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doi:10.3748/wjg.15.2125

World J Gastroenterol 2009 May 7; 15(17): 2125-2131
World Journal of Gastroenterology ISSN 1007-9327

C-type natriuretic-peptide-potentiated relaxation response of gastric smooth muscle in streptozotocin-induced diabetic rats

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Supported by The National Natural Science Foundation of China, No. 30760068
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Received: February 7, 2009 Revised: March 12, 2009
Accepted: March 19, 2009
Published online: May 7, 2009

Abstract

AIM: To study the sensitivity of gastric smooth muscle to C-type natriuretic peptide (CNP) in streptozotocin (STZ)-induced diabetic rats.

METHODS: The spontaneous contraction of a gastric smooth muscle strip was recorded by using physiological methods in rats. The expressions of CNP and natriuretic peptide receptor-B (NPR-B) in gastric tissue were examined by using immunohistochemistry techniques in the diabetic rat.

RESULTS: At 4 wk after injection of STZ and vehicle, the frequency of spontaneous contraction of gastric smooth muscle was significantly reduced in diabetic rats, and the frequency was decreased from 3.10 ± 0.14 cycle/min in controls to 2.23 ± 0.13 cycle/min (n = 8, P < 0.01). However, the amplitude of spontaneous contraction was not significant different from the normal rat. CNP significantly inhibited spontaneous contraction of gastric smooth muscle in normal and diabetic rats, but the inhibitory effect was significantly potentiated in the diabetic rats. The amplitudes of spontaneous contraction were suppressed by 75.15% ± 0.71% and 58.92% ± 1.32% while the frequencies were decreased by 53.33% ± 2.03% and 26.95% ± 2.82% in diabetic and normal rats, respectively (n = 8, P < 0.01). The expression of CNP in gastric tissue was not changed in diabetic rats, however the expression of NPR-B was significantly increased in diabetic rats, and the staining indexes of NPR-B were 30.67 ± 1.59 and 17.63 ± 1.49 in diabetic and normal rat, respectively (n = 8, P < 0.01).

CONCLUSION: The results suggest that CNP induced an inhibitory effect on spontaneous contraction of gastric smooth muscle, potentiated in diabetic rat *via* up-regulation of the natriuretic peptides-NPR-B-particulate guanylyl cyclase-cyclic GMP signal pathway.

Key words: Diabetes; Natriuretic peptide receptor type B; Gastric smooth muscle; Gastroparesis; Spontaneous contraction

Peer reviewer: Leonard R Johnson, Professor, Department of Physiology, University Tennessee College of Medicine, 894 Union Ave, Memphis, TN 38163, United States

Cai YL, Xu DY, Li XL, Qiu ZX, Jin Z, Xu WX. C-type natriuretic-peptide-potentiated relaxation response of gastric smooth muscle in streptozotocin-induced diabetic rats. *World J Gastroenterol* 2009; 15(17): 2125-2131 Available from: URL: http://www.wjgnet.com/1007-9327/15/2125.asp DOI: http://dx.doi.org/10.3748/wjg.15.2125

INTRODUCTION

Gastroparesis (delayed gastric emptying) is frequent in diabetic patients. It is a well-recognized complication of long-standing diabetes. The symptom complex typically associated with gastroparesis occurs in 25%-55% of patients with long-standing type 1 or type 2 diabetes[1,2]. Symptoms of diabetic gastropathy can range from mild dyspepsia to recurrent vomiting and abdominal pain, and may progress to irreversible end-stage gastric failure known as gastroparesis. Gastroparesis seriously affects the quality of life. There is deterioration in glycemic control and incapacitating symptoms such as malnutrition, water and electrolyte imbalance, and aspiration may occur. However, the pathophysiology of diabetic gastropathy

and gastroparesis, including impaired fundic and pyloric relaxation and impaired electrical pacemaking, is still not delineated[3,4]. It is generally considered that diabetic gastropathy and gastroparesis may be due to visceral autonomic neuropathy, hyperglycemia and degeneration of smooth muscle. Several physiological studies have reported that dysfunction of gastric smooth muscle in diabetes is associated not only with neural factors, but also with intracellular signaling pathways[5,6].

