

Regulation of Transepithelial Ion Transport in the Rat Late Distal Colon by the Sympathetic Nervous System

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Summary

The colorectum (late distal colon) is innervated by the sympathetic nervous system, and many colorectal diseases are related to disorders of the sympathetic nervous system. The sympathetic regulation of colorectal ion transport is rarely reported. The present study aims to investigate the effect of norepinephrine (NE) in the normal and catecholamine-depleted condition to clarify the regulation of the sympathetic adrenergic system in ion transport in the rat colorectum. NE-induced ion transport in the rats colorectum was measured by short-circuit current (I_{sc}) recording; the expression of β -adrenoceptors and NE transporter (NET) were quantified by real-time PCR, and western blotting. When the endogenous catecholamine was depleted by reserpine, the baseline I_{sc} in the colorectum was increased significantly comparing to controls. NE evoked downward ΔI_{sc} in colorectum of treated rats was 1.8-fold of controls. The expression of β_2 -adrenoceptor protein in the colorectal mucosa was greater than the control, though the mRNA level was reduced. However, NET expression was significantly lower in catecholamine-depleted rats compared to the controls. In conclusion, the sympathetic nervous system plays an important role in regulating basal ion transport in the colorectum. Disorders of sympathetic neurotransmitters result in abnormal ion transport, β -adrenoceptor and NET are involved in the process.

Key words

Sympathetic nervous system • Colorectum • Ion transport

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Introduction

The late distal colon (colorectum) is the terminal region of the intestine, and performs the functions of water absorption and feces expulsion. Involuntary control of the colon and rectum is regulated by the sympathetic nervous system (Ridolfi *et al.* 2009), through neurons with cell bodies that lie within the dorsal horn of the lumbar spinal cord (T5-L2) (Winge *et al.* 2003). Sympathetic nerve fibers enter the intestinal wall along arteries and terminate in the myenteric and submucosal plexuses and in the mucosa. They control motility, secretion and vasoregulation (Straub *et al.* 2006). The sympathetic regulation of colorectal motility has been demonstrated (Ridolfi *et al.* 2009), but the regulation of colorectal ion transport remains vague.

Neurotransmitters of the sympathetic nerve terminal are ligands at functional receptors to perform biological functions. Norepinephrine (NE) and epinephrine are the active biogenic amines of the sympathoadrenal system and have strong and wide

participation in the regulation and control of many physiological functions. In any particular tissue, the content of NE reflects sympathetic innervation (Martínez-Olivares *et al.* 2006). NE has been reported to promote Na^+ , Cl^- , water absorption in both small intestine and colon (Tapper *et al.* 1981), and evoke K^+ secretion in the rat colon (Schultheiss and Diener 2000). Although epinephrine is a sympathetic neurotransmitter, the necessary concentration to induce maximal secretion is most likely never observed *in vivo*, when the hormone is secreted from adrenal gland, reaching the gut *via* the circulation (Schultheiss and Diener 2000). When NE is released from sympathetic nerve endings within the intestinal wall, it may reach much higher local concentrations. Therefore, the aim of the present study was to investigate the secretory effect of NE in rats with and without reserpine-induced chemical sympathectomy to clarify the regulation of the sympathetic adrenergic system in colorectal ion transport.

Materials and Methods

Tissue preparation

All experiments used male Sprague-Dawley rats (200-250 g) maintained in the animal care facility at the Laboratory Animal Services Center of Capital Medical University. The procedure was approved by the Beijing Administrative Office of Laboratory Animals, in accordance with the Administrative Regulations on Laboratory Animals of Beijing Municipality. The rats had free access to water and food until the day of the experiment. The rats were killed by cervical dislocation. The colorectal segment (1 cm) approximately 2-3 cm away from the anus (around the lymph node) was quickly removed and placed in Krebs-Hensleit solution (K-HS). The serosa, muscularis propria and submucosa were stripped away by hand to obtain the mucosal preparation of the colorectum.

