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BASIC RESEARCH

Role of vasoactive intestinal peptide and nitric oxide in the modulation of electroacupuncture on gastric motility in stressed rats

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Abstract

AIM: To investigate the effects and mechanisms of vasoactive intestinal peptide (VIP) and nitric oxide (NO) in the modulation of electroacupuncture (EA) on gastric motility in restrained-cold stressed rats.

METHODS: An animal model of gastric motility disorder was established by restrained-cold stress. Gastric myoelectric activities were recorded by electrogastroent erography (EGG). VIP and NO concentrations in plasma and gastric mucosal and bulb tissues were detected by radioimmunoassay (RIA). VIP expression in the gastric walls was assayed using avidin-biotin-peroxidase complex (ABC) and image analysis.

RESULTS: In cold restrained stressed rats, EGG was disordered and irregular. The frequency and amplitude of gastric motility were higher than that in control group ($P < 0.01$). VIP and NO contents of plasma, gastric mucosal and bulb tissues were obviously decreased ($P < 0.01$). Following EA at "Zusanli" (ST36), the frequency and amplitude of gastric motility were obviously lowered ($P < 0.01$), while the levels of VIP and NO in plasma, gastric mucosal and bulb tissues increased strikingly ($P < 0.01$, $P < 0.05$) and expression of VIP in antral smooth muscle was elevated significantly ($P < 0.01$) in comparison with those of model group.

CONCLUSION: VIP and NO participate in the modulatory effect of EA on gastric motility. EA at "Zusanli" acupoint (ST36) can improve gastric motility of the stressed rats by increasing the levels of VIP and NO.

Key words: Vasoactive intestinal peptide; Nitric oxide; Gastric motility; Electroacupuncture

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INTRODUCTION

In recent years, along with the extensive research into enteric nerve system (ENS), increasing evidence shows that peptidergic neurotransmitters are the key factors regulating the gastric motility. Our previous research[1-3] showed that electroacupuncture (EA) at acupoints of the Stomach Meridian of Foot-Yangmin may regulate gastric movement, increase blood flow in the microvessels in the gastric mucosa, and exert a protective effect on gastric mucosa. Nitric oxide (NO) and vasoactive intestinal peptide (VIP) participate in the protective effect of EA against gastric mucosal damage. It has been demonstrated that the increase or decrease of NO can lead to electrogastric dysrhythmias[4]. Regular gastric motility in antrum was abolished after traumatic stress in rats. Plasma VIP contents were increased significantly within 2 h after stress[5].The restraint plus water-immersion stress in rats induced obvious enhancement in gastric motility. NO levels of gastric mucosa were decreased significantly, and the gastric mucosal lesion was obvious[6]. VIP may be important in the prevention of gastric mucosal damage induced by cold-restraint stress[7]. Therefore, the aim of this study was to investigate the modulative effect of EA on gastric motility and its relation to NO and VIP in the animal model of restrained-cold stress, in an effort to explore the mechanisms of EA.

MATERIALS AND METHODS

Animal treatment

Eighty Wistar rats (weighing 220 ± 30 g), provided by the Experimental Animal Center of Anhui Medical University, were randomly divided into normal control group, model

group, EA group and non-acupoint group, with 20 animals in each. Each group was further divided into two parts:10 rats measured by radioimmunoassay, and another 10 rats measured by immunohistochemistry. Rats of the last 3 groups were subjected to 24 h of fasting (including water-intake), then anesthetized with 20% urethane (1.0 g/kg), and fastened to an animal board and put into a refrigerator at 4℃ for 2 h after awaking. Bilateral "Zusanli" (ST36) were punctured with filiform needles and stimulated with an EA therapeutic apparatus and using parameters such as frequency of 20-100 Hz, dense-sparse waves, electrical current of 2-3 mA and duration of 30 min. The non-acupoint group was located at the site of the buttock on the bilateral sides. The treatment was given once daily, for 7 consecutive days.

Gastric myoelectric activity

Rats were anesthetized with 20% urethane(1.0 g/kg) and an Ag-AgCl electrode was fixed on the body surface at the projection spot of antrum (1.5 cm below xiphoid process, 0.5 cm leftward). The positive electrogastroenterography (EGG) electrode was connected with the lead column of the surface electrode and the negative electrode was connected with right lower limb, then connected to earth. Gastric myoelectric activities were measured by EGG[8]. Frequency and amplitude of EGG were recorded and analyzed by computer[9].

