INTRODUCTION

Diabetes mellitus (DM) is one of the most important metabolic diseases of people (1, 2). Type 1 or insulin-dependent diabetes mellitus (IDDM) typically in childhood is the result of a frank deficiency of insulin (3). It is due to destruction of pancreatic β cells, most likely the result of autoimmunity to one or more components of those cells (4). In clinical, many of the acute effects of this disease can be controlled by insulin replacement therapy (5, 6). However, the role and the action mechanism of insulin on gastrointestinal (GI) motility are still unclear. The purpose of the present study was to investigate the involvement of cyclooxygenase-2 (COX-2) and prostaglandin E2 in the effects of insulin on gastric emptying in male rats. The normal and streptozotocin (STZ)-pretreated rats were injected intraperitoneally with or without insulin, atropine and specific muscarinic receptor antagonists before examination of measurement of gastric emptying, spontaneous contractile activity of smooth muscle strips, plasma cholecystokinin (CCK), and prostaglandin E2 (PGE2) analysis. Protein expression of COX-2 and insulin receptors (IRs) were analyzed by the technique of western blot. Acute different doses of insulin accelerated gastric emptying. Atropine interrupted the insulin effect on gastric emptying, and muscarinic M1/M3 receptor antagonists interrupted the insulin-reversed gastric emptying in normal and DM rats. Besides, we observed the expression of IRs in GI and found that IR was changed under the insulin and DM treatment, and was also different between STZ-pretreated rats and hyperglycemic rats. Expression of COX-2 in stomach was decreased in DM rats but restored by insulin. The COX inhibitor, indomethacin, decreased the gastric emptying which was induced or reversed by insulin in normal and DM rats, respectively. PGE2 production in stomach corresponded to the COX-2 expression. The contraction of GI smooth muscle stimulated by PGE2, was increased in insulin-pretreated normal and DM rats. We conclude that insulin changed the expression of IRs in stomach in DM rats. The delayed GI motility in diabetes was at least in part due to the COX-2 and PGE2 pathway which associated with decreasing COX-2 and diminishing PGE2 production in stomach. The attenuation of PGE2 production was employed for the index of the reduction of smooth muscle contraction in stomach in diabetes. Insulin stimulated the smooth muscle contraction through the IRs and COX-2 expression plus PGE2 production in rat stomach as well as reversed the delayed gastric emptying via the nervous actions of muscarinic M1 and M3 receptors in DM rats.

Key words: insulin, prostaglandin E2, cyclooxygenase 2, gastrointestinal motility, diabetes mellitus
acetylcholine (ACh) as its neurotransmitter, but other peptides (such as cholecystokinin) may act on the PSNS as a neurotransmitter (25, 26). The ACh acts on two types of receptors, the muscarinic and nicotinic cholinergic receptors (27). Cholinergic-neural system has been shown to be involved in the regulation of blood glucose (28). Many anti-cholinergic agents used in clinical have been found to change GI functions (29). However, the roles and the action mechanisms of muscarinic system on the GI motility are still unclear. Muscarinic acetylcholine receptors, M1 receptor and M3 receptor, are predominantly found to bind to G proteins of class Gαq (30). M1 receptor is Gαq/Giαq-coupled and found to affect the secretion from salivary glands and stomach (31, 32). M3 receptor is Gαq-coupled and mediates an increase in intracellular calcium, it typically causes constriction of smooth muscle (33).

Disordered GI motility is an often overlooked clinical problem (8). Delayed gastric emptying of solid and/or liquid meal in patients with both type I and type II DM occurs in approximately 50% of these patients (34). Delayed gastric emptying is very common in patients with DM (8) and it has no direct correlation to blood sugar control, duration of the disease, and upper gastrointestinal symptoms (35). It has been well-known that the hyperglycemia induced by streptozotocin (STZ) inhibits both gastric emptying (36) and GI transit in rats, but the effect was reversed by supplement of insulin (12, 13). Cholecystokinin (CCK), a GI related peptides, is released mainly from duodenum. It has been shown that CCK inhibits the gastric emptying (37) and plays a key role in the GI tract. Therefore, it is interesting to find out the relationship between DM and GI and to see if it is via the mechanism of CCK secretion.

