Original Article

Effects of selective antagonism of β-adrenoceptor sub-types on motility to noradrenalin in rat colon

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Abstract: Noradrenalin as well as dopamine and adrenalin are involved in regulation of gastrointestinal function including colonic motility and ion transport. Stress is able to enhance colonic motility but the mediated adrenoceptors are not well addressed. In the present study, contraction of isolated longitudinal strips (2×10 mm) in rat distal colon (DC) was measured. Motility of the colon was studied by using strain gauge recording and expression of β adrenoceptors in rat colon was examined by immunofluorescence, RT-PCR, and Western blot. The results indicate that DC manifested automatic rhythm motility, which was greatly enhanced in cold-restrained stress (CRS) rats, from 352±58 to 549±28 mg·min. Addition of NE dose-dependently inhibited colonic motility in both normal (IC₅₀ 2.7×10⁻⁶ M) and stress condition (IC₅₀ was 3.1×10⁻⁶ M), from 120.45±30.21 mg·min in normal group to 89.32±23.17 mg·min in the stress group. The suppressive roles were notably reduced by pretreatment with β₁ or β₃ adrenoceptor antagonist, atenolol (1×10⁻⁵ mol/L) or cyanopindolol (7.5×10⁻⁷ mol/L). The immunofluorescence activity of β-adrenoceptor was widely distributed in the distal colon. An obvious difference of the distribution of the β-adrenoceptors in the smooth muscle layer was observed in different segments of rat distal colon, and protein content of β₃, but not β₁ and β₂-adreceptor increased significantly in CRS rats. There was variation of β-adreceptors in the location and segment. β₃ and β₁-adreceptor involved in NE-induced inhibition on colonic motility, which may play an important role in the formative process of stress-related colon diseases.

Keywords: Colon motility, noradrenalin, stress model, β-adrenoceptor, rat

Introduction

Catecholamines including epinephrine, norepinephrine and dopamine are important neurotransmitters in both the peripheral and central nervous systems. Norepinephrine (NE), an enteric autonomic nerve system transmitter [1] plays important roles in digestion [2-5]. NE involves in the regulation of GI motility, including in stress condition. DA-stimulated colonic ion transport was mediated by β₂-adrenoceptor, not dopamine receptor [6, 7] β-adreceptors have been reported to trigger and mediate colonic relaxation [8, 9], while noradrenalin had opposite effects [10]. In the gastrointestinal tract, acute stress almost immediately stimulates colonic contractile activity, resulting in an enhanced defecation in rats and mice [1]. One of the three prominent mediators of stress response is norepinephrine [11]. However, it is not clear whether β-adreceptors are involved in motility dysfunction in the acute stress model. The aim of the present study is to clarify the role of β-adreceptors in mediating the effect of NE on the colonic motility in stress condition by using strain gauge recording, immunofluorescence, and Western blot. The study will promote understanding of colonic physiology and pathology, and provide more helpful and valuable experimental evidence for the prevention and therapy of human colonic diseases in clinic.

Materials and methods

Materials

The colon strips strain gauge recording experiments were carried out in K-HS contained 117 mmol/L NaCl, 4.7 mmol/L KCl, 24.8 mmol/L NaHCO₃, 1.2 mmol/L KH₂PO₄, 1.2 mmol/L Mg-
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**Table 1. Immunohistochemistry antibodies**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Dilution</th>
<th>Source</th>
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<tr>
<td>β₁</td>
<td>Rabbit Polyclonal</td>
<td>1:70</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>β₂</td>
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<tr>
<td>β₃</td>
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<td>1:70</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Goat/FITC</td>
<td>1:60</td>
<td>Zhongshan golden bridge Biotechnology Co., LTD</td>
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Cl₂6H₂O, 2.5 mmol/L CaCl₂6H₂O, 11.1 mmol/L glucose. The solution was gassed with a gas mixture of 5% CO₂ and 95% O₂. The pH was adjusted to 7.4 with NaOH or HCl. Drugs that were used in the study included dopamine hydrochloride, dimethyl sulfoxide (DMSO), (±)-propranolol hydrochloride, atenolol, ICI 118,551 hydrochloride, R(+)-SCH-23390, sulpiride hydrochloride (Sigma, St. Louis, Mo.), prazosin hydrochloride (Fluka, Buchs, Switzerland), and noradepinephrine bitartrate (Jin Yao Amino Acids Pharmaceutical, Tianjin, China). Stock solutions of dopamine were dissolved in DMSO; others were dissolved in aqueous stock solution.

