Expression of motilin in the hypothalamus and the effect of central erythromycin on gastric motility in diabetic rats

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Abstract: Objective To investigate the expression of motilin-immunoreactive neurons in the hypothalamus and the effect of central administration of erythromycin (EM) on the regulation of gastric motility in diabetic rats. **Methods** The motilin immunoreactive neurons in the hypothalamus and the hippocampus were detected by immunohistochemistry with rabbit anti-motilin polyclonal antibody. To measure the gastric motility, force transducers were surgically affixed to the gastric serosa. A microinjection syringe was connected via a plastic tube to an injection cannula, which was connected with a stainless steel guide cannula. The syringe was inserted into the right lateral cerebral ventricle for microinjecting the chemicals. **Results** Diabetic mellitus was successfully induced in cohorts of rats. Motilin-immunoreactive neurons significantly increased in the paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus in the diabetic rats. Intracerebroventricular (i.c.v.) administration of EM, a motilin receptor agonist, stimulated the gastric motility of diabetic rats. EM (91.56 nmol, i.c.v.) dose-dependently increased the amplitude by $(174.82 \pm 48.62)\%$ ($P < 0.05$), and increased the frequency by $(70.43 \pm 27.11)\%$ ($P < 0.05$) in 5 min. The stimulatory effect lasted more than 15 min to the end of the measurement, and can be blocked partially by the prior treatment of motilin receptor antagonist GM-109. **Conclusion** Motilin-immunoreactive neurons are increased in the PVN and SON of the hypothalamus in diabetic rats. Centrally administered EM may regulate gastric motility by binding to the central motilin receptors, and central motilin might be involved in regulation of gastric motility in diabetic rats.

Keywords: motilin; hypothalamus; erythromycin; gastrointestinal motility; diabetes mellitus; rats

1 Introduction

Motilin is a 22-amino acid peptide secreted from the mucosal endocrine cells in the upper part of the small intestine^[1]. It stimulates gastrointestinal contractility and appears to be involved in regulation of interdigestive motility. Motilin is also present in the central nervous system^[2,3]. Motilin-immunoreactive cells have been observed in various regions of the brain in different species. In addition, the existences of motilin receptor in the cerebellum, hippocampus, thalamus, hypothalamus, and amygdaloid body have been demonstrated^[3]. These findings suggest a neurotransmitter or neuromodulator role of motilin in the brain.

Diabetic gastroparesis, a diabetes complication that occurs in 30%–60% of patients with type I diabetes mellitus^[4,5], is symptomized by delayed emptying of the stomach and impairment in gastric slow waves^[6]. Prokinetic agents, such as erythromycin (EM), are regularly used for the treatment of diabetic gastroparesis^[7]. EM mimics the effect of gastrointestinal hormone motilin by binding to its receptor and acting as a motilin receptor agonist^[8,9]. It has been demonstrated in some *in vitro* experiments that EM may act directly on smooth muscles, and it exhibits the similar animal and tissue specificity as motilin does^[7]. Peripheral administration of motilin or EM (or other motilin mimetics) has a direct effect on the contractility of gastric smooth muscles and improves the gastric motility in diabetic gastroparesis^[8,10-12]. Central nervous system is probably also involved in this peripheral effect of EM. Previous studies have shown that central administration of motilin could increase the food intake of rats^[13]. Recent work from our laboratory has shown that central administration of

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CLC number: R587.2; Q579.2+1; R963

Article ID: 1673-7067(2007)02-0075-08

Document code: A

Received date: 2006-10-12

motilin in conscious rats does stimulate the gastric motility^[14-16]. However, the expression pattern and the significance of central motilin in diabetic rats are still unknown, and the effects of central administration of motilin or EM on gastric motility in diabetic rats remain unclear. In the present study, we used an immunostaining method to detect the expression of motilin immunoreactive neurons in the hypothalamus and so to observe the effect of central EM on the gastric motility in diabetic rats.

