

## Gastric nNOS reduction accompanied by natriuretic peptides signaling pathway upregulation in diabetic mice

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**Supported by** The National Natural Science Foundation of China, No. 31071011; No. 31171107; and the Shanghai Natural Science Foundation, No. 13ZR1423100

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Received: July 4, 2013 Revised: October 14, 2013

Accepted: November 2, 2013

Published online: April 28, 2014

### Abstract

**AIM:** To investigate the relationship between neuronal nitric oxide synthase (nNOS) expression and the natriuretic peptide signaling pathway in the gastric fundus of streptozotocin (STZ)-induced diabetic mice.

**METHODS:** Diabetic mice were induced by injection of STZ solution. Immunofluorescence labeling of HuC/D, nNOS and natriuretic peptide receptor-A, B, C (NPRs) in the gastric fundus (GF) was used to observe nNOS expression and whether NPRs exist on enteric neurons. The expression levels of nNOS and NPRs in the diabetic GF were examined by western blotting. An isometric force transducer recorded the electric field stimulation (EFS)-induced relaxation and contraction in the diabetic GF. An intracellular recording method assessed EFS-

induced inhibitory junction potentials (IJP) on the GF. GF smooth muscles acquired from normal mice were incubated with different concentrations of the NPRs agonist C-type natriuretic peptide (CNP) for 24 h, after which their nNOS expressions were detected by western blotting.

**RESULTS:** Eight weeks after injection, 43 diabetic mice were obtained from mouse models injected with STZ. Immunofluorescence indicated that the number of NOS neurons was significantly decreased and that nNOS expression was significantly downregulated in the diabetic GF. The results of physiological and electrophysiological assays showed that the EFS-induced relaxation that mainly caused by NO was significantly reduced, while the contraction was enhanced in the diabetic GF. EFS-induced IJP showed that L-NAME sensitive IJP in the diabetic GF was significantly reduced compared with control mice. However, both NPR-A and NPR-B were detected on enteric neurons, and their expression levels were upregulated in the diabetic GF. The nNOS expression level was downregulated dose-dependently in GF smooth muscle tissues exposed to CNP.

**CONCLUSION:** These findings suggested that upregulation of the NPs signaling pathway may be involved in GF neuropathy caused by diabetes by decreasing nNOS expression.

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**Key words:** Diabetic gastroparesis; Natriuretic peptides; Nitric oxide synthase; Enteric neuron

**Core tip:** The results demonstrated that the expressions of neuronal nitric oxide synthase (nNOS) and numbers NOS neurons were significantly downregulated while natriuretic peptides (NPs) and the natriuretic peptide receptor-A, B, C (NPRs) signaling pathway were upregulated. C-type natriuretic peptide, an NPRs agonist, inhibited

ited nNOS expression in cultured gastric fundus tissue. These findings suggested that upregulation of the NPs signaling pathway may be involved in gastric fundus neuropathy caused by diabetes, by decreasing nNOS expression. The results are interesting and may represent a molecular mechanism of diabetic gastroparesis.

Lu HL, Huang X, Wu YS, Zhang CM, Meng XM, Liu DH, Kim Y, Xu WX. Gastric nNOS reduction accompanied by natriuretic peptides signaling pathway upregulation in diabetic mice. *World J Gastroenterol* 2014; 20(16): 4626-4635 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i16/4626.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i16.4626>

## INTRODUCTION

Diabetic gastroparesis is a representative diabetic dysmotility, and is associated with dysphagia, heartburn, nausea, vomiting and delayed gastric emptying<sup>[1,2]</sup>. It occurs in up to 50% of patients with type 1 diabetes and in 30% of patients with type 2 diabetes<sup>[3,4]</sup>. Gastroparesis seriously affects patients' quality of life and makes the control of blood glucose more difficult. Although diabetic gastroparesis is a significant health problem, the pathogenesis of this gastric dysfunction and its mechanisms are still not well understood. The gastric motility dysfunction may be caused by several factors, such as hyperglycemia, neuropathy, myopathy and depletion of interstitial cells of Cajal (ICC)<sup>[5,6]</sup>. The enteric nervous system (ENS), composed of excitatory and inhibitory neurons, exists throughout the entire gastrointestinal (GI) tract, which plays an important role in controlling and coordinating the GI tract motility. The ENS has a vital regulatory role in gastrointestinal motility; therefore, it has attracted more attention in recent years. It is generally considered that enteric neuropathy is one of the causative factors of diabetic gastroparesis. Numerous studies have shown that neurons expressing NOS in myenteric plexus are damaged and the number of nNOS immunoreactive positive neurons are significantly reduced<sup>[7]</sup>; both mRNA and protein expression levels of nNOS are downregulated and accompanied by attenuation of NO-induced relaxation in diabetic gastroparesis mice<sup>[8,9]</sup>. To date, the mechanisms of diabetes-induced enteric neuropathy remain unclear, and many investigators have reported that neuronal apoptosis, oxidative stress, advanced glycation end product (AGEs), changes of nerve growth factors and impaired brain-gut interactions may be involved<sup>[9]</sup>. However, whether the effects of important intracellular signaling pathways participate in diabetes-induced enteric neuropathy has not been widely examined.

Atrial natriuretic peptide was isolated from the atrium by de Bold *et al.*<sup>[10]</sup> in 1981. From then on, brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), dendroaspis natriuretic peptide, micrurus natriuretic

peptide (MNP), and ventricular natriuretic peptide have been discovered. These natriuretic peptides are distributed all over the body and exert a variety of biological effects, such as 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 natriuretic peptides (NPs) have been identified<sup>[11-13]</sup>: natriuretic peptide receptor A (NPR-A), NPR-B and NPR-C. They are divided into two major categories. NPR-A and NPR-B are membrane-bound guanylyl cyclase receptors that activate guanylyl cyclase, which catalyzes the formation of cGMP from GTP<sup>[14-16]</sup>. NPR-C, primarily controls NPs concentrations *via* receptor-mediated internalization and degradation, and has been reported in many signaling pathways in the GI tract<sup>[17]</sup>.