Short-circuit current measurement and data evaluation

Briefly, the tissue was fixed in a modified Ussing chamber with a cross sectional area of 0.5 cm^2 , bathed with a volume of 5 ml on each side of the mucosa and short-circuited by a voltage clamp (Physiologic Instruments, San Diego, CA, USA) with a correction for the solution resistance. The short-circuit current (I_{sc}) was continuously recorded and tissue conductance was measured every minute. Drugs were added directly to the basolateral side of the epithelium. The change in I_{sc} (ΔI_{sc})

was calculated as the difference before and after adding drugs and was normalized to the current per unit area of epithelium ($\mu\text{A}/\text{cm}^2$) (Zhang *et al.* 2008).

Catecholamine depletion with reserpine

Intraperitoneal injection of reserpine depletes endogenous catecholamines, producing a nonspecific temporary sympathectomy (Fleming *et al.* 1973). Rats were weighed and intraperitoneally administered with reserpine (Guangdong BangMin Pharmaceutical Co., Ltd, China) at a dose of 5 mg/kg. Eighteen hours after the injection, rats were killed by cervical dislocation and the colorectum was rapidly removed and subjected to I_{sc} recording or β -adrenoceptor expression assays. Controls were intraperitoneally injected daily with sterile 0.9 % saline (1 ml).

RNA extraction and preparation of cDNA

The mucosa of late distal colon was collected in PBS (0.9 % NaCl in 0.01 M sodium phosphate buffer, pH 7.4), which had been treated with 0.1 % diethyl pyrocarbonate (depc-PBS). The tissue was cleaned with depc-PBS and transferred to Trizol (Invitrogen) for extraction of total RNA, which was isolated according to the manufacturer's instructions and stored at -80°C for additional use. Samples of cDNA were generated by reverse transcription following the Superscript first-stand synthesis protocol for RT-PCR (Invitrogen).

Real time polymerase chain reaction (Real-time PCR)

Real-time PCR was used to quantify mRNA encoding β_1 -, β_2 -adrenoceptor and NE transporter (NET) in the colorectal mucosa. Expression of β -actin was used as an internal control for normalization. The specific primers are listed in Table 1. The adrenoceptor and NET transcript levels in the rat colorectal samples were comparatively quantified with the Brilliant SYBR Green QPCR Master Mix kit (Stratagene, La Jolla, CA, USA) using a Light Cycler instrument (Stratagene). Amplifications were performed in a final volume of 20 μl of a commercial reaction mixture according to the manufacturer's instructions. Data were analyzed using the MxPro QPCR software (version 3.0, Mx3000P system, Stratagene).

Western blotting analysis

Tissues were harvested from the colorectal mucosa, washed with PBS, and homogenized in 300 μl of cold lysis buffer containing Nonidet P-40 (1 %), Tris-HCl, (10 mM, pH 8.0), EDTA (1.0 mM), NaCl

Table 1. Sequences of primers.

Primers	GenBank accession number	Primer sequence	Primer location in the sequence
β -actin	NM031144	F: 5'- TTC AAC ACC CCA GCC ATG T - 3' R: 5'- GTG GTA CGA CCA GAG GCA TAC A -3'	460-478 506-527
β_1 -adrenoceptor	NM012701	F: 5'-CAT CAT GGC CTT CGT GTA CCT-3' R: 5'-TGT CGA TCT TCT TCA CCT GTT TCT-3'	714-734 755-778
β_2 -adrenoceptor	NM012492	F: 5'-TTG CCA AGT TCG AGC GAC TAC-3' R: 5'-CAC ACG CCA AGG AGG TTA TGA-3'	372-392 411-431
NET	AB021971	F: 5'- TGG CCA TGT TTT GCA TAA CG -3' R: 5'- GCG AAG GTG TCC AGC AGA GT -3'	193-222 167-186

Table 2. Antibody used in the study.