2 Materials and methods

2.1 Animal One hundred adult Sprague-Dawley male rats (Qingdao Institute for Drug Control), weighing 300–350 g, were used in this study. All rats were housed in the individual cages equipped with filter hoods under the controlled temperature (20 °C) and with a 12:12 h light:dark cycle (lights on 08:00 am). Standard laboratory chow pellets and tap water were available *ad libitum*. All animal experiments were carried out in accordance with the ethic guidelines of Qingdao University for animal care. Ninety of them were used for diabetic induction and sixty-nine rats successfully developed diabetes. Among the sixty-nine diabetic rats, thirty-eight rats showed obvious gastroparesis symptoms, including six died of exacerbation afterwards. The remaining rats were divided into two groups: one group of twenty-two gastroparesis rats were used for gastric motility recording and the rest ten gastroparesis rats were used for immunohistochemical study. Five healthy rats used as control for recording gastric motility, and another five healthy rats as immunohistochemical control.

2.2 Induction of diabetes The animals were given single i.p. injection of freshly prepared alloxan solution dissolved in saline (0.9% NaCl, W/V) at a dose of 12 mg per 100 g body weight^[17]. The diabetic state was ascertained in terms of body weight loss, polyuria, glycosuria, polydipsia, polyphagia, and high blood glucose levels^[18]. Symptoms of diabetes were observed within a week of alloxan injection.

2.3 Surgical procedure The rats used for investigating the effect of central EM on gastric motility were subjected to the following surgical procedures (rats with diabetic gastroparesis, $n = 22$ rats; “healthy” control, $n = 5$ rats).

2.3.1 Cannula implantation in lateral cerebroventricle The animals were anaesthetized with sodium pentobarbital (60 mg per kg body weight, i.p.) and immobilized in a stereotaxic apparatus (Narishige SN-3, Tokyo, Japan) for implantation of a cannula. A stainless steel guide cannula was

inserted into the right lateral cerebroventricle: 1.4 mm lateral to the midline, 0.8 mm posterior to the bregma, and 3.5 mm below the surface of the dura^[19]. The cannula was anchored to the skull and fixed in place by dental cement. A stylet was inserted in the guide cannula, protruding 0.5 mm beyond the tip of the guide cannula. Verification of the correct location of the cannula tip was performed by injection of cresyl violet (10 μ L) at the end of the experiments and then by histological examination of coronal cryosections of the brain. Only animals with the correct placement of cannula were included in the study. A histological check of the site of cannulation and injection did not show obvious signs of inflammation in any investigated experimental animals.

2.3.2 Implantation of force transducer After cannula was implanted into the lateral cerebroventricle, a midline laparotomy was performed. A strain gauge force transducer was sewn onto the serosal surface of gastric antrum along the circular muscles of stomach 3 mm above the pylorus. The connecting wires were tunneled subcutaneously through the anterior abdominal wall along the right side of the trunk, and led outside the skin to the back of the neck for attachment to the recorder. The abdominal wall and skin were closed. Supplement anesthetics were given throughout the surgery as needed. The animals were allowed recover for 3 d before administering chemicals.

2.4 Measurement of the gastric motility in diabetic and normal rats Rats were fasted for 18 h before recording. Gastric motility was recorded on a Polygraph (3066-23; Chengdu Precision Instruments, Sichuan, China) by connecting the transducer lead wires to a bridge circuit. After the baseline was recorded for 30 min, the stylet was first removed from the guide cannula. An injection cannula that connected to a 25- μ L microinjection syringe via a plastic tube was inserted. The syringe needle was lowered 1 mm beyond the tip of the cannula for microinjection of chemicals. The diabetic gastroparesis rats were injected intracerebroventricularly (i.c.v.) with 2 μ L 0.9% saline ($n = 5$ rats as control), 45.78 nmol (50 μ g) EM ($n = 5$ rats), 91.56 nmol (100 μ g) EM ($n = 6$ rats), and 2.95 nmol (2 μ g) GM-109 + 91.56 nmol EM ($n = 6$ rats), respectively. All chemicals were solved in 2 μ L 0.9% saline. For the healthy control groups ($n = 5$ rats), equal volumes of 0.9% saline without EM were given. Twenty-four normal rats were used to examine the effect of i.c.v. injection of EM on the gastric motility. Four groups of normal rats were prepared: i.c.v. injection of 0.9% saline (n

= 6 as control), EM 45.78 nmol ($n = 6$ rats), EM 91.56 nmol ($n = 6$ rats), and EM 91.56 nmol+GM-109 2.95 nmol ($n = 6$ rats). Rats were handled gently during the injection. The amplitude and frequency of gastric motility were measured. For each animal, recordings were made for 1–2 h per day, and lasted several days with at least 2 d interval.