Since atrial natriuretic peptide (ANP) was isolated from atrium by de Bold *et al*[7] in 1981, brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), dendroaspis natriuretic peptide (DNP), micrurus natriuretic peptide (MNP), and ventricular natriuretic peptide (VNP) were found in succession. Natriuretic peptides (NPs) are distributed all over the body besides the heart and exert natriuretic-diuretic, vasorelaxation, and other functions designed to decrease blood pressure and to control electrolyte homeostasis. Three types of single-transmembrane natriuretic peptide receptors (NPRs) for ANP, BNP and CNP have been identified[8,9]; i.e. NPR type A (NPR-A), type B (NPR-B) and type C (NPR-C). NPR-A and NPR-B receptors have membrane-bound particulate guanylate cyclase (pGC), which catalyzes the formation of cGMP from GTP[10-12]. NPR-A preferentially binds ANP and BNP, but has a low affinity for CNP; NPR-B has a much higher affinity for CNP than either ANP or BNP[13]. NPs are also secreted from gastric mucosa[14-16]. Our previous study indicated that CNP relaxes gastric circular and longitudinal smooth muscles in human, rat and guinea-pig stomach, and that NPRs are distributed in rat gastric smooth muscle layer[17-19]. In smooth muscle, CNP activates its cognate NPR-B, which includes an intracellular pGC domain and catalyzes the synthesis of cGMP within the cytosol[20]. CNP and NPR-B have been detected in the stomach[17,21,22]. CNP mRNA expression was increased in the kidney of streptozotocin (STZ)-induced diabetic rats and NPR-B expression was enhanced in vascular smooth muscle in the diabetic mouse[23,24].

However, it is not clear what the relationship is between diabetic gastroparesis and the natriuretic peptide signal pathway. In the present study, the possibility as to whether the natriuretic peptide-dependent cGMP signal pathway is involved in diabetic gastropathy or gastroparesis was investigated in STZ-induced rats.

MATERIALS AND METHODS

STZ-induced diabetic animal model

Male Sprague-Dawley rats (200-220 g) were purchased from the Experimental Animal Center of Yanbian University College of Medicine. Animals were allowed to have free access to food and water. A total of 30 rats were divided into two groups (15 per group): one was the normal control group and another was the diabetic group. All rats were used for the experiment at 4 wk after the injection of STZ and vehicle. Diabetes was induced by a single intraperitoneal injection of STZ (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 mol/L citrate buffer (pH 4.0) at a dose of 65 mg/kg body weight[6]. Control animals received an equal volume of citrate buffer. The glucose concentration in tail-blood was determined at the end of the experiment with a SureStepPlus apparatus (LifeScan, Milpitas, CA, USA). Diabetes was confirmed by measurement of blood glucose concentrations and defined as blood glucose above 350 mg/dL. Animals were treated in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (China).

Organ bath study

Four weeks after treatment with STZ and vehicle, animals were anesthetized with sodium pentobarbital (50 mg/kg, ip) and then the abdomen was opened. The stomach was removed and placed in pre-oxygenated Kreb's Ringer solution at room temperature. The mucosal layer was removed and the strips (about 2.0 mm × 15.0 mm) of gastric antral circular muscle from control and diabetic rats were prepared, respectively. The longer axis of the stomach was cut parallel to the circular muscle fibers. Muscle strips were placed in a 2-mL organ bath containing modified Kreb's Ringer solution at 37℃, aerated with 95% O_2 and 5% CO_2. One end of the muscle strip was anchored to a stationary support, and the other end was connected to an isometric force transducer (Grass FT03C, Quincy, MA, USA). The tension loaded onto each strip was 1.0 g. Isometric contractions were recorded using a computerized data acquisition system (Power Lab/8SP, AD Instruments, Castle Hill, NSW, Australia). The muscle strip was allowed to incubate for at least 40 min before experiments were started. The composition of the modified Kreb's Ringer solution (mmol/L) was as follows: NaCl 120; KCl 4.7; $CaCl_2$ 2.0; $MgCl_2$ 1.2; $NaHCO_3$ 25; KH_2PO_4 1.2; and glucose 14.

