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Research paper

Downregulation of metabotropic glutamate receptors mGluR5 and glutamate transporter EAAC1 in the myenteric plexus of the diabetic rat ileum

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Abstract

Objective: To study the morphologic abnormalities of the myenteric plexus in diabetic rats and to explore the mechanism of their effect on gastrointestinal motility. **Methods:** Forty rats were randomly divided into a diabetic group and a control group, Gastric emptying and small intestine transit rates were measured and histologic and molecular changes in glutamatergic nerves in the ileal myenteric plexus were observed, mGluR5 receptor and EAAC1 transporter changes in the diabetic rats were studied using fluorescence immunohistochemistry and RT-PCR. **Results:**Eighteen weeks after the establishment of the diabetic rats model, gastric emptying and small intestine transit rates were found to be significantly delayed in the diabetic group when compared with the control group. The density of glutamatergic ganglia and neurons in the ileal myenterie plexus were significantly decreased in the diabetic group when compared with control group($P < 0.05$) and the mGluR5 receptors and EAAC1 transporters were downregulated in the diabetic rats($P < 0.05$). **Conclusion:** Decreased glutamatergic enteric ganglia and neurons and decreased mGluR5 receptors and EAAC1 transporters in the intestinal myenteric plexus is one of the mechanisms of diabetic gastroenteropathy in rats.

Key words: Glutamate; mGluR5; EAAC1; Enteric Nervous System; Diabetic gastroenteropathy

INTRODUCTION

Patients with diabetes mellitus commonly experience gastric and intestinal dysfunction. Diabetic gastroenteropathy is characterized by dyspepsia, nausea, vomiting, appetite loss, abdominal pain, abdominal fullness, diarrhea, and constipation. Although these symptoms are common in the general outpatient population at gastroenterology clinics, they are also encountered in up to 75% of diabetic outpatients[1,2]. The pathogenesis of these complications remains poorly understood, although the degree of hyperglycemia appears to be an important determinant in their incidence and severity[3]. At a molecular level, much attention has recently been devoted to the potential role of alterations in the enteric nervous system(ENS).

There are data suggesting that glutamate is a neurotransmitter in the ENS, as demonstrated by the release of glutamate that has been shown to occur in the stimulated guinea-pig ileum[4,5]. Immunoreactivity for glutamate receptors and EAAC1 glutamate transpoters has been detected in the ENS, and glutamate has been shown to co-localize mainly with substance P in the mucosal plexus and with choline acetyltransferase (ChAT) in the myenteric plexus. Excitotoxicity, which is closely associated to glutamate and its receptors, has also been demonstrated in the ENS[6].

The aim of this study was to determine whether morphologic abnormalities of myenteric plexus exist in diabetic rats, and whether the expression of enteric nervous system mGluR5 glutamate receptors and EAAC1 transporters are altered during experimental diabetic gastroenteropathy.

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MATERIALS AND METHODS

Animal model and experiment groups

A total of 40 Male Sprague-Dawley rats, each weighing approximately 100-160 g, were used for all studies. The rats were randomly separated into 2 groups, a control group containing 10 rats, and a diabetic group containing 30 rats. Animals were housed in a room with a 12/12-hour light/dark cycle and an ambient temperature of 22 to 25℃. Control animals were fed a normal chow diet and the diabetes group was fed a high-fat diet, consisting of normal chow diet, 61.5%; fat, 15%; sucrose, 20%; cholesterol, 2%; and chleolate, 1%. After 8 weeks, diabetes was induced by the intraperitoneal (i.p.) injection of streptozotocin(STZ), 30 mg/kg body weight. Control animals received equal volumes of citrate buffer. Animals had free access to food and water after the STZ injection, and both STZ-injected and non-injected animals were continued on their original diets (high fat or normal chow respectively) for the duration of the study. The blood glucose concentration was measured in tail vein blood samples using a glucose oxidase-impregnated test strip, Only rats with fasting plasma glucose(FPG) glucose concentrations higher than 7.8 mmol/L or 140 mg/dL and an oral glucose tolerance test(OGTT) plasma glucose concentration greater than 11.1 mmol/L were considered diabetic and included in the study. After 18 weeks, and after tail vein blood samples were obtained and body weights measured the animals were killed using an overdose of chloral hydrate[7].