**Tissue preparation**

Male SD rats weighing 250-300 g (Laboratory Animal Services Center, Capital Medical University) were housed in group cages under conditions of controlled temperature (22-24°C) and illumination (12-h light cycle starting at 6:00 AM) before experiments are maintained on laboratory chow and water. Protocols describing the use of rats were approved by the Animal Care and Use Committee, Capital Medical University and in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The animals were killed by cervical dislocation.

Isolated distal colons (DC, 1.5 cm to anus) were cut along the mesenteric border into a flat sheet and flushed with Krebs-Henseleit solution (K-HS). The DC segments were pinned flat with the mucosal side up in a Sylgard-lined petri dish containing K-HS. 2×10 mm longitudinal strips in median were obtained by eye scissors and tied with soft thread around both ends for strain gauge measurement. The colon segments were pinned flat with the mucosal side down to be lightly ripped the smooth muscle layers for real-time PCR and Western blot tests.

**Immunohistofluorescence**

Segments of rat colon were embedded in the optimum cutting temperature medium (Mccormick, St. Louis, MO, USA) and frozen in liquid nitrogen immediately. Sections (5 µm) were cut at -20°C in a cryostat microtome (Leica CM-1850, St. Gallen, Switzerland) and thaw-mounted onto Polysine frost plus slides. The sections were fixed for 10 minutes in cold acetone and then washed (3×6 minutes) in 0.3% Triton X-100 phosphate buffer solution (PBST) to eliminate residual fixative. After blocking with 10% normal goat serum (Sigma-Aldrich, St. Louis, MO) for 30 minutes at room temperature, the sections were incubated with primary antibody (Table 1) diluted in 1% cow serum albumin overnight. Omitting the primary antibody was used as the negative control. The sections were then washed in PBST (3×6 min) and incubated with the secondary antibody diluted in 1% cow serum albumin for 1 hour. After the final PBST

**Animal models**

Cold restraint stress (CRS) procedure was similar to that reported by Williams and Barone FC [12, 13]. Rats were fast caught on the back skin, and the four limbs were tied with soft ropes secretly. Then the rats were restricted back to boards by the ropes. The animals were immediately placed in 13±2°C water up to breastbone for 70 minutes. Control animals were allowed to move freely in cages. The rats were killed by cervical dislocation after CRS.

**Strain gauge measurement**

The muscle strips, immersed in 10 ml K-HS chamber maintained at 37°C in the wall water recirculation from a warm reservoir during the experiments, were slinged to strain gauge. The K-HS solution were bubbled with 95% O₂ and 5% CO₂ (V/O₂/V/CO₂) to maintain the pH of the solution at 7.4. Drugs were added directly to the K-HS immersing the colon strips. Tension responses were continuously recorded through the strain gauge to the computer (Australian Power Lab Biology Signal Recording System). The tensions always start at 1 g. After balancing 50 minutes, the value was calculated on tension curve area (g×1 minute) before and after drug administration.
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**Table 2. Western blotting antibodies**

<table>
<thead>
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<td>1:333</td>
<td>ABR</td>
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<tr>
<td>β₂</td>
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<td>Sigma</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Goat/FITC</td>
<td>1:60</td>
<td>ROCKLAND</td>
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wash, the sections were cover slipped and observed under a fluorescence microscope (Olympus BX51, Japan). The samples were incubated with rabbit anti-mouse immunoglobulin (IgG, Santa) for 1 hour at 37°C and stained with 3,3'-diaminobenzidine (DAB) for 3 minutes and hematoxylin for 20 seconds. Samples were observed under a microscope (Olympus BX51, Japan).

