Electroacupuncture relieves irritable bowel syndrome by increasing expression of nucleotide-binding oligomerization domain protein-like receptor family pyrin domain containing 6 in water-avoidance stress mice

Song Yafang, Geng Hao, Chen Lu, Wu Xiaoliang, Yuan Mengqian, Xu Wanli, Guo Jing, Sun Jianhua, Pei Lixia

OBJECTIVE: To investigate the effect of electroacupuncture (EA) on irritable bowel syndrome (IBS) in mice through regulating nucleotide-binding oligomerization domain protein-like receptor family pyrin domain containing 6 (NLRP6).

METHODS: Water-avoidance stress (WAS) mice model was used to investigate the effects and the mechanism of EA. Abdominal withdrawal reflex test, open field test, and intestinal motility test were used to evaluate visceral sensitivity, anxiety, and intestinal motility in mice. The expressions of NLRP6, Mucin-2 (MUC2) and E-cadherin were determined using immunofluorescence and Western blotting assays.

RESULTS: EA significantly upregulated the expression of NLRP6 in the intestine of mice. Moreover, EA increased the expressions of MUC2 and E-cadherin in WAS mice.

CONCLUSION: Our study found that the relief of IBS symptoms by EA may involve the increase in the expression of NLRP6 in WAS mice.

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Keywords: Electroacupuncture; Nod signaling adaptor proteins; Irritable bowel syndrome

INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disease. The incidence of IBS in the world has reached 10%-20%, seriously affecting people’s quality of life. The pathogenesis of IBS is not well understood, and it is considered to be the result of multiple factors such as gastrointestinal motility abnormality, visceral paresthesia, abnormal brain-intestinal regulation, inflammation and mental psychology. Psychosocial stress can alter brain-intestinal interactions and may exacerbate IBS. Despite many new advances in the study of IBS, its treatment is still unsatisfactory. Therefore, there is an urgent need to find targets for
the treatment of IBS and effective clinical treatments. Nucleotide-binding oligomerization domain protein-like receptor family pyrin domain containing 6 (NLRP6) inflammasome as an endogenous or exogenous stress sensor, it has been gradually applied to the study of chronic intestinal diseases in recent years. Activation of NLRP6 recruits apoptosis-associated speck-like protein containing CARD (ASC) and caspase-1. Early studies of NLRP6 found that its primary role was to participate in the pathological process of inflammatory responses. With the deepening of the research, it was found that NLRP6 was widely distributed in the cytoplasm of intestinal epithelial cells (such as intestinal cells, goblet cells) in the small intestine and colon, and was also involved in pathological mechanisms such as immune defense, autoimmune response and tumor formation. The study found that NLRP6 played a negative regulatory role in the host’s immune response and clearance of pathogenic microorganisms. Loss or inhibition of NLRP6 function may result in impaired intestinal anti-inflammatory and self-repairing properties, as well as intestinal flora imbalance. Activation of NLRP6 promotes the secretion of MUC2 and maintains the function of the epithelial defense line. Therefore, NLRP6 may be an effective target for the treatment of IBS.

Methods

Ethics approval

All experimental procedures followed the "Ethical Guidelines and Guidelines for Animal Experiments" and being approved by the Ethics Committee of Nanjing University of Traditional Chinese Medicine.

Drugs and reagents

NLRP6, ASC, Caspase 1, MUC2, and E-cadherin antibodies were purchased from Abcam (Boston, MA, USA). Other reagents were purchased from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China).

Animals

C57/BL6 mice, 8 weeks old, were provided by Model Animal Research Center of Nanjing University. All experimental procedures followed the "Ethical Guidelines and Guidelines for Animal Experiments" and being approved by the Ethics Committee of Nanjing University of Traditional Chinese Medicine.

WAS model

A small platform is placed in the center of the pool made of plexiglass, and then the same room temperature (25 °C) water is injected into the pool. The water surface was 1 cm below the surface of the platform and the mice were placed on a small platform surrounded by water. Leave it for 1 h every day for 10 consecutive days. Subsequently, visceral sensitivity, intestinal motility, anxiety, and depression were evaluated by abdominal withdrawal reflex (AWR) test, open field test, and intestinal motility test.

AWR test

After the mice were anesthetized with ether, the uninflated balloon was coated with paraffin oil and placed in the mouse colorectal. The experiment was started after the mouse woke up and completely adapted to the environment for about 15 min. Four pressures of 20, 40, 60 and 80 mm Hg were used, each pressure lasting 20 s, with an interval of 4 min, and the average of three scores was taken. AWR’s scoring criteria: no significant behavioral change = 0, no or only simple head movement = 1 point, abdominal wall muscle contraction, but not lifted off the table = 2 points; abdominal wall muscle contraction, leaving the table = 3 points.