Gastric emptying and small intestine transit rate

Gastric emptying and small intestine transit distance was measured using a non-absorbed marker, 2% methylene blue, as previously described. Briefly, food was withheld for 24 h before the experiment, with free access to drinking water. All animals received the intragastric administration of 0.4ml 2% methylene blue in saline. After 30 min, the animals were killed and the entire gastrointestinal tract was removed. The gastric contents were rinsed with 4ml of normal saline, which was collected, Methylene blue concentrations were measured with a spectrophotometer after centrifugation, and the gastric pigment remnant rate was determined as[value of diabetic group animal/mean value of control group] × 100(%). The distance from the pylorus to the front of the methylene blue bolus and the ileocecal junction was measured. The rate of transit was determined as [(distance to methylene blue front)/(length of small intestine)] × 100(%).

The LMMP strips preparation

Experiments were performed on longitudinal muscle myenteric plexus(LMMP) strips of ileum. The LMMP strips were prepared from 10 cm sections of terminal ileum as described previously. Segments of distal ileum, at least 10 cm oral to the ileocaecal junction, were obtained. Care was taken to stretch the tissue to its maximal length prior to fixation overnight at 4℃ in 4% paraformaldehyde. The tissue was immersed in 30% sucrose until it sank to the bottom of the container. It was then opened along the mesenteric border, rinsed and pinned flat on a dish containing phosphate buffered saline(PBS). Mucosa, submucosa and circular muscle layers were removed. These whole-mount preparations were stored at 4℃ in PBS

Immunocytochemistry

Longitudinal muscle-myenteric plexus(LMMP) preparations were incubated with Triton X-100(0.5%) for 30 min. and 4% normal donkey serum for 2h at 4℃. After being washed with PBS, the preparations were exposed overnight at 4℃ to anti-mGluR5 polyclonal antibody(Chemicon, USA), diluted 1 : 200, or monoclonal anti-EAAC1 antibody(Chemicon), diluted 1:200. After being washed with PBS, the preparations were incubated with FITC-conjugated secondary antibodies, diluted 1:200, or CY3-conjugated secondary antibody, diluted 1:200. The preparations were washed again with PBS, and then the tissues were mounted on slides using glycerine. The preparations were first observed under a fluorescent microscope(Nikon, Japan), and quantitative analysis was carried out with a confocal microscope(Leica, Germany)

Myenteric ganglia and neuron counting

To count numbers of ganglia and neurons in the myenteric plexus of both control and diabetic rats we used a modification of the myenteric neuron counting method used in previous studies. The numbers of ganglia and neurons in the myenteric plexus were counted in random fields(× 200 magnification) from the ileum for both control and diabetic rats.

RNA isolation and RT-PCR

Total RNA isolated from rat longitudinal muscle with adherent myenteric plexus(LMMP) was prepared by using the Trizol reagent, The concentration and purity were assessed by ultraviolet spectrophotometer. The RNA was reverse transcribed into cDNA by reverse transcriptase MMLV. The cDNA was amplified by PCR with primers corresponding to mGluR5 cDNA. P1 5-GAG CAG ATC AGC AGC GTA GTG-3 ,P2 5-TCA GGT AGG AGG AGC AGA TTGG-3. The length of product was 121 bp. EAAC1 p1 5-CCA CCA CCG TCA TTG CTG TAA TC-3, p2 5-GGC TTG GAC CAG GTT CTC AGG-3. The length of product was 173 bp. β-actin P1 5-CTA TCG GCA ATG AGC GGT TCC-3 P2 5-TGT GTT GGC ATA GAG GTC TTT CG-3.

The length of product was 146 bp. Amplification condition: 5 min of pre-denaturation at 94℃, 30 s of denaturation at 94℃, 30 s of renaturation at 60℃, 1 min of amplification at 72℃. After 30 cycles, a final step of 72℃ was implemented for 10 min. The PCR products were separated on 2% agarose gel and 0.5×TBE. The banded areas were scanned using a UV transilluminator.

Statistical analyses

All data analyses were performed with SPSS 13.0 software. Values are expressed as mean ± SD. Significant differences between diabetic rats and control rats were determined using *t*-test. Values of $P < 0.05$ were considered significant.

RESULTS

Body weight and blood glucose level

The body weight and blood glucose levels of all the animals were measured every two weeks throughout the treatment period. By 4 weeks the mean body weights of the diabetic rats was significantly greater than the mean body weights of the control animals. From 9 weeks to 11 weeks the increase in the mean body weights of the diabetic animals increased more slowly than earlier in the experiment. From 12 to 15 weeks of age there was no difference in mean body weights between the diabetic and control group animals, and from 16 to 18 weeks the mean body weights of the diabetic animals was significantly lower than that of the control group animals(***Fig. 1***).

