

Expression Imbalance of Cholinergic M₂ and M₃ Receptors Contributes to the Motility Reduction of the Small Intestine in Spleen Qi Deficiency

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Abstract

Objective: To study roles of cholinergic M₂ and M₃ receptors in the motility reduction of small intestine (SI) in spleen qi deficiency. **Methods:** 16 male SD rats were randomly divided in the control group and spleen qi deficiency group (model group)—8 rats each group; spleen qi deficiency model of the improper diet and overfatigue was established; the SI propelling rate (SIPR) was used to evaluate the SI motility; ELISA was used to measure concentrations of acetylcholine (ACh), cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) in the SI tissue; immunohistochemistry was employed to detect expressions of cholinergic M₂ and M₃ receptors. **Results:** Compared with those in the control group, SIPR was reduced; expression of M₂ receptors was increased; and expression of M₃ receptors and concentrations of cAMP and PKA were decreased, significantly, in the model group. **Conclusions:** Expression imbalance of cholinergic M₂ and M₃ receptors might contribute to the motility reduction of the SI in spleen qi deficiency.

Keywords

Spleen Qi Deficiency, Small Intestine Motility, M₂ Receptor, M₃ Receptor

1. Introduction

Poor appetite and abdominal distension are common symptoms of spleen qi deficiency, and both of them relate

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to the reduction of gastrointestinal (GI) kinetics. Generally, smooth muscles (SM) of the GI tract are predominantly innervated by adrenergic and cholinergic nerves of the autonomic nervous system (ANS), and the latter was focused in the present study due to its excitatory effect. Acetylcholine (ACh) released by cholinergic nerves can activate muscarinic receptors to evoke the SM contraction [1]. Muscarinic receptors in the GI tract are primarily M_2 and M_3 subtypes. Although M_2 receptors outnumber M_3 receptors by up to 4:1, M_3 subtype is the primary receptor to evoke the SM contraction directly, whereas M_2 subtype is the conditional receptor, and inhibits the synthesis of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA), thereby abrogating the muscle relaxation [2].

Abnormal expressions of M_2 and M_3 subtypes contribute to GI diseases [3] [4]; however, it is still not known whether these two receptors involve in the pathology of spleen qi deficiency. In the present study, based on the rat spleen qi deficiency model of improper diet and overfatigue, the small intestine propelling rate (SIPR) and related cholinergic components were tested, attempting to reveal regulatory roles of M_2 and M_3 receptors in the SI motion of spleen qi deficiency.

2. Materials and Methods

2.1. Animals

Sixteen male SD rats weighing 220 ± 10 g (Liaoning Changsheng Biotechnology Co., Ltd (SCXK (Liao) 2010-0001) were used. Rats were randomly divided into 2 groups of 8 each, the control group and spleen qi deficiency model group (model group). Animals were group housed under the following laboratory condition: temperature $23^\circ\text{C} \pm 1^\circ\text{C}$, humidity 40% - 60%. Animal care procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and every effort was made to minimize the animal suffering.

2.2. Establishment and Evaluation of the Spleen Qi Deficiency Model

Rats in the model group were treated according to the following protocol for 2 weeks: food and water were available *ad libitum* on odd days and fed only with cabbage on even days; forced to swim to fatigue in 35°C - 37°C water every day. Evaluation criterion for spleen qi deficiency is: 1) emaciation: the significant decrease of the body mass; 2) poor appetite: the significant reduction of food and water intake, detected in metabolic cages; 3) mental fatigue: the significant reduction of the motion distance and vertical times in the open field during 5 min, checked with the OFT-100 opening activity experiment system (Chengdu TME Technology Co, Ltd., Chengdu, China); 4) lack of strength: the significant decrease of the forelimb strength, checked with YLS-13A Grip Strength Meter for Rats and Mice (Jinan Yi Yan Technology Development Co., Ltd., Shandong, China).

2.3. Measurement of SIPR

Fasted without water deprivation for 24 h, rats were intragastric administration (1 ml/100g) of 10% carbon powder suspension (5% active carbon and 10% acacia gum), then anesthetized with 10% chloral hydrate (0.35 ml/100g) after 30 min. The SI from the pylorus to the ileocecal junction was carefully taken out, and SIPR was calculated as the following equation: (spreading distance of carbon powder/the SI length) \times 100%.