Immunohistochemistry study

Tissues of normal control and STZ-diabetic rats stomach antrum were fixed in 4% buffered formalin for 24 h, dehydrated in ethanol, and embedded in paraffin. Sections were cut at 5 μm, and mounted on poly-L-lysine-coated slides. Sections were deparaffinized in three changes of xylene, hydrated in a graded ethanol series, and washed in tap water. Endogenous peroxidase activity was blocked by immersing slides in 0.3% H_2O_2 for 30 min. After being washed in phosphate buffered saline (PBS), slides were incubated for 45 min at 37℃ in a humidified container with normal goat serum to block non-specific binding of the primary antibody. The blocking serum was removed by gentle tapping, and slides were incubated for 24 h at 4℃ in a humidified container with either rabbit anti-CNP (1:600, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-NPR-B (1:500, Santa Cruz Biotechnology). After being washed thoroughly in PBS, slides were incubated for 30 min at 37℃ in a humidified container with biotin-labeled goat anti-rabbit serum. After being washed in PBS, the peroxidase-

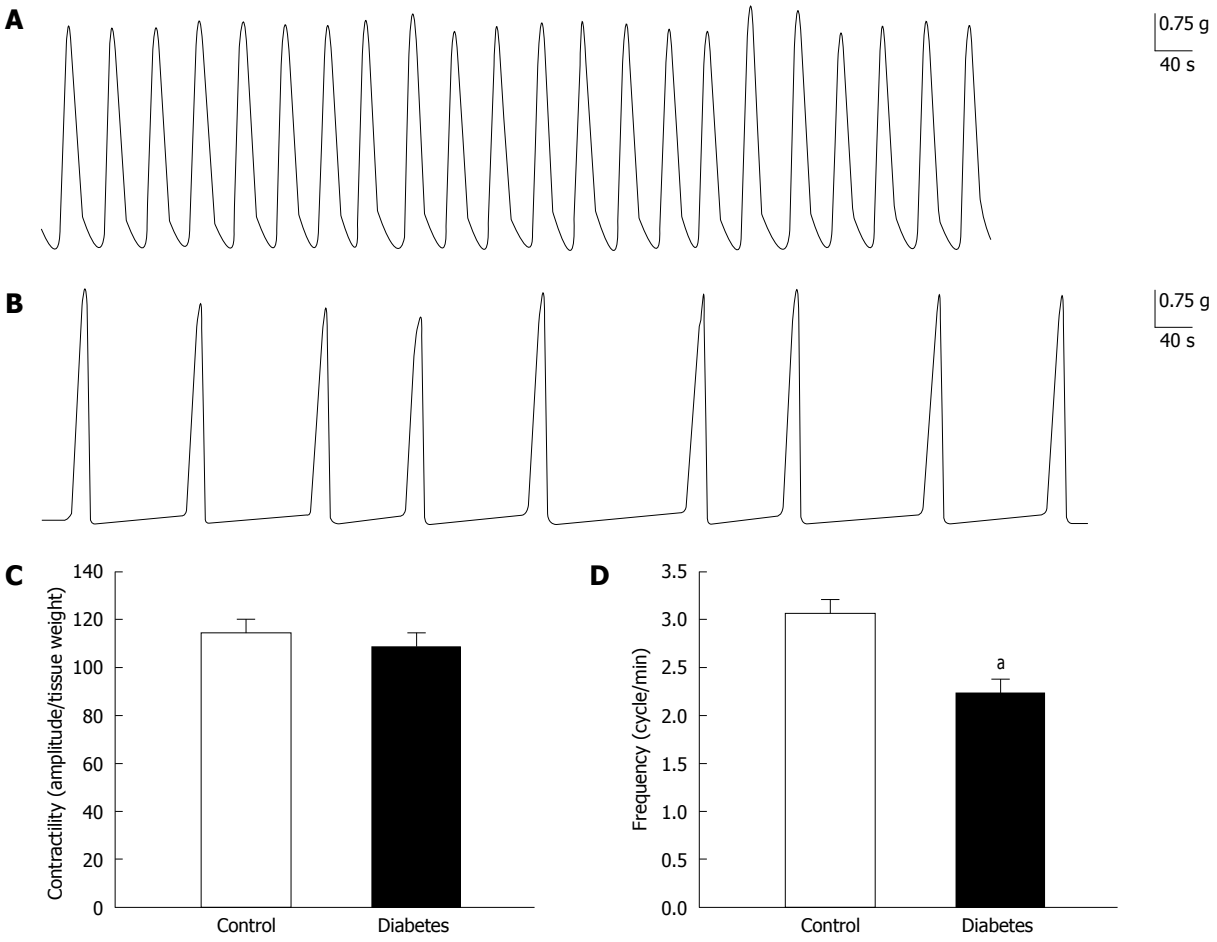

Figure 1 Comparison of gastric smooth muscle contractilities between normal and diabetic rats. A, B: The row traces gastric smooth muscle spontaneous contractions in normal and diabetic rats; C, D: Summary of the contractility in normal and diabetic rats. The contractility per weight of gastric smooth muscle strip was not significantly different between normal and diabetic rats (A-C, $n = 8$, $P > 0.05$). However, the frequency of spontaneous contraction was significantly depressed in diabetic rats (A, B and D, $n = 8$, $^{a}P < 0.01$).

labeled streptavidin complex reagent was added, and the slides were incubated for 30 min at 37℃ in a humidified container. After being washed in PBS, antibody binding was visualized using 3,3'-diaminobenzidine. Slides were washed in running tap water, counterstained lightly with hematoxylin, and mounted in permount. For negative controls, sections were incubated with PBS in place of the primary antibody.

