Electroacupuncture at He-Mu points reduces P2X4 receptor expression in visceral hypersensitivity***

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Graphical Abstract

Abstract

Electroacupuncture at Shangliuxu (ST37) and Tianshu (ST25) was reported to improve visceral hypersensitivity in rats. Colorectal distension was utilized to generate a rat model of chronic visceral hypersensitivity in irritable bowel syndrome. Results showed that abdominal withdrawal reflex scores noticeably increased after model establishment. Simultaneously, P2X4 receptor immunoreactivity significantly increased in the colon and spinal cord. Electroacupuncture and pinaverium bromide therapy both markedly decreased abdominal withdrawal reflex scores in rats with visceral hypersensitivity, and significantly decreased P2X4 receptor immunoreactivity in the colon and spinal cord. These data suggest that electroacupuncture treatment can improve visceral hypersensitivity in rats with irritable bowel syndrome by diminishing P2X4 receptor immunoreactivity in the colon and spinal cord.

Key Words

neral regeneration; traditional Chinese medicine; combination of He-Mu points; electroacupuncture; irritable bowel syndrome; visceral hypersensitivity; P2X4 receptor; acupuncture; grants-supported paper; neuroregeneration
INTRODUCTION

Irritable bowel syndrome is a common chronic functional intestinal disease, mainly presenting with abdominal pain and abnormal defecation, but minimal morphological and biochemical changes[1]. Irritable bowel syndrome belongs to the category of diarrhea, constipation and abdominal pain in Chinese medicine[2]. Presently, the onset mechanism of irritable bowel syndrome remains unclear. Chronic visceral hypersensitivity has been shown to be the main pathophysiological mechanism underlying abdominal pain in patients with irritable bowel syndrome[3-4]. Moreover, irritable bowel syndrome patients extensively suffer from chronic visceral hypersensitivity, with involvement of all levels of the brain-gut axis, as well as various neurotransmitters. Chronic visceral hypersensitivity can occur in the periphery, spinal cord and central nervous system[5-7]. P2 purinergic receptor family members are membrane receptors expressed on various cells, and can selectively combine with extracellular adenosine triphosphate to produce a wide range of biological effects. The P2X receptor is a ligand-gated ion channel that plays an important role in visceral pain, and has been extensively investigated. Acupuncture has been shown to be an effective method for treatment of irritable bowel syndrome[6-9]. However, the mechanism of action of acupuncture on irritable bowel syndrome, and whether acupuncture adjusts visceral hypersensitivity in rats and the potential mechanism, are unknown.

Adenosine triphosphate plays a regulatory role in visceral pain signal transduction and visceral hyperalgesia through P2X and P2Y receptors, and regulates intestinal movement and gastrointestinal secretion[10-11]. Adenosine triphosphate transfers intercellular information via P2 purinergic receptors[12-13]. Burnstock et al[14] proposed that purine signaling may explain the acupuncture mechanism. Adenosine triphosphate injected into human skin can stimulate sensory neurons[15]. A recent study confirmed that subcutaneous injection of adenosine triphosphate activated the P2X receptor in nerve endings[16], and contributed to the release of neurotransmitter from neurons[17]. Acupuncture was also reported to induce skin deformation by lifting, thrusting, and twirling, which did not injure cells, but caused release of adenosine triphosphate from various cell types including osteoblasts, endothelial cells, epithelial cells and glial cells[18]. The combination of thermal and electrical stimulation can reinforce the effects of acupuncture, and lead to the release of adenosine triphosphate. Signals enter the spinal cord through the dorsal root ganglion, and then reach the brain stem via interneurons, including motor neurons of the intestine, lung, heart, artery and reproductive system. Signals are also transmitted to the pain area of the cerebral cortex, resulting in pain suppression[18-20]. The analgesic effect of acupuncture may relate to binding of adenosine triphosphate with purinergic receptors of sensory nerve endings of the skin, causing activation of a signal conduction pathway and regulated pain perception in the cerebral cortex. Recent studies also suggest that P2X may participate in the occurrence of visceral hypersensitivity. For example, P2X4 mRNA expression was detected in the rat intestine, and P2X4 receptor expression was increased in the dorsal commissural nucleus and spinal cord posterior horn in rats with visceral noxious stimulation and colorectal distension-induced irritable bowel syndrome[21-22], indicating that P2X4 may be essential for the increase of visceral hypersensitivity in irritable bowel syndrome. Furthermore, Burnstock et al[14] reported that acupuncture therapy was effective for the treatment of visceral hypersensitivity. Thus, in the present study, we used acupuncture to regulate P2X4 receptor as a potential treatment for visceral hypersensitivity in irritable bowel syndrome patients.