On the other hand, either gastric emptying or cholinergic receptors were related to the smooth muscle contraction (38, 39). PGE2 secretion affects the smooth muscle contraction which was mediated by the COX-1 and COX-2 activation (40). Moreover, COX-2 stimulates smooth muscle contraction in GI tract especially (41). So the western blot analysis was performed to observe the COX-2 expression and direct connection to the smooth muscle contraction in GI tract.

In the present study, we first aimed to examine the effects of insulin on gastric emptying and the nervous action on cholinergic M1 and M3 receptors along with plasma CCK secretion in normal and DM rats. Second, we investigated the changes in contraction of intestinal smooth muscle induced by insulin in DM rats. Finally, we examined the mechanism about expression of IRs and COX-2, and PGE2 production in GI tract under the insulin treatment and the close association with gastric emptying and smooth muscle contraction in DM status.

**MATERIALS AND METHODS**

**Animals**

Male Sprague-Dawley rats weighing 250-350 g were housed in a temperature (22 ± 1°C) and light (6 a.m.-8 p.m.) controlled environment. Tap water and rat chow were given *ad libitum*. Animal protocols were approved by the Institutional Animal Care and Use Committee of National Yang-Ming University. All animals received humane care 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, Taiwan, R.O.C.

**Diabetes induction**

Diabetic hyperglycemia was induced by the intravenous injection of the tail vein with freshly prepared STZ (32 mg/kg, Sigma, St. Louis, MO, U.S.A) solution in saline/0.01 M citrate buffer (pH 4.5). The onset of DM was confirmed by the rapid appearance of polyuria, weight loss, and glycosuria (Combur-Test U, Boehringer Mannheim, Mannheim, Germany).

**Experimental designs**

In the Experiments 1-4, rats were divided into 2-6 groups for the experiment of gastric emptying.

**Experiment 1. Dose effects of insulin on gastric emptying in normal male rats.** Rats were divided into six groups and fasted for 20 h before use. Rats in the first group were injected i.p. with saline. Rats in other groups were injected i.p. with insulin (0-10 IU/kg).

**Experiment 2. Interaction between insulin and atropine on gastric emptying in normal and diabetic male rats.** Normal and diabetic rats were divided into four groups each and fasted for 20 h before use. On the experiment day, rats were injected i.p. with saline, insulin (0.25 IU/kg), atropine (5 mg/kg) or insulin plus atropine, respectively. Diabetes was induced by the intravenous injection of the tail vein with freshly prepared STZ (32 mg/kg). Some STZ-induced diabetic rats were divided into four groups and fasted for 20 h before use. Finally, the plasma glucose and (CCK) was measured by RIA after decapitation.

**Experiment 3. Role of muscarinic receptors in the effects of insulin on GI motility in normal and diabetic male rats.** The normal or DM rats were divided into six groups and fasted for 20 h before use. Diabetes was induced by the intravenous injection of the tail vein with freshly prepared STZ (32 mg/kg). Either normal or STZ-induced diabetic rats were injected i.p. with saline, insulin (0.25 IU/kg), pirenzepine (a M1 receptor antagonist, 15 mg/kg), 4-DAMP (a M3 receptor antagonist, 3 mg/kg), insulin plus pirenzepine, or insulin plus 4-DAMP, respectively.

**Experiment 4. Role of NSAIDs in the effects of insulin on GI motility in normal and diabetic male rats.** The normal or DM rats were divided into eight groups and fasted for 20 h before use. Diabetes was induced by the intravenous injection of the tail vein with freshly prepared STZ (32 mg/kg). Either normal or STZ-induced diabetic rats were injected i.p. with saline, insulin (0.25 IU/kg), indomethacin (the COX general inhibitor, 40 mg/kg), insulin plus indomethacin, respectively.