Antibody	Immunizing antigen or conjugation	Host species	Dilution	Source/Catalog No.
β_1 -adrenoceptor	a peptide mapping at c-terminus of β_1 -adrenoceptor of mouse origin	Rabbit	1:500	Santa Cruz / sc-568
β_2 -adrenoceptor	a peptide mapping at c-terminus of β_2 -adrenoceptor of mouse origin	Rabbit	1:500	Santa Cruz / sc-570
NET	22 amino acid peptide sequence mapping to the 1 st extracellular domain of rat NET	Rabbit	1:500	Chemicon / AB5066P
Actin	a peptide corresponds to amino acid residues 20-33 of actin with N-terminal added lysine	Rabbit	1:10,000	Sigma / A5060
Goat anti-rabbit IgG	IRDye TM 800	Goat	1:10,000	Rockland / 611-132-122

(150 mM), SDS (1 %), sodium orthovanadate (1 mM), deoxycholic acid (0.5 %), phenylmethanesulfonyl fluoride (1.0 mM), aprotinin (5 μ g/ml), and leupeptin (5 μ g/ml), all purchased from the Sigma Chemical Co. (St. Louis, MO, USA). The total tissue homogenates were sonicated to dissolve them completely and then centrifuged at 12,000 rpm for 45 min at 4 °C. The supernatant was collected. After SDS/PAGE on 10 % gels, the proteins (80 μ g) were electroblotted onto a nitrocellulose (NC, Millipore) membrane for 20 min at 15 mV. To minimize non-specific protein binding, the NC membrane was treated with blocking buffer containing 5 % non-fat dry milk in Tris-sodium chloride buffer (TBS, including 10 mM Tris-HCl, 500 mM NaCl, pH 7.5) for 1 h at room temperature. The blot was incubated overnight at 4 °C with polyclonal primary antibodies to the β_1 - or β_2 -adrenoceptor or the NET. After a series of wash steps in TBST (TBS containing 0.05 % Tween 20), the blot was incubated with

a secondary antibody to rabbit IgG for 1 h at room temperature (Table 2). The blot was finally washed with TBS, scanned in the Odyssey Infrared Imager (LI-COR, Nebraska, USA), and analyzed with the Odyssey software package (version 1.2). Beta-actin was used as an internal control.

Solutions and drugs

The K-HS contained (mM): NaCl 117, KCl 4.7, NaHCO₃ 24.8, KH₂PO₄ 1.2, MgCl₂·6H₂O 1.2, CaCl₂·2H₂O 2.5 and glucose 11.1. The solution was gassed with a gas mixture of 5 % CO₂ and 95 % O₂. The pH was adjusted to 7.4 with 1 M HCl. Indomethacin and dimethyl sulfoxide (DMSO) were obtained from Sigma. Stock solutions of indomethacin were dissolved in DMSO, with a final DMSO concentration that never exceeded 0.1 % (vol/vol). Norepinephrine bitartrate was purchased from Jin Yao Amino Acids Pharmaceutical

Co., Ltd (Tianjin, China).

Statistics and data analysis

Data are presented as the mean \pm SEM, n refers to the number of rats per group. Statistical analyses were performed by one-way ANOVA followed by the Newman-Keuls test or Student's paired or unpaired t-test. Statistics and graphs were generated using GraphPad Prism, version 4.0 (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

The regulation of ion transport in the rat colorectum by the sympathetic nervous system

After approximately 30 min of stabilization in the Ussing chamber with K-HS, indomethacin (10 μ M) was routinely added to the basolateral side to abolish the effects of endogenous prostaglandin (Zhang *et al.* 2008). The main neurotransmitter of the sympathetic nervous system, NE (10 μ M) evoked an ΔI_{sc} of -30.1 ± 3.8 μ A/cm² (Fig. 1B,C), suggesting that the sympathetic nervous system could regulate the transepithelial ion transport in rat colorectum.

Effect of catecholamine depletion on ion transport in the rat colorectum

To investigate the sympathetic regulation of ion transport in the colorectum, we used reserpine to exhaust the endogenous catecholamine stores and thus exclude the effects of the sympathetic nervous system. The reserpine-treated rats were feeble, and the feces were shapeless (Fig. 1A). The baseline I_{sc} in catecholamine-depleted rats was increased 2.8-fold relative to the control ($n=11$, $P < 0.05$); the transepithelial resistance (R_{te}) was not significantly altered (Table 3). The NE-induced ΔI_{sc} was increased from -30.3 ± 1.6 to -54.4 ± 4.6 μ A/cm² in catecholamine-depleted rats, which was 1.8-fold higher than the control ($n=4$, $P < 0.01$) (Fig. 1B,C).

