

Original Article

Interleukin-6 (IL-6) mediated the increased contraction of distal colon in streptozotocin-induced diabetes in rats via IL-6 receptor pathway

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Abstract: Colonic dysmotility occurs in diabetes and blood plasma interleukin (IL)-6 levels are significantly elevated in type 1 diabetes mellitus. The aim of this study was to investigate whether IL-6 and the IL-6 receptor pathway mediates colonic dysfunction in type 1 diabetes mellitus. Male SD rats were treated with a single intraperitoneally injected dose of streptozotocin (STZ), and those displaying sustained high blood glucose were selected as diabetes mellitus models. Longitudinal muscle strips of colon were prepared to monitor colonic contraction in vitro. Contractile responses of strips of colon were recorded following treatment with IL-6 in control animals, and following anti IL-6 antibody treatment in STZ-induced diabetes in rats. Concentration of IL-6 in plasma and colon were determined by ELISA. Expressions of IL-6 α -receptor and IL-6 β -receptor in colon tissues were determined by immunohistochemistry or Western blot analysis. The non-diabetes rats treated with IL-6 and the untreated diabetes rats showed increased contraction of distal colon, whereas the diabetes rats treated with anti-IL-6 antibody showed decreased contraction of distal colon compared with the untreated diabetes rats. The IL-6 levels of plasma but not colon increased in diabetes rats. The expression of IL-6 α -receptor increased in diabetes rats. These results indicate that diabetes rats show an increase in the contractions of distal colon partly via the IL-6-IL-6 receptor pathway.

Keywords: Diabetes mellitus, colon motility, IL-6, IL-6 receptor

Introduction

Colonic dysmotility occurs in diabetes and patients exhibit diarrhea or constipation [1-5]. Diabetic patients with severe constipation exhibit a decrease in colonic motility [6, 7]. In some diabetic animal models, colonic contractions have been shown to be decreased compared with normal animals [8-10]. In contrast, other reports have shown an increase in colonic contraction in diabetic animals [11, 12]. It is unclear why colonic motility might be decreased or increased in diabetes mellitus.

Type 1 diabetes is associated with increased cytokine-mediated inflammation. Blood plasma interleukin (IL)-6 levels are significantly elevated in type 1 diabetic subjects [13-16]. IL-6 has been reported to be involved in the contraction of GI tract [17-22]. Whether IL-6 is involved in

the dysmotility of colon in type 1 diabetes mellitus is still unknown.

IL-6 exerts its biological action by binding to two types of membrane receptors, namely IL-6 α -receptor (IL-6R α) and the gp130 molecule (IL-6 β -receptor) [23, 24]. IL-6 binds to IL-6R α on the cell membrane of target cells and this complex in turn associates with gp130 and induces signal transduction via phosphorylation of Stat3. IL-6R α is expressed by specific cells, such as neutrophils, monocytes/macrophages, hepatocytes, and in certain lymphocyte phenotypes, whereas gp130 is widely expressed on the cell membrane of various cell types [25, 26]. IL-6 receptor proteins have been reported to be expressed in colon [27]. The IL-6 receptor has been shown to be a target for prevention of coronary heart disease [28]. Whether the IL-6 receptor is a target of colonic dysfunction

tion in type 1 diabetes mellitus is still uncertain.

We hypothesize that IL-6 might play a role in the colonic dysmotility of type 1 diabetes. The IL-6 pathway is activated or enhanced either by activating the receptor or by increasing expression of the receptor.

Materials and methods

Animal preparation

Male SD rats, weighing 180 ± 10 g, were housed in a temperature (22°C)-controlled environment. The use and treatment of animals followed the guidelines of the International Animal Care and Use Committee of Tongji University. All animals were cared for in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals, published by the National Science Council, China.

For induction of diabetes mellitus, intact four-week-old male SD rats were subjected to a single injection of streptozotocin (60 mg/kg) in sodium citrate buffer (0.1 M, pH 4.2). Control rats were injected with the sodium citrate buffer alone. Body weight and blood glucose levels were measured the day the STZ was injected. Blood glucose levels were measured by using glucose test strips (Bayer, Elkhart, IN). The second week after STZ injection, blood glucose levels were measured again to confirm the development of hyperglycemia. Six weeks after the STZ was injected, animals were fasted overnight with free access to water. Body weight and blood glucose levels were measured, and the rats were killed by decapitation.

Immediately following decapitation, both the proximal colon (1 cm from the ileocaecal sphincter) and the distal colon (above the pelvic brim) were excised and put in Krebs solution (composition in mM: NaCl 118.5, KCl 4.8, KH_2PO_4 1.2, MgSO_4 1.2, CaCl_2 1.9, NaHCO_3 25.0, and glucose 10.1). The segments of colon were opened along the mesenteric border and pinned mucosa side up. The mucosa was removed by sharp dissection.