**RNA extraction and preparation of cDNA**

The colon segments were 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). After the wall of each piece of gut was opened, 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 with 1 μg of each total RNA, 500 ng of oligo (dT) 12-18 primers, 10 nmol dNTPs, DEPC sterile distilled water incubate at 65°C for 5 minutes, then place on ice for at least 1 minute and 4 μl 5X first strand buffer including 250 mM Tris-HCl (pH 8.3), 375 mM KCl and 15 mM MgCl₂, 40 U of RNase OUT, and 200 U of SuperScript III RT, 0.1 μmol DTT, was added in a 20 μl reaction volume. Following brief centrifugation, the tractions were incubated at 50°C for 50 minutes, then at 70°C for 15 minutes. The completed reverse transcription reactions were stored at -20°C and used for PCR without further treatment.

**Real-time PCR**

Real-time PCR was used to quantify mRNA encoding β-adrenoceptor in the rat different colon segment smooth muscle respectively. Expression of β-adrenergic receptors was normalized to that of 28S rRNA, a housekeeping gene that is not thought to be subject to regulation. Transcripts encoding β-adrenergic receptors in samples of rat colon were comparatively quantified by real-time PCR with the Brilliant SYBR Green QPCR Master Mix kit (Stratagene) using a Light Cycler instrument (Stratagene). The specific primers are listed in Table 2.

Amplifications were performed in a final volume of 20 μl of a commercial reaction mixture (Stratagene). The primers for the amplification of cDNA encoding β-actin, β₁, β₂ and β₃-adrenoceptors were used at a final concentration of 0.2 μM, 0.25 μl of the cDNA prepared from tissue was added to the mixture. A reference dye ROX was used to normalize for non-PCR related fluorescence fluctuations occurring well to well or over time. Within the instrument, the reaction mixture was first incubated at 95°C for 10 minutes to denature the template DNA. Amplification was then performed for 40 cycles, each involving denaturation at 95°C for 30 seconds, annealing for 1 minute at 60°C, and elongation at 72°C for 30 seconds. The appearance of double-stranded DNA was quantified by measuring the fluorescence of SYBR Green after each step of annealing. A melting point analysis was finally performed to improve the sensitivity and specificity of amplification reactions detected with the SYBR Green I dye; samples were incubated at 95°C for 1 minute, at 55°C for 30 seconds and then from 55 to 95°C with a transition rate of 0.2°C·sec⁻¹. Data were analyzed with computer assistance using the MxPro-Mx3000P software.

**Western blotting for adrenoceptors**

Tissue was harvested from the mucosa of rat distal colon, washed with PBS, and homogenized in 300 μl cold lysis buffer, pH 7.5, containing Nonidet P-40 (1%), TRIS-HCl (10 mM, pH 8.0), EDTA (1.0 mM), NaCl (150 mM), EGTA (2.0 mM), 10% SDS (0.1%), sodium orthovanadate (1 mM), deoxycholic acid (0.5%), phenylmethylsulfonyl fluoride (1.0 mM), aprotinin (5 μg/ml), and leupeptin (5 μg/ml), all purchased from Sigma. Total tissue homogenates were sonicated to dissolve completely and then centrifuged at 12,000 rpm for 30 min at 4°C to separate the membrane-containing fraction (pellet) from the cytosol. Proteins (100 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis. The separated proteins were electroblotted onto nitrocellulose membrane (NC membrane, Millipore), and then the mem-
brane was washed for 10 minutes with TBST (20 mM TRIS-Cl pH 7.5, containing 0.15 M NaCl and 0.05% Tween 20) and immersed in blocking buffer containing 5% nonfat dry milk in TBST for 1 hour at room temperature. The blot was washed with TBST and finally incubated overnight at 4°C with polyclonal primary antibodies to β₁ (Affinity BioReagents, USA; diluted 1:500 in 5% nonfat dry milk), β₂ (Santa Cruz Biotechnology, Santa Cruz, Calif.; diluted 1:500 in 5% nonfat dry milk) or β₃ (Santa Cruz Biotechnology, Santa Cruz, Calif.; diluted 1:500 in 5% nonfat dry milk). After washed in TBST, the blot was incubated with secondary antibody to rabbit IgG (Rockland) for 1 hour at room temperature. The blot was finally washed with TBST, scanned by infrared rays with the Odyssey Infrared Imager (LI-COR, Nebraska, USA), and analyzed by Odyssey software (version 1.2).