Many studies have also examined the potential mechanisms of acupuncture in relieving visceral hypersensitivity. For example, acupuncture at Tianshu (ST25) and Shangjuxu (ST37) were reported to provide significant curative effects for irritable bowel syndrome[23]. Tianshu and
Shangjuxu are acupoints indicated for intestinal diseases such as diarrhea and abdominal pain. Tianshu belongs to Mu points, which are the twelve points located at the chest or abdomen, and which are closely related to the zang-fu organ. Shangjuxu belongs to the lower confluent point, which is the confluent point of the three yang meridians of the hand located at the lower extremities. A combination of He-Mu points refers to the clinical point selection methods that combine points of the lower He-sea point of six-fu organs (a collective term for gall bladder, stomach, small intestine, large intestine, urinary bladder, and sanjiao) with the front Mu point. Yang and Yan investigated the spectral peak of differential protein expression in the stomach after acupuncture at He point and Mu point using nano-two dimensional-liquid chromatography, and concluded that the therapeutic mechanism of acupuncture on stress ulcers may relate to a decrease in the contents of five types of polypeptides. Electroacupuncture at Shangjuxu has therapeutic effects on irritable bowel syndrome and reduces visceral hypersensitivity. Moreover, the effects were better than that of single electroacupuncture and single sham acupuncture, which was identical to the results of abdominal withdrawal reflex scores. Acupuncture at Tianshu and Shangjuxu reduced visceral hypersensitivity in a rat model of colorectal stimulation-induced irritable bowel syndrome, elevated pain threshold and diminished abdominal withdrawal reflex scores in rats with visceral hypersensitivity. Acupuncture exerted therapeutic effects by altering 5-hydroxytryptamine and c-Fos gene expression in the colon, spinal cord and brain of rats with irritable bowel syndrome, downregulating adrenocorticotropic hormone, vasoactive intestinal peptide and enkephalin in the hypothalamus, and reducing the number of mast cells and substance P and prokineticin-1/prokineticin receptor-1 expression in the mucous membrane of the colon. Positron emission tomography revealed that electroacupuncture at Tianshu also reduced abdominal pain, abdominal distension and abdominal discomfort by diminishing the glucose metabolic rate in the brain.

Only a few studies have addressed the regulatory effect of acupuncture on purinergic receptors (no studies for the P2X<sub>4</sub> receptor), and it remains unclear whether Burnstock’s hypothesis can be verified. In the present study, we sought to explore the mechanism of electroacupuncture at He-Mu points in the prevention and treatment of visceral hypersensitivity in irritable bowel syndrome. We examined: (1) whether the P2X<sub>4</sub> receptor is involved in visceral hypersensitivity in irritable bowel syndrome, (2) whether the P2X<sub>4</sub> receptor is expressed in the rat colon and spinal cord, and (3) the regulatory effect of acupuncture on P2X<sub>4</sub> receptor expression. Pinaverium bromide has frequently been used to treat irritable bowel syndrome-related abdominal pain, defecation disorder and intestinal discomfort. Thus, the present study utilized pinaverium bromide as a reference treatment.

RESULTS

Quantitative analysis of experimental animals
A total of 32 neonatal rats were equally and randomly assigned to control, model, electroacupuncture and pinaverium bromide groups. A rat model of chronic visceral hypersensitivity was produced in the model, electroacupuncture and pinaverium bromide groups. After model induction, rats of the electroacupuncture group underwent electroacupuncture bilaterally at Shangjuxu and Tianshu. Rats of the pinaverium bromide group were intragastrically administered pinaverium bromide. All rats were included in the final analysis.

Electroacupuncture at He-Mu points decreased chronic visceral hypersensitivity
Abdominal withdrawal reflex scores were significantly increased with increasing colorectal distension (2.66, 5.32, 7.98 and 10.64 kPa; P < 0.01). After electroacupuncture at Shangjuxu and Tianshu, abdominal withdrawal reflex scores were significantly lower compared with the model group (P < 0.05), and were similar to those in rats undergoing intragastric administration of pinaverium bromide (P > 0.05; Figure 1).

[Graph showing the effect of electroacupuncture at He-Mu points on abdominal withdrawal reflex (AWR) scores in rats with chronic visceral hypersensitivity.]