Nitric oxide (NO), identified as a biological signaling molecule in the 1980s, is a major nor-adrenergic, non-cholinergic (NANC) inhibitory neurotransmitter, which mediates smooth muscle relaxation. It is synthesized by NO synthase (NOS) and its three isoforms (eNOS, nNOS and iNOS) are expressed in many tissues, including endothelium, vascular smooth muscle, specific segments of the nephron and the heart<sup>[18,19]</sup>. It has been reported that nNOS is expressed on inhibitory neurons and plays an important role in regulation of NO production in the GI tract. NO binds to soluble GC and increases cGMP levels. Many studies have reported the relationships between NPs and NOS. CNP interacts with the NPR-C receptor coupled *via* G proteins leading to the activation Ca<sup>2+</sup>-calmodulin dependent endothelial NOS (eNOS), and subsequent increasing in NO production would induce the reduction in cardiac myocyte contractility<sup>[20]</sup>. By contrast, it has been demonstrated that BNP can increase iNOS and eNOS expression in the rat myocardium and cultures of cardiomyocytes, respectively<sup>[21-22]</sup>.

Our previous studies demonstrated that both NPR-A and NPR-B were distributed in the rat gastric smooth muscle layers and that CNP caused relaxation of the gastric circular and longitudinal smooth muscle tissues in stomachs of humans, rats and guinea pigs<sup>[23-25]</sup>. Recently, we also reported that the NPs/NPRs signaling pathway is upregulated in the gastric antrum and corpus smooth muscle layers, which may be involved in diabetes-induced loss of gastric ICC *via* decreasing the production of mSCF indirectly<sup>[26]</sup>. However, it is not clear whether natriuretic peptides play a role in diabetes-induced neuropathy. In this study, we investigated whether the NOS neurons are damaged and the relationships between CNP/NPRs signaling pathways and nNOS expression in the GF of STZ-induced diabetic mice.

## MATERIALS AND METHODS

### STZ-induced diabetic mouse model

Male imprinting control region (ICR) mice (5-wk-old) used for this study were purchased from the Experimen-

tal Animal Center of Shanghai Jiaotong University School of Medicine. One hundred mice were randomly divided into two groups: the control group and the diabetic model group. Mice in the diabetic model group were fasted overnight and intraperitoneally injected with STZ (Sigma-Aldrich, Saint Louis, MO, United States) solution. STZ was prepared freshly in 0.1 mol/L ice-cold citrate buffer (pH = 4.0) and used at a dose of 200 mg/kg body weight. Mice in the control group were intraperitoneally injected with the same volume of 0.1 mol/L citrate buffer. The animals had free access to food and water, and were maintained under standard housing conditions (room temperature 24–27 °C; humidity 60%–65%) with a 12 h light and dark cycle. After two months, blood glucose and body weight of each mouse were measured. Blood withdrawn from mouse tail vein after fasting for 8 h and the blood glucose concentration was measured with One-touch blood glucose monitoring system (Johnson and Johnson Medical Company, New Brunswick, NJ, United States). A mouse was declared diabetic when its blood glucose concentration was above 16 mmol/L.

### Tissue preparation

Whole stomachs were quickly excised from the mice and placed in a Sylgard base dish with pre-oxygenated Krebs solution (containing in 118.1 mmol/L NaCl, 4.7 mmol/L KCl, 1.0 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 1.0 mmol/L MgSO<sub>4</sub>, 25.0 mmol/L NaHCO<sub>3</sub>, 2.5 mmol/L CaCl<sub>2</sub>, and 11.1 mmol/L glucose), which was equilibrated with 95% oxygen and 5% CO<sub>2</sub>. The mesenteric fat was removed, and the stomach was cut along the greater curvature and pinned to the Sylgard base with the mucosa facing upward. Mucosal and submucosal layers were carefully removed under a dissecting microscope and the smooth muscle layers in the GF were used for immunohistochemistry and other experiments.

### Immunohistochemistry

Smooth muscle tissues (10 mm × 10 mm) from the GF were fixed with ice-cold paraformaldehyde (4% w/v) for 25 min. These tissues were then washed in 0.1 mol/L phosphate buffered saline (PBS) overnight at 4 °C. To reduce non-specific antibody binding, they were preincubated in 5% bovine serum albumin (Sigma) for 1 h at room temperature before incubation with the rabbit anti-nNOS antibody (1:1000; Cell Signaling Technology, Danvers, MA, United States) and mouse anti-HuC/HuD antibody (A-21271, Abcam, Burlingame, CA, United States). To achieve greater penetration during labeling, incubation solutions with the primary antibody were mixed with Triton-X 100 (0.5%; Sigma). Tissues were incubated in the primary antibodies for 48 h at 4 °C. Following washing in 0.1 mol/L PBS overnight at 4 °C, tissues were incubated with the corresponding secondary antibody (DyLight 488 conjugated anti-rabbit IgG and DyLight 549 conjugated anti-mouse IgG, 1:400, CoWin Biotech, China) for 1 h at 25 °C. Tissues were washed in 0.1M PBS for 4 h before being mounted on a slide glass with an anti-fading agent

(Molecular Probes, Mississauga, Ontario, Canada) and examined using a confocal microscope (TCS-SP2; Leica Microsystems, Wetzlar, Germany).