Drugs

CNP (rat CNP-22), STZ, cGMP antibody and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, US). CNP was dissolved in distilled water (1 mmol/L) and further diluted in the superfusion buffer to the concentrations indicated in the text.

Statistics analysis

The staining index was calculated from the staining intensity and area by means of image analysis software, in three areas per section, three sections per group, and weak, medium and strong CNP and NPR-B staining intensities graded as 1, 2 and 3 points according to Feng J Lai's method[25]. The contractility = amplitude of spontaneous contraction (g)/gastric smooth muscle strip weight (g). Inhibitory percentages = amplitude in control - amplitude decreased by CNP/amplitude in control × 100%. Staining index = staining intensity × staining area. Data were expressed as mean ± SE. Statistical significance was evaluated by *t* test. Differences were considered significant when $P < 0.05$.

RESULTS

Change in body weight and plasma glucose

Rats were used for experiments at 4 wk after injection with STZ. At the time of the experiment, all STZ-treated rats exhibited hyperglycemia; their blood glucose concentrations (478.0 ± 27.9 mg/dL) were significantly higher than those of the non-diabetic control rats (108.9 ± 11.4 mg/dL, $n = 8$, $P < 0.001$) and the body weights of the diabetic rats (209.7 ± 8.0 g) were significantly lower than those of the control rats (247.4 ± 13.1 g, $n = 8$, $P < 0.05$).

The spontaneous contraction of gastric smooth muscle

To determine the extent of gastric motility impediment in diabetic rats the spontaneous contractions of gastric smooth muscle strips were observed in control and