Statistics methods

Motility index (mg·min⁻¹) is the area under the tension curve from the baseline. Nonlinear regression figure Y axis “ratio of motility index to the original” was calculated from every time motility index of different NE dose respectively divided by the corresponding initial motility index. Data from at least three independent experiments performed in triplicates are presented as the mean ± SE. Error bars in the scatterplots and the bar graphs represent SE. Data were examined to determine whether they were normally distributed with the One-Sample Kolmogorov-Smirnov test. If the data were normally distributed, comparisons of measurement data between two groups were performed using independent sample t test and the comparisons among three or more groups were first performed by one-way ANOVA test. If the results showed significant difference, when the data were skewed distribution, comparisons were performed by nonparametric test. Measurement data between two groups were performed using nonparametric Mann-Whitney test. Statistics and graphs were generated using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, U.S.A.). Statistical analysis between two samples was tested using a Student’s paired or unpaired t-test and F test of nonlinear regression comparison. “n” refers to the number of tissue preparations used from different animals. Statistical tests were two-tailed and a p value of less than 0.05 was considered statistically significant.

Results

Appearance of segmental discrepancy of motility in rat distal colon

As shown in Figure 1A, the distal colon was defined as the 7 cm long segment proximal to the lymph node, (typically situated 3 cm away from the anus) as reported elsewhere [14]. The distal colon was split into four segments termed DC₃ (next to lymph node), DC₂, DC₁, and DC, respectively. Because the motility format on the segment around lymph node (DC₃) is very different from the other three segments and also the variation from DC₃ to DC₄ is gradually prominent, the DC₃ and DC₄ were therefore chosen to investigate the segmental discrepancy of the rat distal colonic motility and the possible underlying mechanism. Similar to our previous investigated there were segmental discrepancy of the rat distal colonic ion secretion [15]. As shown in Figure 1B, there was segment discrepancy of the rat distal colonic motility in term of frequency and amplitude. As Figure 1C shows that the motility frequency in segment d (1.69±0.15, n=6) is obviously higher than that in segment a (1.22±0.08, n=8, P<0.05) by 27.8%, segment b (1.11±0.07, n=8, P<0.01) by 34.3% and segment c (0.97±0.06, n=8, P<0.001) by 42.6%. As Figure 1D indicates, the motility index segment d (85.25±11.35 mg·min⁻¹, n=8) is much lower than that in other segment a (277.9±27 mg·min⁻¹, n=9, P<0.001) by 269.7%, segment b (251.4±35.75 mg·min⁻¹, n=9, P<0.001) by 194.9%, segment c (277.9±27 mg·min⁻¹, n=9, P<0.001) by 225.9%. All these results indicate that there is segmental discrepancy of motility in rat distal colon.

Norepinephrine-induced inhibition of rat DC motility in normal and CRS condition

Previous studies have shown that NE could inhibit distal colon spontaneous rhythm motility [16, 17]. It has also been reported that NE could inhibit distal colon ion secretion [7]. To test whether extrinsic NE contribute to the appearance of segmental discrepancy of motility in rat distal colon. As Figure 2A shown, NE inhibits the motility of distal colon with the dose-dependent curves on different segment colon strips; segmental a and b curves had almost same tendency (P>0.05, n=5 pairs), but both segmental c and d curves were different from the others (P<0.05-0.01, n=5-8). As the
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result of Figure 2B indicated that comparing the NE dose curves with the initial motility index ratio to the original 100%, NE inhibitory effects on a and b segments were less than c and d segments about 72.59% and 51.25% at the concentration of NE in $10^{-7}$ mol ($P<0.05$, $n=6-7$) respectively. It also had the same tendency at the concentration of NE in $10^{-6}$ mol, segmental a and b were about 59.75% decreased and segmental c and d were about 42.88% inhibition ($P<0.05$, $n=6-7$) respectively. All these investigations illustrate that NE can induce dose-dependent inhibition of rat distal colon motility in normal conditions with segmental differences.