We also used frozen tissue sections to verify whether NPRs were expressed on the myenteric plexus. Small GF tissues were collected after the mice were killed by cervical dislocation. The samples were fixed with 4% paraformaldehyde overnight at 4 °C and sectioned at 5 µm thickness. The sections were then blocked with 10% goat serum in PBS for 1 h at room temperature. The blocking solution was removed and primary antibody solution added (NPR-A, ab70848, 1:100; NPR-B, ab14357, 1:200; HuC/D, A-21271, Abcam), before being incubated overnight at 4 °C. The sections were washed three times in PBS (10 min per time), followed by 1 h incubation in secondary antibody solution (DyLight 488 conjugated anti-mouse IgG and DyLight 549 conjugated anti-rabbit IgG, 1:400, CoWin Biotech, China). The negative control group was created by incubating sections without primary antibodies. Sections were then washed in 0.1 mol/L PBS for 15 min three times before being mounted on a slide glass. These slices were visualized and photographed under a fluorescence microscope (Olympus IX71, Tokyo, Japan).

### Isometric tension measurement

Fresh GF smooth muscle strips (approximately 2 mm × 8 mm) were acquired by cutting along the circular axis from the gastric smooth muscle tissue. After a silk thread (USP 5/0) was attached to both ends of the muscle strips, the strips were mounted along the circular axis in 10 mL organ baths containing warmed (37 °C) and oxygenated (95% O<sub>2</sub>: 5% CO<sub>2</sub>) Krebs solution. An isometric force transducer (RM6240C, Chengdu Instrument Factory, China) that was connected to an amplifier recorded the isometric contraction measurements. The muscle strips were incubated at the appropriate tension for 40 min before the experiment. To observe the excitatory and inhibitory signals in the GF, electric field stimulation (EFS) was applied. Muscle strips were subjected to EFS at 1, 5, 10, 15, 20 and 25 Hz under a constant voltage was 50 V. The pulse width was 0.5 ms, and the duration of stimulation was 10 s. At every interval, 4 min were allowed for recovery of spontaneous activity. After each series of stimulations, the bath solution was exchanged. At the conclusion of each experiment, 50 mmol/L KCl was used to normalize the differences among the readings for each sample.

### Intracellular microelectrode recording

Muscle strips (approximately 5 mm × 10 mm) dissected from the GF were isolated and pinned onto the base of a Sylgard-coated chamber, circular muscle side up, and continuously perfused with warmed (37 °C) and oxygenated Krebs solution. Strips were allowed to equilibrate for approximately 2 h before the recording commenced. Experiments were carried out in the presence of nifedipine (1 µmol/L) to minimize the movement of



muscles. Glass microelectrodes filled with 3 mol/L KCl (30–60 M $\Omega$  of resistance) were inserted into the cells. Membrane potentials were recorded using a standard electrometer (Duo 773, WPI Inc., Sarasota, FL, United States). EFS was applied in this experiment under a constant voltage of 50 V. The pulse width was 0.5 ms, the duration of stimulation was 20 ms and the slow inhibitory junction potentials (IJP) of circular smooth muscle in normal and diabetic GF were recorded.

### Tissue incubation

Smooth muscle tissues in the GF were obtained from normal mice as mentioned above. They were washed using sterile PBS three times for five minutes each. To study the relationship between natriuretic peptides (NPs) and nNOS expression in the GF, these tissues were exposed to DMEM containing 0.5% FCS and different concentrations of CNP ( $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  mol/L) for 24 h. These tissues were then cleaved into protein sample solutions and detected by western blotting.

### Western blotting analysis

Protein samples were extracted from the smooth muscle tissues in the GF as recommended by the manufacturer of RIPA buffer (Beyotime chemical Co., Jiangsu, China). They were mixed with  $2 \times$  loading buffer and in a 100 °C water bath for 10 min before a protein assay (Bio-Rad, Hercules, California, United States) was used to determine the protein content. Equivalent amounts of protein (normally 40  $\mu$ g per lane) and pre-stained markers were separated by 10% SDS-PAGE and electro transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, United States). Membranes were then blocked in Tris buffered saline-Tween 20 (TBS-T) with 5% (w/v) non-fat dry milk for 2 h at room temperature. The membranes were incubated overnight at 4 °C with rabbit anti-nNOS polyclonal antibody (1:1000; Cell Signaling Technology, Boston, MA, United States), rabbit anti-NPR-A antibody (1:400; sc-25485, Santa Cruz Biotechnology, Dallas, Texas, United States), rabbit anti-NPR-B antibody (1:300; sc-25486, Santa Cruz Biotechnology, United States), rabbit anti-NPR-C antibody (1:400; sc-25487, Santa Cruz Biotechnology) or rabbit anti-GAPDH monoclonal antibody (1:1000; Cell Signaling Technology). After washing three times (five minutes each) with TBS-T, the membranes were incubated with the alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (1:1000; CoWin Biotech, Beijing, China) for 1 h at room temperature. Following removal of the secondary antibody, membranes were washed three times and BCIP/NBT Phosphatase Substrate System (KPL Inc., Gaithersburg, MD, United States) was used to detect the signals on the blots. The image from each western blotting was quantitatively analyzed by using Quantity One software (Bio-Rad) and normalized by the GAPDH signal.

### Ethics

This study was carried out in strict accordance with the

recommendations in the Guide for the Care and Use of Laboratory Animals of the Science and Technology Commission of PRC (STCC Publication No. 2, revised 1988). The Committee on the Ethics of Animal Experiments of Shanghai Jiaotong University School of Medicine approved the protocol (permit number: Hu 686-2009).

### Statistical analysis

The data were expressed as the mean  $\pm$  SE. Analysis of differences between multiple groups of data was performed with one-way ANOVA, followed by a post hoc Bonferroni test. For comparison between two data sets, a paired or unpaired Student's *t*-test was used. Differences were considered to be significant at a *P* value less than 0.05.

## RESULTS

### Changes in blood glucose concentration and body weight

Two months after injection of STZ, the majority mice exhibited hyperglycemia. Plasma glucose concentrations of 43 of the STZ-treated mice were above 16 mmol/L and were thus defined as diabetic. Their mean blood glucose concentration was  $23.6 \pm 1.9$  mmol/L ( $n = 43$ ), which was significantly higher than the control group ( $5.9 \pm 0.6$  mmol/L,  $n = 43$ ,  $P < 0.01$ ). Their average body weight was  $20.5 \pm 0.6$  mg, which was significantly lower than the control group ( $31.7 \pm 0.6$  mg,  $n = 43$ ,  $P < 0.01$ ).