2.5 Immunohistochemistry Coronal sections (8 μ m) of the forebrain were cut on a cryomicrotome (LEICA, CM, 1900-V5.0). For immunocytochemistry staining, sections were first treated with a solution containing 25% methanol and 3% H₂O₂ (V/V) in 0.01 mol/L PBS for 15 min to block endogenous peroxidase activity, and then treated with PBS containing 10% normal goat serum and 1% Triton X-100 for 30 min to prevent non-specific binding of secondary antibodies. Next, sections were incubated for 24 h with a rabbit polyclonal antibody against Motilin (Research Diagnostic Inc., Flanders, NJ) diluted 1:50 in PBS containing 10% normal goat serum, 0.1% bovine serum albumin, 0.05% thimerosal and 0.01% sodium azide (PBSA). The sections were then incubated for 2 h with biotin-conjugated goat anti-rabbit immunoglobulin G (Beijing Zhongshan Biotechnology Co. Ltd., China) diluted 1:100 in PBSA, and immersed for 2 h in a horseradish peroxidase conjugated streptavidin complex diluted 1:100. The immunoreaction was visualized by DAB staining (DAB Substrate Kit; Beijing Zhongshan Biotechnology Co. Ltd., China) for 5 min and observed under microscope. The reaction was terminated by rinsing the sections with distilled water. Method-specificity of the immunostaining was assessed by omitting the primary antiserum. Mounted sections were viewed and photographed with an Olympus BX50 microscope. All images were processed by using Adobe Photoshop7.01 software (Adobe Systems, San Jose, USA).

2.6 Cell counting Representative sections of the mid-brain including the paraventricular nuclei (PVN) and the supraoptic nuclei (SON) of the hypothalamus and the hippocampus were selected from the diabetic gastroparesis group ($n = 10$ rats) and the control group ($n = 5$ rats). From

each section, the total number of motilin-positive neurons in the selected nucleus was counted. In total, 3 sections per nucleus (9 sections per animal) in each animal group were used for counting. Counting was performed on an Olympus BX50 microscope and analyzed with the AnalySis image analysis software (Compix, INC. USA).

2.7 Statistics Data were presented as mean $\pm$ SEM. Statistical analysis between the control and the treated groups was performed by a Student's *t*-test. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Induction of diabetes Alloxan is an oxidized product of uric acid and tends to selectively destroy the islet cells of the pancreas when administered to experimental animals, producing diabetic mellitus. The diabetic state was ascertained and confirmed in terms of body weight loss, polyuria, glycosuria, polyphagia and blood glucose levels^[18,20] (Tab. 1). Symptoms of diabetes in the alloxan-treated Sprague-Dawley rats were observed within a week of alloxan injection. The body weight of the diabetic rats decreased significantly ($P < 0.001$), while the blood sugar (serum glucose) and urine sugar level increased markedly ($P < 0.001$) compared with those in the control group rats. After two weeks of injection, the diabetic rats (54%) showing symptom of gastroparesis such as lacking of appetite and abdominal bloating were used in this study. The gastric motility frequency and amplitude of these rats were much lower than that of the control group (Fig. 3A). No migrating motor complex (MMC) was observed in these rats (Fig. 3B–E).

3.2 Motilin expression in the PVN and SON of diabetes rats Quantitative analysis revealed a significant increase of motilin immunoreactive neurons in the PVN and SON in diabetic rats (Fig. 1) compared with those in the control group. The motilin immunoreactive neurons increased more dramatically in the PVN (Fig. 2). Motilin immunoreactive neurons showed no statistical significant difference in the hippocampal region between the diabetic rats and the

Tab. 1 Changes of physiological parameters in alloxan-treated rats (means $\pm$ SEM)

