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Original Article

Alterations of Colonic Contractility in Long-term Diabetic Rat Model

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Background/Aims
Dysfunction of the gastrointestinal tract occurs in about 76% of patients who are diabetic for more than 10 years. Although diabetes-related dysfunctions of the stomach such as gastroparesis have been extensively studied over the recent years, studies about the mechanism underlying colonic symptoms in long-term diabetes models are rare. Therefore, the goal of our study was to clarify the nature of colonic dysfunction in a long-term diabetic rat model.

Methods
The characteristics of colonic smooth muscle were investigated in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, an animal model of type 2 diabetes. These results were compared to those obtained from Long-Evans Tokushima Otsuka (LETO) control rats.

Results
Spontaneous contractility of the proximal colon was significantly decreased in the diabetic rats compared to the controls, while the spontaneous contractility of the distal colon was not. The number of interstitial cells of Cajal networks in the proximal colon was greatly decreased in diabetic rats compared to the controls. Contractility of the proximal colon in response to carbachol, an acetylcholine receptor agonist, was significantly weaker in the diabetic rats. In addition, the degree of relaxation in response to nitric oxide in the proximal colon of diabetic rats also appeared to be attenuated.

Conclusions
The results from our study suggest that the decrease of interstitial cells of Cajal network, cholinergic receptors, and neuronal nitric oxide synthase in the proximal colon plays important roles in diabetes-related dysfunction of colon.
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Key Words
Colon; Diabetes mellitus; Gastrointestinal motility; Interstitial cells of Cajal

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Conflicts of interest: None.

Introduction

Diabetes and its long-term complications, including cardiovascular, renal, neurologic and ophthalmic diseases, represent a major cause of morbidity and mortality throughout the world.[1] It has been reported that 76% of outpatients with diabetes have one or more gastrointestinal (GI) symptoms such as nausea, vomiting, early satiety, abdominal pain, constipation, diarrhea and fecal incontinence.[2-8] The prevalence of diabetes-related GI symptoms may negatively impact the patient's quality of life.[9]

There have been many reports about upper GI symptoms, especially gastroparesis, in diabetic patients.[6,8,10-13] However, few studies about the mechanism underlying colonic symptoms in long-term diabetes models have been conducted and produced conflicting results.[14,15] In many reports, these GI symptoms are thought to be results of abnormal GI motility that, in turn, may be a manifestation of diabetic autonomic neuropathy involving the GI tract.[3,5,6,8] Increasing evidence, however, indicates that GI symptoms in diabetic patients are multifactorial.[16,17] Hyperglycemia can impair gastric and small intestinal motility[18,19] and autonomic neuropathy cannot be considered as the only reason for GI motility disorders.[20]

Interstitial cells of Cajal (ICC) have been identified as a pacemaker and mediators of neurotransmission in the GI tract, and so play a central role in GI motility.[21] The involvement of ICC in diabetic gastroenteropathies was recognized relatively recently.[13,21] Since then, several studies have reported the depletion of ICC networks in both diabetic patients and various diabetic animal models.[11,14]

In the present study, we analyzed Otsuka Long-Evans Tokushima Fatty (OLETF) rats older than 1 year as a long-term type 2 diabetes mellitus (DM) model. The clinical and pathological features of diabetes in OLETF rats closely resemble those of humans with type 2 DM.[22] We evaluated colonic motility, the cholinergic/nitrergic response, and ICC networks in OLETF rats to clarify the pathogenesis of the lower GI symptoms associated with type 2 DM.

Materials and Methods

Experimental Animals

Six male OLETF and 6 male Long-Evans Tokushima Otsuka (LETO) rats older than 60 weeks were used. All animals were allowed free access to water and food, and were maintained in a controlled environment with alternating 12 hour light/dark cycles. All animal procedures were approved by the Institutional Guideline Committee for Animal Experiments, Keimyung university school of medicine.

Tissue Preparations

Tissue specimens were collected from the proximal (about 1 cm from the cecum) and distal (about 1 cm from the rectosigmoid junction) colon. The mucosal layer was peeled off and transferred to a dissecting dish containing oxygenated (95% O_2 and 5% CO_2) Krebs solution with the following composition: 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM CaCl, and 11 mM glucose. Colonic circular muscle strips (2 × 10 mm) were prepared.