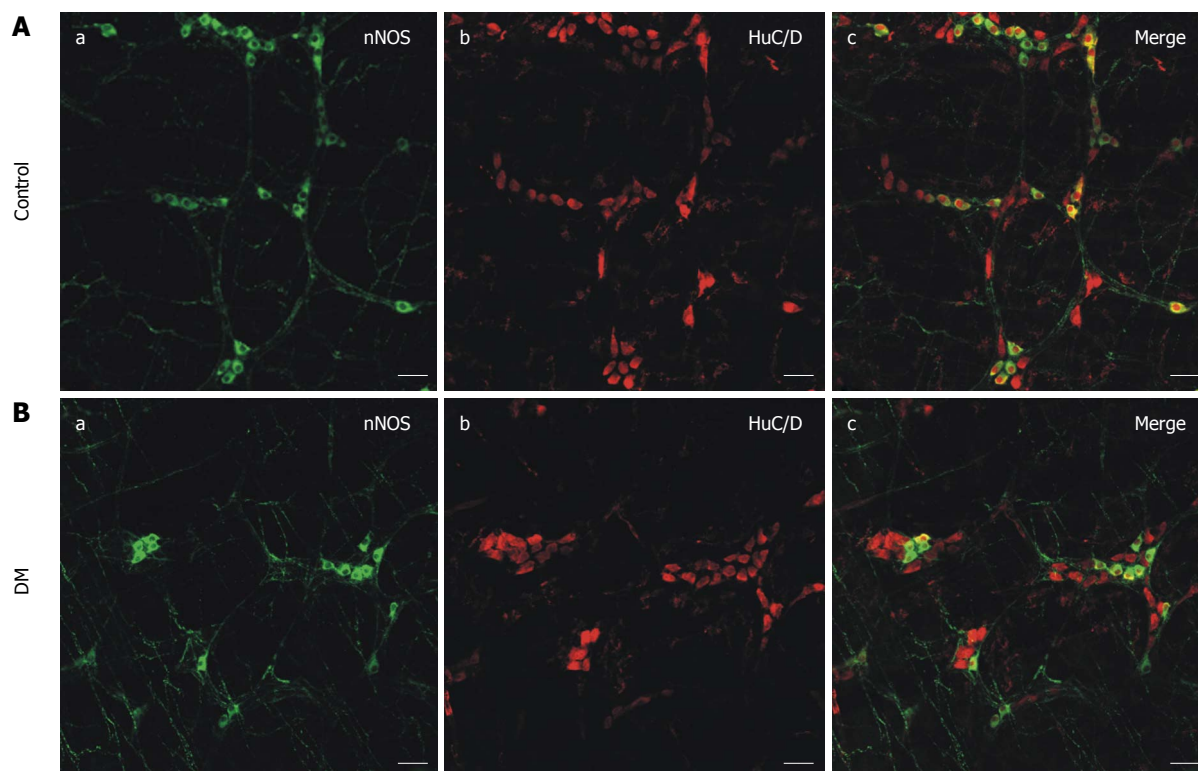
### Distribution of NOS neurons and nNOS expression in gastric fundus smooth muscle tissues

To determine whether diabetes-induced neuropathy had occurred, myenteric neurons were observed in the control and STZ-induced diabetic mice. According to other reports, specific anti-nNOS antibodies and anti-HuC/D antibodies were used to detect NOS neurons and all myenteric neurons in gastric fundus smooth muscle, respectively<sup>[27–29]</sup>. Green fluorescence showed nNOS and red fluorescence showed HuC/D. Fewer nNOS immunopositive cells were detected in the diabetic mice (Figure 1B) compared with the control mice (Figure 1A) and the NOS neurons were significantly damaged in the STZ-induced diabetic mice (Figure 1B), as observed from the typical merged images (Figure 1).

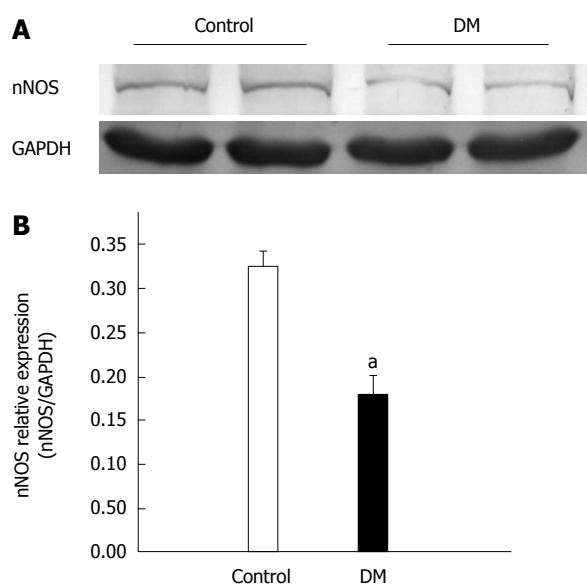
The protein expression level of nNOS in gastric fundus smooth muscle tissue was further analyzed by western blotting. The results showed that the nNOS expression level in STZ-induced diabetes was  $0.17 \pm 0.03$ , which was significantly lower than that of control mice ( $0.33 \pm 0.02$ , Figure 2,  $n = 7$ ,  $P < 0.05$ ).