	<i>n</i>	Body weight (g)	Food intake (g/day)	Water intake (mL/day)	Blood sugar (mmol/L)	Urine volume (mL/day)	Urine sugar (mg/mL)
Diabetic rats	69	241.3 $\pm$ 10.7*	38.8 $\pm$ 1.4 [#]	103.1 $\pm$ 5.0 [#]	24.7 $\pm$ 1.7 [#]	93.2 $\pm$ 6.7 [#]	26 $\pm$ 1.6
Control rats	10	372.0 $\pm$ 12.4	18.9 $\pm$ 1.5	26.3 $\pm$ 1.2	6.2 $\pm$ 1.6	7.9 $\pm$ 1.0	n.d.

n.d.: no detectable. Blood sugar (serum glucose) level was determined by the glucose dehydrogenase method. * $P < 0.01$, [#] $P < 0.001$ vs Control rats.

Fig. 1 Microphotograph of motilin immunoreactivity in neurons of the paraventricular nuclei (PVN, A-D) and supraoptic nuclei (SON, E-H) in the hypothalamus. Scale bar: 100 μ m in A, C, E, G; 20 μ m in B, D, F, H.

Fig. 2 Histogram showing the significant increase of motilin immunoreactive neurons in PVN and SON of the hypothalamus in diabetic rats. For each nucleus, the mean number of motilin expression neurons from three sections was calculated. ** $P < 0.05$ vs control. Open bars, normal rats (control); solid bars, diabetic rats.

control's (data not shown).

3.3 Effect of i.c.v. administrated EM on the gastric motility of diabetic rats

I.c.v. administration of EM produced a marked increased in gastric motility (Fig. 3B–D). Administration of 50 μ g of EM resulted in an increases of the amplitude and frequency of gastric motility within 5 min by $(50.13 \pm 14.02)\%$ ($n = 5$, $P < 0.05$) (Tab. 2) and $(25.13 \pm 18.27)\%$ ($n = 5$, $P < 0.05$) (Tab. 3), respectively. Increasing the dose of injected EM led to further increase in gastric motility; i.c.v. microinjection of 100 μ g of EM increased the amplitude by $(174.82 \pm 48.62)\%$ ($P < 0.05$) in 5 min, by $(184.38 \pm 64.88)\%$ ($P < 0.05$) in 10 min, and by $(58.62 \pm 26.52)\%$ in 15 min (Tab. 2). Similarly, the frequency also increased by $(70.43 \pm 27.11)\%$

Fig. 3 Effects of i.c.v. administration of EM on gastric motility in diabetic rats. A: Gastric motility of the healthy control rats before and after i.c.v. administration of 2 μ L of 0.9% saline; B: i.c.v. administration of 2 μ L of 0.9% saline to the diabetic rats; C: i.c.v. administration of 45.78 nmol EM to the diabetic rats; D: i.c.v. administration of 91.56 nmol EM to the diabetic rats; E: GM-109 (2.95 nmol) was i.c.v. administrated to the diabetic rats 5 min before microinjection of EM (91.56 nmol).

Tab. 2 Effects of i.c.v. administration of EM and (GM-109+EM) on the amplitude of gastric motility in diabetic rats (% , mean $\pm$ SEM)

Group	<i>n</i>	Time after administration (min)			
		5	10	15	20
Control	5	-6.65 $\pm$ 17.41	-14.7 $\pm$ 13.87	-18.87 $\pm$ 23.17	-18.13 $\pm$ 25.04
45.78 nmol EM	5	50.13 $\pm$ 14.02*	42.53 $\pm$ 15.76	26.33 $\pm$ 30.30	4.87 $\pm$ 23.57
91.56 nmol EM	6	174.82 $\pm$ 48.62*	184.38 $\pm$ 64.88*	58.62 $\pm$ 26.52*	53.48 $\pm$ 23.40
2.95 nmol GM109+ 91.56 nmol EM	6	-10.15 $\pm$ 34.34	-16.32 $\pm$ 34.49	-9.28 $\pm$ 63.66	-29.49 $\pm$ 27.43

Percentage of the amplitude change was derived from the equation: amplitude change = (amplitude after microinjection – amplitude before microinjection) / amplitude before microinjection $\times$ 100%. **P* < 0.05 vs Control.