Open field test

The mice were gently placed in the middle of the test chamber (50 cm × 50 cm × 50 cm), and the activity of the mice was recorded within 5 min. The computer analysis system recorded the residence time of the mice in the central area within 5 min, reflecting anxious behavior in mice.

Intestinal motility test

The number of bowel movements was recorded by intragastric administration of 1.5% methylcellulose (containing 0.75 mg phenol red) at 250 μL each.

Interventions


Tianshu (ST 25): The xiphoid and pubic symphysis joints are divided into 13 parts, the next 8: 5 is the Shenque (CV 8), 0.5 cm beside it.

Zusanli (ST 36): The mouse hindlimb, under the knee joint, in the muscle groove 0.3 cm below the humerus head.

The mouse was fixed with a special mouse rack and
rubber band. The acupoint skin was sterilized with conventional 75% alcohol. 0.19 mm × 10 mm sterile acupuncture needles were inserted in a depth of 3 mm in the Tianshu (ST 25), Zusanli (ST 36) points. HANS-200 type electro-acupuncture instrument, oscilloscope monitoring current intensity 0.5 mA, was using density wave (2 / 15 Hz) once a day, electro-acupuncture for 15 min, total treatment for 7 d.

**Western blotting**
Tissues were lysed with protein concentrations detected by the BCA (Bicinchoninic Acid) method. According to the molecular weight of target protein, an appropriate concentration of SDS-PAGE gel was prepared. 30 μg Protein sample was loaded, electrified, subjected to vertical electrophoresis, properly separated and transferred to a PVDF membrane. Subsequently, the membrane was blocked for more than 1 h, and incubated with primary antibodies overnight at 4 °C and with secondary antibody at room temperature for 2 h. Chemiluminescence imaging was performed by using gel imaging system software. The primary antibodies included GAPDH (1: 5000), NLRP6 (1: 1000), ASC (1: 1000), Caspase-1 (1: 1000), E-cadherin (1: 1000) and MUC2 (1: 1000).

**Immunofluorescence staining**
The samples were fixed in 4% paraformaldehyde for 24 h. The gradient ethanol was dehydrated, paraffin-embedded, slicer sections of 5 μm thickness and citrate buffer for antigen retrieval. Block in 5% bovine serum albumin (BSA), primary antibody at 4 °C overnight, secondary antibody at 37 °C for 60 min. Corresponding in DAPI, then washed three times. Observe and shoot and choose three fields of view.

**Statistical analysis**
Analysis of variance (ANOVA) or t test was conducted to test the differences between groups. Results are expressed as mean ± standard deviation. P < 0.05 was considered as statistically significant. All analyses were performed with GraphPad Prism Version 5.01 (GraphPad Software Inc., San Diego, CA, USA).

**RESULTS**

**EA attenuated WAS-induced intestinal disorders in mice**
The results showed that WAS group had higher AWR scores than control group with colorectal distention pressures of 20, 40, 60 and 80 mm Hg, while EA significantly decreased the AWR scores (Figure 1A). Moreover, the residence time of WAS mice in the central area was significantly increased compared to the control group, while EA significantly decreased the residence time in the central area (Figure 1B). It suggested that EA could significantly inhibit the anxiety caused by WAS. Next, we performed the intestinal motility test (Figure 1C).

**EA promoted the activation of NLRP6 inflammasome in WAS mice**
Western blotting revealed that EA significantly increased the expression of NLRP6 compared with that of the WAS group (Figure 2A). Moreover, EA significantly increased the expressions of ASC and caspase 1 (Figure 2A). Similarly, immunofluorescence exhibited that EA significantly increased the expressions of NLRP6, ASC and caspase 1 compared with those of the WAS group (Figure 2B-2D).

**EA increased the expression of MUC2 in WAS mice**
Immunofluorescence results showed that EA significantly increased the expressions of MUC2 compared with those of the WAS group (Figure 3A). Furthermore, Western blotting results confirmed that the expression of MUC2 was significantly decreased in WAS mice compared with the control group, which was attenuated by EA treatment (Figure 3B).

**EA elevated the expression of E-cadherin in WAS mice**
Immunofluorescence results showed that EA significantly increased the expressions of E-cadherin compared with those of the WAS group (Figure 4A). Furthermore, Western blotting results confirmed that the expression of E-cadherin was significantly decreased in WAS mice compared with the control group, which was attenuated by EA treatment (Figure 4B).