Functional Studies

One side of the colon strip was pinned to the floor of the recording chamber and the opposite side was connected to an isometric force transducer (Grass Technology, West Warwick, RI, USA). The strips were allowed to equilibrate for 60 minutes under an initial tension of 0.5 g. During this period, the bath solution was changed at every 10 minutes with aerated Krebs solution (95% O_2 and 5% CO_2). The bath temperature was maintained at 37 ± 0.5°C. Carbachol (1 nM-1 μM; Sigma- Aldrich, St. Louis, MO, USA), a cholinergic agonist, was used to evaluate the cholinergic system in the proximal colon. L-NG- nitroarginine methyl ester (L-NAME, 100 μM; Sigma-Aldrich, St. Louis, MO, USA) was used to evaluate the effect of nitric oxide on colonic motility. Electrical field stimulation (EFS; 1-10 Hz, 120 V, 0.5 ms for 30 seconds) was applied between 2 platinum plates using a Grass S48 stimulator (Grass Technology) to induce a neuronal response. Tetrodotoxin (TTX, 1 μM; Tocris Bioscience, Avonmouth, Bristol, UK) was used to confirm that the EFS-induced responses were neuronal. At the beginning of each EFS experiment, the strips were pretreated with atropine (1 μM; Sigma-Aldrich).

Fluorescent Immunohistochemistry

Colonic tissues were fixed in 4% paraformaldehyde and washed for 5 minutes with phosphate-buffered saline (PBS, pH 7.4) with a 3% dehydroxide solution. Sections were preincubated with blocking solution (Invitrogen, Carlsbad, CA, USA) for 30 minutes before being incubated with the following antibodies at room temperature: anti-ICC (c-Kit; Santa Cruz Biotechnology,

CA, USA), anti-M_2 (Abcam, Cambridge, UK), anti-M_3 (Santa Cruz Biotechnology), and anti-nNOS (Abcam). After the sections were incubated for 1.5 hours with the primary antibodies, they were washed with PBS before being incubated with: an Alexa Fluor 488 goat anti-rabbit (Invitrogen) or Alexa Fluor 546 goat anti-mouse (Invitrogen) secondary antibodies for 1.5 hours at room temperature. After being washed with PBS, the specimens were counter-stained with 4',6-diamidino-2-phenylindole (DAPI) and mounted. The immuno-stained tissues were observed by confocal laser scanning microscope (LSM 5 EXCITER; Carl Zeiss, Jena, Germany), using a C-Apochromat objective lens (×40). The confocal images shown are digital composites of Z-series scans of 30 optical sections (1.0 μm thick) through a depth of full or partial thickness of the musculature. Image analysis was completed with LSM 5 EXCITER software (Carl Zeiss).

Statistical Methods

The results are expressed as the mean ± SEM. The SPSS statistical package (version 14.0; SPSS Inc, Chicago, IL, USA) was used for statistical analyses. The area under the curve (AUC) was calculated using LabChart 7 software (AD Instrument, Colorado Springs, CO, USA). Differences between the 2 groups were analyzed using Student's *t* test. A *P*-value less than 0.05 was considered to be statistically significant.

Results

Spontaneous Contractile Activity

Spontaneous contractility of the proximal and distal colon was examined. Spontaneous contractility of the proximal colon significantly decreased in diabetic rats compared to the control rats, while that of the distal colon was not (Fig. 1A and 1B). The AUC of spontaneous contractility significantly decreased for the diabetic rats compared to the controls (Fig. 1C). Therefore, we performed subsequent experiments to study the proximal colon.

Identification of the Interstitial Cells of Cajal Network

Identification of ICC was made with antibodies against the receptor tyrosine kinase, Kit.[21] We observed Kit-positive cells in the proximal colon. The ICC network of the proximal colon was greatly decreased in the diabetic rats compared to the control rats (Fig. 2).