**Experiment 5. Effects of insulin on expression of IRs on stomach in normal and diabetic male rats.** The normal or DM rats were injected i.p. with saline or insulin (0.25 IU/kg) once per day for 3 days. Rats were fasted for 20 h before use. On the experimental day, rats were decapitated. Proteins from stomach were extracted by lysis buffer. Protein of IRs was analyzed by the analysis of Western blot.

**Experiment 6. Effects of insulin on expression of IRs in stomach in normal, diabetic and hyperglycemic male rats.** The normal rats were divided into two groups. One group was injected i.p. with 20% dextrose (2g/kg) once per day for 3 days and another was i.p. with saline for vehicle. The group 3 was DM rats those were injected i.p. with saline for three days. All rats were fasted for 20 h before use. On the experimental day, rats were decapitated. Proteins from stomach and colon were extracted by lysis buffer. Protein of IRs was analyzed by the analysis of Western blot.

**Experiment 7. Effects of insulin on expression of COX-2 enzyme in stomach of normal and diabetic male rats.** The normal or DM rats were injected i.p. with saline or insulin (0.25 IU/kg) once per day for 3 days. Rats were fasted for 20 h before use. On the experimental day, rats were decapitated. Proteins from stomach were extracted by lysis buffer. Protein of COX-2 enzyme was analyzed by the analysis of Western blot.
Experiment 8. Effects of insulin on the stomach PGE2 concentration in normal and diabetic male rats. The normal or DM rats were injected i.p. with saline or insulin (0.25 IU/kg) once per day for 3 days. Rats were fasted for 20 h before use. On the experimental day, rats were decapitated. The plasma and tissue samples of stomach were collected and acidified by addition of 2 M HCl to pH of 3.5 for PGE2 EIA.

Experiment 9. Effects of PGE2 on stomach smooth muscle contraction in normal and diabetic male rats. The normal or DM rats were injected i.p. with saline or insulin (0.25 IU/kg) once per day for 3 days. Rats were fasted for 20 h before use. On the experimental day, rats were decapitated. The segments of the stomach were quickly removed for experiment of spontaneous contractile activity of smooth muscle strips.

Measurement of gastric emptying

All animals were used after a 20 h fast. On the day of experiment, animals were received i.p. injection of drugs. Fifteen min later, all rats were orally ingested with radioactive Na251CrO4 containing 10 % charcoal via a PE-205 tubing directly into the stomach. Fifteen min after administration of the liquid meal, rats were decapitated. The small intestine was divided equally into ten segments. The radioactivities in the stomach and 10 segments of small intestine were counted by an automatic gamma counter (1470 Wizard, Pharmacia, Turku, Finland). Gastric emptying was determined by measuring the amount of radiolabeled chromium contained in the small intestine as a percentage of the initial amount received.

Measurement of spontaneous contractile activity of smooth muscle strips

Rats were fasted for 20 h and divided into 4 groups. Two groups of them were DM groups (induced by STZ, 32 mg/kg). They were individually received i.p. injection of normal saline (1 ml/kg) or/and insulin (0.25 IU/kg) 30 min before decapitation. Stomach tissues were quickly removed and cultured in Kreb’s solution. The stomach was collected along the mesentery after decapitation. Muscle strips which parallel to the longitudinal fibers were cut into small pieces (3×7 mm) and the mucosa on each strip was removed gently. The muscle strips were suspended in a thermostatically controlled (37°C) tissue chamber containing 5 ml Kreb’s solution and bubbled continuously with 95% O2 and 5% CO2. The composition (in mM) of the Kreb’s solution included NaCl 119, KCl 4.75, KH2PO4 1.2, NaHCO3 25, MgSO4 1.5, CaCl2 2.5 and glucose 11. One end of the strip was fixed to a hook at the bottom of the chamber and another end was connected to an external isometric force transducer. After being stabilized for 30 min, PGE2, dose-response curves were constructed by applying different concentrations (10^-6~10^-5 M) at 5-min intervals. Spontaneous contractile activity of muscle strips (under a initial tension of 1 g) was simultaneously recorded by the PowerLab data acquisition system with Chart software (ADInstruments).