As data were not normally distributed and the median values were used, intergroup comparison was performed using the least significant difference t-test. *P < 0.01, vs. control group; †P < 0.05, vs. model group; ‡P < 0.01, vs. the previous time point.
Electroacupuncture at He-Mu points diminished P2X₄ receptor immunoreactivity in the colon of rats with chronic visceral hypersensitivity

Immunohistochemistry revealed that P2X₄ receptor immunoreactivity was increased in the colon of rats with irritable bowel syndrome ($P < 0.01$). Compared with the model group, P2X₄ receptor immunoreactivity was significantly lower in the electroacupuncture and pinaverium bromide groups ($P < 0.01$; Figure 2).

Electroacupuncture at He-Mu points diminished P2X₄ receptor immunoreactivity in spinal cord tissue of rats with chronic visceral hypersensitivity

Immunohistochemistry revealed that P2X₄ receptor immunoreactivity was increased in the spinal cord of rats with irritable bowel syndrome ($P < 0.01$). Compared with the model group, P2X₄ receptor immunoreactivity was significantly lower in the electroacupuncture and pinaverium bromide groups ($P < 0.01$; Figure 3).

**DISCUSSION**

Hypotonic pressure, electrostimulation and mechanical
pressure are sensitive signals of abrupt release of adenosine triphosphate from cells\textsuperscript{39}. Adenosine triphosphate transfers information via binding to membrane P2 purinergic receptors (P2X and P2Y) to produce extensive biological effects\textsuperscript{40}. The P2X and P2Y receptor subtypes are determined according to their pharmacological properties. P2X receptors are ligand-gated, non-selective, cation channels that contain seven subtypes (P2X1–7). After activation by adenosine triphosphate, these receptors are permeable to Na\textsuperscript{+}, K\textsuperscript{+} and Ca\textsuperscript{2+}. P2Y receptors are G protein-coupled, and nine subtypes have been cloned from human tissues, including P2Y\textsubscript{1–2, 4, 6, 11–15}.

The P2X receptor is extensively expressed in cells of nerve tissue, smooth muscle, intestine, skin, bladder and myocardium of mammals\textsuperscript{41–43}. Burnstock and Wood\textsuperscript{44} proposed that adenosine triphosphate secreted from various cell types exerts effects on pain sensation by activating corresponding receptors on sensory nerve endings. In that study, epithelial cells of the ureter, bladder and intestine were confirmed to release adenosine triphosphate after distention stimulation. After activation, P2X receptors become permeable to Na\textsuperscript{+}, K\textsuperscript{+} and Ca\textsuperscript{2+} ions, resulting in a rapid increase in intracellular Ca\textsuperscript{2+} concentration and activation of downstream signaling\textsuperscript{44}. Extracellular adenosine triphosphate binds to and activates the P2X\textsubscript{4} receptor, resulting in aberrant pain signals\textsuperscript{45}. P2X receptors were also suggested to play a role in formation of visceral hypersensitivity, as P2X receptors, in particular P2X\textsubscript{2} and P2X\textsubscript{3} receptors, are involved in conduction and modulation of nociceptive information in both the peripheral and central nervous systems, and as P2X\textsubscript{4} receptor expression in spinal cord microglia is associated with abnormal pain\textsuperscript{46–47}. However, there are few studies examining the relationship of the P2X\textsubscript{4} receptor with visceral pain. Wang and colleagues\textsuperscript{48} verified that the P2X\textsubscript{4} receptor is widely distributed in the rat nervous system. Ma et al\textsuperscript{49} also reported that the P2X\textsubscript{2} and P2X\textsubscript{4} receptors were expressed on neurons and fibers extensively distributed in the rat medulla, suggesting a binding site for the regulatory effects of adenosine triphosphate on cardiovascular activity, respiratory activity and sensation of pain. Furthermore, Kanazawa et al\textsuperscript{41} found that P2X\textsubscript{4} expression was increased in the spinal cord posterior horn in patients with irritable bowel syndrome. Low-grade inflammation in the intestinal tract with irritable bowel syndrome can activate spinal cord microglia, resulting in upregulated P2X\textsubscript{4} receptor expression and cell activation. Simultaneously, activation of the P2X\textsubscript{4} receptor by adenosine triphosphate released from sensory neurons produced an inward current and an increase in intracellular Na\textsuperscript{+} and Ca\textsuperscript{2+} concentrations\textsuperscript{50}; this signal was transmitted to downstream signaling molecules by activating P38MAPK, resulting in visceral hypersensitivity. Finally, low-dose ketamine was reported to inhibit microglial activation, reduce P2X\textsubscript{4} receptor expression and diminish visceral hypersensitivity\textsuperscript{51}.

