Effect of endogenous insulin-like growth factor and stem cell factor on diabetic colonic dysmotility

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Abstract

AIM: To investigate whether the reduction of stem cell factor (SCF) is mediated by decreased endogenous insulin-like growth factor (IGF)-1 in diabetic rat colon smooth muscle.

METHODS: Sixteen Sprague-Dawley rats were randomly divided into two groups: control group and streptozotocin-induced diabetic group. After 8 wk of streptozotocin administration, colonic motility function and contractility of circular muscle strips were measured. The expression of endogenous IGF-1 and SCF was tested in colonic tissues. Colonic smooth muscle cells were cultured from normal adult rats. IGF-1 siRNA transfection was used to investigate whether SCF expression was affected by endogenous IGF-1 expression in smooth muscle cells, and IGF-1 induced SCF expression effects were studied. The effect of high glucose on the expression of endogenous IGF-1 and SCF was also investigated.

RESULTS: Diabetic rats showed prolonged colonic transit time (252 ± 16 min vs 168 ± 9 min, P < 0.01) and weakness of circular muscle contraction (0.81 ± 0.09 g vs 2.48 ± 0.23 g, P < 0.01) compared with the control group. Endogenous IGF-1 and SCF protein expression was significantly reduced in the diabetic colonic muscle tissues. IGF-1 and SCF mRNA expression also showed a paralleled reduction in diabetic rats. In the IGF-1 siRNA transfected smooth muscle cells, SCF mRNA and protein expression was significantly decreased. IGF-1 could induce SCF expression in a concentration and time-dependent manner, mainly through the extracellular-signal-regulated kinase 1/2 signal pathway. High glucose inhibited endogenous IGF-1 and SCF expression and the addition of IGF-1 to the medium reversed the SCF expression.

CONCLUSION: Myopathy may resolve in colonic motility dysfunction in diabetic rats. Deficiency of endogenous IGF-1 in colonic smooth muscle cells leads to reduction of SCF expression.

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Key words: Diabetes; Gastrointestinal motility function; Insulin-like growth factor-1; Stem cell factor; Smooth muscle cell

Core tip: Endogenous insulin-like growth factor (IGF)-1 levels in diabetic rat colonic tissues were decreased. Hyperglycemia may be involved in initiating this change. In colonic smooth muscle cells, IGF-1 had a direct effect on increasing stem cell factor mRNA and protein levels mediated by extracellular-signal-regulated kinase 1/2 signaling.
INTRODUCTION

Approximately 75% of diabetic patients with any type of diabetes mellitus (DM) present gastrointestinal symptoms[1,2], particularly those who have poor glycemic control. Constipation and the use of laxatives are relatively common in patients with DM[3]. This disorder involves the depletion of interstitial cells of Cajal (ICCs), dystrophic changes of smooth muscle cells (SMCs), and impairment of enteric nervous systems[4-6]. ICCs are electrical active cells that generate and propagate electrical slow waves and serve as a bidirectional interface between nerves and smooth muscle[7-9]. ICCs damage has been shown in the stomach, jejunum, and colon of patients suffering from either type 1 or type 2 diabetes. ICCs depletion has been demonstrated in both patients with DM and laboratory animal models[9], and ICCs depletion is a consequence of stem cell factor deficiency[9].

It is well established that ICCs survival and function depend on the activation of c-kit, a receptor tyrosine kinase integral to ICCs. SCF, as a c-kit ligand, is produced locally within the tunica muscularis[10-12].

In vitro experiments showed insulin-like growth factor-1 (IGF-1) prevented ICC deletion in long-term cultured smooth muscle[13]. In intact tissues, a significant proportion of tissue IGF-1 is actually produced by SMCs[14]. Therefore, local IGF-1 signaling may play a significant role in SCF expression. The aim of this research is to study the local colonic IGF-1 expression in DM rats and the endogenous IGF-1 effects on SCF production.