Processing of plasma for measurements of blood glucose, plasma CCK and PGE2 concentrations

The concentration of blood glucose was an indicator of the stable experimental design. On experimental days, rat blood samples were measured for blood glucose (Accu-Chek Advantage II, Mannheim, Germany) immediately and then collected and mixed with EDTA (1 mg/ml of blood) plus aprotinin (500 kiu/ml of blood) after decapitation. Plasma was immediately prepared by centrifugation at 1000 x g for 30 min at 4°C and used for measurement of plasma CCK and PGE2 concentrations.

CCK radioimmunoassay (RIA)

The plasma samples were acidified with an equal volume of 1% trifluoroacetic acid (TFA) and then centrifuged at 2600 x g for 20 min at 4°C. The SEP-PAK C18 cartridge (Waters Associates, Milford, MA, U.S.A.) was equilibrated with 60% acetonitrile in 1% TFA (1 ml), followed by 1% TFA (3 ml, three times), and then the supernatant from the treated plasma sample was applied. After being washed with 1% TFA (3 ml, twice), the peptide (bound material) was slowly eluted with 3 ml of 60% acetonitrile in 1% TFA. The eluant was collected, lyophilized in a Speed Vac concentrator (Salvant Instruments, Farmingdale, NY, U.S.A.), then stored at -80°C and reconstituted with the appropriate assay buffer before measurement by radioimmunoassay (RIA). The CCK concentration in the extracted sample was measured by RIA using a rabbit anti-CCK antiserum supplied by Dr. K. Y. Francis Pau (Irvine, CA, U.S.A.) and [3H-CCK purchased from Amersham International Plc. In this RIA system, a known amount of unlabeled CCK in a total volume of 0.3 ml of 0.1% gelatin-PBS was incubated at 4°C for 24 h with 100 µl of anti-CCK antiserum, 1: 2,000 dilution in normal rabbit serum and 100 µl of [1H]CCK (~8,000 cpm). Two hundred µl of anti-rabbit gamma-globulin (ARGG) was then added, and incubation continued at 4°C for 24 h. The assay tubes were then centrifuged at 1,000 x g for 20 min. The pellet was dissolved in 400 µl of 1N NaOH, and 80 µl of 5 N HCl was added. The sample was mixed with 3 ml of liquid scintillation fluid, and the radioactivity counted in an automatic gamma counter (Wallac 1409, Pharmacia, Turku, Finland).

Western blot (immunoblotting)

All animals were used after a 20 h fast. On the experimental day, rats were decapitated. Proteins from stomach were extracted by lysis buffer and analyzed by the analysis of Western blot. The proteins (20 µg each) were separated by 12% SDS-PAGE and then transferred onto polyvinylidenefluoride (PVDF) membranes. Membranes were blocked with 5% nonfat milk then incubated with primary antibodies (of IR: Santacluz sc-57342 ; COX-2: Santacluz sc-1745; GAPDH: Santacluz sc-22233). Membranes were washed four times with TBS-T and then incubated with secondary antibody (Santacluz). Finally, immunoreactive bands were detected by chemiluminescence.

PGE2 enzyme immunoassay (EIA)

The tissue samples were lysed by lysis buffer in advance. The lysed tissue and plasma samples were acidified by addition of 2 M HCl to pH of 3.5 and at 4°C for 15 min. Samples were centrifuged at 10000 xg for 2 min to remove precipitates. The C18 reverse phase column was prepared by washing with 10 ml of ethanol followed by 10 ml of deionized water. The sample was applied under a slight positive pressure to obtain a flow rate of about 0.5 ml/ min. The column was washed with 10 ml of water, followed by 10 ml of 15% ethanol, and finally 10 ml hexane. The sample was eluted from the column by addition of 10 ml ethyl acetate and analyzed immediately by PGE2 EIA kit.