Tissue culture

Four full-thickness muscle strips (2×8 mm) were cut along the longitudinal axis. Silk thread was attached to both ends of the muscle strips,

and the each strip was mounted in a 5 ml organ bath. The organ baths contained aerated (5% CO_2 , 95% O_2) Krebs solution maintained at 37°C . Strips were adjusted in length to an initial tension of 1 gram, and were allowed to stabilize for 60 minutes before experimental procedures were initiated. Isometric tension was measured using force transducers (JH-2B, Beijing, China). Force signals were amplified with a SMUP-PC amplifier (Fudan University, Shanghai, China), and recorded on the MFlab system (Fudan University, Shanghai China).

Experimental protocols

Colonic strips were divided into 4 groups of at least 6 rats each: colonic strips from normal rats [either treated or untreated with IL-6 (0.1 $\mu\text{g}/\text{ml}$)], colonic strips from diabetic rats [either treated or untreated with anti-IL-6 antibody (0.1 $\mu\text{g}/\text{ml}$)]. Strips treated with IL-6 or anti-IL-6 antibody were exposed for at least 30 min. All strips were exposed to carbachol (1 μM) for at least 3 min first, and finally the strips were exposed to high potassium Krebs solution (composition in mM: NaCl 34.6, KCl 90, KH_2PO_4 1.2, MgSO_4 1.2, CaCl_2 1.9, NaHCO_3 25.0, glucose 10.1 and pH 7.4).

Detection of IL-6 in plasma and colon by ELISA

The colon segments were homogenized in phosphate buffer containing 0.05 % Tween 20, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 IU aprotinin A. These homogenates, and blood samples, were centrifuged at $3,000 \times g$ for 10 min. The supernatants were assayed for IL-6 using ELISA system (Cytoscreen, Biosource International, Camarillo, CA). The assay is a solid-phase sandwich-type system that utilizes specific anti-rat IL-6 antibody coated onto the wells of microtiter plates. The samples (50 μl) and standards were pipetted in triplicate into appropriate microtiter wells, and the assay was performed according to manufacturer's instructions. The sensitivity of this IL-6 ELISA system is 0.7 pM, and the upper limit of detection is 150 pM.

Expression of IL-6 α -receptor and IL-6 β -receptor in colon tissues by Western blot analysis

Tissue samples of about 0.5 g were excised from colon, cut into pieces of about 0.25 cm^3 , and then ground into a cell suspension with a