MATERIALS AND METHODS

Animals

Male SD rats (weighing 200-250 g) were used for the experiments. Smooth muscle cells were obtained from SD rat colons. Diabetic rats (streptozotocin, STZ-D, n = 8) were induced by a single intraperitoneal injection of streptozotocin (STZ, 60 mg/kg body weight) dissolved in a citrate buffer, and age-matched control rats (n = 8) received equal volumes of buffer by ip injection. Diabetes was confirmed 1 wk later by measurement of tail vein blood glucose levels with an AccuChek Compact Plus glucometer (Roche, IN, United States). Rats with final blood glucose levels > 16.7 mmol/L were included in the study. At 8 wk after STZ administration, all the experimental rats were sacrificed by cervical dislocation. Food and water were given ad libitum. All animals were maintained in a controlled environment with alternating 12 h light/dark cycles. All animal care, use, and experimental protocols were approved by the Institutional Animal and Use Committee of Nanjing Medical University.

Distal colonic transit

Eight weeks after STZ injection, colonic transit was measured using a bead expulsion test as described[14]. A glass bead (5 mm in diameter) was inserted through the anus, and pushed with a plastic rod into the distal colon for a distance of 3 cm, while each rat was under transient ethyl ether anesthesia. The time until bead expulsion was measured.

In vitro studies of colonic smooth muscle contractility

Eight weeks after STZ injection, freshly obtained full-thickness distal colon tissues (about 5 cm from the anus) from control and diabetic rats were stored on ice for less than 1 h and then immersed in warm, oxygenated (95% O2 and 5% CO2) Krebs solution (in mmol/L: 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1 NaH2PO4, 1.2 MgCl2, 11 D-glucos and 25 NaHCO3). The mucosal layers were removed by micro-dissection under a magnifying glass and discarded. Circular muscle strips (2 mm × 10 mm) were prepared. After connecting to an isometric force transducer (Alcott-Biotech), the strips were allowed to equilibrate for 30 min under an initial tension of 0.5 g. The bath temperature was maintained at 37 °C. Contractile responses to acetylcholine (Ach, 1 μmol/L, Sigma-Aldrich, St. Louis, MO, United States) were obtained. At least three muscle strips from the distal colon of each rat were used for muscle experiments. The mean of the three or more muscle strips exposed to Ach 1 μmol/L was determined for each rat.

Cell culture and treatment

SMCs were isolated from colonic tissues and cultured as described previously[15]. Briefly, whole SD rat colons were dissected, and mucosa and serosa were quickly removed from muscle tissues. After digestion with type II collagenase, SMCs were cultured in DMEM medium (Gibco) with 10% fetal bovine serum. Cells of passage 2 or 3 were used for the experiments. Before the experiments, cells were serum starved for 24 h and then stimulated with different concentrations of IGF-1 (R and D Systems, Minneapolis, MN, United States) for various time periods. Phosphorylated extracellular-signal-regulated kinase 1/2 (ERK1/2) and phosphorylated Akt were measured after IGF-1 (100 ng/mL) treatment for 15 min. ERK1/2 inhibitor PD98059 (50 μmol/L) and phosphoinositide 3-kinase inhibitor (PI3K) inhibitor LY294002 (50 μmol/L) (Cell Signaling, Danvers, MA, United States) were added to the medium 30 min before IGF-1 treatment. Culture medium containing 25 mmol/L D-glucose was used for high glucose stimulation, and mannitol (5 mmol/L) D-glucose plus 19.5 mmol/L mannitol was used for osmotic controls.

siRNA transfection

IGF-1 siRNA was synthesized by GenePharma (Shanghai GenePharmaCo, China). The sequence for IGF-1 siRNA was as follows: anti-IGF-1-sense 5’-GCAG-
GCAAAGACCUCATT-3', and anti-IGF-1-antisense 5'-UGUAGGCUUGUUUCGCGT-3'. Negative control siRNA: sense 5'-UUCUCCGAACGUGACUCCGUTT-3', and antisense 5'-ACGUACGUGUACGGAGAATT-3'. Primary colonic smooth muscle cells were grown in six-well plates to 30% confluence, washed with serum-free medium immediately before transfection, and 800 μL serum-free medium was added to each well. siRNA (4 μg) was mixed with 10 μL of X-tremeGENE siRNA transfection reagent (Roche) in 200 μL serum-free medium. The mixture was incubated for 20 min at room temperature and then added to cells, according to the manufacturer's instructions. Serum was added 4 h later to a final concentration of 10%. Cells were harvested at 72 h after transfection.