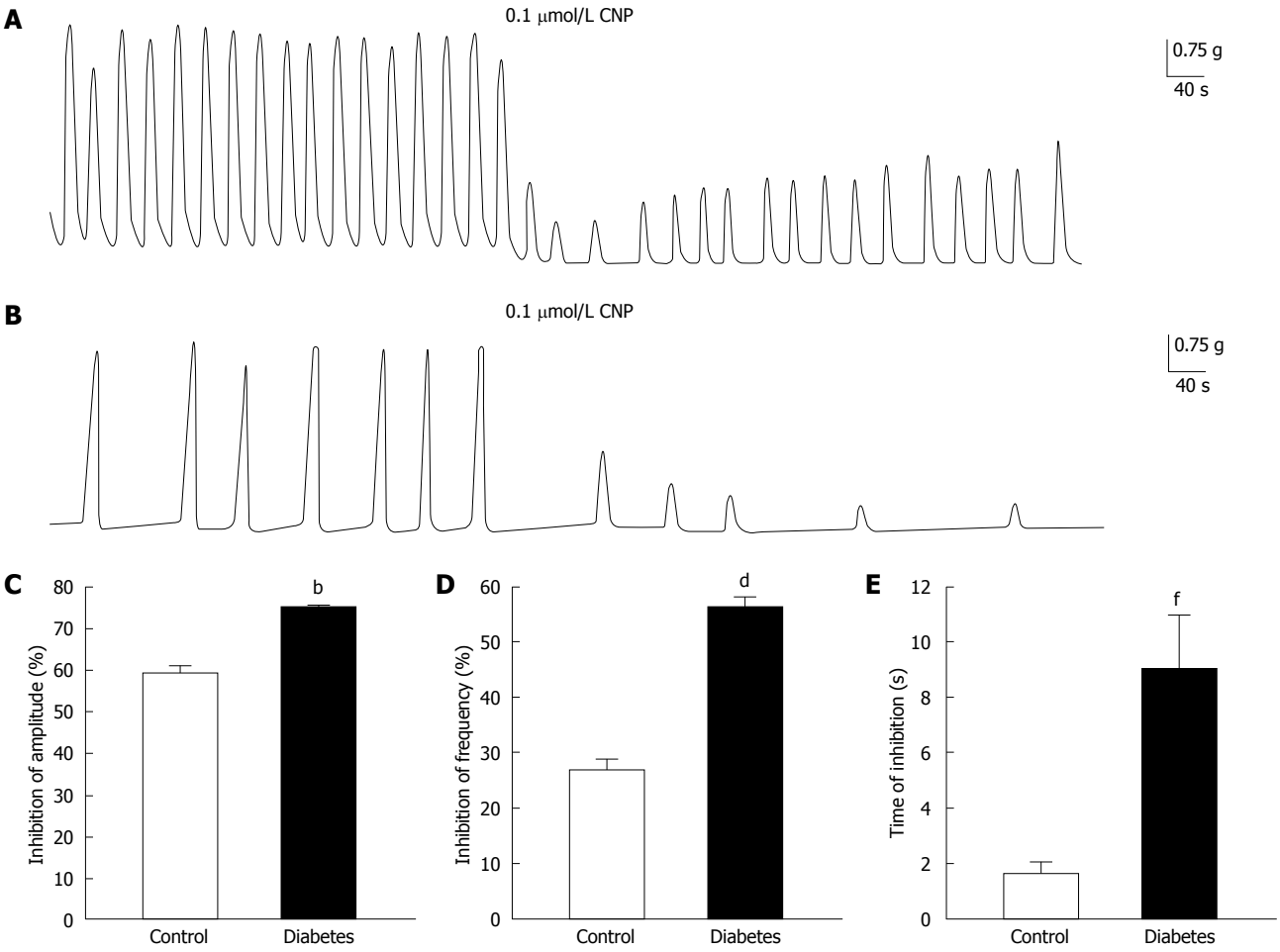

Figure 2 The sensitivity of gastric smooth muscle to CNP. A, B: The row traces gastric smooth muscle spontaneous contractions in response to CNP in normal and diabetic rats; C-E: Summary of the contractility in response to CNP in normal and diabetic rats. CNP induced relaxation of gastric antral smooth muscle in control and diabetic rats (A, B). However, CNP-induced inhibition of spontaneous contraction was potentiated in diabetic rats, and the amplitude (C, $n = 8$, $^{b}P < 0.01$) and frequency (D, $n = 8$, $^{d}P < 0.01$) of spontaneous contraction were more potentially suppressed by CNP in diabetic rats. The inhibition time of CNP of spontaneous contraction was significantly prolonged in diabetic rats (E, $n = 8$, $^{f}P < 0.01$).

diabetic rats. At 4 wk after injection of STZ and vehicle, the spontaneous contraction was recorded in gastric smooth muscle strips of normal and diabetic rats. In order to compare the contractilities of gastric smooth muscle between normal and diabetic rats, the amplitudes of spontaneous contraction of gastric smooth muscle were normalized by every muscle strip weight. The frequency of spontaneous contraction was significantly decreased in diabetic rats, while the amplitude of spontaneous contraction was not significantly affected in diabetic rats (Figure 1A and B). The frequency of spontaneous contraction was decreased from 3.10 ± 0.14 cycle/min of the control to 2.23 ± 0.13 cycle/min (Figure 1D, $n = 8$, $P < 0.01$), however, the contractilities were 115.18 ± 8.69 and 109.34 ± 6.54 in normal and diabetic rats, respectively (Figure 1C, $n = 8$, $P > 0.05$).