Tab. 3 Effects of i.c.v. administration of EM and (GM-109+EM) on the frequency of gastric motility in diabetic rats (% , mean $\pm$ SEM)

Group	<i>n</i>	Time after administration (min)			
		5	10	15	20
Control	5	-5.60 $\pm$ 4.34	-9.94 $\pm$ 14.20	-9.74 $\pm$ 6.68	-13.01 $\pm$ 13.06
45.78 nmol EM	5	25.13 $\pm$ 18.27*	18.62 $\pm$ 15.07	20.48 $\pm$ 14.18	12.25 $\pm$ 18.89
91.56 nmol EM	6	70.43 $\pm$ 27.11*	123.15 $\pm$ 58.55*	70.53 $\pm$ 25.18*	56.22 $\pm$ 91.34
2.95 nmol GM109+91.56 nmol EM	6	1.95 $\pm$ 36.64	1.34 $\pm$ 17.54	3.25 $\pm$ 27.99	3.68 $\pm$ 33.88

Percentage of the frequency change was derived from the equation: frequency change = (frequency after microinjection – frequency before microinjection) / frequency before microinjection $\times$ 100%. **P* < 0.05 vs Control.

($P < 0.05$) in 5 min, by $(123.15 \pm 58.55)\%$ ($P < 0.05$) in 10 min, and by $(70.53 \pm 25.18)\%$ ($P < 0.05$) in 15 min (Tab. 3). Both the amplitude and frequency showed dose-dependent increase in 5 min, but the dose-dependent increase of frequency did not reach a statistical significance ($P > 0.05$).

Effects of i.c.v. administration of EM or GM-109+ EM on the normal rats were shown in Fig. 4. I.c.v. infusion of EM in doses of 9.16, 45.78, and 91.56 nmol caused dose-dependent increases in the amplitude of gastric motility within 10 min by $(-5.72 \pm 3.05)\%$, $(56.25 \pm 8.27)\%$, and $(83.88 \pm 18.25)\%$, respectively. These responses were blocked by

pretreatment with the motilin receptor antagonist GM-109.

3.4 Antagonistic effects of GM-109 on EM-induced response GM-109 is a motilin receptor antagonist, and was shown to be able to abolish the excitatory effect of motilin in mice^[21]. To test whether the effect of motilin mimetics on gastric motility in diabetic mellitus was mediated by the motilin receptor in the brain, 2 μg of GM-109 (i.c.v.) was microinjected 5 min before the i.c.v. infusion of EM. The excitatory effect of EM on gastric motility was partially blocked by the motilin receptor antagonist GM-109 (Fig. 3E), and this effect lasted for more than 15 min to the end of the measurement.

Fig. 4 Effects of i.c.v. administration of EM on gastric motility in normal rats. A: Gastric motility of the normal control rats before and after i.c.v. administration of 2 μL of 0.9% saline. B: i.c.v. microinjection of 9.16 nmol EM to the normal rats; C: i.c.v. administration of 45.78 nmol EM to the normal rats; D: i.c.v. administration of 91.56 nmol EM to the normal rats; E: GM-109 (2.95 nmol) was i.c.v. administrated to the normal rats 5 min before the microinjection of EM (91.56 nmol).

4 Discussion

The alteration of neuropeptide expression such as neuropeptide Y (NPY) and somatostatin in the brain of diabetic rats has been reported^[22-25]. However, the hypothalamic motilin-containing neurons in the diabetic rats have not been investigated. Recent work from our laboratory demonstrated that central administration of motilin increased gastric motility in conscious rats. These observations suggested that the hypothalamus was a main target of motilin action^[14-16]. In fact, a high expression of motilin

peptide in the hypothalamic nuclei has been demonstrated^[26], and a large amount of motilin mRNA in the brain has also been observed^[27]. In this study, we reported that diabetic mellitus led to a significant increase of motilin immunoreactive neurons in the PVN and SON of the hypothalamus. Hypothalamus is the center of endocrine and autonomic nerve regulation, and the relay station of afferent and efferent information of food intake and energy homeostasis network. The nuclei of medial hypothalamus such as PVN and SON participate in regulation of gastric motility and food intake. The increased motilin expression observed in

this study suggested the involvement of central motilin in regulation of gastric motility in diabetic rats. The increases of motilin expression in both PVN and SON observed were consistent with the earlier demonstrated functional link between these two hypothalamic nuclei^[28-30]. In the present study, the motilin immunoreactive neurons in the hypothalamus of the diabetic rats were mostly in the PVN and was relatively strong compared with staining in the SON. The PVN seems in a position to integrate mainly interoceptive information such as gastrointestinal fill and availability of substrate and metabolites with information from adipose stores, and to organize appropriate endocrine and autonomic responses.