Measurement of VIP and NO concentrations

At the end of each experiment and after decapitation of the rat, 3 mL of blood sample was taken and put into a test tube containing 30 μL of 10% EDTA-Na_2 (an anticoagulant) and 40 μL of trasylol, and mixed evenly. The blood sample was refrigerated at 4℃-8℃ for 15 min, and then centrifuged at 3500 r/min, 4℃ for 5 min to separate the plasma, which was put into a 1 mL tube, stored in the refrigerator at -20℃. The gastric mucosal and bulb tissues were weighed and added to 0.5 mL of 1 mol/L acetic acid and mixed evenly in a homogenizer to obtain a homogenate. The extracted homogenate was added to 0.5mL of 1 mol/L NaOH and centrifuged at 3000 r/min, 4℃ for 30 min. The supernatant fluid was collected and stored at -20℃.

The concentrations of VIP in the plasma and supernatant were determined by radioimmunoassay. The gastric mucosal and bulb tissues were boiled in 2-3 mL of normal saline for 3 min. The NO contents in blood and mucosa of gastric antrum and bulb tissues were assayed according to the method by Green *et al*[10]. The test kit was supplied by Beijing Huaying Bio-technique Institute.

Immunocytochemistry and image analysis

The expression of VIP in gastric wall was assayed using avidin-biotin-peroxidase complex[11]. Rats were anesthetized with intraperitoneal injection of sodium pentobarbital (30 mg/kg). Thoracotomy and aortic cannulation were performed. After flushing of the blood with saline, the rats were perfused iv rapidly with 400 mL of fixative (4% paraformaldehyde and 0.5% glutaraldehyde) and then with slow iv drip infusion for 1 h. Antral tissues were fixed in 4% paraformaldehyde for 3 h and then immersed into 20% cane sugar fluid for 12 h at 4℃ in a refrigerator. The frozen tissue was serially sectioned at 30 μm thickness and mounted onto glass slides. Changes in VIP distributions in antral smooth muscle were observed by immunocytochemistry and image analysis. VIP monoclonal antibody was purchased from Sigma Chemical, USA. SABC kit was purchased from Boshide Biotechnology Co, Wuhan. Detection was carried out according to the instruction of the kit. Five visual fields in three sections of each tissue, were randomly selected and observed under the light microscope and analyzed with LeicaQ500IW image analysis system for the integral optical density (IOD).

Table 1 Effect of EA on gastric electroactivity of antrum under restrained-cold stress in rats (mean ± SD)

Groups	*n*	Frequency (cpm)	Amplitude (μv)
Control	20	3.07 ± 0.55 (0.18)	363.21 ± 42.41 (0.12)
Model	20	4.26 ± 0.58[b] (0.14)	428.54 ± 56.23[b] (0.13)
EA	20	3.41 ± 0.48[d] (0.14)	378.62 ± 24.38[d] (0.06)
Non-acupoint	20	4.12 ± 0.36 (0.09)	407.25 ± 65.42 (0.16)

[b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* model group. Numerical value in brackets is variation coefficient.

Statistical analysis

Data were expressed as mean ± SD, and analysed by SPSS 11.0 software. Analysis of variance and *t* test were used for intergroup comparison. $P < 0.05$ was considered statistically significant. $P < 0.01$ was considered statistically obviously significant.

RESULTS

Gastric myoelectric activity

EGG was regular with frequencies of 3.07 cycle per min (cpm) and amplitudes of 360-370 μv in the control group. In rats with gastric motility disorder induced by cold restraining stress, EGG was disordered and irregular. There was an obvious difference in frequency and amplitude of EGG between the model group and control group ($P < 0.01$). Following EA at "Zusanli" (ST36), the frequency and amplitude of gastric motility were obviously decreased ($P < 0.01$). In the non-acupoint group, the two parameters had no obvious difference compared with those of model group ($P > 0.05$) (Table 1).