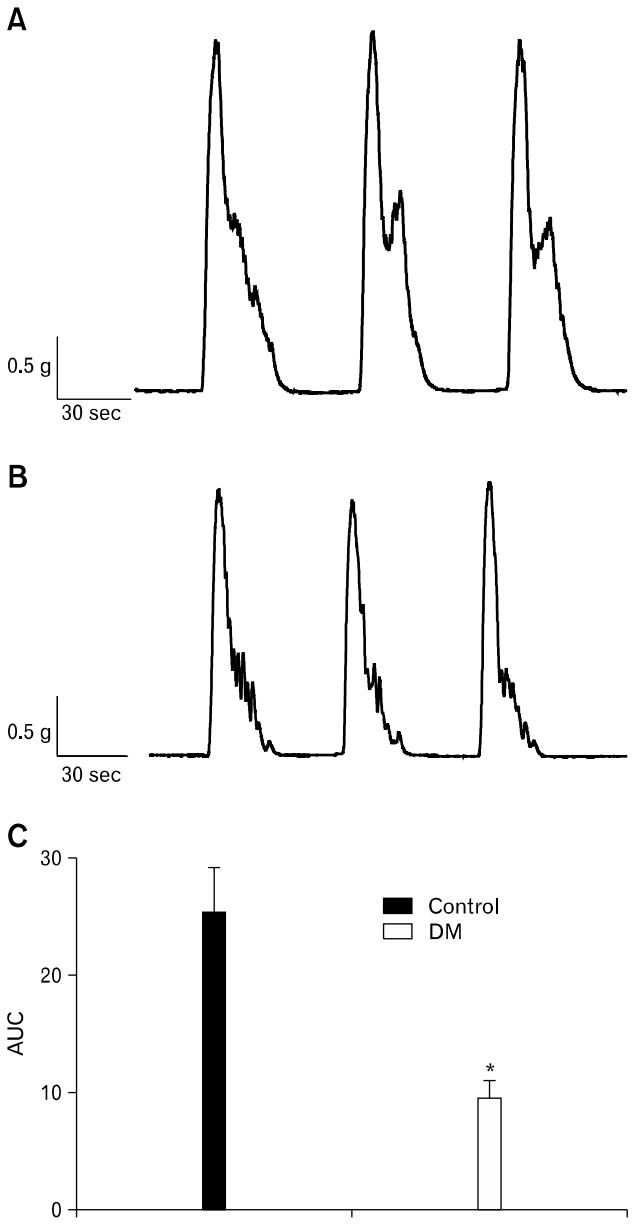

Figure 1. Spontaneous contractility of the proximal colon in control (A) and diabetes mellitus (B) rats. The degree of contractility was expressed as the area under the curve (AUC) (C). Values are expressed as the means ± SEM (n = 6). *$P < 0.05$ compared to the control. DM, diabetes mellitus.

Response to Cholinergic Agonist

Muscle strips from control rats responded to carbachol with a progressive increase of phasic contractile activity in a dose-dependent manner. The AUC was significantly decreased for diabetic rats compared to the control rats (Fig. 3).

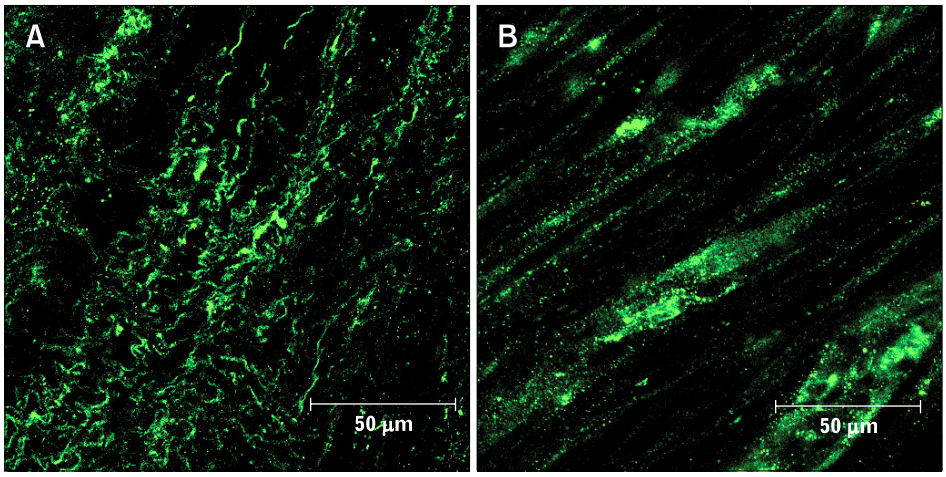

Figure 2. Network of interstitial cells of Cajal in control (A) and diabetes mellitus (B) rats. Immunofluorescence corresponding to for c-Kit (green) was prepared in the proximal colon.