Statistical analysis

The data were expressed as mean ± S.E.M. The treatment means were tested for homogeneity using one-way analysis of variance (ANOVA), and the significance of any difference between means tested using Duncan’s multiple range test. A difference between two means was considered to be statistically significant when P was less than 0.05.
RESULTS

Effects of insulin on gastric emptying and its correlation with blood glucose and muscarinic system in normal and DM rats

The fasted normal range of blood sugar was between 80-100 mg/dl. The level of blood glucose was decreased (about 50 mg/dl) by the i.p. injection of insulin (0.25 IU/kg) and rose markedly (almost 400 mg/dl) after intravenous injection of the tail vein with freshly prepared STZ (32 mg/kg). Insulin suppressed the high level blood glucose in DM rats and returned the value to the levels with no difference from normal range (data not shown). Fig. 1 shows the dose effects of acute administration of insulin on gastric emptying in male rats. Insulin at doses of 0.25, 0.5, 1, 5 and 10 mg/kg significantly (P<0.05) increased gastric emptying comparing with controls (Fig. 1). Administration of atropine (5 mg/kg) in vivo maintained a normal range of blood glucose level (Fig. 2A, the middle panel), but restored the higher level of gastric emptying induced by insulin back to the control level (Fig. 2A, the upper panel). However, insulin did not change the level of plasma CCK in response to atropine, although restored the lower gastric emptying to control levels (Fig. 2A, the lower panel). Administration of STZ (32 mg/kg) in rats inhibited gastric emptying, and insulin significantly restored (P<0.05) the inhibition of gastric emptying. Atropine significantly inhibited (P<0.05) the gastric emptying in DM rats. However, treatment of insulin combined with atropine significantly restored the inhibition of gastric emptying in DM model (Fig. 2B, upper panels). Atropine did not affect the blood glucose in DM rats (Fig. 2B, the middle panel). Atropine increased plasma CCK in normal rats but insulin significantly reduced (P<0.05) the higher plasma CCK concentration in DM rats (Fig. 2A and B, lower panel). *, ** P<0.05 and P<0.01 as compared to the control group respectively. +, P<0.05 as compared with insulin-treated group. #, P<0.05 as compared with atropine-treated group.

Fig. 1. Effects of acute administration of insulin on rat gastric emptying: Insulin at doses of 0.25, 0.5, 1, 5 and 10 mg/kg significantly increased gastric emptying comparing with controls (P<0.05 as compared to control level).

Fig. 2. Effects of atropine on the insulin-enhanced gastric emptying in normal and DM rats: Administration of atropine (5 mg/kg) in vivo maintained a normal range of blood glucose level (Fig. 2A, the middle panel), but inhibited the gastric emptying (P<0.01) and reduced the higher level of gastric emptying induced by insulin back to the control level (Fig. 2A, the upper panel). Administration of STZ (32 mg/kg) in rats inhibited gastric emptying, and insulin significantly restored (P<0.05) the inhibition of gastric emptying. Atropine also significantly inhibited (P<0.05) the gastric emptying in DM rats. Treatment of insulin combined with atropine significantly restored the inhibition of gastric emptying in DM model (Fig. 2B, upper panels). Atropine did not affect the blood glucose in DM rats (Fig. 2B, the middle panel). Atropine increased plasma CCK in normal rats but insulin significantly reduced (P<0.05) the higher plasma CCK concentration in DM rats (Fig. 2A and B, lower panel). *, ** P<0.05 and P<0.01 as compared to the control group respectively. +, P<0.05 as compared with insulin-treated group. #, P<0.05 as compared with atropine-treated group.
2B, upper panels). On the other hand, insulin treatment in STZ rats significantly reduced (P<0.05) the higher plasma CCK concentration in DM rats (Fig. 2B, lower panel). Treatment with M₁ and M₃ receptor antagonists, pirenzepine (15 mg/ml/kg) and 4-DAMP (3 mg/ml/kg), respectively, decreased the insulin-induced up-regulation of gastric emptying (P<0.01) (Fig. 3A). Either M₁ or M₃ receptor antagonist reduced the effects of insulin in normal and DM rats (Fig. 3A and B).