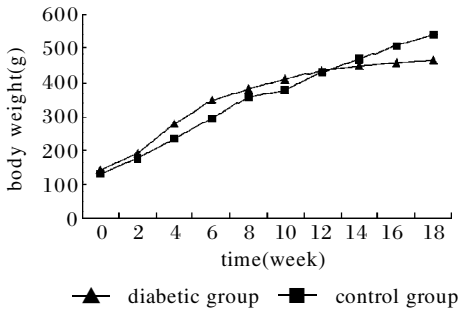


Fig. 1 Comparison of body weights of the control rats and the diabetic rats

Mean blood glucose concentrations were significantly higher in all the diabetic animals when compared to the control animals($P < 0.01$). From 9 weeks, the mean blood glucose concentration in the diabetic rats was significantly greater than that of control animals. Blood glucose of the control rats and the diabetic rats are illustrated in ***Fig. 2***.

Gastric emptying and small intestine transit rate

Gastrointestinal motility abnormalities occurs in diabetic rats, the major characteristics being a delayed

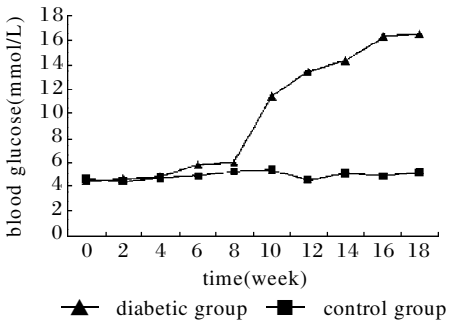


Fig. 2 Comparison of blood glucose of the control rats and the diabetic rats

gastric emptying and decreased intestinal transit. The mean gastric pigment remnant rate in the diabetic group was significantly greater than that in the control group, indicating that gastric emptying was significantly decreased. The intestinal transit rate in the diabetic rats was significantly decreased compared with the value of control group($P < 0.05$)(***Table 1*** ***Fig. 3***).

Table 1 **Comparison of gastric emptying and small intestine transit rate** (Means ± SE)

Group	Gastric pigment remnant rate(%)	Small intestine Transit rate(%)
Diabetic group	127.96 ± 11.93	36.93 ± 8.99
Control group	99.54 ± 6.76	44.87 ± 6.95

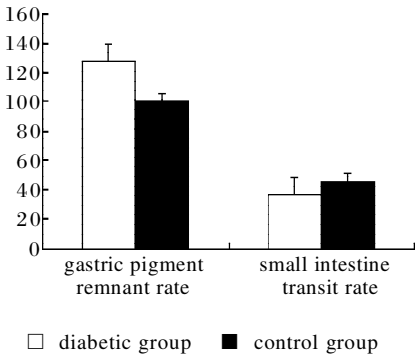


Fig. 3 Comparison of gastric pigment remnant rate and small intestine transit rate of the control rats and the diabetic rats

Immunofluorescence staining

Immunofluorescence staining for mGluR5 and EAAC1 showed immunoreactivity in the myenteric plexs of the ileum of both the control and the diabetic rats. The mGluR5 and EAAC1 immunofluorescence staining showed immunoreactivity in neurons and fibers. However the expression in diabetic rats was significantly less than in control rats($P < 0.05$)(***Table 2***).

Counting myenteric ganglia and neurons

The general structure of the plexus was analysed. Ganglia were considered as agglomerates of three or more juxtaposed nerve cell bodies separated by about

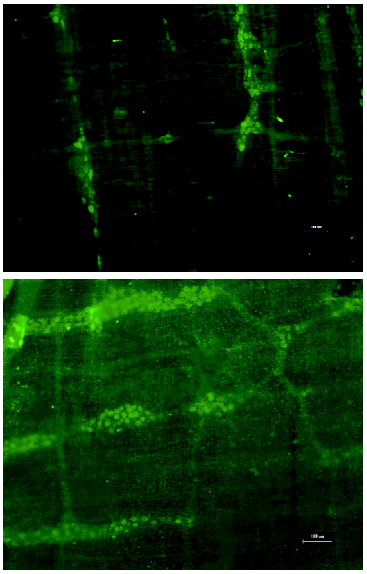
Table 2 **Comparison of the optical density in the diabetic group and the control group**

(Means ± SE)

	Diabetic group (IOD)	Control group (IOD)
mGluR5	145.23 ± 28.78	167.23 ± 30.56
EAAC1	124.23 ± 18.46	146.23 ± 29.74

50 μm, which is the diameter of a cell body and its surrounding nerve fiber varicosities. To quantitatively analyze the general myenteric neuronal population the density of ganglia and the number of cells per ganglion (including isolated neurons or pairs of neurons) was counted. The spatial density of ganglia and neurons was significantly altered in the diabetic animals($P < 0.05$).