IL-6 and colonic contraction of diabetes

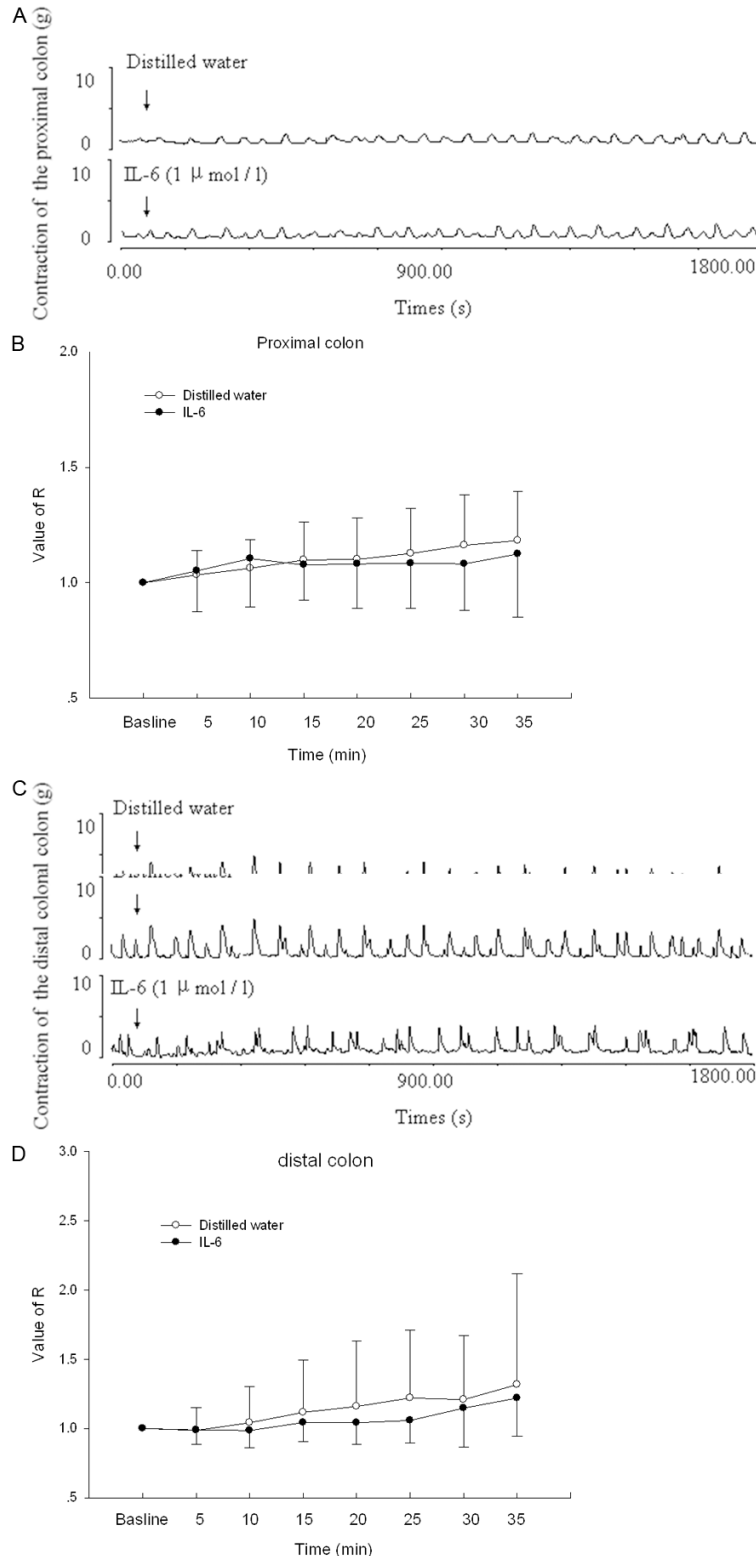


Figure 1. Effects of IL-6 on the spontaneous contraction of colon in control rats. A. The contractile responses produced by either the distilled water or IL-6 on proximal colonic smooth muscle strips. IL-6 was applied at points marked by the arrows. B. Statistical data of tension induced by IL-6 on proximal colon. C. The contractile responses produced by IL-6 on distal colon. IL-6 was applied at points marked by the arrows. D. Statistical data of tension

induced by IL-6 on distal colon. Control means the contraction of colon treated with distilled water (50 μ L) in Krebs solution. $n = 10$.

blade. Crude total proteins were extracted, and protein concentrations were measured by the bicinchoninic acid method. Fifty micrograms of protein were separated on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to a PVDF membrane. After being incubated for 1.5 h in a buffer with bovine serum albumin, the blot was incubated with an IL-6Ra antibody (1:500) at 37°C for 1 h and then at 4°C overnight, followed with incubation of a horseradish peroxidase-conjugated secondary antibody at a ratio of 1:2,000 for 2 h. After enhanced chemiluminescence, the blot was subject to autoradiography. The expression of gp130 proteins from the same samples were performed similarly with the antibody against gp130 that were used at a 1:200 dilution.

Immunohistochemistry

Immediately after the animals were killed, a segment of distal colon was removed and soaked in 4% paraformaldehyde for 12 h. The fixed tissue was rinsed for 100 min and was dehydrated, cleared and mounted in wax. The tissue was sectioned into 4 μ m sections. Sections were stained using a two-step method. Activity of endogenous peroxidase was blocked with 3% hydrogen peroxide. After three rinses in PBS, 10% normal rabbit serum (NRS) was applied for 15 min, and then the sections were incubated with primary rabbit anti-IL-6 α