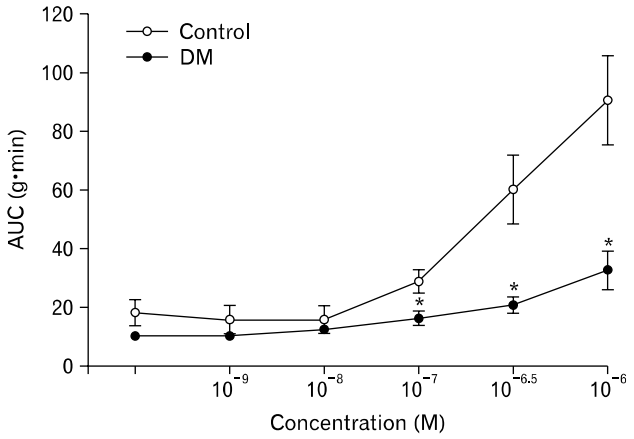

Figure 3. Effects of carbachol on the contractility of the proximal colon in control and diabetes mellitus rats. Values are expressed as the means ± SEM (n = 6). *$P < 0.05$ compared to the control value at the same concentration of carbachol. AUC, area under the curve.

Expression of Muscarinic Receptors

M_2 and M_3 muscarinic receptors preferentially expressed in GI smooth muscles[23,24] were observed in the proximal colon with immunofluorescence. Expressions of receptors relative to the number of nuclei decreased in the diabetic rats compared to the control rats (Fig. 4A and 4B). Co-localizations presented as expression level decreased in the diabetic rats compared to the control rats (Fig. 4C and 4D).

Response to Electrical Field Stimulation

EFS (1, 2, 5 and 10 Hz; 120 V; 0.5 ms) was applied for 30 seconds. In the proximal colon, on-relaxation was noted during EFS. As the EFS frequency increased, the AUC of on-relaxation decreased in the control rats (Fig. 5A and 5C), whereas this response was attenuated in the diabetic rats (Fig. 5B and 5C). To further investigate whether the appearance of on-relaxation during EFS resulted from nitrergic neurotransmission to the smooth muscles, L-NAME was administered for 10 minutes before recording the EFS-induced responses. L-NAME, a non-selective nitric oxide synthase (NOS) inhibitor, prevented the appearance of on-relaxation during EFS in the both the control and diabetic rats (Fig. 5A-5C). EFS caused on-relaxation followed by off-contraction; the amplitude of off-contraction increased in the control rats (Fig. 5A and 5D) whereas this response was attenuated in the diabetic rats (Fig. 5B and 5D). L-NAME also prevented the appearance of off-contraction after EFS in the both the control and diabetic rats (Fig. 5A, 5B and 5D).

Expression of Neuronal Nitric Oxide Synthase

Non-adrenergic, non-cholinergic relaxation is primarily mediated by nitric oxide in the colon,[25] and endogenous neuronal nitric oxide synthase (nNOS) plays an important role in regulating intestinal motility.[26] Expression of nNOS relative to the number of nuclei decreased in the diabetic rats compared to the controls (Fig. 6A and 6B). Co-localizations presented as expression level were decreased in the diabetic rats compared to the control rats (Fig. 6C).

Discussion

This observational animal study showed that decreases of the ICC network, cholinergic receptors and nNOS expression in the proximal colon play important roles in diabetes-related colonic dysfunction.