There was a significant reduction in the mean number of ganglia in LMMP preparations. The maximum number of ganglia found in LMMP preparations of diabetic rats was 7, while in control rats it was 13. There was a significant reduction in the mean number of neurons per ganglion. The maximum number of neurons per ganglion found in diabetic animals was 105 neurons, whereas in the control rats it was 135 neurons(***Fig. 4***).

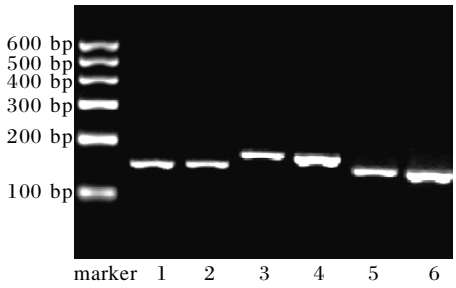


A:Diabetic rat; B:Control rat

Fig. 4 Expression of mGluR5 neurons in the myenteric plexus of the ileum(× 200)

mGluR5 and EAAC1 mRNA levels

Because the quality of RNA affects the result greatly, RNA was assessed by spectrophotometry. The RNA quality was considered acceptable as the ratios of values at 260nm and 280nm were above 1.80. Agarose gel electrophoresis of RT-PCR products, imaging and strap analysis showed the products to be distinct and consistent with predictions of the primers. When normalized to β-actin, the levels of mGluR5 and EAAC1 mRNA were significantly lower in RNA samples from the ileal myenteric plexus of diabetic rats than samples obtained from control rats($P < 0.05$)(***Fig. 5***).



Lanes 1 and 2: β-actin; 3:EAAC1 of diabetic group; 4:EAAC1 of control group; 5:mGluR5 of diabetic group; 6:mGluR5 of control group

Fig. 5 Electrophoresis image of RT-PCR products of mGluR5 and EAAC1

DISCUSSION

The enteric nervous system(ENS) is a complex network of neurons and glial cells located within the gastrointestinal tract, also named the "brain of the gut" which can function independently of the central nervous system (CNS)[8]. This system controls gastrointestinal motility, exocrine and endocrine secretions as well as the microcirculation[9]. The myenteric plexus controls gastrointestinal motility by means of well organized circuits which are comprised of sensory neurons, interneurons and motor neurons in the muscular layers[10]. Overall, more than 20 candidate neurotransmitters regulating gastrointestinal functions have now been identified in enteric neurons, including acetylcholine, vasoactive intestinal polypeptide and nitric oxide[11].

mGluRs are G protein-coupled receptors that are highly expressed throughout the CNS. To date, eight metabotropic glutamate receptors(mGluR1-8) have been cloned and are classified into three major groups. Group I mGluRs includes mGluR1 and mGluR5. The mGluRs are widely distributed throughout the CNS and play important roles in regulating cell excitability and synaptic transmission at excitatory and inhibitory synapses[12] .

Glutamate transporters participate in regulating extracellular glutamate concentration and preventing over-stimulation of glutamate receptors[13]. To date, at least five membrane glutamate transporter subtypes have been cloned, including GLAST(EAAT1), GLT-1 (EAAT2), EAAC1(EAAT3), EAAT4, and EAAT5. GLAST and GLT-1 are associated with glial cells, whereas EAAC1 is considered a neuronal glutamate transporter[14].

