenteric nerve plexus (Fig. 4B). After heat exposure, the expression pattern of TRPA1 in mucosal epithelial cells was different compared with that in control, with enhanced expression observed in the myenteric nerve plexus. Furthermore, increased positive staining was observed in the luminal side of the mucosa, which appeared to be concentrated in the cells of the luminal side (Fig. 4B).

*Cell viability detection using a CCK-8 assay.* Caco-2 cells were treated with different concentrations of NE for 6 and 24 h, respectively. Treatment with increasing concentrations of NE for 6 h did not affect cell viability (Fig. 5A). However, the cell viability gradually increased as the NE concentration elevated from 10 to 100 $\mu$M in the 24 h group, but there was no statistical difference when cells were treated with 120 and 160 $\mu$M NE compared with that in control (Fig. 5B).

*Protein expression levels of Occludin, ZO-1, TRPA1 and PAR-2 after the administration of NE.* To examine the regulatory effect of NE on the expression levels of tight junction proteins, as well as PAR-2 and TRPA1, 10, 60 and 120 $\mu$M were selected
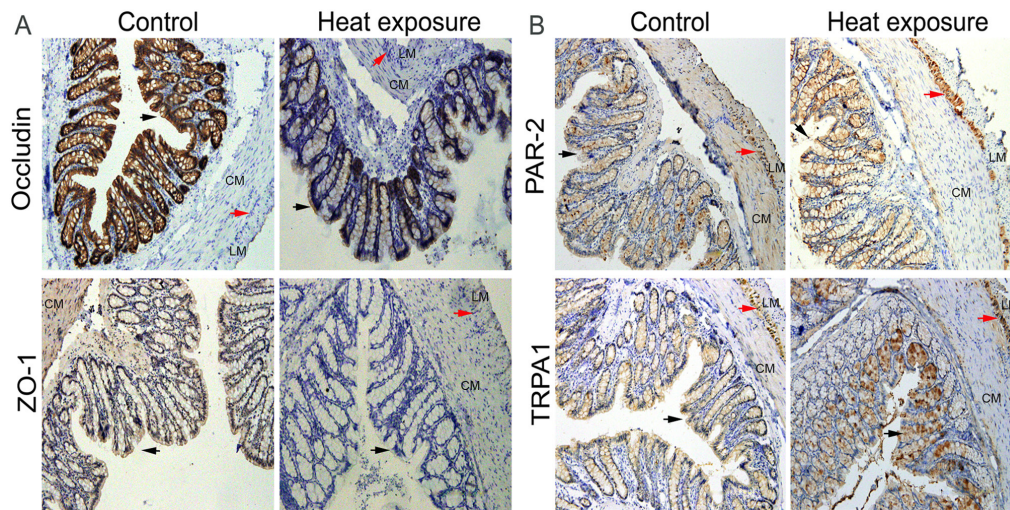
Figure 4. Expression levels of Occludin, ZO-1, TRPA1 and PAR-2 in the rat distal colon tissue sections in control and heat exposure groups. The figures (A, B) shown are representative of three different animals. Magnification, x200. Black arrows indicate the musca. Red arrows indicate the myenteric plexus. ZO-1, zonula occludens-1; PAR-2, protease-activated receptor 2; TRPA1, transient receptor potential ankyrin 1 channel; CM, circular muscle; LM, longitudinal muscle.
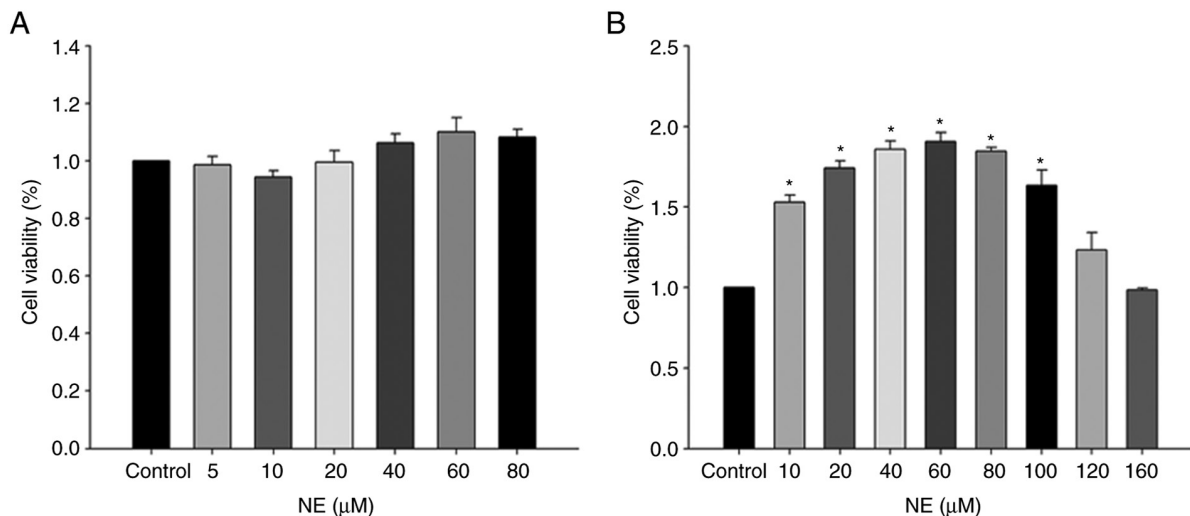