Previous investigation showed that CRS could enhance the spontaneous contractions [18, 19], however the different segments colon strips reacting to CRS is still vague. In order to give the direct proof of the different segments effect on CRS, the spontaneous motility of distal colon strips were recorded. As shown in Figure 2C, CRS could make the spontaneous contractions enhancement in all segments. As Figure 2D shows, the motility index ratio to the control group is enhancement from 315.3±44.6 to 549.3±83.24 mg·min$^{-1}$ ($P<0.05$, $n=7$) about 74.2% in segmental a. There was also an increase in segmental b and c from 251.4±35.8 to 541.7±39.2 mg·min$^{-1}$ about 115.5% ($P<0.001$, $n=7$) and 266.4±22.7 to 448.0±61.3 mg·min$^{-1}$ by 68.2% ($P<0.05$, $n=7$) respectively, and there was enhancement in segmental d from 85.3±11.4 to 404.2±49.1 mg·min$^{-1}$ about 374.1% ($P<0.001$, $n=7$).

In order to further confirm the hypothesis that NE could suppress the different segment colon motility in CRS condition, segmental a, b, c and d of CRS were compared as control and treatment with NE all the curves in different segment were recorded simultaneously (Figure 2E). Just as Figure 2F and 2G shown, they had almost same the tendency with the same colon segment in same conditions with NE-dose curve, none of the three segments changed
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Figure 2. NE inhibits different segment strip motility of rat colon. NE dose curves of different segment colon strips in the motility index; segment a and b curves were almost same, P>0.05, n=5 pairs; both c and d segment curves were different from the others, segment d curves was lower than others. P<0.05, n=5 pairs (A). Comparing the NE
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dose curves with the initial motility index ratio to the original 100%, NE inhibitory effects on a and b segments were less than c and d segments in the concentration of $10^{-7}$ and $10^{-6}$ mol/L, $P<0.05$, $n=5$ pairs, $**P<0.05$, comparing d to the other segments (B). CRS influence on motility of different segments colon strips (C). The motility indexes were significant enhancement than the control group (C). The motility in the CRS were enhanced in all segments, especially segment d, $**P<0.01$, $***P<0.001$, comparing motility index of CRS rat colon to the control one; $\bullet P<0.05$, comparing motility index of CRS rat colon to the other CRS's, only b segment was different to d (D). NE inhibitory effect on different segment colon motility in the CRS condition, the motility indexes were significant enhancement than the control group (C). Comparing NE dose curves (%) in segment a and b of control and CRS rat colon, the tendency were same, $P>0.05$, $n=5$ pairs (F). Comparing control and CRS rat colon NE dose curves of segment c, they were different, $P<0.05$, $n=5$ pairs (G). Comparing control and CRS rat colon NE dose curves of d segments, they were different at the concentration of NE $10^{-7}$ mol/L, $p<0.05$, $n=5$ pairs (H). The control NE dose curve of d segment rat colon strip was different from the CRS's, $P<0.001$, $n=7$ pairs.

Figure 3. Influence of different receptor antagonists on NE inhibitory on rat different colon strip motility. Pretreatment with $\beta_1$ adrenoceptor antagonist, atenolol $1.0\times10^{-5}$ M, or $\beta_3$-adrenoceptor antagonists, cyanopindolol $7.5\times10^{-7}$ M, there were notable differences in NE dose lines of distal colon motility with the control group at the concentration of NE from $10^{-7}$-$10^{-6}$ mol/L ($P<0.05$-$0.01$, $n=6$) (A). Pre-treatment with $D_1$ antagonist (SCH-23390, $10^{-5}$ mol/L), $D_2$ antagonist (sulpiride, $10^{-5}$ mol/L), or $\beta_3$ antagonist (ICI 118,551, $1\times10^{-5}$ mol/L) respectively, there were no difference with the NE-induce inhibition in distal colon ($P>0.05$, $n=7$) (B). Comparing the motility index ratio to the original (%) pretreatment with $\beta_1$ adrenoceptor antagonist, atenolol at concentration of $1\times10^{-5}$ mol/L or $\beta_3$-adrenoceptor antagonists, cyanopindolol $7.5\times10^{-7}$ mol/L respectively, the NE-dependence dose curves with $\beta_1$ or $\beta_3$-antagonist shifted to the right ($P<0.05$-$0.01$, $n=7$), while pre-treatment with $\beta_2$ adrenoceptor antagonists, ICI 118,551 at concentration of $1\times10^{-6}$ mol/L the NE dose- curves showed only a partial right shift ($P>0.05$, $n=7$) (C). The NE-dependence dose curves with $D_2$ antagonist (sulpiride, $10^{-5}$ mol/L), antagonist had no shift ($P>0.05$, $n=7$) (D), $*P<0.05$, $**P<0.01$, $***P<0.001$, comparing d to the control group.