The sensitivity of gastric smooth muscle to CNP

To determine the role of the natriuretic peptide signal pathway in diabetic gastroparesis, the effect of CNP on spontaneous contraction was observed in normal and diabetic rats. CNP significantly inhibited the spontaneous contractions in both groups (Figure 2A and B), however, the inhibitory effect was potentiated in diabetic rats. The amplitude of spontaneous contraction was suppressed by 58.92% ± 1.32% and 75.15% ± 0.71% in normal and diabetic rats, respectively (Figure 2C, $n = 8$, $P < 0.01$). The frequency of spontaneous contraction was decreased by 26.95% ± 2.82% and 53.33% ± 2.03% in normal and diabetic rats, respectively (Figure 2D, $n = 8$, $P < 0.01$). The time of CNP-induced inhibition (inhibition time) was measured as the time from starting to reduce the amplitude of spontaneous contraction to starting to recover from peak inhibition. The inhibition time was prolonged from 1.43 ± 0.80 min of control to 8.95 ± 2.07 min in diabetic rats (Figure 2E, $n = 8$, $P < 0.01$).

CNP and NPR-B expression in gastric tissues

Since the CNP-induced inhibition of spontaneous contraction was potentiated in diabetic rats, the expressions of CNP and NPR-B in gastric tissues were further confirmed. There was no CNP immunopositive expression in negative controls of normal and diabetic rats (Figure 3A and B). The CNP immunopositive brown granules were mainly expressed in gastric muscle layers of normal and diabetic rats (Figure 3C and D),

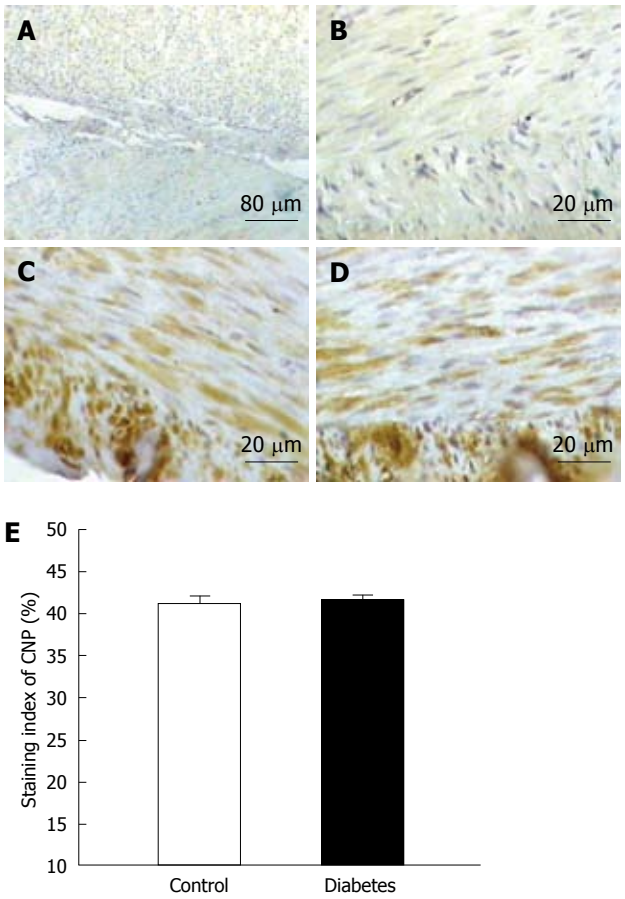

Figure 3 CNP expression in gastric tissues in normal and diabetic rats. A-D: CNP expression in gastric smooth muscle in normal and diabetic rats. In negative controls CNP was not expressed in normal and diabetic rats (A, B) and the CNP immunopositive brown granules were mainly expressed in gastric muscle layers of normal and diabetic rats (C, D); E: Summary of CNP expression in normal and diabetic rats. The staining indexes were not significantly different between normal and diabetic rats (E, n = 8, P > 0.05). Scale bars = 80 μm (A), 20 μm (B-D).