### Change of NOS neuron function in diabetic GF

To further confirm whether NOS neurons were damaged and to observe the functional changes in the GF caused by diabetes, physiological and electrophysiological methods were applied. EFS (50 V, 0.5 ms pulse width,

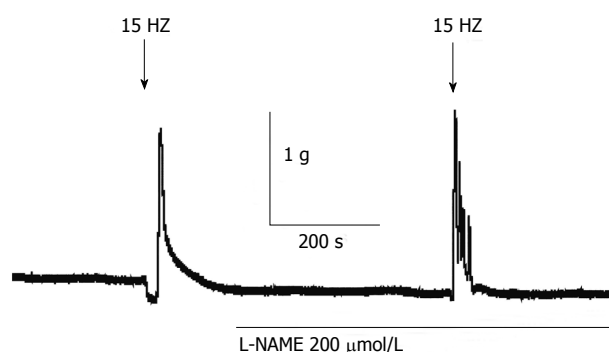


**Figure 1** Whole mount immunostaining of nitric oxide synthase and HuC/D in gastric fundus smooth muscle tissue. Neuronal nitric oxide synthase (nNOS) staining is used to show NOS neurons, while HuC/D staining is used to label all enteric neurons in the control and diabetic mice (DM) groups. Bar = 50  $\mu$ m.



**Figure 2** Expression of nitric oxide synthase in gastric fundus smooth muscle tissues. A: Representative bands of nitric oxide synthase (nNOS) protein expression in control and diabetic mice (DM) groups; B: The nNOS expression level in gastric fundus smooth muscle tissue was significantly decreased in the DM group ( $n = 7$ ,  $^aP < 0.05$  vs control).

10 s duration, 15 Hz) induced a relaxation and following contraction of gastric fundus smooth muscle strips in normal mice. However, the EFS-induced relaxation disappeared in the presence of 200  $\mu$ mol/L L-NAME, an NOS inhibitor (Figure 3,  $n = 5$ ). The results showed that EFS-induced relaxation in the GF was caused by NO

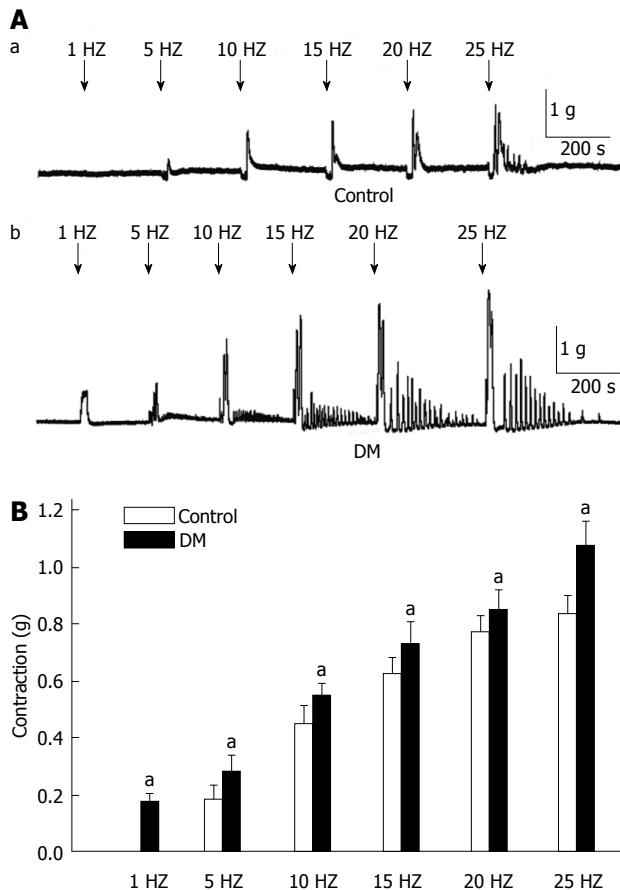


**Figure 3** Electric field stimulation-induced relaxation and contraction in gastric fundus smooth muscle of normal mice. A typical raw trace showing that electric field stimulation EFS (15 Hz)-induced relaxation was completely blocked by L-NAME ( $n = 5$ ).

synthesized by NOS neurons.

Different frequencies of EFS (1, 5, 10, 15, 20, 25 Hz) were applied in the GF, respectively (Figure 4A). In the diabetic mice, the EFS-induced relaxation was almost completely inhibited while the excitability contraction amplitude was significantly enhanced compared with control mice ( $0.18 \pm 0.02$ ,  $0.28 \pm 0.04$ ,  $0.55 \pm 0.03$ ,  $0.73 \pm 0.05$ ,  $0.86 \pm 0.05$  and  $1.1 \pm 0.07$  g in diabetic mice, and  $0$ ,  $0.18 \pm 0.04$ ,  $0.45 \pm 0.03$ ,  $0.63 \pm 0.03$ ,  $0.77 \pm 0.03$  and  $0.83 \pm 0.04$  g in controls, respectively, Figure 4B,  $n = 8$ ,  $P < 0.05$ ).

An intracellular recording technique was used to determine the EFS-induced IJP on gastric fundus smooth muscle tissues (Figure 5A and B). The duration of the



**Figure 4** Electric field stimulation-induced contraction of gastric fundus smooth muscle was recorded in control and diabetic mice. A: Typical raw traces of fundus smooth contractions induced by electric field stimulation (EFS) at different frequencies in control and diabetic groups; B: The amplitude of contraction from two groups and the smooth muscle contraction was more sensitive to EFS in the diabetic mice (DM) group ( $n = 8$ ,  $^aP < 0.05$  vs control).