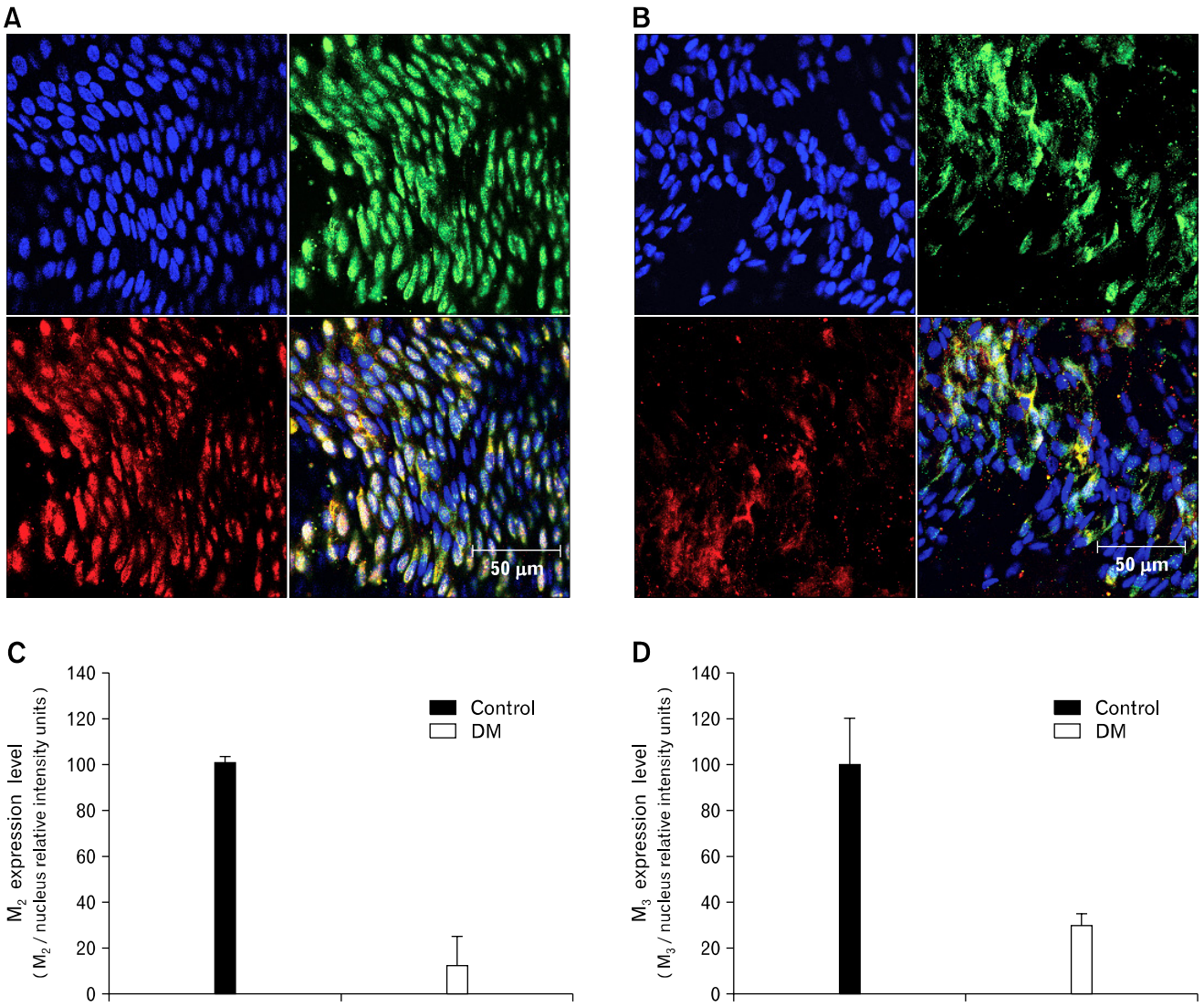

Figure 4. Expression of M_2 and M_3 muscarinic receptors in control (A) and diabetes mellitus (DM) (B) rats. Blue: nuclei, Green: M_2 receptors, Red: M_3 receptors (representative data from n = 6). Co-localization of M_2 and nuclei in the control and DM rats (C). Co-localization of M_3 and nuclei in control and DM rats (D). Values are expressed as the means ± SEM (n = 6).