IL-6 and colonic contraction of diabetes

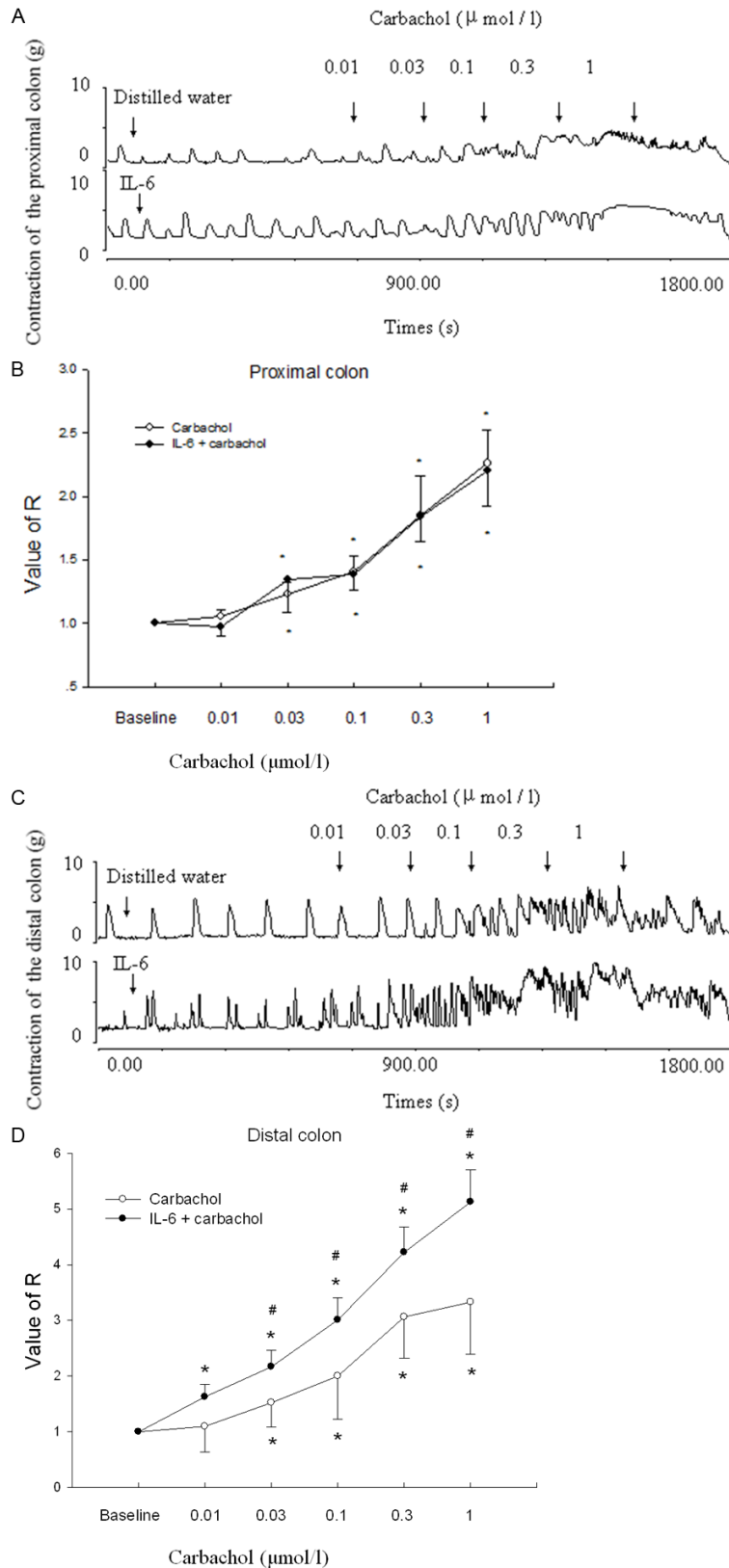


Figure 2. Effects of IL-6 on carbachol-induced contraction of colon in rats. A. The contractile responses produced by carbachol on proximal colonic smooth muscle strips pretreated with IL-6 in control rats. Carbachol was

applied at points marked by the arrow. B. Statistical data of tension induced by the carbachol on proximal colon pretreated with IL-6. C. The contractile responses produced by carbachol on distal colonic smooth muscle strips. Carbachol was applied at points marked by the arrows. D. Statistical data of tension induced by the carbachol on distal colon. Note that when distal colon is pretreated with IL-6, the carbachol-induced contraction increased. Control means the carbachol-induced contraction of colon treated with distilled water (50 μL) in Krebs solution. * $P < 0.05$ compared with Krebs solution. # $P < 0.05$ compared with control. $n = 10$.

receptor antibody (diluted 1:100 in PBS) overnight in a humid chamber at 4°C. After the sections were washed, they were incubated with polymer peroxidase-anti-rabbit serum (ZSGB-BIO, Beijing, China) for 30 min at room temperature. After several rinses, peroxidase was revealed by a 3, 3'-diaminobenzidine tetrahydrochloride substrate kit (ZSGB-BIO). Negative controls were performed without primary antibody.

Chemicals

Streptozotocin (STZ) and carbachol were obtained from Sigma Co. Ltd. (St. Louis, MO, USA). Recombinant rat IL-6 and anti-rat IL-6 antibody were purchased from Preprotech Inc. (USA). Recombinant anti-rat-IL-6 α -receptor and anti-rat-IL-6 β -receptor antibody were purchased from Biosynthesis biotechnology Company (Beijing, China). STZ was dissolved in sodium citrate buffer (0.1 M pH 4.2). The antibody was prepared in TBST (0.1% Tween 20, 50 mM Tris, and 150 mM NaCl).