compared to the control groups ($P>0.05$, $n=7$). But as in Figure 2H shown segmental d, the change tendency motility were significantly different with the control ones, there was marked decrease at the same concentration of NE $10^{-7}$ mol/L from $34.9\pm5.5\%$ to $15.6\pm2.9\%$ about 55.3% ($P<0.01$, $n=11$). All these data indicate that NE might also be involved in CRS-induced the spontaneous contractions enhancement and the appearance of segmental discrepancy in CRS condition. All these changes may play an important role, which could explain that the number of feces enhanced in the stress condition [20].
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Specify the difference between the NE-induced inhibition of DC motility with β-adrenoceptor or DA-receptor antagonist

Just as Figure 3A shown, comparing the pretreatment with either β₁ adrenoceptor antagonist, atenolol at concentration of 1×10⁻⁶ mol/L or β₂-adrenoceptor antagonists, cyanopindolol 7.5×10⁻⁷ mol/L to the control, there were markedly differences in NE dose lines of distal colon motility. Furthermore, statistically, β₂ adrenoceptor antagonist had stronger prevention to NE inhibition at the concentration of from 10⁻⁷ to 10⁻⁶ mol/L on the rat distal colon spontaneous contractions from 67.4±8.3% to 97.8±3.3% (P<0.05, n=9) and from 23.8±3.6% to 84.8±3.2% (P<0.01, n=9) than that of β₁-adrenoceptor antagonist from 67.4±8.3% to 83.4±3.6% (P>0.05, n=6) and from 23.8±3.6% to 52.5±3.9% (P>0.05, n=6). NE at concentration more than 1×10⁻⁶ M, could almost inhibit by 80% distal colonic idiopathic rhythmicity motility as control no matter whether pretreated with β₁ or β₂-adrenoceptor antagonists. As shown in Figure 3B, pretreatment of β₂-adrenoceptor antagonists, ICI 118,551 at concentration of 1×10⁻⁶ mol/L, D₁ antagonists, SCH-23390, at concentration of 1×10⁻⁶ mol/L or D₂ antagonists, sulpiride, at concentration of 10⁻⁵ mol/L respectively, all the antagonist curves had the same manner with control group (P>0.05, n=9). As shown in Figure 3C, pretreatment with β₂ antagonist (ICI 118,551, 10⁻⁶ mol/L), it had the depressant effect on the motility index ratio to the original in some degree (P>0.05, n=6), but with pretreated with β₁ or β₂-adrenoceptor antagonists, the motility index ratio to the original was higher than the control (P<0.001, n=6). As shown in Figure 3D, all these results indicate that β₂ and β₂-adrenoceptor antagonists might prevent the NE-induce inhibition in distal colon. The results suggest that pretreatment with D₁ antagonist (SCH-23390, 10⁻⁵ mol/L), D₂ antagonist (sulpiride, 10⁻⁵ mol/L), or β₁ antagonists (ICI 118,551, 1×10⁻⁵ mol/L), the motility index ratio has few different, but no statistically significant change to the control group only with the different concentration of NE and has independence with the NE-induce inhibition in distal colon.