A previous study reported higher rates of GI symptoms among 149 patients with type 2 DM who were referred to a university clinic compared to community controls. In this study, the duration of diabetes was the only factor independently associated with GI symptoms.[7] Therefore, our experiments were performed on male OLETF and LETO rats older than at least 60 weeks as an animal model of long-term diabetes. The clinico-pathological characteristics of OLETF rats resemble those of humans with type 2 DM including late onset of hyperglycemia, a chronic disease course and resultant end stage.[22]

In the present study, we initially examined colonic motility of both the proximal and distal colon. However, contractility of the distal colon was not different between the 2 groups. Therefore, we performed subsequent experiments to evaluate the proximal colon. The degree of contractility was estimated with several indicators such as the amplitude, frequency and AUC. Although the frequency of spontaneous contraction seemed to be higher in the diabetic rats and the amplitude of the contraction waves seemed to be larger in control group, these differences were not statistically significant. Therefore, we used the AUC as an indicator of contractility; by doing this, a significant difference between the 2 groups was observed.

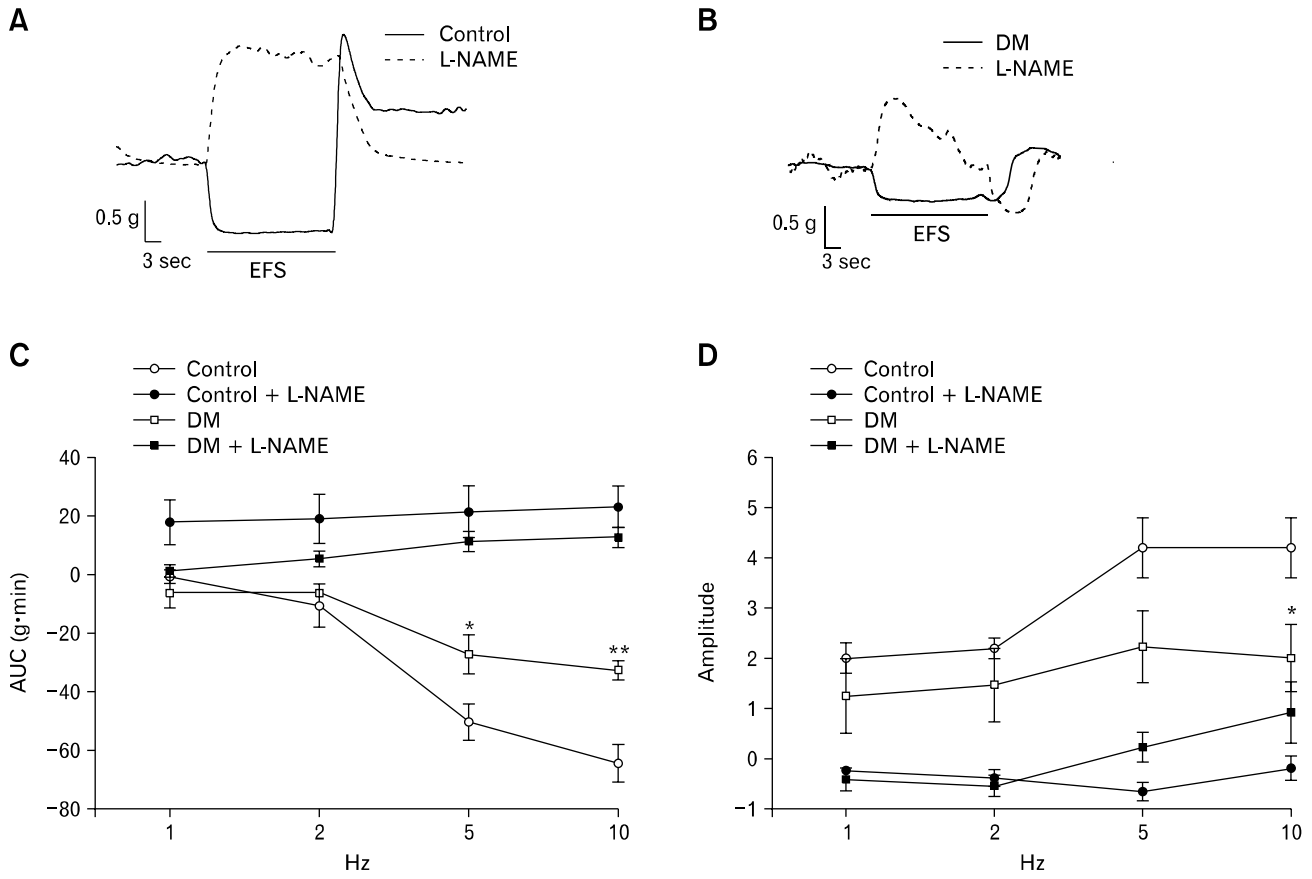

Figure 5. Response to electric field stimulation (EFS) in the proximal colon. (A) EFS (10 Hz) induced normal patterns of on-relaxation and off-contraction response in control rats. These responses disappeared after treatment with L-NG-nitroarginine methyl ester (L-NAME). (B) In diabetes mellitus (DM) rat, the degrees of on-relaxation and off-contraction were less than those observed in the control. Both responses also disappeared after treatment with L-NAME. (C) Response of on-relaxations of the proximal colon in the control was more dependent on the EFS frequency than that of DM rats. (D) Response of off-contractions of the proximal colon in the control and DM rats. Values are expressed as the means ± SEM (n = 6). $*P < 0.05$ compared to the control value at the stimulation of same frequency. $**P < 0.001$ compared to the control value at the stimulation of same frequency. AUC, area under the curve.