Table 3. The basic I_{sc} values in the colorectal mucosa of rats.

Group	n	Basal I_{sc} μ A/cm ²	Transepithelial resistance (R_{te}) $\Omega \cdot \text{cm}^2$
Control	11	21.7 \pm 4.8	78.2 \pm 12.0
Reserpine	11	81.8 \pm 8.0 *	72.9 \pm 8.2

Data are expressed as mean \pm SEM; * $P < 0.05$.

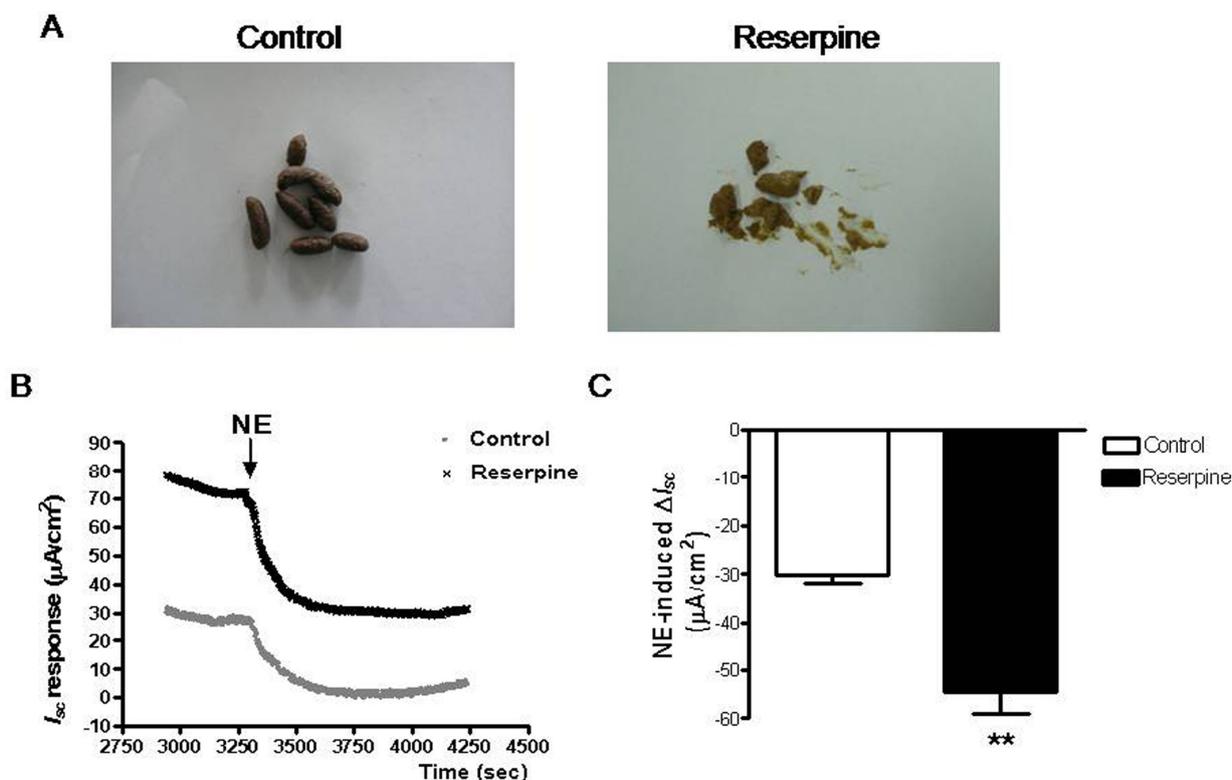


Fig. 1. NE-evoked ion transport in the mucosa of the late distal colon in control and reserpine-treated rats. **A.** Feces shapes in different rat groups. **B, C.** NE (10 μ M) induced I_{sc} response in the colorectal mucosa of control and reserpine-treated rats ($n=11$). Data are expressed as mean \pm SEM; ** $P < 0.01$.