2.4. Concentration Measurement of ACh, cAMP and PKA in the SI Tissue

About 2 cm SI from the pylorus was taken out and cleaned with 4°C saline, and the tissue homogenate was prepared for ELISA. Concentrations of ACh, cAMP and PKA (kits were purchased from Beijing Boosen Biological Technology Co., Ltd., Beijing, China) were determined by an auto analyzer (iMark, Bio-Rad, USA).

2.5. Expressions of M_2 and M_3 Receptors

Immunostaining of M_2 and M_3 receptors was performed using the polink-2 plus HRP detection system for rabbit primary antibody with commercially available kits (Beijing Boosen Biological Technology Co., Ltd., Beijing, China). Sections cut at 4 μm thickness from the SI tissue embedded in paraffin blocks were deparaffinized with xylene, hydrated in ethanol, incubated in 3% H_2O_2 for 10 min, washed 3 times for 2 min with PBS. Sections were incubated with anti- M_2 receptor (1:100) or anti- M_3 receptor (1:100) in PBS at 4°C for 24 h, washed with

PBS 3 times for 2 min, and Reagent 1 was added at 37°C for 10 - 20 min, washed with PBS 3 times for 2 min, followed with Reagent 2 in the same way. Sections were added with diaminobenzidine tetrahydrochloride for 5 min, counterstained with hematoxylin, dehydrated, and mounted. PBS was used to replace primary antibodies as the negative control. Mean optic density (MOD) was measured with BI-2000 system (Chengdu TME Technology Co, Ltd., Chengdu, China).

2.6. Statistical Analysis

The data were expressed as mean \pm S.D., and statistical analysis was performed between groups by ANOVA using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ or less was considered to show statistical significance.

3. Results

3.1. Model Evaluation of Spleen Qi Deficiency

Table 1 showed that all items in the model group were significantly less than those in the control group. The reduced body mass meant “emaciation”, decreases of food and water intake suggested “poor appetite”, the reduction of the motion distance and vertical times in the open field implied “mental fatigue”, and the decrease of forelimb strength represented “lack of strength”. These results confirmed that spleen qi deficiency model of improper diet and overfatigue was established successfully.

3.2. Changes of SIPRs

SIPRs in the control and model group were $64.31\% \pm 5.31\%$ and $46.75\% \pm 4.41\%$, respectively ($n = 8$, $p < 0.01$, **Figure 1**).

3.3. Changes of Related Cholinergic Components

Table 2 showed that, compared with those in the control, expression of M_2 receptor was increased significantly, expression of M_3 receptor and concentrations of cAMP and PKA were decreased markedly, but the ACh level remained unchanged, in the model group.

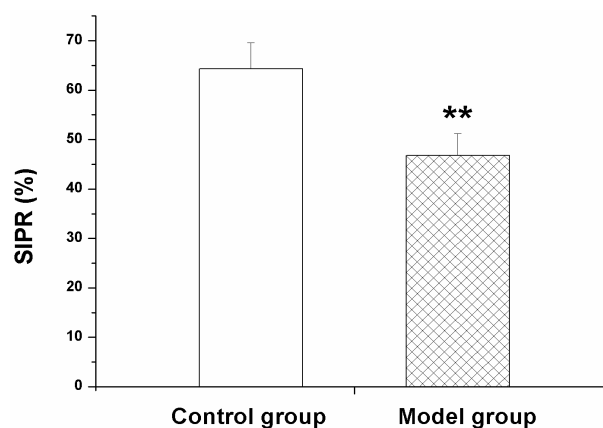


Figure 1. SIPRs of rats. Values are means \pm S.D. ($n = 8$). ** $p < 0.01$ versus the control.

Table 1. Model evaluation of rat spleen qi deficiency.

Group	Body mass (g)	Food intake (g)	Water intake (ml)	Motion distance (cm)	Vertical times	Forelimb strength (g)
Control group	371.47 \pm 22.46	32.31 \pm 9.61	89.4 \pm 24.0	9512.90 \pm 1881.59	20.6 \pm 5.9	1363.54 \pm 105.83
Model group	239.78 \pm 12.10**	21.05 \pm 5.21*	59.3 \pm 8.1**	4930.35 \pm 1253.18**	15.3 \pm 3.6*	1215.54 \pm 127.72*

Values are means \pm S.D. ($n = 8$). * $p < 0.05$, ** $p < 0.01$ versus the control.