Figure 5. Influence of NE on relative Caco-2 cell viability. The relative viability of Caco-2 cells treated with different concentrations of NE for (A) 6 or (B) 24 h. *P<0.05 vs. Control. NE, norepinephrine.

as the final NE concentration to stimulate Caco-2 cells for 6 and 24 h. In the 6 h treatment group, Occludin expression was significantly decreased when 10 and 60 $\mu$M NE were applied compared with that in control (n=4; P<0.05; Fig. 6A and B). After 24 h treatment, 10 $\mu$M NE also significantly downregulated Occludin expression compared with that in control (n=4; P<0.05; Fig. 6A and B). Although administration of 60 $\mu$M NE also decreased Occludin expression in the 24 h group, the difference was not statistically significant (Fig. 6A and B). Occludin expression was not significantly affected by treatment with 120 $\mu$M NE for 6 h, 120 $\mu$M NE appeared to have reduced Occludin expression after 24 h, however, there was no significant difference (Fig. 6A and B). Significant upregulation of ZO-1 expression was observed after administration of 10 $\mu$M NE for 6 h compared with that in the control group (n=4; P<0.05), but its expression was downregulated by 10 $\mu$M NE after 24 h treatment compared with that in the control group (P<0.05; n=4; Fig. 6A and C). PAR-2 protein expression

was significantly increased after 10 $\mu$M NE treatment for 6 h (P<0.05; n=4) and 24 h (P<0.05; n=4) compared with that in control, but not after 60 $\mu$M NE treatment (Fig. 6A and D). However, 120 $\mu$M NE treatment for 6 h increased PAR-2 expression (P<0.05; n=4). There were no statistical significant changes in TRPA1 protein expression when different concentrations of NE were applied to the Caco-2 cells for the different time periods (Fig. 6A and E).