IL-6 and colonic contraction of diabetes

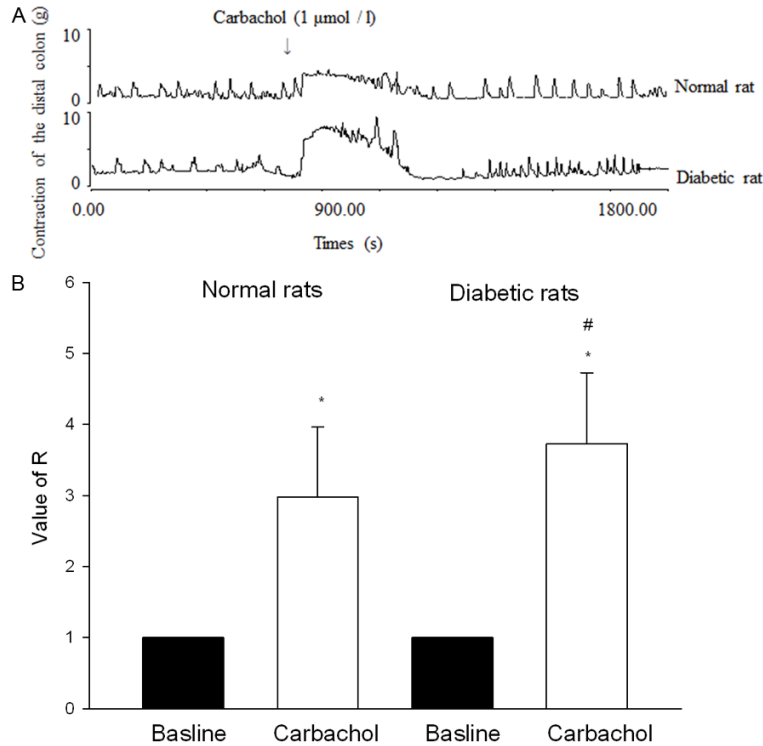


Figure 3. Effects of carbachol on the contraction of distal colonic strips in diabetic rats. A. The contractile responses produced by carbachol. Carbachol was applied at points marked by arrows. B. Statistical data of tension induced by carbachol. Carbachol-induced contractions of distal colonic strips were higher in diabetic rats than in normal rats. Normal rats mean the rats injected with the sodium citrate buffer. * $P < 0.05$ compared with Krebs solution. # $P < 0.05$ compared with normal rats. $n = 10$.

Carbachol was prepared in double distilled water.

Statistical analysis

The peak forces of colonic phasic contraction were measured using an MFlab system (Fudan University, Shanghai, China). In each experiment, the peak forces of contractions were evaluated at 0.5 min before and after drug administration. Mean peak forces for the 1 min period before drug administration was taken as the baseline. The value of the force after drug treatment was normalized to the baseline value. The ratio of force post treatment to baseline force was expressed as the ratio R, so that the baseline for each experiment was equal to 1. Western blots were evaluated by determining the value of the gray, the value of ratio is the value of the gray scale division between the diabetes rats and normal ones. Data were presented as means \pm SD. Statistical analysis was performed by means of Student's

paired t-test for comparisons between two groups and repeated-measures comparison on the same specimen with SigmaStat 3.5 software (SPSS Inc., Chicago, IL, USA). A probability level of $P < 0.05$ was considered to be statistically significant.

Results

Effects of IL-6 on the spontaneous contraction of proximal and distal colonic motility

In strips from control rats, forces recorded after IL-6 (0.1 $\mu\text{g/ml}$) treatment were not significantly different from forces recorded after control treatment with distilled water ($P > 0.05$) (Figure 1A-D).

Effects of IL-6 on carbachol-induced contraction of proximal and distal colonic motility in control rats

Carbachol (0.01-1 μM) increased the contraction of proximal and distal colonic strips that were either treated with IL-6 or distilled water ($P <$

0.05, $n = 10$) (Figure 2A-D). In the proximal colon, carbachol-induced contractions were not different from contractions in strips not treated with IL-6 ($P > 0.05$) (Figure 2A and 2B). In the distal colon, after the strips were treated with IL-6 for 1 h, carbachol-induced contraction was greater than in the untreated strips ($P < 0.05$) (Figure 2C and 2D).

Effects of carbachol on the contraction of distal colonic strips in diabetic rats

Carbachol evoked contractions of distal colonic strips in both the diabetic and normal rats ($P < 0.05$, $n = 10$) (Figure 3A and 3B). The force of contraction in diabetic colon was significantly greater than the normal rats ($P < 0.05$, $n = 10$) (Figure 3B).

Effects of anti-IL-6 antibody on the contraction of distal colonic strips in diabetic rats

Anti-rat IL-6 antibody (2 ng/ml) had no effect on the contraction of distal colonic strips ($P >$