Recent studies have confirmed that acupuncture exhibits therapeutic effects on ulcerative colitis\textsuperscript{52–55}, Crohn disease\textsuperscript{56–58} and irritable bowel syndrome\textsuperscript{23, 59}. Tianshu, the Front-Mu point of the large intestine, and Shangjuxu, the Lower He-Sea point of the large intestine, have been widely used in the clinic\textsuperscript{60–61}. Different manipulations or quantity of stimulus have positive regulatory effects on gastrointestinal functions, and show good therapeutic effects on diarrhea or constipation\textsuperscript{60–61}. Previous studies demonstrated that acupuncture at Tianshu and Shangjuxu produces strong effects on irritable bowel syndrome, and can regulate immune function of the patients\textsuperscript{62–63}. Experimentally, acupuncture at Tianshu and Shangjuxu effectively diminished abdominal withdrawal reflex scores in rats with visceral hypersensitivity in irritable bowel syndrome, elevated pain threshold and regulated expression of multiple cytokines\textsuperscript{64}.

In summary, we found that visceral hypersensitivity was increased in rats with irritable bowel syndrome, with concurrent elevation in P2X\textsubscript{4} receptor expression in the colon and spinal cord. The effects of acupuncture at Tianshu and Shangjuxu on visceral hypersensitivity were comparable to those of Western medicine, as both acupuncture and Western medicine effectively improved the clinical symptoms in this model. Acupuncture at He-Mu points also effectively reduced P2X\textsubscript{4} receptor expression in the colon and spinal cord of rats with visceral hypersensitivity, suggesting a potential mechanism of action of treatment of visceral hypersensitivity in irritable bowel syndrome.

**MATERIALS AND METHODS**

**Design**

A completely randomized controlled animal study.

**Time and setting**

Model preparation and treatment were conducted in 2011 at the Experimental Animal Center of Shanghai Medical College of Fudan University in China. Sample collection and index detection were performed at the Third-Level

**Materials**

**Experimental animals**
A total of 32 male specific pathogen-free Sprague-Dawley neonatal rats aged 8 days were provided by the Experimental Animal Center of Shanghai Medical College of Fudan University in China. Groups of eight neonatal rats were housed with a lactating rat in a quiet laboratory at 22 ± 2°C and humidity of 60 ± 5% with a 14 hour light/10 hour dark cycle, avoiding strong light. Lactating rats were allowed free access to food and water. All protocols were in accordance with *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[65].

**Drugs**
Pinaverium bromide tablets were purchased from Solvay (Shanghai) Company, Ltd., China, approval No. H20080414 (50 mg/tablet, lot No. 613173). Pinaverium bromide was suspended in double distilled water, and made into 5 mg/mL pinaverium bromide suspension.

**Methods**

**Establishment of rat models of visceral hypersensitivity in irritable bowel syndrome**
A rat model for visceral hypersensitivity in irritable bowel syndrome was induced by performing colorectal distension daily in 8–21 day-old rats according to a previously published method[66]. An in-house made saccule of 20.0 mm length and 3.0 mm diameter was slowly inserted into the descending colon. Saccule distension (0.2 mL) was performed for 1 minute. Air was exhausted and the saccule was pulled out. One hour later, the same stimulus was repeated. After stimulation, the rats were housed for 2–3 weeks. Stool character was observed and abdominal withdrawal reflex was scored to confirm the success of model induction.

**Electroacupuncture treatment**
After model establishment, in the electroacupuncture group, electroacupuncture was performed at *Tianshu* (0.2 cm lateral to the intersection of upper 8/13 and lower 5/13 from xiphoid to symphysis) and *Shangjuxu* (intersection of upper 6/16 and lower 10/16 of lateral condyle ofibia and lateral malleolus, 0.1 cm lateral to crista anterior tibiae) at equal pace[67]. Needling depth was 5 mm. The disposable aseptic needles (performance standard No. GB2024-94, 0.25 mm diameter × 25 mm length) were purchased from Cloud & Dragon Medical Device Co., Ltd. (Wujiang, Jiangsu Province, China). A Han’s acupoint nerve stimulator (model HANS100A; Nanjing Gensun Technology, Nanjing, Jiangsu Province, China) was used with a frequency of sparse and dense waves of 2/100 Hz, a current of 2 mA, for 20 minutes, once a day, for 7 consecutive days.