### Western blotting
Distal colonic tissues were dissected from the mucosa and serosa. The remaining tissues were mainly muscle layers and used for Western blotting[10]. Tissues and cultured cells were lysed and centrifuged at 12000 g for 20 min. Protein samples were run on a 12% polyacrylamide gel in Tris-HCl. Proteins were then transferred to nitrocellulose membranes for 1 h at 100 V. The membranes were blocked with 5% (w/v) skim milk for 1 h at room temperature, and probed with primary antibodies at 4 °C overnight, and then washed in Tris-buffered saline with 0.1% Tween 20 for 5 min three times. The membranes were probed with corresponding horseradish peroxidase-conjugated secondary anti-rabbit and anti-goat antibodies at 1:2000 dilutions. Protein bands were detected with enhanced chemiluminescence Western blotting reagents (Thermo Fisher Scientific, Rockford, IL, United States). The resulting bands were scanned with an Epson 2400 printer and analyzed using the ImageJ program. All the antibodies used in this study, except for IGF-1 (Abcam, Cambridge, MA, United States) and SCF (Santa Cruz, CA, United States), were from Cell Signaling Technology.

### Real-time polymerase chain reaction
Total RNA was isolated from colonic muscle layers and isolated SMCs, and quantified using a Spectrophotometer Nanodrop 2000 (Thermo Scientific). RNA (500 ng) was submitted for cDNA synthesis using Prime Script RT Master Mix (TaKaRa). The levels of isolated mRNA were measured by real-time polymerase chain reaction (PCR) using SYBR Premix Ex Taq (TaKaRa) according to the manufacturer's instructions. PCR was initiated at 95 °C for 30 s, followed by 40 cycles of denaturing at 95 °C for 5 s, annealing and extending at 60 °C for 30 s. After PCR, a dissociation curve was constructed at 60-95 °C. PCR was performed in triplicate. The oligonucleotide primers used were as follows: IGF-1, forward, GGCAATTTGGGATGAGTTTG, reverse, GCTCTTGCCATGTCAGTGTG; SCF, forward, TTCCGTGGTAAATGGCTTTGC; reverse, CAACTGCCTTGTAAGACTTGCA; and 18S rRNA, forward, GCCGAAGCATTGGCAAGAA, reverse, GGCAATTTGGTCTCGGAAC. Results were shown to be relative to 18S rRNA mRNA.

### Statistical analysis
The results are expressed as the mean ± SE. The SPSS statistical package (version 14.0; SPSS Inc, Chicago, IL, United States) was used for statistical analysis. The differences between the two groups were analyzed using Student’s t test, and differences between the three groups were analyzed using analysis of variance. A P value < 0.05 was considered statistically significant.

### RESULTS

#### Effects of diabetes on colonic motility function
All STZ-induced rats showed hyperglycemia at the time of experiments, with their blood glucose concentrations being significantly higher than those of the age-matched rats which were not injected with STZ (n = 8) (Figure 1A). The body weight of STZ-treated (STZ-D) rats was significantly lower than the age-matched normal rats (n = 8) (Figure 1B).

The distal colon transit time was significantly increased in STZ-D group 8 wk after induction of diabetes (Figure 1C). Colonic circular smooth muscle strips from STZ-D rats showed weak contractility to Ach (Figure 1D). The maximum contractile tension of the STZ-D group was significantly reduced compared with control group (n = 8 each group) (Figure 1E).

#### SCF and IGF-1 expression in STZ-D rat distal colonic tissue
Eight weeks after STZ administration, the rats were sacrificed and distal colonic tissues were harvested. Because the mucosa/submucosa and serosa had been removed, the protein and mRNA were mainly from the muscle layer. The protein levels of SCF and IGF-1 were significantly decreased in STZ-D rat distal colons (Figure 2A). SCF and IGF-1 mRNA levels were consistent with the protein results. SCF mRNA levels of diabetic rats were lower than those of control rats, and IGF-1 mRNA levels were also decreased in diabetic rat colonic tissues (Figure 2B).