IL-6 and colonic contraction of diabetes

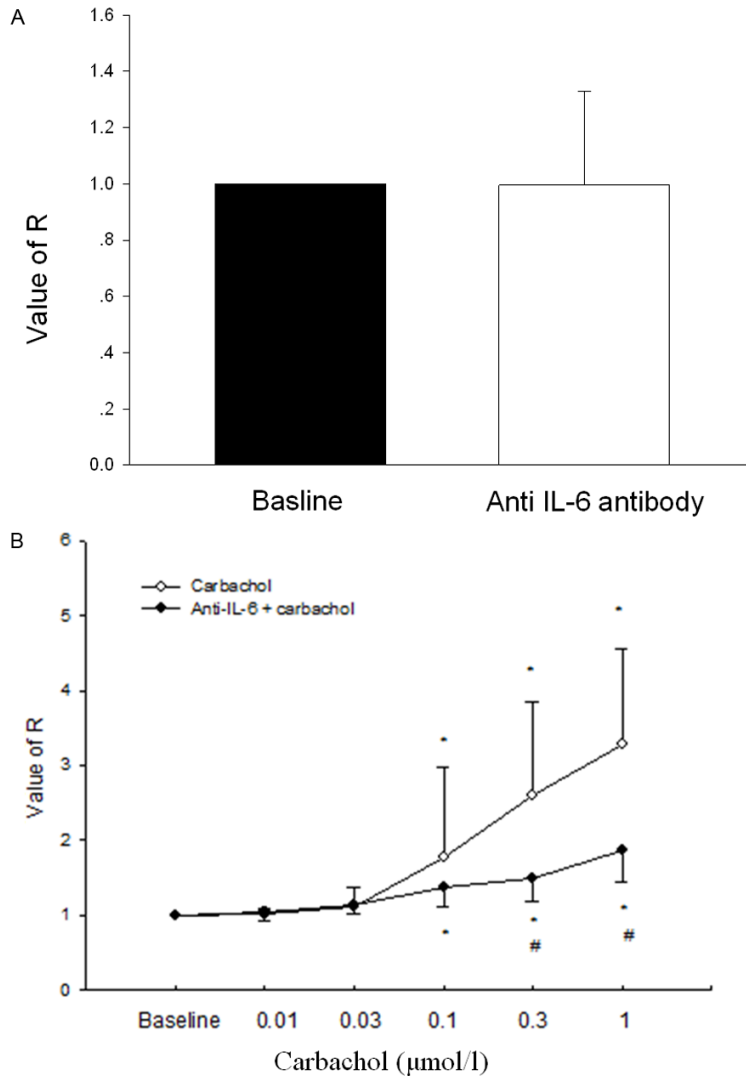


Figure 4. Effects of anti-IL-6 antibody on carbachol-induced contraction of distal colonic motility in diabetic rats. A. Anti-IL-6 antibody showed no effect on spontaneous contraction of colon. B. The contractile responses produced by carbachol pretreated with anti-IL-6 antibody in rats. Note that when distal colon is pretreated with anti-IL-6 antibody, the carbachol-induced contraction decreased. Control means the carbachol-induced contraction of colon treated with distilled water (50 µL) in Krebs solution. * $P < 0.05$ compared with Krebs solution. # $P < 0.05$ compared with control. $n = 5$.

0.05, $n = 5$) (Figure 4A). Carbachol (0.1-1 µM) increased contraction of distal colonic strips in diabetic rats that were either treated with anti-IL-6 antibody or distilled water ($P < 0.05$, $n = 5$) (Figure 4B). The force of contraction following treatment with anti-IL-6 antibody was significantly lower than the force observed after treatment with distilled water ($P < 0.05$, $n = 5$) (Figure 4B).

IL-6 concentration of plasma and colon in rats

In diabetic rats, the concentration of IL-6 in plasma was higher than that of normal rats ($P < 0.05$, $n = 8$) (Figure 5A and 5B). There was no difference in IL-6 content in colon tissue.

IL-6 α -receptor and IL-6 β -receptor levels in colon tissues by the Western blot analysis and immunohistochemical assay

There were two bands of IL-6 α -receptor protein in tissue from diabetic rats but only one band in tissue from normal rats (Figure 6A). Expression of IL-6 α -receptor proteins was significantly higher in diabetic rats than in normal rats ($P < 0.05$, $n = 8$) (Figure 6A-C). Expression of IL-6 β -receptor protein in colon of diabetic rats was almost the same with that of normal rats, but the bands showed a little higher ($P > 0.05$, $n = 8$) (Figure 6D and 6E).

IL-6 α -receptor immunoreactivity was detected in myenteric nerve plexus in the colon (Figure 6F).

Discussion

The present study demonstrates that IL-6 had no effect on the spontaneous contraction of colon in normal rats. IL-6 increased the carbachol induced contraction of distal colon in normal rats. The carbachol evoked contractile responses of distal colon was increased in diabetic rats. Treatment with anti-IL-6 antibody decreased the carbachol-induced contraction of distal colon in diabetic rats. IL-6 concentration in plasma but not in colon was increased in diabetic rats. The expression of IL-6 receptor α