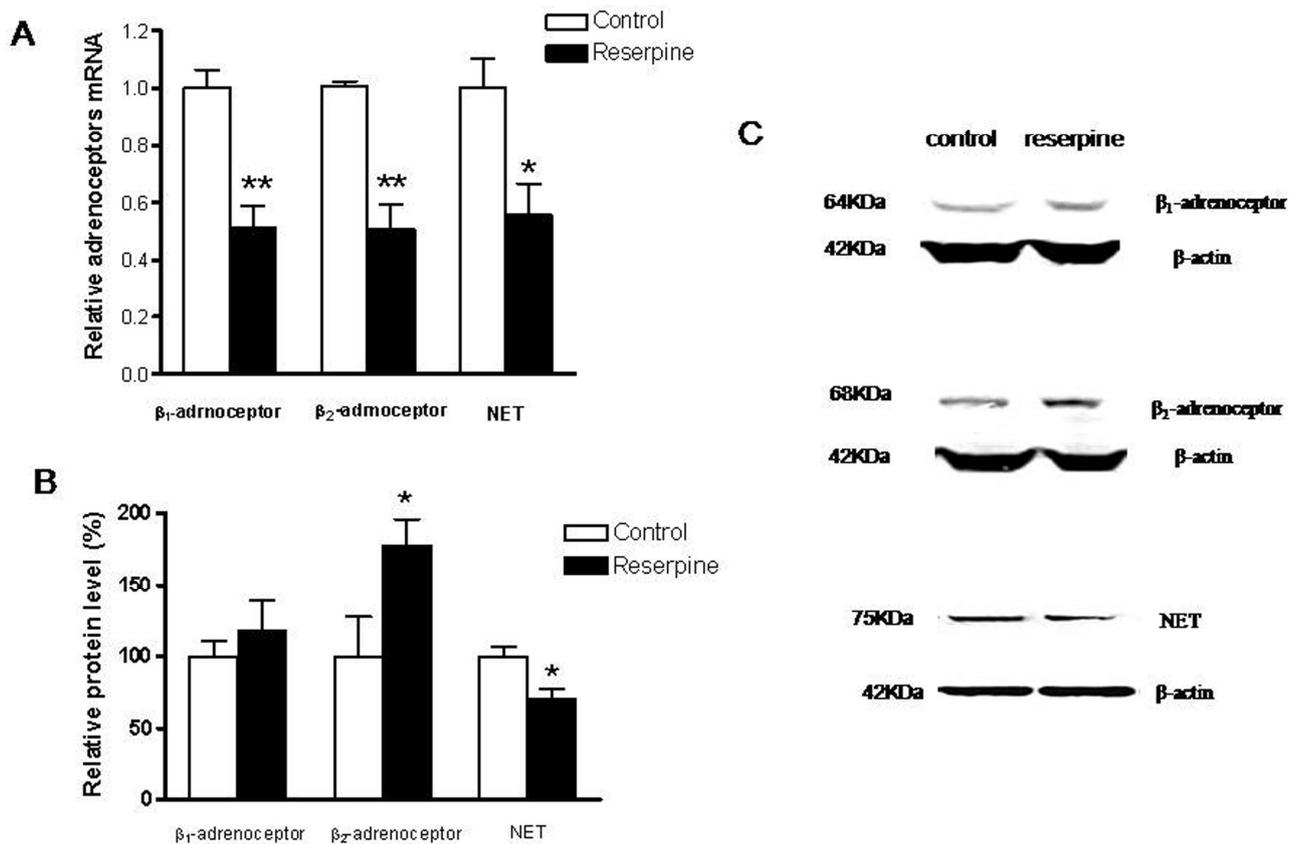


Fig. 2. β -adrenoceptor expression in the colorectal mucosa in control and reserpine-treated rats. **A.** The mRNA level of β -adrenoceptors and NETs in the colorectal mucosa of control and reserpine-treated rats. **B, C.** The protein level of β -adrenoceptors (n=4) and NETs (n=5) in the colorectal mucosa of control and reserpine-treated rats. β -actin was the internal control for normalization. Data are expressed as mean \pm SEM; * P<0.05; ** P<0.01.