Effect of EA on VIP and NO concentrations

In cold restraining stress rats, VIP and NO concentrations in plasma and mucosal and bulb tissues were obviously decreased ($P < 0.01$). Following EA of "Zusanli" (ST36), the concentrations of VIP and NO rose obviously ($P < 0.01$, $P < 0.05$) in comparison with those of the model group. In the non-acupoint group, the levels had no obvious difference compared with those of model group (Table 2).

Table 2 Effect of EA on VIP and NO concentrations under restrained-cold stress in rats (Mean ± SD)

Groups	*n*	NO			VIP		
		Plasma ($\mu mol \cdot L^{-1}$)	Antrum (ng/mg)	Bulb tissues (ng/mg)	Plasma ($\mu mol/L$)	Antrum (ng/mg)	Bulb tissues (ng/mg)
Control	10	28.35 ± 1.32	45.23 ± 2.11	34.51 ± 3.24	8.80 ± 2.55	1.95 ± 0.62	0.48 ± 0.07
Model	10	19.24 ± 0.95[b]	28.01 ± 1.42[b]	23.48 ± 2.62[b]	5.16 ± 1.58[b]	1.12 ± 0.46[b]	0.37 ± 0.05[b]
EA	10	25..36 ± 1.61[d]	44.51 ± 0.89[d]	31.72 ± 1.06[d]	14.35 ± 3.47[d]	1.63 ± 0.59[a]	0.64 ± 0.10[d]
Non-acupoint	10	20.13 ± 1.78	29.76 ± 1.14	28.67 ± 1.06	4.87 ± 1.48	1.09 ± 0.41	0.39 ± 0.05

[b]$P < 0.01$ *vs* control group; [a]$P < 0.05$, [d]$P < 0.01$ *vs* model group.

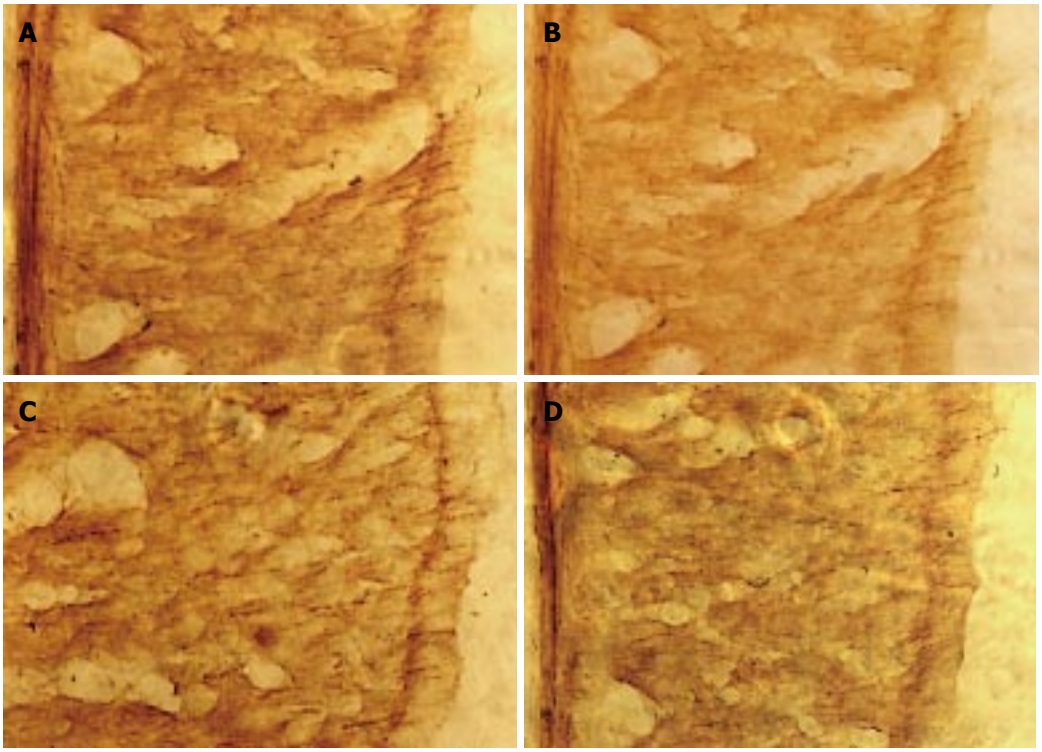

Figure 1 Microscopic photography of VIP-positive fibers of gastric smooth muscle in antrum (× 400). **A**: Moderate immunoreactive staining of VIP-positive nerve fibers in control group (× 400); **B**: Weak immunoreactive staining of VIP-positive nerve fibers in model group (× 400); **C**: Strong immunoreactive staining of VIP-positive nerve fibers in EA group (× 400); **D**: Moderate immunoreactive staining of VIP-positive nerve fibers in non-acupoint group (× 400).