**Abdominal withdrawal reflex scores**
Colorectal distension was conducted within 60 minutes after the last electroacupuncture. The self-made saccule was connected to a T-valve. One end was connected to a 10 mL syringe and the other end was connected to a blood pressure monitor (model XJ11D; Shanghai Medical Instruments Co., Ltd., Shanghai, China). Stimuli at 2.66, 5.32, 7.98 or 10.64 kPa (20, 40, 60 or 80 mmHg, respectively) were given after the saccule was inserted. Each stimulus lasted for approximately 20 seconds with a five-minute interval. Each stimulus was repeated three times, and an average value (integer) was calculated as the final score. Reactions were scored in accordance with a previously published method[66]: 0 = no behavior reaction; 1 = movement standstill and transient head movement; 2 = contraction of abdominal muscles during stimuli; 3 = contraction of abdominal muscles during stimuli; 4 = body raised, elevation of pelvic cavity and scrotum.

**Sample collection**
The rats were anesthetized with 10% chloral hydrate (0.3 mL/100 g). The chest was opened, and 100 mL of saline was rapidly perfused into the ascending aorta through left ventricle intubation, followed by perfusion with PBS (pH 7.4) containing 4% paraformaldehyde. A total of 2–3 cm colon 5 cm above the anus and L4–S3 segment of the spinal cord were fixed in PBS (pH 7.4) supplemented with 4% paraformaldehyde, followed by paraffin imbedding and slicing (4 μm thick).

**Immunohistochemistry for P2X4 receptor expression in rat colon and spinal cord**
After dewaxing and hydrating, tissue was incubated in rabbit anti-rat P2X4 polyclonal antibody (1:400; Alomone Labs, Israel) at 37°C for 2 hours, and then washed in 0.01 mol/L PBS (pH 7.2–7.6) five times, for 5 minutes each. Using a goat anti-rabbit/rat immunohistochemistry kit (Gene Tech Co., Ltd., Shanghai, China), tissues were incubated with the A liquid for 5 minutes at room temperature in a wet box, washed in 0.01 mol/L PBS three times at 5 minutes each, followed by visualization in 3,3′-diaminobenzidine/H2O2. Staining time was controlled under a light microscope (Olympus, Tokyo, Japan). Staining was terminated by PBS 0.01 mol/L or distilled water. A wash with
0.01 mol/L PBS (pH 7.4) was performed between each step. Sections were mounted on gel-coated slides, dehydrated through a graded alcohol series, permeabilized with xylene, and then mounted. Images were collected from three fields randomly selected under a 400 × light microscope. The average value of integral absorbance was calculated using Motic Med 6.0 image analysis (Beijing Maikeaodi Image Technique Co., Ltd., Beijing, China).

Statistical analysis
All data were analyzed using SPSS 18.0 software (SPSS, Chicago, IL, USA). Normally distributed measurement data were presented as mean ± SD. Non-normally distributed data were presented as median values. One-way analysis of variance was employed for intergroup comparison if normally distributed and with homogenous variance. Non-parametric tests were used if heterogeneity of variance was present. Least significant difference t-test or Dunnett’s t-test were utilized for paired comparison among multiple means. A value of P < 0.05 was considered statistically significant.

Research background: Numerous clinical studies have demonstrated that electroacupuncture at Shangjuxu (ST37) and Tianshu (ST25) improve irritable bowel syndrome and mitigate visceral hypersensitivity. It is assumed that acupuncture can signal the affected receptor of the local Shu point, which is transmitted to the center via cell signaling pathways to regulate the internal environment of the body.

Research frontiers: We hypothesized that the analgesic effect of acupuncture relates to adenosine triphosphate signaling via a purinergic receptor on sensory nerve endings of the skin, and subsequent activation of a signal conduction pathway resulting in adjustment of pain sensation in the cerebral cortex.

Clinical significance: Our investigation addressing the effects of electroacupuncture at He-Mu points on P2X4 receptor expression in the colon and spinal cord of rats with chronic visceral hypersensitivity verified that electroacupuncture could adjust purinergic receptor expression, providing theoretical evidence for electroacupuncture as a treatment strategy for irritable bowel syndrome and acupuncture analgesia.

Academic terminology: Abdominal withdrawal reflex score is a scoring method that measures visceral pain after colorectal distension with a sac cue. The score includes five grades (0–4), with a higher score indicating higher sensitivity.

Peer review: Recent studies have reported that P2X4 receptor expression is increased in the colon and spinal cord after visceral noxious stimulation, indicating that P2X4 may be an important factor for enhanced visceral sensitivity. Our study found a potential pathogenetic mechanism of visceral hypersensitivity, and provided a target for clinical treatment strategies.

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