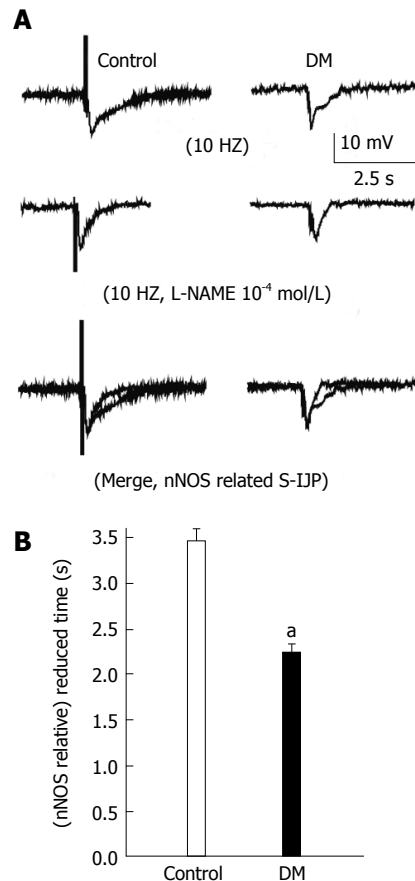
L-NAME sensitive, NO-mediated IJP in diabetes was  $2.3 \pm 0.07$ , which was significantly reduced compared with the control ( $3.4 \pm 0.08$ , Figure 5C,  $n = 9$ ,  $P < 0.05$ ).

#### Expressions of NPRs in gastric fundus smooth muscle tissues

To examine whether there were significant changes in NPRs expression in the diabetic GF, total homogenate of GF tissues was used and analyzed by western blotting. Figure 6A shows that NPRs were detected in the GF. The expression levels of NPR-A, NPR-B, NPR-C in diabetic and control mice were  $0.68 \pm 0.03$ ,  $0.94 \pm 0.03$  and  $0.43 \pm 0.03$ , and  $0.54 \pm 0.03$ ,  $0.7 \pm 0.02$  and  $0.20 \pm 0.02$ , respectively. The expression levels of NPRs were all upregulated in STZ-induced diabetic mice (Figure 6B,  $n = 8$ ,  $P < 0.05$ ).

#### Effect of NPRs on nNOS expression in cultured tissue

NPRs were overexpressed in diabetic GF smooth muscle; therefore, the role of NPRs in diabetes-induced neuropathy should be investigated. Firstly, we tried to evaluate whether NPRs were expressed on myenteric neurons. Cryosection staining results revealed that both NPR-A

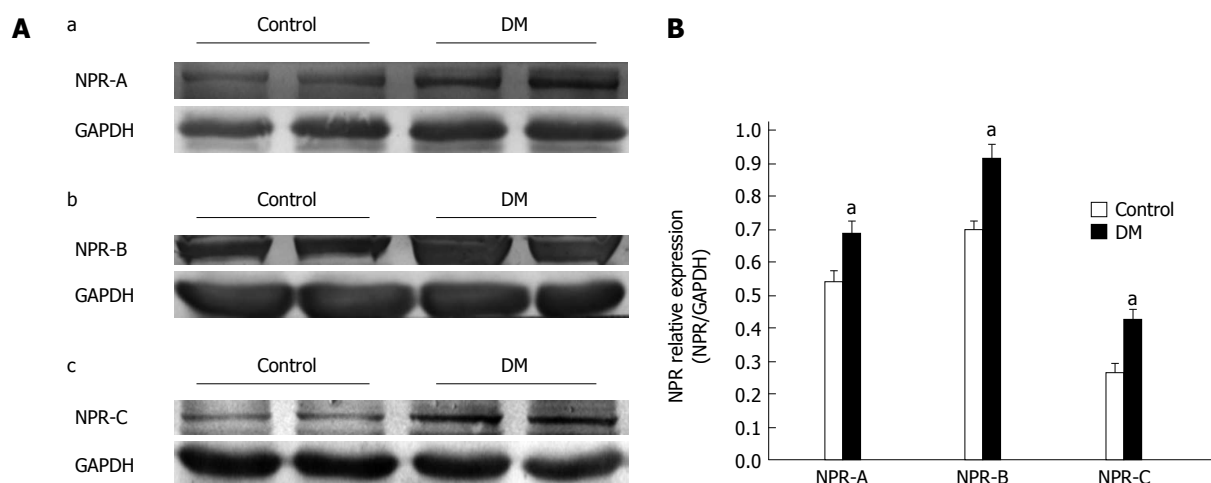


**Figure 5** Slow inhibitory junction potentials of gastric fundus smooth muscle were recorded in normal and diabetic mice. A: Representative raw traces of slow inhibitory junction potentials (slJP) elicited by electric field stimulation in control and diabetic mice (DM) groups; B: Summarized data showing that the NO-mediated duration of slJP was significantly decreased in the DM group ( $n = 9$ ,  $^aP < 0.05$  vs control).