Effects of catecholamine depletion on the expression of β -adrenoceptor and NET in colorectal mucosa

β_1 - and β_2 -adrenoceptors were reported to mediate the effect of NE-induced ion transport in the colorectum (Zhang *et al.* 2008); therefore, to further investigate the sympathetic regulation of the colorectum, the expression of β -adrenoceptors was investigated in catecholamine-depleted rats. The β_1 -adrenoceptor mRNA expression level was significantly decreased by 48.9% (n=4, P<0.01) (Fig. 2A), and no significant change was found in the protein level (Fig. 2B,C). The β_2 -adrenoceptor mRNA expression level was decreased by 49.7% (n=4, P<0.01) (Fig. 2A), but the protein level was increased (n=4, P<0.05) (Fig. 2B,C).

The NET reuptakes NE to repackage or metabolize, and supports efficient noradrenergic signaling and presynaptic NE homeostasis (Matthies *et al.* 2009), which is essential for phenotypic specification. It is a hallmark protein of noradrenergic neurons (Fan *et al.* 2009). Therefore, we examined the expression of NET

in catecholamine-depleted rats. The NET mRNA level was reduced by 44.3% compared to the controls (n=3, P<0.05) (Fig. 2A), and the protein level was also reduced by 22.5% (n=5, P<0.05) (Fig. 2B,C). These results indicate that the sympathetic regulation is attenuated in the catecholamine-depleted colorectum.

Discussion

The present study investigates the regulation of ion transport by the sympathetic nervous system in the rat colorectum. The sympathetic nervous system regulates gastrointestinal blood flow, electrolyte transport, mucous secretion, and motility, among other aspects of physiology (Gershon and Erde 1981, Vanner and Surprenant 1996). The gut mucosa is extensively innervated by noradrenergic neurons and a high concentration of catecholamines can be potentially achieved in the vicinity of the epithelial cells (Gershon and Erde 1981). Under physiological conditions, the

colonic mucosa close to the anus (colorectum) usually plays an important role in water and electrolyte absorption (Kunzelmann and Mall 2002); however, there are few studies reporting the regulation of colorectal ion transport by the sympathetic nervous system.

Sympathetic innervation regulates intestinal fluid and electrolyte transport by providing tonic inhibition of secretory fluxes and mediates a pro-absorptive phenotype *via* adrenoceptors (Carey and Zafirova 1990). NE is a primary neurotransmitter of the sympathetic nervous system, and can evoke transepithelial ion flux in the rat colorectum *via* β -adrenoceptors located on the mucosa (Zhang *et al.* 2008, 2010). Thus, the effect of NE on colorectal secretion indirectly reflects extent of sympathetic nervous system innervation in the colorectum. Though NE induces ion transport in the rat distal colon, the effect of NE is larger in the colorectum than in other parts of distal colon (Zhang *et al.* 2010), indicating that sympathetic innervation in the colorectum is augmented compared to other parts of the colon. Liu *et al.* (1997) and Lam *et al.* (2003) found α_2 -adrenoceptor takes part in the intestinal ion transport. We previously have reported that α -adrenoceptors are not involved in the ion transport induced by NE, as the antagonist of α -adrenoceptor phentolamine did not block the I_{sc} invoked by NE (Zhang *et al.* 2010), which is different from theirs. The main reason might be the differential tissue preparations. The mucosa-only preparation was used in our study, but the mucosa-submucosa preparation was used in theirs. It has been reported that α_2 -adrenoceptors were more distributed in the submucosal plexus, and the functions were more depended on the plexus (North *et al.* 1985, Xia *et al.* 2000).