Table 2. Changes of related cholinergic components.

Group	ACh ($\mu\text{mol}/\text{mg}$)	Expression of M_2 receptors	Expression of M_3 receptors	cAMP (mmol/mg)	PKA (pg/ml)
Control group	3.42 ± 0.40	0.117 ± 0.004	0.181 ± 0.004	13.52 ± 0.60	0.463 ± 0.045
Model group	3.37 ± 0.51	$0.215 \pm 0.004^{**}$	$0.130 \pm 0.002^{**}$	$10.63 \pm 0.80^*$	$0.320 \pm 0.051^*$

Values are means \pm S.D. ($n = 8$). * $p < 0.05$, ** $p < 0.01$ versus the control.

4. Discussion

According to the basic theory of traditional Chinese medicine, spleen dominates the transformation and transportation, which includes the whole GI function and related regulatory mechanisms. As a matter of fact, the SI is one of the main performers of spleen qi. The SI can receive the chyme from the stomach and digest it into fine nutrients and waste, the former is sent upward, the latter is conveyed into the large intestine. Therefore, the SI motility plays an important role in spleen qi.

Spleen qi can be damaged by various causes, for example, improper diet, overfatigue, cold and dampness, etc. Patients with spleen qi deficiency exhibit poor appetite, abdominal distension, emaciation, mental fatigue and lack of strength, lack of qi and no desire to speak, yellowish complexion, and so on. The main pathology of spleen qi deficiency is the reduction of digestion and absorption, especially the hypofunction of the SI. In the present study, the rat model of spleen qi deficiency of improper diet and overfatigue was successfully established, and the SI motility was evaluated with SIPR. Results showed that SIPR was significantly reduced in the model group, suggesting the SI motility was decreased in spleen qi deficiency.

Among regulatory mechanisms of the SI motion, the cholinergic activity of ANS plays a classic excitatory role, which was focused in the present study. Cholinergic M_2 and M_3 receptors express widely in the GI tract, and they can bind with ACh to evoke the SM contraction. Although the number of M_2 receptors is much greater than that of M_3 receptors, the contractile response of SM to ACh is mediated extensively by the minor M_3 subtype. M_3 receptors stimulate phospholipase C via Gq/11-type G proteins, inducing the hydrolysis of phosphatidylinositol 4,5-bisphosphate and then liberates inositol 1,4,5-trisphosphate to release Ca^{2+} from intracellular stores [5], resulting in the SM contraction through activation of the contractile proteins. In contrast, M_2 receptors play a conditional role in concert with M_3 receptors to modulate contraction [2]. Activation of M_2 receptors inhibit the elevation of the adenylate cyclase activity to decrease synthesis of cAMP and PKA, thereby abrogating muscle relaxation, and opens non-selective cation channels to increase Na^+ influx, elevating the SM excitability [6] [7]. However, M_3 receptors exert a permissive role over the M_2 receptor mediated cation current [8]. Hence, it is thought that M_3 receptors directly evoke the SM contraction, whereas M_2 receptors play an indirect role in the SM contraction.

The imbalance of activities mediated by M_2 and M_3 subtypes occurs in various GI diseases, however, it is not clear that such an imbalance occurs in spleen qi deficiency. In the present study, it was found that ACh concentration in the SI tissue of model rats was unchanged, which suggested that excitability of cholinergic nerves might be not altered in spleen qi deficiency, but expression of M_2 receptors was increased and expression of M_3 receptors was decreased, indicating that the imbalance of activities mediated by M_2 and M_3 subtypes occurred in spleen qi deficiency. Such an imbalance might induce the abnormal response of SM to ACh, resulting in the reduction of the SI motility. As M_3 receptors evoke the SI contraction directly, the down-regulated expression of M_3 receptors contributed greatly to the motility reduction of the SI. In contrast, the up-regulated expression of M_2 receptors might enhance SM responses to ACh, for example, elevating the SM excitability, decreasing intracellular cAMP and PKA levels. Although these responses might compensate some loss of the SI motility, such activities were limited because the effect of M_2 receptors on the SM contraction is indirect and controlled by M_3 receptors.

5. Conclusion

In conclusion, expression imbalance of cholinergic M_2 and M_3 receptors contributed to the motility reduction of the SI in spleen qi deficiency.

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