Specify distribution and protein content of β-adrenoceptors in DC

β-adrenoceptors distribution was investigated by means of the immunohistochemistry and protocols in molecular biology. As Figure 4A and 4B show, there was segmental variability on the distribution of β-adrenoceptors subtype. Also, β-adrenoceptors were located in the myenteric nerve plexus, smooth muscle layer and epithelial mucosa [7, 21] in distal colon and β₂-adrenoceptors and mustered in the epithelial mucosa and submucosa.

The Western blot was used to investigate the effect of CRS on the protein content of β-adrenoceptors in rat DC. As shown in Figure 4C, more protein content of β-adrenoceptors was observed in the smooth muscle compared with epithelia (P<0.05-0.01, n=9). Whereas, CRS rat β₁-adrenoceptors had a little enhancement compared to control (P>0.05, n=8), the β₂-adrenoceptors had significant increased about 38.11% (P<0.05, n=8), but not β₂-adrenoceptors. All these results indicate that β₂-adrenoceptors expression might be enhanced in the CRS condition.

Discussion

Exposure to physical and psychological stressors is largely recognized to alter gastrointestinal functions in humans and in rodents. In rodents, stress produces gastric ulceration [22], inhibits gastric motility and emptying and promotes intestinal propulsive motor activity [1], enhances epithelial permeability to macromolecules in the small and large intestine [4, 23] increases visceral pain in response to rectal distension [24]. Stress is also associated with development, progression, and relapse of inflammatory bowel diseases in humans, and with intestinal inflammatory processes in animal models [25]. Our previous study revealed that NE is able to suppress transepithelial ion transport in rat late distal colon [26] and β₂-adrenoceptors mediate the process [24]. Adrenoceptors have also been found in peripheral organs, including the gastrointestinal tract [27, 28]. However, it is not clear whether there is a difference with which β adrenoceptors (s) mediated NE-induced motility in rat distal colon. The functional role β₂-adrenoceptor in inhibiting human colonic motility has been suggested by the authors of several in vitro functional studies carried out on human muscle-strip preparations from circular and taenia coli colonic SMC [8, 29]. It has been assumed that adrenergic agonists inhibit gastrointestinal motility by interacting with a heterogeneous smooth muscle population of β-adrenoceptors,
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Figure 4. Representative immunofluorescence images of rat colon in non-stressed rats. β-adrenoceptors were identified with monoclonal antibody to rat. The distribution of the β-adrenoceptor subtypes had variability in various segment (green as β-adrenoceptor, blue as nucleus and red as NF, yellow as merge) (A). DAB staining images of rat colon. LM represents the outer longitudinal muscle layer; CM represents inner circular muscle layer; and the black line arrow represents cells in which β-adrenoceptors are expressed (representative data from n=5, n represents animal number) (B). Quantitative evaluation of the protein content of β-adrenoceptors in rats exposed to cold-restraint stress for 100 minutes at 18°C. The bar graph shows quantification of bands by densitometric analysis (C). Each bar represents the mean ± SEM for 8 rats per group (D). *P<0.05, **P<0.01, comparing CRS to the control group.
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namely $\beta_1$, $\beta_2$, and $\beta_3$ [30]. CRS increased the automatic rhythm motility of DC in our experiments too much to let the water in content absorbed completely as normal, so that the colon content inclined to trigger peristalsis pushing feces. So, CRS rats have more and looser bowels than control [13] as other stress [10, 23].

In our current study, it has been demonstrated that $\beta_1$ and $\beta_2$-adrenoceptors, but not $\beta_3$ receptors, mediate NE-induced colon motility inhibition in the distal colon of rats, as evidenced by the following.

1. Pretreatment with $\beta_1$ or $\beta_2$-adrenoceptors antagonists, the NE-induced colon motility inhibition in the distal colon of rats was much more blocked than that of pretreatment with $\beta_3$; 2. Addition with $\beta_2$-adrenoceptors antagonists or $D_1$ or $D_2$ receptors antagonists respectively, there are no obvious differences with the control group; 3. There were $\beta$-adrenoceptor subtypes location in the colon epithelial mucosa and myenteric nerve plexus and the protein content of $\beta_1$ and $\beta_3$ adrenoceptors, but not $\beta_2$, which might be increased in the CRS condition.