A common way to study the sympathetic contribution to regulatory responses is through the use of sympathectomy. The catecholamine-depleting effect of reserpine is caused by an irreversible blockage of the catecholamine storage mechanism in the amine storage granules of catecholamine-containing cells (Martínez-Olivares *et al.* 2006), producing a nonspecific temporary sympathectomy (Fleming *et al.* 1973). When the endogenous catecholamine was depleted by reserpine, the basic I_{sc} in the colorectal mucosa was much greater than that in controls, suggesting that the loss of sympathetic control is able to enhance the baseline mucosal secretion. Both sympathetic and parasympathetic systems play an important role in the regulation of the balance of intestinal secretion. After reserpine-sympathectomy,

parasympathetic dominance play uncompetitive role in the intestinal basic secretion, abolishing net sodium movement and inducing net chloride secretion (Keely *et al.* 2011). Furthermore, the exogenous NE induced I_{sc} increase is related to the up-regulated β receptors (mainly β_2 -adrenoceptors) and down-regulated NET, which is an adapted response to the depletion of endogenous catecholamine. Because the mRNA levels are not always consistent with the protein levels, the low mRNA levels of β -adrenoceptors might be the consequence of a negative feedback regulatory mechanism (Tian *et al.* 2008) or a direct influence on the transcription and half-life of the mRNA (Bergann *et al.* 2009).

NET is a 12-transmembrane protein and a member of the Na/Cl-dependent monoamine transporters, and it is localized presynaptically on noradrenergic nerve terminals. Reuptake of released NE by NET in the synapse is the major physiological mechanism by which released NE is inactivated (Barker and Blakely 1995). Sympathetic neurons are a prominent source of NET, and their axons project to the gut; therefore, NET functionality has a strong role in dictating the distribution of sympathetic innervation (Mayer *et al.* 2006). It has been reported that no significant difference exists in the NET mRNA level across any specific region of normal mouse bowel, from the stomach to distal colon and rectum (Li *et al.* 2010). In the present study, reserpine-induced catecholamine depletion not only exhausted the endogenous NE but also reduced the expression of NET in the colorectum of the rat. This is different from what we observed with the adrenergic receptors. On one hand, the limited NE reuptake may have directly led to the down-regulation of NET; on the other hand, to maintain the NE concentration in the synaptic space, the down-regulation of NET may have been an adaptive alteration. All the above reflect a weak sympathetic innervation on the colorectal mucosa.

Abnormalities of the autonomic nervous system have been associated with several disease processes of the colon and rectum. The colorectum is the vulnerable part in inflammatory bowel diseases such as colorectal inflammation, ulcers and even tumors, and β -adrenoceptors localized here can play an important role in the trans-epithelial ion transport in physiological and pathophysiological conditions that alter catecholamine levels (Zhang *et al.* 2010). Decreased colonic NE release was found in inflammatory bowel disease (IBD) and Crohn's disease (Swain *et al.* 1991, Straub *et al.* 2006). However, in ulcerative colitis, the density of the

adrenergic nerve fibers and sympathetic activities was significantly increased (Kyosola *et al.* 1977, Furlan *et al.* 2006). Although the dysfunction of the sympathetic nervous system is not the causative agent in these intestinal diseases, it contributes to some clinical symptoms. It is reported that inflammation-induced inhibition of NE release could reduce the sympathetic repression of secretomotor neurons, which leads to a state of neurogenic secretory diarrhea (Straub *et al.* 2006). In our study, almost all of the catecholamine-depleted rats had a shapeless stool. The possible reasons might be the enhanced basic mucosal secretion, which was caused by loss of sympathetic control and a predominant parasympathetic activity. Of course, the diarrhea in catecholamine-depleted rats was not just due to the abnormal regulation of basal ion transport in the mucosa. It has been reported that sympathetic nervous system also

takes part in the colonic motility (Ridolfi *et al.* 2009) and immune regulation (Straub *et al.* 2006, Cervi *et al.* 2013), therefore these influence should not be ignored.

In summary, the sympathetic nervous system plays an important role in regulating ion transport in the colorectum. Disruption of sympathetic neurotransmitters resulted in abnormal ion transport and both β -adrenoceptor and NET were involved in the process.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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