#### Effects of IGF-1siRNA on SCF expression
siRNA knockdown of endogenous IGF-1 reduced SCF mRNA levels in SMCs. and SCF protein levels also showed a significant parallel reduction of IGF-1 levels compared with the controls (Figure 3).

#### IGF-1 increased expression of SCF in SMCs through ERK1/2 pathway
IGF-1 elicited a dose-dependent increase in the expression of SCF in rat colonic SMCs. The concentration of 50 ng/mL significantly increased the expression of SCF (P < 0.01). One hundred ng/mL and 150 ng/mL IGF-1 showed a maximal effect on the expression of SCF (P < 0.01); therefore, 100 ng/mL was used in subsequent experiments (Figure 4A).

IGF-1 induced maximum SCF expression in SMCs at
els had almost returned to baseline (0 h) (Figure 4B).

To determine whether IGF-1-induced SCF expression
was mediated by Akt-dependent or ERK1/2-dependent mechanisms, we measured the phosphorylation of Akt and ERK1/2. Both of them could be phosphorylated by IGF-1 (100 ng/mL). We then used a PI3K/Akt inhibitor (LY-294002, 50 μmol/L) and an ERK1/2 inhibitor (PD-98059, 50 μmol/L) before IGF-1 (100 ng/mL) was added to the media. Sixteen hours after IGF-1 stimulation, the ERK1/2 inhibitor significantly inhibited IGF-1-induced SCF expression \( (P < 0.01) \) while the PI3K/Akt inhibitor showed no effect (Figure 4C).

**Figure 4** Insulin-like growth factor-1-induced stem cell factor expression through the extracellular-signal-regulated kinase 1/2 pathway in smooth muscle cells. A: Insulin-like growth factor-1 (IGF-1) effects on the expression of stem cell factor (SCF) as a function of concentration after treatment with IGF-1 for 24 h. \( \beta P < 0.01 \) vs 0 ng/mL IGF-1; \( \beta P < 0.01 \) vs 50 ng/mL IGF-1; B: IGF-1 effects on SCF expression as a function of time. \( \beta P < 0.05, \beta P < 0.01 \) vs 0 h; C: Phosphorylation of extracellular-signal-regulated kinase 1/2 (ERK1/2) and Akt after 100 ng/mL IGF-1 treatment for 15 min; D: Effects of PD98059 and LY294002 on SCF expression by IGF-1 treatment. \( \beta P < 0.01 \) vs the no treatment group; \( \beta P < 0.01 \) vs IGF-1 treatment group. Western blotting represent 3 independent experiments. α-Tubulin was used as a loading control (each bar represents the mean ± SE of 3 independent experiments).

**High glucose suppresses endogenous IGF-1 and SCF expression**

Compared with cultures for 24 h in normal glucose (5 mmol/L) medium, cultures in DMEM containing 25 mmol/L glucose significantly inhibited IGF-1 expression in cultured colonic SMCs. Mannitol was used as an osmotic control which did not have similar inhibitive effect (Figure 5A). High glucose concentrations also suppressed SCF expression, which was reversed by the addition of exogenous IGF-1 (100 ng/mL) (Figure 5B).

**DISCUSSION**

Intestinal transit is often disturbed in diabetes (rapid or slow)[17], and symptoms such as diarrhea and constipation occur more frequently than in the general population[18]. The prevalence of GI symptoms was found to be more
IGF-1 and IGF-1 may induce SMCs to produce SCF. Both insulin and IGF-1 receptors. Therefore, both insulin and IGF-1 express insulin or IGF-1 receptors mature ICCs were probably indirect because ICCs do not observe pro-survival effects of insulin and IGF-1 on gastric tunica muscularis tissues could reverse SCF reduction et al. Decreases in IGF-1 signaling have been considered to be responsible for the decrease in SCF levels. Horváth et al. have shown that IGF-1 treatment of murine gastric tunica muscularis tissues could reverse SCF reductions in long-term organ culture, and decreased levels of SCF were correlated with smooth-muscle atrophy. The observed pro-survival effects of insulin and IGF-1 on mature ICCs were probably indirect because ICCs do not express insulin or IGF-1 receptors, while SMCs express both insulin and IGF-1 receptors. Therefore, both insulin and IGF-1 may induce SMCs to produce SCF.