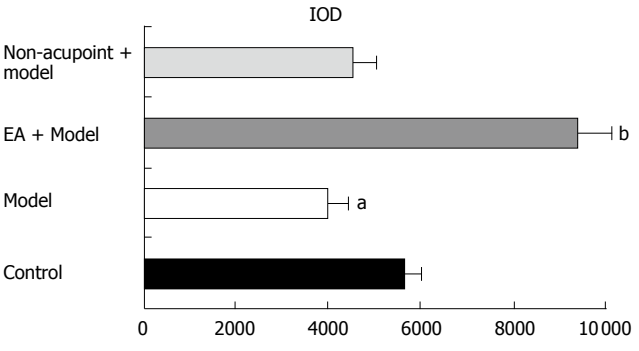

Figure 2 IOD analysis of the effect of EA on VIP immunoreactivity. [a]$P < 0.05$ *vs* control group; [b]$P < 0.01$ *vs* model group. Data are mean ± SD, $n = 10$.

Detection of VIP by immunocytochemistry

Immunocytochemistry results showed that positive immune reaction for VIP in fibers presented as a series of beads or filaments in brown color, with a background showing no staining or stained yellow. The positive reaction was usually located in the muscle layer and submucosa. In the gastric wall muscle layer of model group, positive immune reaction for VIP fibers appeared as lighter staining and lower density. In gastric wall muscle layer of the EA group, positive immune reaction for VIP fibers appeared as obviously dense staining (Figure 1). In the model group, computer image analysis showed that the expression of VIP in gastric wall declined obviously in comparison with the control group (3979.31 ± 582.10 *vs* 5646.25 ± 458.79, $P < 0.05$), while those of VIP in gastric wall in the EA group increased obviously in comparison with model group (9420.50 ± 897.56 *vs* 3979.31 ± 582.10, $P < 0.01$). However, no obvious changes of the densities of VIP in gastric wall in the non-acupoint group were found. This indicated that the effect of EA of "zusanli" can enhance the expression of VIP in gastric wall (Figure 2).

DISCUSSION

Brain gut peptides (BGPs) distribute extensively in the brain and the gastrointestinal tract. It has been demonstrated that some BGPs including gastrin (GAS), motilin (MTL), substance P(SP), VIP and somatostatin(SS) participate in gastrointestinal motility, secretion and absorption. Recent data revealed that BGPs are partially responsible for the regulatory effect of EA

on gastrointestinal tract activity, and EA can generate apparent changes in many bioactive substances such as GAS, MTL, SP, VIP, *etc.*, and the effect of EA of "Zusanli" (ST36) is of high specificity[12,13]. Acupuncture at the stomach channel of foot-Yangming can increase the levels of NO and NOS in gastric mucosa and the NOS-positive nerves in antral myenteric plexus[14,15]. In recent years, it has been found that decrease in gastric mucosal blood flow (GMBF) and hyperactivity of gastric motility play an important role in inducing gastric mucosal lesions under stress conditions[16]. During stress, the gastric smooth muscle contracts intensively, resulting in disturbance of the blood circulation, decrease of GMBF and increased permeability of the vascular wall. As a result, gastric ulcer occurs[17,18]. The results of the present study showed that in cold-restraining stressed rats, the gastric mucosal lesion was obvious. EGG was disordered and irregular. The frequency and amplitude of gastric motility were higher than that in control group ($P < 0.01$). VIP and NO levels in plasma and mucosal and bulb tissues were obviously lowered ($P < 0.01$). Following EA of "Zusanli"(ST36), the frequency and amplitude of gastric motility were lowered ($P < 0.01$), while the concentrations of VIP and NO were increased obviously ($P < 0.01$ or $P < 0.05$) in comparison with those of the model group and the expression of VIP-positive nerve fibers in gastric smooth muscle was elevated significantly ($P < 0.01$ or $P < 0.05$) in comparison with model group. The abnormal gastric motility caused by cold-restraining stress can be improved by EA and the mechanism of EA may be related to the endogenous changes of NO and VIP.