In the proximal colon of the diabetic rats, the ICC network was greatly decreased compared to that of the controls. Additionally, expression of cholinergic receptors and nNOS was decreased in the diabetes rats. However, we could not estimate the change in the number of ICC. To estimate the number of ICC or to observe the whole network of ICCs, it is necessary to dissect the specimen horizontally through the myenteric plexus. However, we could not perform this procedure because the specimen was not thick enough. Instead, we observed the morphology and degree of c-Kit expression with confocal microscopy.

ICC are mesenchymal cells that are found throughout the muscular coat of the GI tract. These cells perform functions critical for normal GI motility including the generation and propagation of electrical slow waves and mediation of bidirectional communication between the autonomic nervous system and smooth muscle cells.[27] It is also well established that ICC serve as pacemaker cells responsible for promoting spontaneous contractions of the smooth muscle coats.[28,29] ICC deficiencies and ultrastructural changes in GI muscle have been reported in diabetic animal models.[13,30] A similar deficiency of ICC has also been described in a case report of an insulin-dependent DM patient[31] and a study of 7 patients with DM.[32] It remains unclear whether this ICC deficiency in colonic smooth muscle is related to the lower rate of GI symptoms associated with type 2 DM. Since ICC are GI pacemaker cells, it can be postulated that impairment of the ICC network can variably affect to the contractile pattern of smooth muscles (eg, dysrrhythmia, absence or decrease). Although we could observe only decreased contractility, further