IGF-1 is an endogenous growth factor that plays a central role in the growth and development of visceral and vascular smooth muscle. Existing evidence suggests that mesenchymal cells, including α-smooth muscle actin-positive myofibroblasts and smooth muscle cells, are primary sources of locally expressed IGF-1 in the intestine. Local intestinal IGF-1 has autocrine effects on the growth of human intestinal muscle cells. In this study, we found that endogenous IGF-1 was decreased in diabetic colonic tissues. Meanwhile, SCF expression in colonic tissue was paralleled by endogenous IGF-1, which suggested that a lack of endogenous IGF-1 might lead to low levels of SCF expression.

IGF-1 binds to the cellular membrane IGF-1 receptor leading to stimulation of proliferation and inhibition of apoptosis through PI3K/Akt and mitogen-activated protein kinase pathways. In the present study, IGF-1 could cause both ERK1/2 and Akt phosphorylation, but IGF-1 promoted SCF expression mainly through ERK1/2-dependent signaling, not the PI3K/Akt pathway. Both type 1 and type 2 diabetes patients have hyperglycemia as a basic feature, and chronic hyperglycemia is associated with dysfunction, damage, and failure of several organs. This study showed that high glucose impaired the production of trophic factors in colonic SMCs. Whether the mechanism underlying the effects is oxidative stress caused by high glucose remains to be determined.

The current study is an extension of those described by Horváth et al. The significant differences between that study and the current one are: (1) we showed evidence supporting the presence of dysfunction of smooth muscle contractility in animals, which is more compelling evidence of myopathy than just a reduction of myh11 mRNA levels; (2) in cell culture studies, we demonstrated that IGF-1 had a direct effect on increasing SCF mRNA and protein levels, and this effect was mediated...
by ERK1/2 signaling; additionally; and (3) we found that endogenous IGF-1 levels in diabetic rat colonic tissues were decreased, and that hyperglycemia may be involved in initiating this change.

In conclusion, we have demonstrated that the myopathy may resolve in colonic motility dysfunction in diabetic rats. Deficiency of endogenous IGF-1 in SMCs caused reduction in SCF expression, which is a critical developmental, growth, and survival factor for ICCs.

COMMENTS

Background
Most diabetic patients experience gastrointestinal symptoms, particularly those who are under poor glycemic control. Constipation is relatively common in patients with diabetes mellitus, and involves the depletion of interstitial cells of Cajal (ICCs), dystrophic changes of smooth muscle cell, and impairment of enteric nervous system.

Research frontiers
ICCs are the primary electrical pacemakers for rhythmic contractile activity. ICC depletion has been demonstrated in both patients with diabetes mellitus and laboratory animal models, and is a consequence of stem cell factor (SCF) deficiency. Cell culture experiments have shown that addition of insulin-like growth factor-1 (IGF-1) prevented ICC depletion in long-term cultured smooth muscle cells.

Innovations and breakthroughs
This study showed evidence supporting the presence of dysfunction of smooth muscle contractility in animals, which is compelling evidence of myopathy. In cell culture studies, the authors demonstrated that IGF-1 had a direct effect on increasing SCF mRNA and protein levels, and this effect was mediated by extracellular-signal-regulated kinase 1/2 signaling; and that endogenous IGF-1 levels in diabetic rat colonic tissues were decreased, and that hyperglycemia may be involved in initiating this change.

Applications
The results of this study suggest that compensation of endogenous IGF-1 is a potential therapeutic option that could prevent the development of diabetic colonic dysmotility.

Terminology
SCF is a cytokine that binds to the c-Kit receptor. SCF can exist both as a transmembrane protein and a soluble protein. This cytokine plays an important role in hematopoiesis, spermatogenesis, and melanogenesis. In gastrointestinal tract, SCF is mainly produced by smooth muscle cells and neurons.

Peer review
This is a good descriptive study in which authors showed that myopathy may resolve in colonic motility dysfunction in diabetic rats, and deficiency of endogenous IGF-1 in colonic smooth muscle cells caused reduction of SCF expression. The results are interesting and suggest that endogenous IGF-1 is a potential therapeutic target for diabetic colonic dysmotility.

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