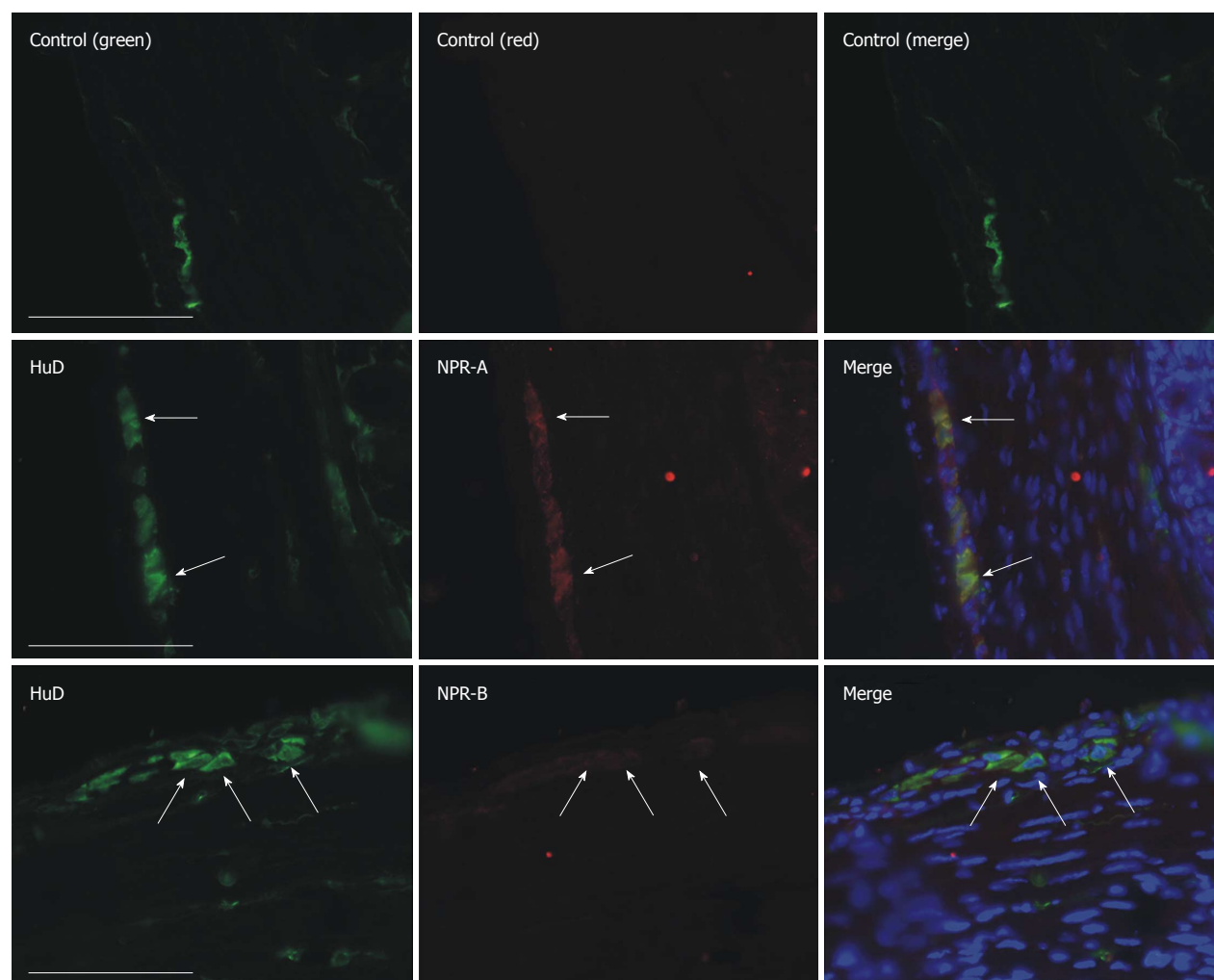
and NPR-B were detected on myenteric neurons (red fluorescence, Figure 7). To further investigate the relationship between upregulation of NPRs and nNOS expression, GF smooth muscles were exposed to different concentrations of CNP, a NPRs agonist and the nNOS expression levels were detected. The nNOS expression levels were  $0.49 \pm 0.02$  in the control and  $0.47 \pm 0.02$ ,  $0.35 \pm 0.04$ ,  $0.28 \pm 0.03$  in the presence of  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  mol/L CNP, respectively. CNP significantly reduced nNOS expression in cultured GF tissues in a concentration-dependent manner (Figure 8B,  $n = 7$ ,  $P < 0.05$ ).

## DISCUSSION

Gastroparesis is a syndrome characterized by delayed gastric emptying in the absence of mechanical obstruction of stomach. It is a well-recognized chronic complication of long-standing diabetes and affects patients' digestion and absorption functions seriously. Although diabetic gastroparesis (DGP) is a significant health problem, the pathogenesis of gastric dysfunction is still not well understood. The mutual cooperation and coordination between ENS, ICC, and smooth muscle play an



**Figure 6** Natriuretic peptide receptors expression in gastric fundus smooth muscle tissues in control and diabetic mice. A: Representative bands of natriuretic peptide receptors (NPRs) protein expression in control and diabetic mice (DM) groups; B: Summarized data showing that the levels of NPRs expression in the DM group were significantly decreased ( $n = 8$ ,  $^aP < 0.05$  vs control).

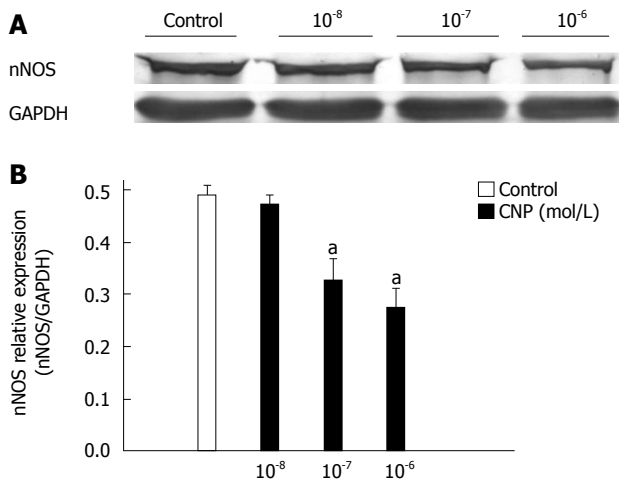


**Figure 7** Natriuretic peptide receptors-A and B immunofluorescence staining in gastric fundus smooth muscle tissue of normal mice. Myenteric neurons were labeled by an anti-HuD antibody and natriuretic peptide receptors (NPRs) were labeled by an anti-NPR-A, B antibody. Bar = 100  $\mu$ m.

important role in maintaining normal gastrointestinal motility. Several studies have reported that DGP may be caused by many factors, such as the depletion of ICC,

diabetes-induced neuropathy and damage to NOS neurons<sup>[30-33]</sup>. High-density NOS neurons are primarily involved in gastric receptive relaxation and pyloric sphinc-





**Figure 8** Effects of C-type natriuretic peptide on the nitric oxide synthase expression in gastric fundus smooth muscle tissues of normal mice. A: Representative bands of nitric oxide synthase (nNOS) protein expression in control and C-type natriuretic peptide (CNP) groups; B: Summarized data shows that CNP significantly inhibited nNOS expression in a dose-dependent manner in cultured gastric fundus smooth muscle tissue ( $n = 7$ ,  $^aP < 0.05$  vs control).

ter relaxation, which is extremely important for normal gastric emptying<sup>[34-37]</sup>. The first part of this study was focused on whether the NOS neurons were destroyed in STZ-induced diabetic mice. Our results indicated that the relative fluorescence intensity of nNOS was much weaker in diabetic GF smooth muscle compared with that of the control (Figure 1). The nNOS expression detected by western blotting revealed a consistent decrease in the protein level (Figure 2). The results suggested that the number of NOS neurons was significantly decreased and further detection showed nNOS expression levels to be significantly downregulated in diabetic mice compared with the control.