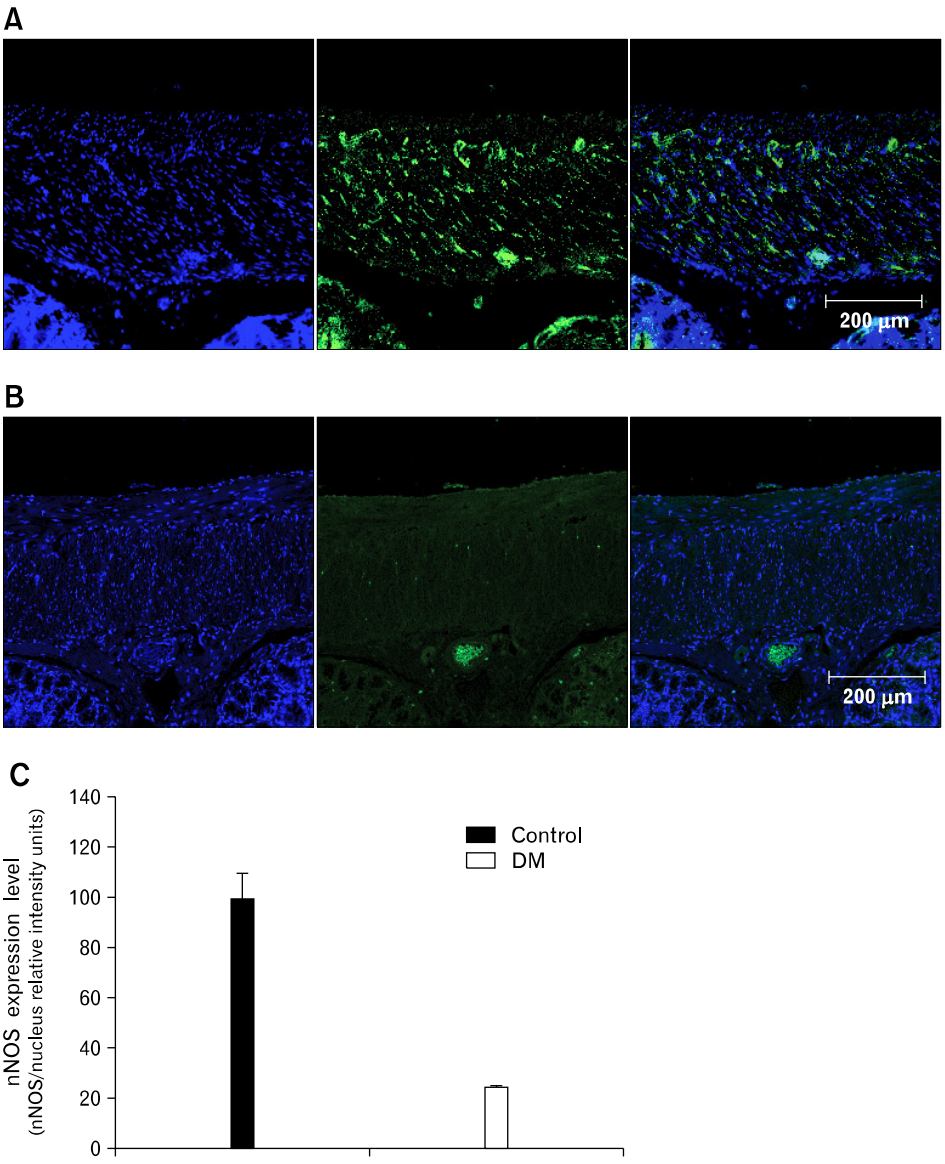

Figure 6. Expression of neuronal nitric oxide synthase (nNOS) in the control (A) and diabetes mellitus (DM) (B) rats. Blue: nuclei, Green: nNOS (representative data from n = 6). Co-localization of nNOS and nuclei in the control and DM rats (C). Values are expressed as the means ± SEM (n = 6).

investigation may induce other abnormalities.

ICC within the GI tract express a number of neurotransmitter, their receptors and modulators including acetylcholine receptors (M_2 and M_3), tachykinin receptors (NK1 and NK3), vasoactive intestinal peptide-1 and nNOS.[33-38] In GI smooth muscles, M_2 and M_3 muscarinic receptors are preferentially expressed with a preponderance of the former subtype.[23,24] The contractile response to externally administered agonists or cholinergic nerve stimulation has been shown to be promoted by either M_2 or M_3 receptors.[39,40] Little is known about the alteration of these muscarinic receptors in the long-term diabetic rat model. Endogenous nNOS plays an important role in non-adrenergic, non-cholinergic intestinal relaxation.[25,26] Neuronal NOS deficiencies in the GI muscle layer and/or delayed gastric emptying have been observed in both diabetic animal models[41-44] and patients with DM.[31]

Our study had several limitations. First, the number of animals we studied was small because it was difficult to maintain old diabetic rats as well preserved condition. Second, experiments were not performed to examine the distal colon only because no significant difference in simple contractility was observed between the 2 groups. Third, as mentioned above, we could not estimate changes in number of ICC. Despite these limitations, the results from this study still indicated the involvement of several mechanisms in diabetic colonic dysmotility in our long-term diabetic rat model which resemble humans with type 2 DM.

In conclusion, we showed that spontaneous contractility, cholinergic responses and nitrergic responses in the proximal colon were attenuated in a long-term diabetic rat model. We speculated that the loss of the ICC network might have decreased spontaneous contractility. We also hypothesized that decreased numbers of M_2/M_3 receptors and nNOS expression might have blunted the cholinergic and nitrergic responses, respectively. These results suggest that decreases of the ICC network, the number of cholinergic receptors and nNOS expression in the proximal colon affect DM-related symptoms of the lower GI in long-term diabetic rats. Further investigation is required to identify the precise mechanism underlying lower GI symptoms in patients with diabetes.

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