To further study the effect of central motilin on gastric motility in diabetic rats, the gastric motility in both conscious diabetic rats (Fig.3) and normal rats (Fig. 4) was observed. EM is a known non-peptide agonist of motilin receptor as shown in gastro-intestinal studies^[10] and easier to handle. I.c.v. administration of EM produced an increase in the amplitude and frequency of gastric motility in both diabetic and normal rats (Tab. 2, 3; Fig. 3, 4). Both the amplitude and frequency increases showed a dose-dependency, but the dose-dependency of frequency increase did not reach a statistical significance in this study. The effect of EM on gastric motility in diabetic rats can be partially blocked by prior injection of motilin antagonist GM-109 (Fig. 3E), indicating that the above effect of EM on gastric motility was mediated by motilin receptor in the central nervous system. It is worth to note that the amplitude and frequency of gastric motility in the diabetic rats were lower than those in the normal rats before EM treatment. The changes in the amplitude and frequency of gastric motility in response to i.c.v. injection of EM in the normal rats were marked, whereas i.c.v. injection of EM had a weaker effect on gastric motility in the diabetic rats. The reason for above differences between the normal and the diabetic rats might be due to the diabetic gastroparesis, and the diabetic gastroparesis was improved but not recovered completely by EM treatment. It has been shown that motilin receptor has been found exist in the hippocampus, thalamus, hypothalamus and amygdaloid body of the brain^[3], and a recent study showed that EM and motilin share a common binding site in motilin receptor^[31]. EM and its various derivatives have been clinically used to treat diabetic gastroparesis^[7]. Peripheral administration of EM improves the

gastric disorder in diabetic gastroparesis^[3,4]. In conclusion, motilin immunoreactive neurons increased in the PVN and SON of hypothalamus in diabetic rats, centrally administered EM regulates gastric motility by binding to the central motilin receptors, and our data suggest that central motilin might be involved in the regulation of gastric motility in diabetic rats.

Acknowledgements: This work was supported by the National Natural Science Foundation of China (No. 30370467) and by the Bilateral Scientific and Technological Cooperation between Flanders and China (Contract BIL01/13).

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糖尿病大鼠下丘脑中胃动素的表达及中枢注射红霉素对胃运动的影响

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摘要: 目的 以往的研究表明胃动素存在于大鼠的中枢神经系统, 在中枢注射胃动素具有促进清醒大鼠胃运动的作用。但糖尿病大鼠中枢胃动素表达含量及中枢注射胃动素对糖尿病大鼠胃运动的潜在作用目前尚未报导。本研究探索糖尿病大鼠胃动素免疫阳性神经元在下丘脑的表达, 观察侧脑室微量注射胃动素受体激动剂红霉素(erythromycin, EM)对正常和糖尿病大鼠胃运动的作用。方法 采用免疫组织化学方法测定下丘脑胃动素免疫阳性神经元的分布特征。在胃窦浆膜层植入应力传感器, 测定清醒大鼠胃运动的幅度和频率。结果 糖尿病大鼠下丘脑室旁核和视上核胃动素免疫阳性神经元数量明显高于正常对照大鼠($P < 0.05$)。脑室内微量注射EM可以明显促进糖尿病大鼠的胃运动。注射91.56 nmol的EM五分钟后, 大鼠胃窦运动幅度升高($174.82 \pm 48.62\%$) ($P < 0.05$), 运动频率加快($70.43 \pm 27.11\%$) ($P < 0.05$)。在脑室内微量注射胃动素拮抗剂GM-109后, 再注射EM, 其促胃运动效应可被部分阻断。结论 糖尿病大鼠中枢胃动素对胃运动有一定的调制作用, 而且脑室内微量注射的EM是通过脑内的胃动素受体介导而发挥其促胃动力效应。

关键词: 胃动素; 下丘脑; 红霉素; 胃运动; 糖尿病大鼠