IL-6 and colonic contraction of diabetes

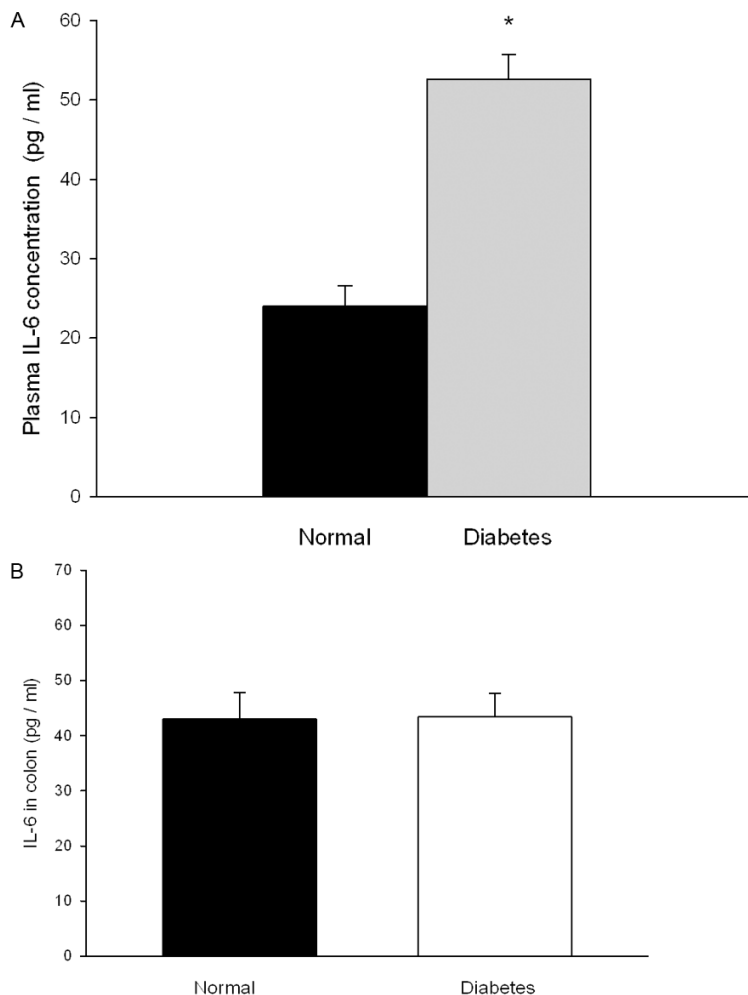


Figure 5. IL-6 concentrations of plasma and colon. A. Concentration of IL-6 in plasma. B. Concentration of IL-6 in colon. Normal rats means the rats injected with the sodium citrate buffer. * $P < 0.05$ compared with normal rats. $n = 8$.

increased in diabetic rats. IL-6 receptor α was shown to be located in the myenteric nerve plexus of the colon.

Others have shown that IL-6 can either increase or decrease colonic motility, depending on the experimental model. IL-6 was increased in dextran sulfate sodium (DSS)-induced colitis, and carbachol-induced contractions of colon were significantly increased [29]. In a depression rat model, exogenous IL-6 facilitated the contraction of the colon [30]. In a rodent model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis, IL-6 increased and the frequency of bowel movements increased [31]. In contrast, carbachol-induced contraction was reported to be lower in the colon of rats treated with both IL-1 β and IL-6 [20]. IL-6 was expressed

both in the muscle and mucosal layer in the 2, 4, 6-trinitrobenzenesulphonic acid (TNBS)-induced colitis mouse model, and the carbachol-induced contraction of colon decreased [32]. In postoperative ileus mice, mRNA expression of IL-6 increased and colonic transit time increased [33]. Reduced colonic motility in multiple organ dysfunction syndrome (MODS) rats was reported to be related to the increase of IL-6 in the colon muscularis [19]. Treatment with antibiotics inhibited contraction of the colon, but reduced lipopolysaccharides (LPS)-elicited production of IL-6 [34].

In our study, we observed a direct effect of IL-6 on the spontaneous and carbachol-induced colonic contraction in normal rats. We found that IL-6 increased the carbachol-induced contraction of distal colon but not spontaneous not the IL-6 from colon increased the colonic motility in type 1 diabetes.

The pleiotropic cytokine IL-6 belongs to a group of cytokines that share the ability to use the signal transducer molecule gp130. In classic signaling, IL-6 first binds to a non-signaling membrane-bound IL-6 α -receptor (IL-6R α), which in turn associates with and activates the signal-transducing β -receptor chain gp130 [24]. In the present study, we found that the protein level of IL-6R α increased in the colon of diabetic rats. The two bands of IL-6R α in Western blot analyses suggested that both IL-6R α and phosphorylated IL-6R α existed in the colon of diabetic rats. We further found that although the IL-6 β -receptor protein of colon in diabetic rats was almost the same as that of normal rats, the molecular weight was a little different. IL-6 β -receptor might also be phosphorylated. The IL-6R pathway has been reported to be relevant to coronary heart disease [41]. IL-6R α is involved in obesity-associated resistance to insulin [42]. Our