The regulation of gastric motility is a complex process related to neural activity and gastrointestinal hormone. NO and VIP, two main inhibitory neurotransmitters of the nonadrenergic noncholinergic (NANC) nerve of the intrinsic intestinal nerve system, were extensively distributed in the brain and gastrointestinal tract. They play an important role in the modulation of the function of the digestive tract. Immunocytochemistry showed that VIP-positive fibers were closely distributed around the nNOS-positive neurons. The results suggested that nNOS-positive neurons might have close morphological relationship with VIP-positive or AChE-positive neurons. They might be coordinated in the regulation of the function of the digestive tract and nitric oxide might regulate the activity of both myenteric neurons and smooth muscle[19]. NO is involved in NANC nerve-induced relaxation and the participation of VIP (and related neuropeptides) cannot be excluded in causing relaxation of mouse gastric fundus muscle strips. Experimental findings support the idea that VIP directly stimulates the production of NO by increasing nNOS activity and thereby activating soluble guanosine cyclase in smooth muscle[20]. It has been found that 65% of NOS containing immune active nerve fibers also have VIP immune activity, and 75% of VIP containing immune active nerve fibers also possess NOS immune activity in alimentary tract smooth muscle neurons, nerve fibers and submucosa in cats[21]. In the rabbit stomach circular muscle layer, VIP combined with the specific receptor can lead to intracellular Ca^{2+} content increase in stomach muscle cells. Microinjection of VIP into the dorsal vagal complex(DVC) evokes increases in gastric motor activity[22]. Extracellular VIP also stimulates stomach muscle cells to produce NO, and increase the content of inner cAMP and cGMP, inducing muscle relaxation. Recent studies in gastric muscle strips of rabbits, rats, and guinea pigs showed that VIP-induced relaxations were inhibited by NOS inhibitors, suggesting the cascade pathway, in which VIP was proposed to be the primary neurotransmitter, inducing relaxation partially via activation of adenylyl cyclase and partially via stimulation of NO production[23-25].

NANC nerve excitement by electric field stimulation induces muscle relaxation of gastric fundus. The underlying mechanism has been attributed to the low frequency stimulation which acts through NO production and VIP release[26,27]. Keef *et al*[28] proposed that NOS and VIP-like immunoreactivities are co-localized in enteric neurons and varicose fibers in the circular muscle layer, and that NO and VIP are co-transmitters, released in parallel from enteric inhibitory nerves. We conclude that NOS and VIP are extensively distributed in the nerve cells and nerve fibers of the whole alimentary tract from esophagus to anus. In the meantime, coexistence could also be seen. Zhang *et al* put forward that the mechanism of cooperative effects of VIP and NO may be as follows: NO and VIP act together as NANC nerve inhibitory transmitters. When NANC nerves excite, they induce release of VIP and increase of NO production. NO and VIP through interaction accelerate production of themselves[29]. NO, besides producing smooth muscle relaxation, may enhance the release of VIP, which in turn may further stimulate NO formation[30]. In contrast, the ineffectiveness of NOS inhibitors on the relaxations induced by VIP in many gastrointestinal tissues supports the idea that there is no interaction between NO and VIP in the gastrointestinal tract[31]. Thus there is still controversy about the interaction between NO and VIP and the underlying mechanisms of this interaction remain to be resolved.

The results of this study suggest that gastric motility disorders during restrained-cold stress may be partially mediated by release of BGPs. Changes of NO and VIP levels in plasma and mucosal and bulb tissues may be related with the gastric motility. The regulative effect of EA on gastric motility is closely correlated with the increase or decrease of NO and VIP. EA at "Zusanli" acupoint (ST36) is capable of improving gastric motility function of the animal model by increasing the levels of VIP and NO. These data imply that NO and VIP may have a synergistic modulative effect on gastric mucosal blood flow and gastric motility. The modulative mechanism of acupuncture on gastroenterotract may be through the intricate neuro-endocrine-immune network.

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