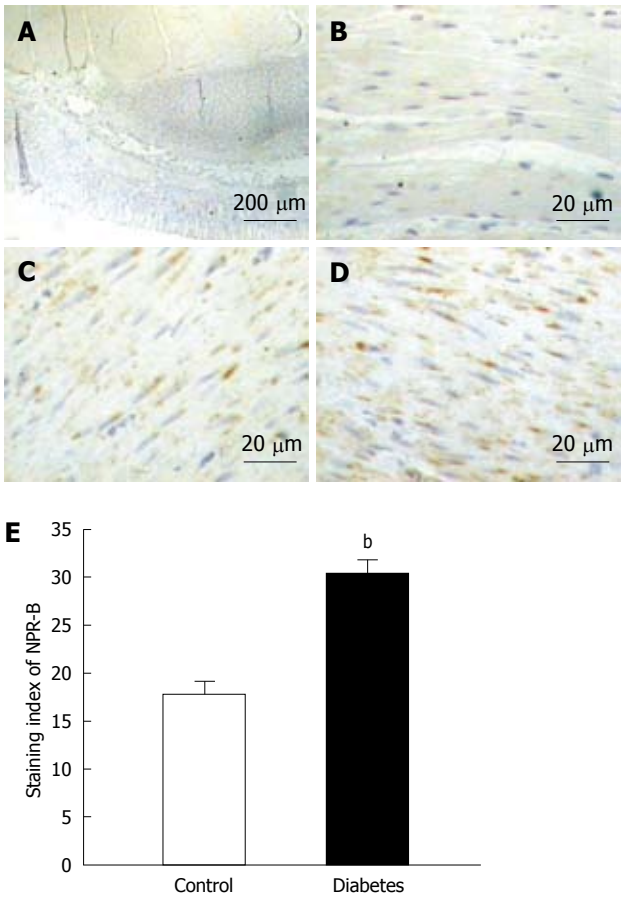

Figure 4 NPR-B expression in gastric tissues in normal and diabetic rats. A-D: NPR-B expression in gastric smooth muscle in normal and diabetic rats. There was no NPR-B immunopositive expression in negative controls of normal and diabetic rats (A, B). The NPR-B immunopositive brown granules were expressed in gastric antral smooth muscle in normal and diabetic rats. The staining was deeper in diabetic rats (C, D); E: Summary of NPR-B expression in normal and diabetic rats. The staining indexes were increased significantly in diabetic rats (E, n = 8, ^bP < 0.01). Scale bars = 200 μm (A), 20 μm (B-D).

and the staining indexes were not significantly different between normal and diabetic rats (Figure 3E, n = 8, P > 0.05). There was no NPR-B immunopositive expression in negative controls of normal and diabetic rats (Figure 4A and B). The NPR-B immunopositive brown granules were expressed in gastric antral smooth muscle in normal and diabetic rats, however the staining was deeper in diabetic rats (Figure 4C and D). The staining indexes were increased from 17.63 ± 1.49 in controls to 30.67 ± 1.59 in diabetic rats, and there were significant differences between normal and diabetic rats (Figure 4E, n = 8, P < 0.01).

DISCUSSION

The effects of CNP on gastrointestinal motility have been described by some reports: relaxant effect on chick rectum muscle strip[26] and guinea pig cecum circular smooth muscle[27], and inhibitory effect on rabbit colon[28]. We previously reported that CNP significantly inhibited spontaneous contraction of gastric smooth muscles in rats, guinea pigs and humans[17]. Although previous studies demonstrated that spontaneous activity of the smooth muscle in the gastrointestinal tract was attenuated in diabetic-model animals[29-31], no studies were made of the relationship with the NPR-pGC-cGMP signal pathway. In our present study, at 4 wk after injection of STZ and vehicle, the frequency of spontaneous contraction was significantly depressed in diabetic rats (Figure 1A and B), while the amplitude of spontaneous contraction was not significantly affected in diabetic rats (Figure 1C). CNP induced relaxation of gastric antral circular smooth muscle in normal and diabetic rats, however the relaxation response induced by CNP was significantly potentiated in diabetic rats (Figure 2). The results indicate that the gastric smooth muscles were more sensitive to CNP in the diabetic rats than in the normal rats, and they suggest that the NPs-NPR-B-pGC-cGMP signal pathway may be upregulated in STZ-induced diabetic rat.