IL-6 and colonic contraction of diabetes

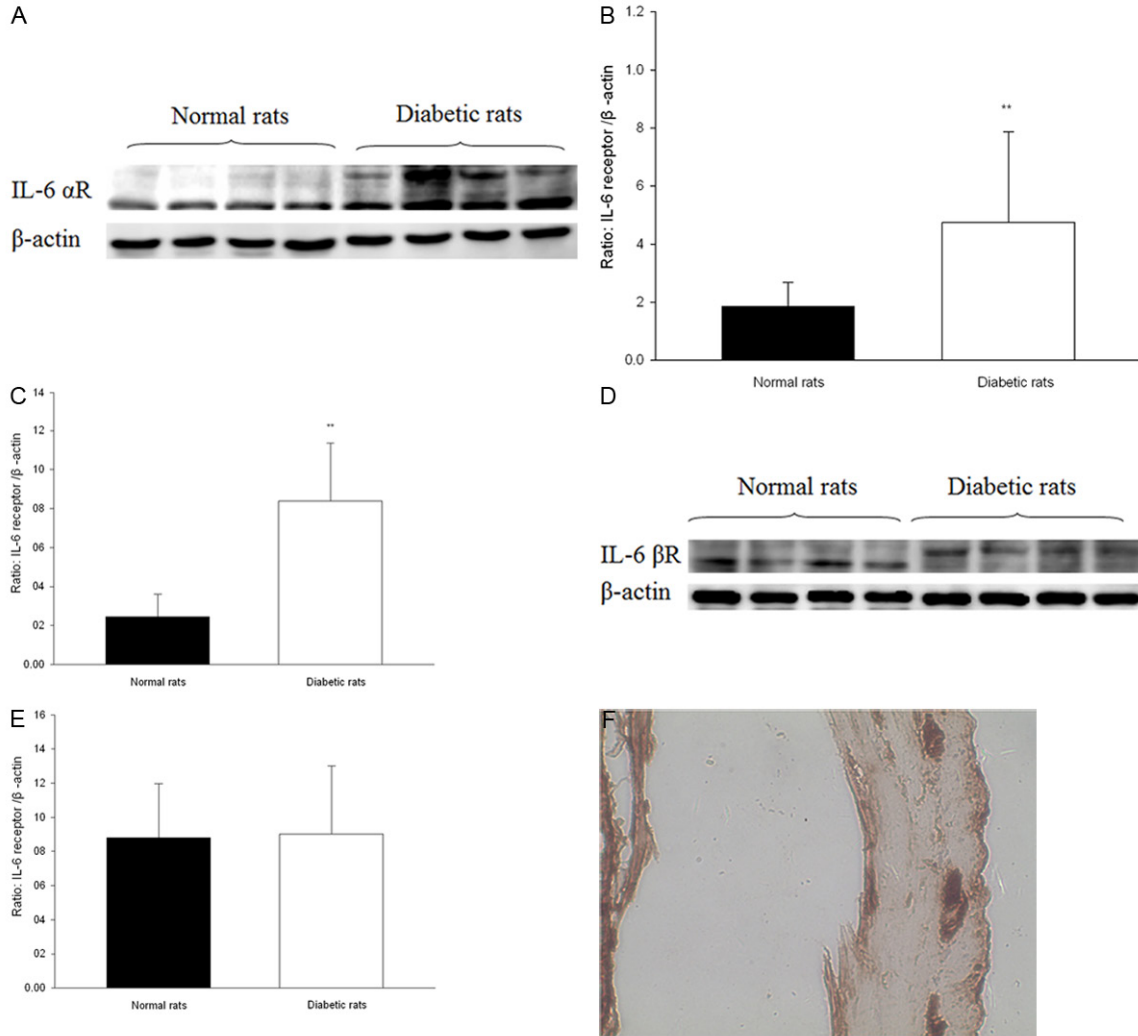


Figure 6. Expression of IL-6 receptors in colon from diabetic and normal rats. A. Representative immunoblot analyses of IL-6 α -receptor. The upper panels show major protein band, β -actin is internal control. B, C. Statistical analysis of the expression of IL-6 α -receptor. D. Representative immunoblot analyses of IL-6 β -receptor. The upper panels show major protein band, β -actin is internal control. E. Statistical analysis of the expression of IL-6 β -receptor. F. IL-6 α -receptor immunoreactivity expressed in colon ($\times 40$). * $P < 0.05$ compared with internal control. $n = 8$.

findings suggested that the IL-6R pathway is relevant to colonic dysmotility in type 1 diabetes. IL-6R α might be a target for prevention of colonic dysmotility in type 1 diabetes.

Our study showed that IL-6R α was expressed in the mucosal layer and myenteric plexus of colon. The mucosa was removed carefully when we recorded the contraction of muscle strips. IL-6 might act on the IL-6R α of myenteric plexus.

In conclusion, we demonstrated for the first time that IL-6 mediated the increased contraction of longitudinal muscle strips from distal

colon in type 1 diabetes rats via the IL-6R pathway. The present data support the hypothesis that IL-6 receptor is regarded as a target for prevention of colonic dysmotility in type 1 diabetes.

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Disclosure of conflict of Interest

None.

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IL-6 and colonic contraction of diabetes

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IL-6 and colonic contraction of diabetes

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