*Immunofluorescence.* The present study also evaluated the expression levels of tight junction proteins, in addition to PAR-2 and TRPA1 by immunofluorescence after the application of 10 $\mu$M NE for 6 and 24 h. Occludin-positive staining was located on the cell membrane of Caco-2 cells, which was reduced by NE treatment in both 6 and 24 h groups (Fig. 7A). ZO-1 was also found to be expressed on the surface of Caco-2 cells, where it was found that 6 h treatment with NE increased the expression of ZO-1, but 24 h treatment
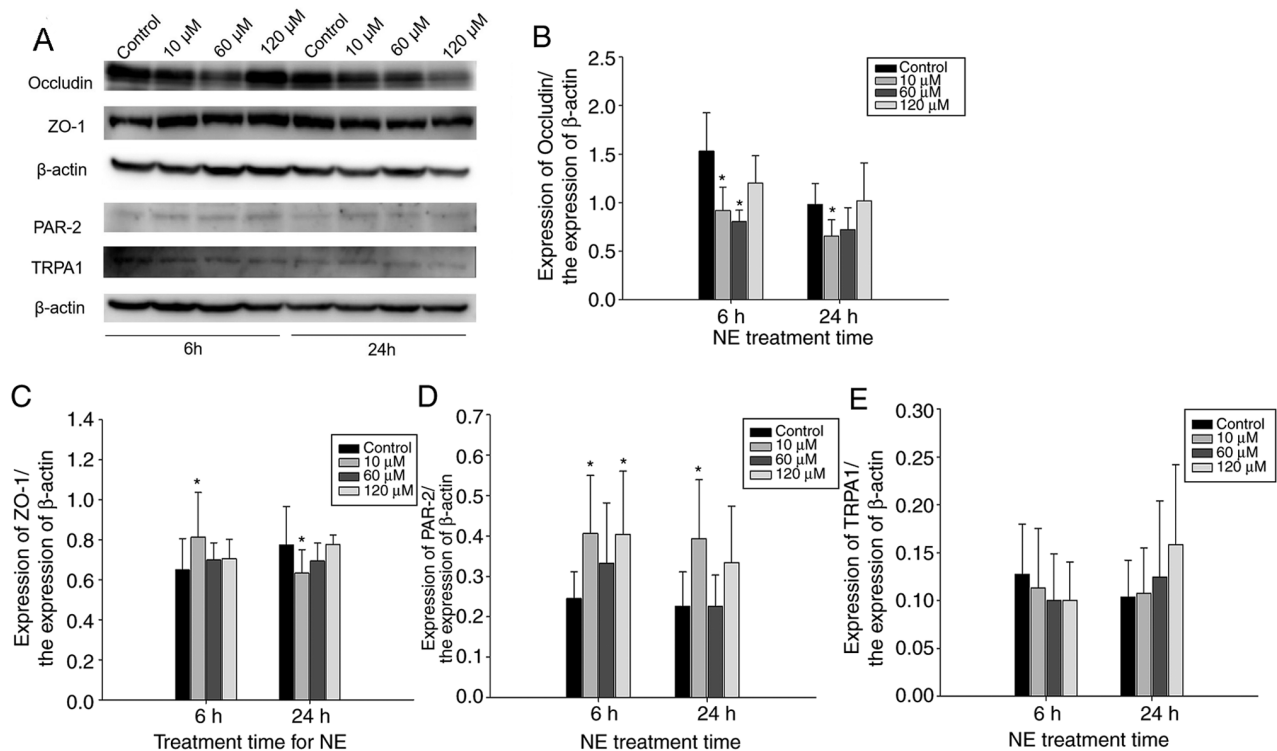
Figure 6. Expression levels of Occludin, ZO-1, PAR-2 and TRPA1 in Caco-2 cells after treatment with NE. (A) Representative western blotting images from four independent experiments. Comparison of the relative expression levels of (B) Occludin, (C) ZO-1, (D) PAR-2 and (E) TRPA1 in colon of control and NE-treated cells. *P<0.05 vs. Control. Data are presented as the mean ± SD. NE, norepinephrine; ZO-1, zonula occludens-1; PAR-2, protease activated receptor 2; TRPA1, transient receptor potential ankyrin 1 channel.

reduced its expression (Fig. 7B). PAR-2 was observed on the cell membrane, where its staining was stronger in NE-treated 6 h and 24 h cells (Fig. 7C). The staining pattern of TRPA1 was different compared with that of the aforementioned proteins (Fig. 7D). The positive staining was discontinuous, which became more obvious after NE treatment for 6 and 24 h. It appeared that TRPA1 gathered on the cell membrane of Caco-2 cells, which was similar with the results observed from immunohistochemistry in the rat colon mucosal epithelium.

## Discussion

In the present study, heat stress induced by heat exposure attenuated body weight gain. Histological examination of the rat colon tissues after heat exposure demonstrated that there was no obvious damage to the rat colonic mucosa, but the protein expression levels of tight junction proteins Occludin and ZO-1 were decreased. These findings were consistent with a previous study, which reported that psychological stress reduced brain and intestinal expression levels of tight junction proteins, including Claudin 5, Occludin, α-actin and ZO-1 (1),However, whether elevated NE levels regulated the expression levels of tight junction proteins was not previously investigated (1). The present study observed elevated plasma NE levels and found that the administration of 10 $\mu$M NE for 6 and 24 h downregulated the expression of Occludin in Caco-2 cells, whilst NE treatment upregulated ZO-1 expression after 6 h treatment but reduced ZO-1 expression after 24 h. These data indicated that NE directly regulated the expression levels

of tight junction proteins, which can contribute to altered gut permeability under stress.