**Effects of DM and insulin on the expression of IRs and COX-2 enzymes in rat stomach**

Expression of IRs was decreased significantly in the stomach of DM rats (Fig. 4A). Administration of insulin increased the expression of IRs in stomach in normal rats (Fig. 4A). However, insulin reversed the decreased expression of IRs in DM rats. COX-2 enzyme expression was decreased significantly (P<0.05) in the stomach of DM rats (Fig. 5A). Administration of insulin increased the expression of stomach COX-2 in both normal and DM rats (Fig. 5A and B).

**Effects of DM and hyperglycemia on the expression of IRs enzymes in rat stomach**

The IRs expression in antrum was decreased in DM rats (P<0.05) but unchanged at 20% dextrose treatment as compared to normal rats (Fig. 4B).

**Effects of DM and insulin on stomach and plasma PGE₂ concentrations**

In rat stomach, the concentration of PGE₂ was increased by the administration of insulin. In DM rats, the concentrations of stomach PGE₂ was significantly reduced (P<0.05), but restored by insulin replacement (Fig. 6). The concentration of plasma PGE₂ was not altered in the DM rats as compared to normal rats (data not shown). Insulin did not affect the level of plasma PGE₂ in normal rats, but enhanced (P<0.05) that in DM rats (data not shown).

**Effects of PGE₂ on the contractile activity of rat stomach smooth muscle**

The contraction of stomach smooth muscle stimulated by PGE₂ was increased in normal rats. However, the action was

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**Fig. 3.** Effects of muscarinic M₁/M₃ receptor antagonists on the insulin-stimulated gastric emptying in normal and DM rats (panel A) and DM rats (panel B). * * P<0.05 and P<0.01 as compared to the control levels. +, P<0.05 as compared to insulin-treated levels.

**Fig. 4.** Effects of DM and insulin on the expression of IRs in rat stomach: Expression of IRs was decreased significantly in the stomach of DM rats (panel A, P<0.05). Administration of insulin increased the expression of IRs in stomach in normal rats (panel A, P<0.05). However, insulin reversed the decreased expression of IRs in DM rats. Effects of DM and hyperglycemia on the expression of IRs enzymes in rat stomach: The expression of IR in antrum was decreased in DM rats (P<0.05) but maintain the original level at 20% dextrose treatment (panel B). Protein of IRs was analyzed by the analysis of western blot. * P<0.05 as compared to the normal group.
exhibited strongly when pretreated with insulin, especially in 10^{-5} M (P<0.01). On the other hand, the effect of PGE₂ on stomach smooth muscle contraction in DM rats was not a significant stimulation. But the stimulatory effect of PGE₂ was restored when pretreated with insulin plus DM (Fig. 7).

**DISCUSSION**

Diabetes is a popular disease in which the body does not produce or properly use insulin. In clinical, diabetic patients usually suffer from GI disorders including nausea, vomiting, bloating, and fullness about dysfunction of gastrointestinal tract (42, 43). Many pregnancy women also have the type 1 diabetes related to the disorder of GI hormone secretion and gene expression (44, 45). However, when blood glucose returns to normal with insulin supplements, the patients have a brief respite in most symptoms above. The relationship between insulin and GI tract in diabetes is an interesting topic. The gastric emptying might be decreased by hyperglycemia and increased by replacement of insulin. The present results showed that administration of insulin (0.25~10 IU/kg) dose-dependently increased gastric emptying from 56% to 98%.