By evaluating the direct myogenic contribution of $\beta$-adrenoceptors in mediating rat colonic smooth muscle relaxation, our study supports the functional role of $\beta$-adrenoceptors in both myenteric nerve plexus, epithelial, and smooth muscle layers. The presence of $\beta$-adrenoceptors in human colonic SMC was strongly suggested by the concentration-dependent relaxation exerted by the selective antagonist [31]. The plasma concentrations of norepinephrine at 4 and 8 hours after the last stressor were significantly greater than the control values prior to the start of the stress protocol and at 1 hour after the end of the last stressor [32]. Just as the previous investigation the contractile responses in circular muscle strips after the last stressor of the heterotypic chronic stress protocol were significantly greater than those in strips taken from sham-treated controls [32].

Therefore, a common receptor target or a common signaling pathway might exist between dopamine- and norepinephrine-induced colonic motility. Non-selective and selective adrenoceptor antagonists were used to investigate whether adrenoceptor(s) was involved in nor-epinephrine-induced colon motility [33]. As others reported work that both $\beta_1$ and $\beta_3$ adrenoceptors could inhibit colon peristalsis motility [25] and base tone [34], it was found that NE, sympathetic nerve transmitter, can inhibit rat colon automatic rhythm motility through $\beta_1$ and mainly $\beta_2$-adrenoceptors so as to cause less peristalsis motility pushing feces.

In the immunohistochemistry investigation, the results show that there was segmental variability in distribution of $\beta$-adrenoceptors, which indicated that constriction or secretion-mediated by $\beta$-adrenoceptors may have segmental variability in different segments. $\beta$-adrenoceptors mainly distributed in both DC smooth muscle as human [34, 35], give direct evidence of NE decreasing rat DC automatic motility [21]. But $\beta_1$- and $\beta_3$-adrenoceptors also appeared at and worked on colon neurons [36, 37], whether their functions in the NE inhibition on rat colon motility through the neurons need to be investigated. As it is seen that $\beta$-adrenoceptors also congregated at apical mucosa epithelium, coordinating with other forward work [38] most likely to modulate liquid secretion lubricating feces.

Many studies have proved $\beta_1$, $\beta_2$ and $\beta_3$-adrenoceptors mRNAs appearing in colon smooth muscle of human [29, 35, 39] and rat colon [40]. Our results further demonstrate that mRNAs encoding $\beta_1$- and $\beta_3$-adrenoceptors appear in smooth muscle of rat DC [31]. As the data suggest that the adrenal medulla releases norepinephrine in peripheral circulation in stimulation [41]. The content $\beta_2$-adrenoceptors protein in DC of CRS rats was up-regulated in the Western blot, which gave the protective structure basement to help NE effectively correcting [42] of CRS rats colon to normal as a kind of self-cure mechanism [43]. So $\beta_1$-adrenoceptors might be the main active factor to inhibit rat colon motility in both normal and CRS rat, additionally $\beta_1$-adrenoceptors could compensate $\beta_2$-adrenoceptors as consistency as previous report [44]. The existence of $\beta_2$-adrenoceptors is now generally accepted, and these receptors have been shown to be abundant in a number of gastrointestinal smooth muscle preparations [45]. Data in the previous section indicate that smooth muscle might have hyper-reactivity to NE increases in a time dependent manner after the acute stressor.
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The Western blotting results also demonstrated that the β₃-adrenoceptor protein in CRS rat DC smooth muscle was higher than that of the control group statistically.

In conclusion, NE can inhibit rat DC strip motility through β₁ and mainly β₃-adrenoceptors, CRS stimulated rat DC motility while the up-regulation of β₃-adrenoceptors in DC smooth muscle of CRS rats should be a compensatory mechanism that help NE to mitigate the stronger motility caused by CRS to normal condition [46].

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Disclosure of conflict of interest

None.

Abbreviations

DA, Dopamine; CRS, cold restraint stress; NE, noradrenalin; DC, distal colon; PC, proximal colon; GI, gastrointestinal; PD, Parkinson's disease.

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