Three types of single-transmembrane NPRs for ANP, BNP and CNP have been identified[8,9], i.e. NPR-A, NPR-B and NPR-C. NPR-A and NPR-B have membrane-bound pGC which catalyzes the formation of cGMP from GTP[10-12]. NPR-A preferentially binds ANP and BNP, but has a low affinity for CNP, NPR-B

has a much higher affinity for CNP than either ANP or BNP[13]. CNP mRNA expression was increased in the kidney of STZ-induced diabetic rats and NPR-B expression was enhanced in vascular smooth muscle in the diabetic mouse[23,24].

In smooth muscle, CNP generally causes relaxation by eliciting membrane-bound pGC-mediated cGMP production[32]. Moreover, many experiments also demonstrated that CNP cognate receptors were distributed in gastrointestinal smooth muscle[23,24,28]. In our present study the NPR-B immunopositive brown granules were increased in the gastric antral smooth muscle of diabetic rats (Figure 4). However, the CNP expression in gastric muscle was not significantly different from normal rats (Figure 3). These results suggest that the NPs-NPR-B-pGC-cGMP signal pathway may be involved in diabetic gastropathy *via* increasing of the NPR-B expression. Furthermore, the data are compatible with the idea that up-regulation of the NPs-NPR-B-pGC-cGMP signal pathway may be an important factor which hastens or induces the disorder of gastric motility, and occurs concomitantly with development of gastrointestinal dysfunction, for example, gastroparesis. Thus, every stage of the NPs-NPR-B-pGC-cGMP signal pathway may be a potential target for investigating the mechanism of diabetic gastropathy or gastroparesis and preventing diabetic gastrointestinal dysfunction.

In summary, this study has demonstrated that diabetes firstly induces frequency depression of gastric motility but not contractility. The CNP-induced relaxation response is potentiated in STZ-induced diabetic rats, and this is related to increased NPR-B expression in the gastric smooth muscle. These results suggest that the NPs-NPR-B-pGC-cGMP signal pathway plays an important role in diabetic gastropathy or gastroparesis.

COMMENTS

Background

A common gastrointestinal complication of diabetes is gastroparesis. However, the pathogenesis is not clear yet. A recent study has indicated that atrial natriuretic peptide (ANP) is secreted from gastric mucosa and plays an inhibitory role in the regulation of gastrointestinal motility, but the effect of the natriuretic peptides (NPs) signal pathway on diabetic gastroparesis has not been reported.

Research frontiers

NPs are distributed all over the body besides the heart, for example, the gastrointestinal tract and enterochromaffin cells in gastrointestinal mucosa secrete NPs. However, the many functions of NPs in the gastrointestinal tract in physiological and pathophysiological conditions need to be explored. In the present study, the possibility as to whether the NPs/cGMP signal pathway is involved in diabetic gastroparesis was investigated in streptozotocin-induced diabetic rats.

Innovations and breakthroughs

Recent reports have highlighted the pathogenesis of diabetic gastroparesis. This is the first study to report that the expression of NP receptor type B in gastric tissue is increased and the sensitivity of gastric smooth muscle to C-type NP (CNP) is significantly enhanced in the diabetic rat. This study suggests that the NPs/cGMP signal pathway may be involved in diabetic gastroparesis.

Applications

By understanding that the NPs/cGMP signal pathway may be involved in diabetic gastroparesis, this study may represent a future strategy for therapeutic or preventive intervention in the treatment of patients with diabetes.

Terminology

Gastroparesis (delayed gastric emptying) is frequent in diabetic patients. Symptoms of diabetic gastropathy can range from mild dyspepsia to recurrent vomiting, abdominal pain and may progress to gastric failure known as gastroparesis. NPRs are natriuretic peptide receptors for ANP, brain natriuretic peptide and CNP.

Peer review

It is an interesting article pointing to a novel mechanism that may explain diabetic changes in gastric function. The results showed are logical, attractive and congruent. In many ways the work is interesting and quite novel and is probably worthy of publication.

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