In patients with septic shock, NE use is associated with increased enterocyte damage (28), where the reason for this could be the direct regulation by NE on tight junction proteins. In a previous study on vascular endothelial cells, the inhibitory effect of angiotensin II on Occludin and ZO-1 expression was identified (29). In addition, our unpublished data also revealed the significant inhibitory action of 6 h NE treatment on the expression levels of Occludin and ZO-1 in thoracic aortic endothelial cells. Taken together, these findings indicate a direct regulatory effect of this stress hormone on tight junction proteins in epithelial cells. Although there have been a few reports that evaluated the direct regulatory effect of NE on the tight junction proteins (25,30), it has been suggested that in bovine aortic endothelial cells, treatment with NE to concentrations ranging from normal to pathophysiological circulating plasma levels significantly impedes trypan blue dye-bovine serum albumin conjugate diffusion, compared with that in untreated controls (31). The difference between the present study and this previous report in the barrier-modulating effects of NE may be due to the dose and cell type. As a chronic stress hormone, NE promotes tumor progression by stimulating $\beta_2$-adrenoreceptors in oral cancer (32). In the present study, treatment of Caco-2 cells with different concentrations of NE for 6 h did not affect cell viability, but NE increased cell viability 24 h after treatment. This finding was in accordance with a previous observation that 24 h NE treatment enhanced cell viability and invasion of pancreatic cancer cells (33).
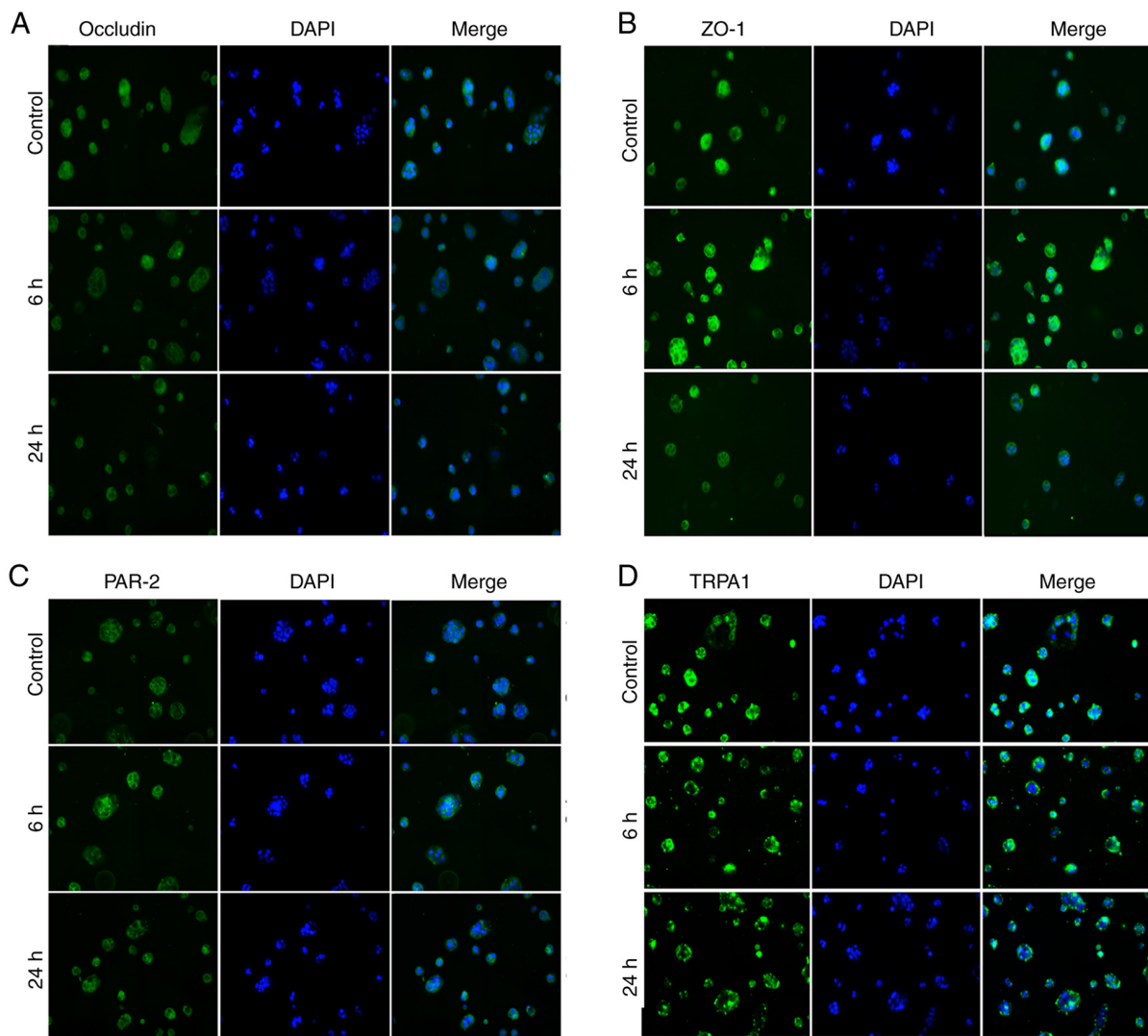
Figure 7. Immunofluorescence staining of Occludin, ZO-1, PAR-2 and TRPA1 in Caco-2 cells after 10 $\mu$M NE application. Staining of (A) Occludin, (B) ZO-1, (C) PAR-2 and (D) TRPA1. Magnification, x400. NE, norepinephrine; ZO-1, zonula occludens-1; PAR-2, protease-activated receptor 2; TRPA1, transient receptor potential ankyrin 1 channel.

