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

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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.

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

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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\cite{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\cite{5,6},

Since atrial natriuretic peptide (ANP) was isolated from atrium by de Bold et al\cite{7} in 1981, brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), dendoraspinatriuretic peptide (DPN), microcur 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\cite{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\cite{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\cite{13}. NPs are also secreted from gastric mucosa\cite{14,15}. 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\cite{16-19}. In smooth muscle, CNP activates its cognate NPR-B, which includes an intracellular pGC domain and catalyzes the synthesis of cGMP within the cytosol\cite{20}. CNP and NPR-B have been detected in the stomach\cite{21,22,23}. 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\cite{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\cite{25}. 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°C, 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\(_2\)PO\(_4\) 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\(_2\)O\(_2\) for 30 min. After being washed in phosphate buffered saline (PBS), slides were incubated for 45 min at 37°C 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°C 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°C in a humidified container with biotin-labeled goat anti-rabbit serum. After being washed in PBS, the peroxidase-
labeled streptavidin complex reagent was added, and the slides were incubated for 30 min at 37°C 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
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, \( p < 0.01 \)) and frequency (D, \( n = 8, p < 0.01 \)) 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, \( n = 8, p < 0.01 \)).

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 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 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, \( n = 8, p < 0.01 \)) and frequency (D, \( n = 8, p < 0.01 \)) 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, \( n = 8, p < 0.01 \)).

Figure 2  The sensitivity of gastric smooth muscle to CNP. A, B: The raw 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, \( n = 8, p < 0.01 \)) and frequency (D, \( n = 8, p < 0.01 \)) 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, \( n = 8, p < 0.01 \)).

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).
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, \( n = 8, P < 0.01 \)). Scale bars = 80 μm (A), 20 μm (B-D).

**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 NPRs-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[22]. Moreover, many experiments also demonstrated that CNP cognate receptors were distributed in gastrointestinal smooth muscle[23,24,26]. 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.
link atrial natriuretic peptide and somatostatin secretion in the antrum of the stomach. *Regul Pept* 2003; 110: 101-106

17 **Guo HS**, Jin Z, Jin ZY, Li ZH, Cui YF, Wang ZY, Xu WX. Comparative study in the effect of C-type natriuretic peptide on gastric motility in various animals. *World J Gastroenterol* 2003; 9: 547-552

18 **Guo HS**, Cui X, Cui YG, Kim SZ, Cho KW, Li ZL, Xu WX. Inhibitory effect of C-type natriuretic peptide on spontaneous contraction in gastric antral circular smooth muscle of rat. *Acta Pharmacol Sin* 2003; 24: 1021-1026

19 **Guo HS**, Cai ZX, Zheng HF, Li XL, Cui YF, Wang ZY, Xu WX, Lee SJ, Kim YC. Role of calcium-activated potassium currents in CNP-induced relaxation of gastric antral circular smooth muscle in guinea pigs. *World J Gastroenterol* 2003; 9: 2054-2059

20 **Potter LR**, Hunter T. Guanylyl cyclase-linked natriuretic peptide receptors: structure and regulation. *J Biol Chem* 2001; 276: 6057-6060

21 **Stepan H**, Leitner E, Bader M, Walther T. Organ-specific mRNA distribution of C-type natriuretic peptide in neonatal and adult mice. *Regul Pept* 2000; 95: 81-85

22 **Rambotti MG**, Giambanco I, Spreca A. Detection of guanylate cyclases A and B stimulated by natriuretic peptides in gastrointestinal tract of rat. *Histochern* 1997; 29: 117-126

23 **Christoffersen C**, Bartels ED, Nielsen LB. Heart specific up-regulation of genes for B-type and C-type natriuretic peptide receptors in diabetic mice. *Eur J Clin Invest* 2006; 36: 69-75

24 **Shin SJ**, Wen JD, Lee YJ, Chen IH, Tsai JH. Increased C-type natriuretic peptide mRNA expression in the kidney of diabetic rats. *Endocrinol* 1998; 158: 35-42

25 **Kim SZ**, Kim SH, Park JK, Koh GY, Cho KW. Presence and biological activity of C-type natriuretic peptide-dependent guanylate cyclase-coupled receptor in the penile corpus cavernosum. *J Urol* 1998; 159: 1741-1746

26 **Sudoh T**, Minamino N, Kangawa K, Matsuho H. C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain. *Biochem Biophys Res Commun* 1990; 168: 863-870

27 **Itaba S**, Chijiwa Y, Matsuzaka H, Motomura Y, Nawata H. Presence of C-type natriuretic peptide (CNP) in guinea pig caecum: role and mechanisms of CNP in circular smooth muscle relaxation. *Neurogastroenterol Motil* 2004; 16: 375-382

28 **Kim JH**, Jeon GJ, Kim SZ, Cho KW, Kim SH. C-type natriuretic peptide system in rabbit colon. *Peptides* 2001; 22: 2061-2068

29 **Xue L**, Suzuki H. Electrical responses of gastric smooth muscles in streptozotocin-induced diabetic rats. *Am J Physiol* 1997; 272: G77-G83

30 **Takano H**, Imaeda K, Koshita M, Xue L, Nakamura H, Kawase Y, Hori S, Ishigami T, Kurono Y, Suzuki H. Alteration of the properties of gastric smooth muscle in the genetically hyperglycemic OLETF rat. *J Auton Nerv Syst* 1998; 70: 180-188

31 **Ordog T**, Takayama I, Cheung WK, Ward SM, Sanders KM. Remodeling of networks of interstitial cells of Cajal in a murine model of diabetic gastroparesis. *Diabetes* 2000; 49: 1731-1739

32 **Carvajal JA**, Germain AM, Huidobro-Toro JP, Weiner CP. Molecular mechanism of cGMP-mediated smooth muscle relaxation. *J Cell Physiol* 2000; 184: 409-420

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