**DISCUSSION**
In this study, EA significantly increased the expression of NLRP6 in WAS mice. Moreover, EA suppressed the expression of MUC2, as well as the expressions of E-cadherin in WAS mice. Taken together, EA significantly improved IBS may be related to its upregulation of NLRP6 expression.
NLRP6 is highly expressed in the small and large intestine, and plays a key role in maintaining intestinal homeostasis. The study found that IBS is associated with inhibition of NLRP6, and activation of NLRP6 significantly attenuates intestinal dysfunction caused by WAS. Similarly, our study showed that NLRP6 were significantly reduced in WAS mice, and EA significantly increased the expression of NLRP6 (Figure 2A, 2B). The activated NLRP6 can also promote extracellular secretion of MUC2 to maintain the function of the inner mucosa and intestinal homeostasis. Therefore, the up-regulation of EA on MUC2 in WAS mice may be through activation of NLRP6. Functional instability in the intestinal homeosta-
sis and destruction of the epithelial defense line promotes the development of IBS. E-cadherin is a major component of adherens junctions. Loss of E-cadherin expression results in destruction of intestinal epithelial defense line and exacerbation of intestinal diseases.20 Studies have reported that E-cadherin protein expression was significantly lower in IBS and was associated with abdominal pain and symptom duration.21,22 Our study found that the expression of E-cadherin was significantly decreased in WAS mice, which was attenuated by EA treatment (Figure 4B). Recent research proves that the fluorescence signal of NLRP6 overlapped the fluorescence signal of E-cadherin, suggesting that the expression of E-cadherin may be regulated by NLRP6.23 Our research has indeed found that EA could upregulate NLRP6 and E-cadherin. Therefore, by activating NLRP6, EA up-regulated MUC2 and E-cadherin, and protect the intestinal epithelial barrier.

Clinically, EA has been recognized as an effective treatment for patients with IBS, but there are differences in efficacy.24 This may be due to the acupuncture treatment relies on Traditional Chinese Medicine (TCM) theory, which requires the patient’s TCM diagnosis combined with different acupoint stimulation to achieve the best therapeutic effect. Visceral hypersensitivity is an important feature of IBS. Our study found that EA can significantly inhibit visceral hypersensitivity induced by WAS (Figure 1A, 1B), while inhibiting anxiety and intestinal motility disorders caused by WAS (Figure 1C, 1D), suggesting that EA has a good therapeutic effect on IBS mice. Many studies have suggested that the mechanism of acupuncture is mediated by opioid receptors, adrenergic and serotoninergic pathways. Zhou et al.25 found that EA alleviated stress-induced visceral hypersensitivity through an opioid system in rats, which could be blocked by naloxone. Camilleri et al.26 demonstrated the effectiveness of 5-HT3 receptor antagonists in the treatment of IBS. Our study demonstrates that EA treatment of IBS is through activation of NLRP6. However, this study is only a preliminary exploration of the relationship between EA and NLRP6, and the precise mechanism requires further study.

In summary, our findings suggest that EA increases the expressions of MUC2 and E-cadherin to improve IBS by activating NLRP6. This study may provide some insight into the mechanism underlying EA’s action on IBS.
Figure 2 EA promoted the activation of NLRP6 inflammasome in WAS mice
A1, A2: the NLRP6, ASC and caspase-1 expressions in mice treated with electroacupuncture (EA) were detected by Western blotting. GAPDH was used as internal control. *P < 0.01, versus control group; †P < 0.05, versus model-WAS group (n = 6). B1-B9: the NLRP6 expressions in mice treated with EA were detected by immunofluorescence (green) (scale bar, 10 μm). B1-B3: control group; B4-B6: model-WAS group; B7-B9: EA treatment group. B1, B4, B7: NLRP6; B2, B5, B8: DAPI; B3, B6, B9: Merge. C1-C9: the ASC expressions in mice treated with EA were detected by immunofluorescence (green) (scale bar, 10 μm). C1-C3: control group; C4-C6: model-WAS group; C7-C9: EA treatment group. C1, C4, C7: ASC; C2, C5, C8: DAPI; C3, C6, C9: Merge. D1-D9: the Caspase-1 expressions in mice treated with EA were detected by immunofluorescence (green) (scale bar, 10 μm). D1-D3: control group; D4-D6: model-WAS group; D7-D9: EA treatment group. D1, D4, D7: Caspase 1; D2, D5, D8: DAPI; D3, D6, D9: Merge. 1: control group; 2: Model-WAS group; 3: EA group. The control group, the water-avoidance stress model group with EA group. The acupuncture points Tianshu (ST 25) and Zusanli (ST 36) were stimulated in the EA treatment group. HANS-200 type electro-acupuncture instrument, oscilloscope monitoring current intensity 0.5 mA, was using density wave (2 / 15 Hz) once a day, EA for 15 min, total treatment for 7 d. NLRP6: the nucleotide oligomerization domain-like receptor family 6; ASC: apoptosis-associated speck-like protein containing CARD.
ACKNOWLEDGEMENTS

We are thankful to Experimental Animal Center of Nanjing University of Chinese Medicine for providing necessary facilities.

REFERENCES