There is no spontaneous rhythmic contraction in the GF, therefore, electric field stimulation (EFS) was used to induce relaxation and contraction. Firstly, we tried to study whether NO is involved in EFS-induced response in GF smooth muscle tissues (Figure 3). Different frequencies of EFS were applied on the GF of diabetic and control mice. The results showed that the EFS-induced relaxation was significantly reduced while contraction was enhanced in the diabetic GF (Figure 4). Junction potentials (JPs) occur spontaneously and can be evoked by EFS. Output from the enteric nervous system to the gastric smooth muscle can be detected as neuromuscular excitatory and inhibitory junction potentials (EJPs and IJPs)<sup>[38]</sup>. The IJP has both rapid and slow components. The rapid component of the IJP is mediated by P2Y<sub>1</sub> receptors and is widely considered to be transmitted by ATP<sup>[39,40]</sup>. The slow component of the IJP is nitrogen, and can be blocked by NOS inhibitors<sup>[41]</sup>. In this experiment, IJPs were evoked by EFS on GF smooth muscles and the slow component (NO component) duration was significantly reduced in diabetic mice compared with the control (Figure 5). This result might imply that diabetes

causes serious neuropathy, especially NOS neurons damage, resulting in reduced NO production, further inducing abnormal excitability contraction and reduced sIJP duration time.

The NPs system is a local endocrine system in the gastrointestinal tract. It plays an important role in regulation of motility, secretion and absorption. Our previous studies showed that NPs can induce smooth muscle relaxation and the NPs signaling pathway participates in diabetes-induced ICC damage<sup>[23-26]</sup>. Many studies have reported the relationships between NPs and NOS<sup>[20-22]</sup>. In this study, we tried to evaluate whether NPs are involved in NOS neuron damage. Firstly, the protein expression levels of NPRs in diabetic GF smooth muscle were detected by western blotting. The results showed that the expression levels of three types of natriuretic peptide receptor (NPR-A, B, C) in diabetes were much higher than in control mice (Figure 6). Secondly, we observed the distribution of NPRs on GF enteric nerve system in frozen sections by immunohistochemistry. The results showed that there were many NPR-A, B proteins expressed on myenteric neurons (Figure 7). To investigate the relationship between upregulated NPRs and nNOS expression, GF smooth muscles were incubated with different concentrations of CNP and then the nNOS expression level was detected. The results showed that CNP decreased nNOS expression in a concentration-dependent manner (Figure 8). We can conclude that NPs may be involved in diabetes-induced neuropathy *via* decreasing nNOS expression.

In summary, we found that the number of NOS neurons was reduced and nNOS expression was down-regulated, while the NPRs expressions were upregulated in GF smooth muscle of STZ-induced diabetic mice. Diabetes-induced NOS neuron damage resulted in poor production of NO, which eventually caused abnormal excitability contraction and damaged relaxation in diabetic GF. Diabetes-induced upregulation of the NPs signaling pathway may be involved in NOS neurons injury.

## COMMENTS

### Background

Diabetic gastroparesis is a common complication of diabetic dysmotility. It is generally considered that enteric neuropathy is one of the causes of diabetic gastroparesis. Numerous studies have shown that neurons that synthesize the nitric oxide synthase (NOS) in the myenteric plexus were damaged and the number of nNOS immunoreactive neurons were significantly reduced; however, the mechanism of diabetes-induced enteric neuropathy remain unclear.

### Research frontiers

The natriuretic peptides (NPs) system is a local endocrine system in the gastrointestinal tract. It plays an important role in regulation of motility, secretion and absorption. Previous studies have reported that NPs can induce smooth muscle relaxation and the NPs signaling pathway participates in diabetes-induced interstitial cells of Cajal damage. In this study, the authors demonstrated that upregulation of the NPs signaling pathway might be involved in gastric fundus neuropathy caused by diabetes *via* decreasing nNOS expression.

### Innovations and breakthroughs

Recent reports have highlighted the importance of damage to NOS neurons accompanied by upregulation of NPs/NPRs/cGMP signaling pathway in the diabetic gastric fundus. C-type natriuretic peptide (CNP), a NPRs agonist, inhib-



ited nNOS expression in cultured gastric fundus tissue. This is the first study to report the relationship between NPs/NPRs signaling pathway and NOS neuron damage in STZ-induced diabetic gastric fundus tissues.

### Applications

By understanding the mechanism by which NOS neurons are damaged in diabetic gastroparesis, this study may represent a future strategy for therapeutic intervention in the treatment of patients with diabetic gastroparesis.

### Terminology

The NPs are a family of three polypeptide hormones termed atrial natriuretic peptide, brain natriuretic peptide, and CNP. In gastrointestinal tract NPs are involved in gastrointestinal motility, absorption and secretion.

### Peer review

The authors examined numbers of NOS neurons, and the expressions of nNOS and natriuretic peptide receptor-A, B, C (NPRs) in diabetic gastric fundus. The results demonstrated that the numbers of NOS neurons and the expression of nNOS were significantly downregulated while the NPs/NPRs signaling pathway was upregulated. CNP, a NPRs agonist, inhibited nNOS expression in cultured gastric fundus tissue. The results are interesting and may represent a molecular mechanism of diabetic gastroparesis.

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P- Reviewer: Feng CG S- Editor: Zhai HH  
L- Editor: Stewart GJ E- Editor: Liu XM





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ISSN 1007-9327



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