In autonomic nervous system, the PSNS secrets ACh which acts on the cholinergic receptors (46). The important site of muscarinic receptors has been found in endothelial cells of blood vessels (47). It has been known the cholinergic receptors mediated smooth muscle contraction (48). The muscarinic receptors can be blocked by atropine. We also showed that administration of atropine (a peripheral muscarinic receptor antagonist) decreased the gastric emptying strongly. Our study partly showed that administration of atropine in normal male rats increased the levels of plasma CCK but decreased the gastric emptying. It proved that the effects of cholinergic receptors were related to the GI tract mostly. When treatment of insulin plus atropine, their restored effects on gastric emptying mean that insulin affect GI motility via an action on the muscarinic receptors. However, insulin did not change concentration of

**Fig. 5.** Effects of DM and insulin on the expression of COX-2 enzymes in rat stomach: COX-2 enzyme expression was decreased significantly in the stomach of DM rats (panel A, P<0.05). Administration of insulin increased the expression of stomach COX-2 in both normal and DM rats (panel A, P<0.05). Administration of insulin increased the expression of COX-2 in both normal and DM rats (panel A, P<0.05). Protein of COX-2 was analyzed by the analysis of western blot. *P<0.05 as compared to the normal group.

Effects of COX inhibitor NSAID, indomethacin, on gastric emptying treated with insulin in normal and DM rats: The gastric emptying decreased in DM rats and reversed by insulin. Administration of indomethacin, the stimulation and reversal of insulin on gastric emptying in DM rats were attenuated, respectively. (panel B, P<0.05).

**Fig. 6.** Effects of DM and insulin on stomach PGE₂ concentration: The concentration of PGE₂ was increased by the administration of insulin in stomach. In DM rats, the concentrations of stomach PGE₂ was significantly reduced (*P<0.05), but restored by insulin replacement.* P<0.05 as compared to the normal group.

**Fig. 7.** Effects of PGE₂ on the contractile activity of rat stomach smooth muscle: The contractions of stomach smooth muscles stimulated by PGE₂ in normal and DM rats. However, the action was exhibited strongly when pretreated with insulin. *P<0.05 as compared to the control group at corresponding level of PGE₂.
plasma CCK. We suggested that insulin did not affect CCK secretion in itself. In STZ-DM rats, the gastric emptying was decreased. Insulin reversed the effect of STZ on gastric emptying and atropine decreased the restored action of insulin in DM rats. On the other hand, insulin decreased the stimulation of STZ on concentration of plasma CCK to normal range. Therefore, we suggested that insulin restored gastric emptying at least partly via some association with cholinergic receptors. Insulin attenuated the plasma CCK levels to normal range in DM rats but atropine maintained the higher secretion of CCK in either normal or DM rats. The difference between the phenomena meant that the reverse of insulin on gastric emptying in DM rats partly depended on the plasma CCK secretion, but atropine did not alter the CCK production by itself. It meant that the block of cholinergic receptor did not affect the CCK secretion levels under the insulin treatment in DM rats.

Five subtypes of muscarinic receptors have been determined including M₁, M₂, M₃, M₄, and M₅ receptors. M₁ receptor was found to affect the secretion from salivary glands and stomach (31, 32). M₁ receptor was mainly responsible for smooth muscle contraction and increased endocrine-exocrine gland secretions in stomach (33). M₁ receptor was abundant in heart (46). Mᵣ and M₃ receptors were unclear yet mainly. Some studies have shown that effects on inflammation and proliferation may be associated with Mᵣ and M₃ receptors (49). Muscarinic receptor activation by the release of Ach from vagal nerves thus mainly leads to release of gastrin and inhibition of somatostatin release, which together with the direct muscarinic effects on the parietal and chief cells to increase gastric acid production (49). In the present study, pirenzepine (a M₁ receptor antagonist, 15 mg/kg) and 4-DAMP (a M₁ receptor antagonist, 3 mg/kg) were used to repeat the measurement of gastric emptying. Our data showed that both pirenzepine and 4-DAMP were similar to the atropine to reduce gastric emptying. However, some studies have shown that when pirenzepine at 15 mg/kg inhibited gastric acid secretion in M₁-receptor knockout mice (50). However, activation of M₅ receptors evoked a gastric smooth muscle relaxation via a NO-mediated mechanism (49).