Functional mGluRs are also expressed outside the CNS, in nodose ganglia, dorsal root ganglia, taste buds, osteoblasts, pancreatic islets, murine thymocytes, and

the retina[15]. The mGluRs are also found in the ENS[10]. Glutamate receptor ligands affect intestinal motility and secretion. Evidence indicates, therefore, that glutamate receptors may play a role in the modulation of enteric reflexes in the guinea pig small intestine, and activation of group I mGluRs depolarizes submucosal neurones[16]. Fast excitatory neurotransmission in the ENS is largely mediated by acetylcholine acting at nicotinic acetylcholine receptors. Several authors have proposed that glutamate can act as an excitatory neurotransmitter in the gut. Enteric ganglia contain glutamatergic neurons[5]. Various subtypes of glutamate receptor were identified by immunohistochemistry in rat and guinea-pig gut. In addition, a subset of enteric neurons was found to be EAAC1 immunoreactive. EAAC1-immunoreactive neurons were also found within ganglia.

Diabetic gastroenteropathy is often the result of damage to several cell types. In diabetic rats both function and structure of the myenteric plexus change. Previous studies reported neuronal apoptosis in the myenteric plexus of STZ-treated rats[5,17]. Gastrointestinal dysfunctions as complications of diabetes are mutually related to not only a single factor, but to multiple factors. Hyperglycemia studies are inadequate for revealing related alterations in neurons and diabetic gastrointestinal dysfunctions. Thus, in order to determine the physiological function of the ENS in diabetes further studies are required.

Our study quantitates the density of glutamategic ganglia and neurones in the myenterie plexus of ileum and the expression variants of mGluR5 and EAAC1 in diabetic rats. The actual physiological role of mGluR5 and EAAC1 in the diabetic rat ENS has not yet been established.

We used immunofluorescence and confocal microscopy to study the spatial density of ganglia and neurons in whole-mount preparations. It is relatively easy to establish the spatial density of neurons in whole-mount preparations with a fair degree of accuracy because of the longitudinal and circumferential uniformity of the pattern of the myenteric plexus in the ileum. Whole-mount preparations were stretched for counting so that the neurons almost formed a cellular monolayer within the ganglia, thus no cells would be missed being counted because of cellular overlap.

There was a statistically significant reduction in ganglia and the mean number of cells per ganglion. Quantitative analysis was carried out with a confocal microscope and it was found that the expression of mGluR5 and EAAC1 and the IOD values were significantly lower in the diabetic rat ileum compared to control animals. Using RT-PCR we found that mGluR5 and EAAC1 mRNA were also significantly reduced in the diabetic animals. These alterations may possibly contribute to the diabetic gastrointestinal dysfunctions seen in these experiments.

Previous studies have reported excessive exposure to glutamate causes cell death("excitotoxicity") in CNS neurones. Excitotoxicity consists of necrosis and apoptosis and is thought to occur via a breakdown in ionic homeostasis mediated by NMDA and non-NMDA glutamate receptor subtypes. Other studies demonstrate that excitotoxicity occurs in the ENS. Both necrosis and apoptosis were observed in myenteric neurons after exposure to glutamate. The process of excitotoxicity in the ENS appears similar to that observed in the CNS[18].

It seems likely that there is rapid uptake of glutamate in the bowel, mediated by high affinity glutamate transporters, including the EAAC1 transporter, EAAC1 participates in regulating extracellular glutamate concentration and preventing over-stimulation of glutamate receptors. Preliminary data indicate that EAAC1 appears to have a diverse array of physiologic and metabolic functions in the mammalian central nervous system. Knockdown of EAAC1 expression reduces glutamate uptake, and causes moderate neurodegeneration[6]. Mice deleted of EAAC1 develop an age-related neurodegenerative disorder, suggesting an important role for EAAC1 in neuronal death processes[19]. Because excitotoxicity is dependent on Ca^{2+} influx, neurons can be protected from excitotoxicity by Ca^{2+} buffers. Glutamate has been found to increase Ca^{2+} in enteric neurons by metabotropic mGluR5 glutamate receptors. It seems likely that when and EAAC1 was significantly reduced in the diabetic rat, the glutamate concentration increased, and excessive exposure to glutamate produced neuronal cell loss[20].

Thus the mechanism responsible for the gastrointestinal dysfunctions seen as complications of diabetes may be a result of decreased mGluR5 and EAAC1 expression in the myenteric plexus The decreased number of neurons in the diabetic rats may be due to excitotoxicity caused by EAAC1-mediated glutamate uptake dysfunction, and excessive exposure to glutamate causing cell death.

In summary, damage to the ENS is one of the probable causes of diabetic gastroenteropathy. Our data indicate that excitotoxicity may be occuring in the ENS of diabetic rats, and this appears to be mediated by enteric metabotropic mGluR5 glutamate receptors and EAAC1 glutamate transporters.

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