The activation of PAR-2 and mast cell is involved in increased epithelial permeability due to changes in tight junction proteins under heat stress (11,34,35), PAR-2 modulates Ve-Cadherin expression to affect human vascular endothelial barrier function (13), such that activation of PAR2 changes the localization of the tight junction proteins and increases barrier permeability (12). However, it has also been reported that treatment with a PAR-2 agonist prevents the downregulation of tight junction proteins after *P. aeruginosa elastase* treatment in human nasal epithelial cells (36). According to the present study, expression of PAR-2 in colonic epithelial cells was increased after heat exposure, which may be a reason for the altered expression of tight junction proteins in the heat-exposed rats colon. Furthermore, PAR-2 expression was upregulated after application of 10 $\mu$M NE for both 6 and 24 h, whereas NE level was increased in heat-exposed rats. These findings suggested that NE can regulate the expression of PAR-2 under stress.

TRP channels are non-selective cation channels that act as biosensors for environmental and noxious stimuli, including capsaicin and allicin, in addition to changes in temperature and conditions inside the cell (14,15). The TRPA1 receptor is highly expressed in the intestinal mucosa and can be activated by oxidative stress products, where the cell damage signals can induce oxidative stress (19), which implicates its possible association with intestinal disfunction. Cold stress increases ROS production by TRPA1 activation in A549 cells (37). In addition, upregulation of TRPA1 expression and function on vagal afferents is associated with stress-exaggerated visceral mechanonociception after antral cold (4˚C) stress (38). TRPA1 also mediates cigarette smoke extract-induced damage of bronchial and alveolar epithelial cells via the modulation of oxidative stress, inflammation and mitochondrial damage (39). It has been reported that substance P may initiate the earliest changes observed in blood-brain barrier permeability (40). In addition, it has also been documented that TRPA1 mediates the development of gastric mucosal and duodenal lesions in a water immersion restraint stress rat model by promoting the release of substance P (22,41). Since TRPA1 is involved in $Ca^{2+}$ influx and increases in tight junction permeability (42), TRPA1 may contribute to damage in epithelial barrier function by

regulating oxidative stress induced by stress. The present study demonstrated that the expression of TRPA1 was changed, with increased positive staining observed in the luminal side of the mucosa in heat exposed rats, which was consistent with previous results reported following water avoidance stress (43). Furthermore, the expression pattern was altered after heat exposure and the expression of TRPA1 was concentrated on the luminal surface. The present study investigated the regulatory mechanism mediated by NE on expression of TRPA1. However, there was no change in its protein expression level after treatment with NE for either 6 or for 24 h (Fig. 6D). It was found that higher levels of TRPA1 were gathered or recruited onto the cell membrane to form dot staining after NE treatment. This may be the reason for the failure in detecting changes in protein expression using western blotting, therefore further investigation is required to confirm the role of NE on the expression of TRPA1. Furthermore, in the present study, the detailed regulatory mechanisms of NE on tight junction proteins and PAR-2 function were not investigated. Further experiments are needed to address these questions in the future.

In conclusion, the present study demonstrated the changed expression levels of tight junction proteins, PAR-2 and TRPA after heat exposure, which was implicated in intestinal barrier function under stress. The present results suggested that NE directly regulated the expression of tight junction proteins and PAR-2 *in vitro*. It was also indicated that NE may be directly responsible for the altered levels of tight junction proteins and PAR-2 under stress.

## Acknowledgements

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

YL contributed to the conception and design of the study and completed the western blotting and immunohistochemistry experiments. HM contributed to cell culture and acquisition of data and performed IF analysis. SN established the animal model. XL performed the Cell Counting Kit-8 assay. LN contributed to the interpretation of data and revised the article for important intellectual content. GL contributed to the conception and design of the study. YL and GL can authenticate the raw data of this study. All authors read and approved the final version of the manuscript.

## Ethics approval and consent to participate

The experimental procedures were approved by the Animal Ethics Committee of the Ningxia Medical University and Use Committee (Yinchuan, China) and were performed in accordance with the Guidelines of the Council of the Physiological Society of China.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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