Gastric acid secretion, one mechanism of gastrointestinal motility, was not correlated completely with gastric emptying (51, 52). It may be concerned with pharmacology (53), endocrinology (54) or neurology (55). Administration of insulin plus pirenzepine or 4-DAMP, the returned effects of gastric emptying were reconstructed. It means that the insulin affects GI gastric emptying via an action depending mainly on the muscarinic M₁ and M₅ receptors, but it still exists other pathways between insulin and gastric emptying. In DM rats, the stimulatory effects of insulin on gastric emptying were also reduced by the administration of pirenzepine and 4-DAMP. Therefore, we suggested that insulin restored gastric emptying at least in part by a mechanism associated with the muscarinic M₁ and M₅ receptors in diabetes.

Although insulin is thought of as a popular hormone, it is difficult to keep balance between blood glucose control and side effects. However, no studies have examined the effects of insulin on distribution and expression of IRs in GI tract of DM rats. In the present study, we further observed the strong expression of IRs under insulin treatment in stomach. Insulin affected the expression of IRs in stomach of DM rats. We believed the down-regulation of IRs expression in diabetic rats was caused by STZ treatment. In Fig. 2B the middle panel, the group 2 column showed that when treatment with insulin at dose 0.25 IU/kg in DM rats, the blood glucose level was low down but not to the normal range. Moreover, the blood glucose levels stimulated by dextrose were called hyperglycemia. The blood glucose value was very high (>300 mg/dl more) in either DM or hyperglycemic rats. In DM rats, the β-cells were damaged by STZ treatment to lack production of insulin. Relatively, insulin secretion was stimulated in hyperglycemia. However, the expression of IRs in antrum was decreased in DM rats but not in the hyperglycemic rats. It suggested that the decreased IR expression in DM rats was mainly due to the deficiency of insulin caused by STZ administration.

COX is a kind of enzyme that can convert AA to PGH₂ and control the symptoms of inflammation and pain (18). PGH₂ converts to PGE₂ via the PGE synthase (56). PGE₂ can stimulate the smooth muscle contraction (57). COX-1 is considered as a constitutive enzyme, being found in most cells and tissues (58). COX-2 is undetectable in most normal tissues (20). As an inducible enzyme, COX-2 is almost activated under stimulation (59). It has been known that COX-2 mediated the production of inflammatory eicosanoids in the joints but sparing the endogenous protective eicosanoids in the stomach (60). Many studies have pointed out that COX-2 up-regulates the vascular smooth muscle contractile hyperreactivity (21). The inducible enzyme COX-2 exerts its action at inflammatory site of the joints and muscles chiefly (60). In our study, we found that the expression of COX-2 in stomach was decreased in DM rats. Insulin stimulated the expression of COX-2. Pretreatment of insulin and STZ, COX-2 expression was reversed to the normal range.

Indomethacin, the COX general inhibitor, was an irritative activity of antiinflammatory agents (61). It has been known that indomethacin pretreatment existed different reactive oxygen-scavenging system (ROSS) activity (62). Cytotoxicity of reactive nitrogen oxide species (RNOS) causes cellular damage and leads to the disorders with smooth muscle proliferation (62). It has been well known that administration of indomethacin once daily for 2 wk enhances development of gastric mucosal damage (63). It also increases the gastric emptying and induces gastric lesions (64) as well as gastric ulcers (65) with time-course. It was established that NSAIDs increased the vulnerability of the GI mucosa for the development of peptic lesion and many serious ulcer complications, including erosions, inflammation, ulceration, bleeding and perforation (60). But it has no effect at dose 40 mg/kg within 30 min short-time after administration of indomethacin. In the present study, treatment of indomethacin decreased the stimulation of insulin in gastric emptying in both normal and DM rats. We found that indomethacin attenuated the reversal by insulin of the delayed gastric emptying in diabetic rats. Authentically, insulin affected gastric emptying in DM rats through the COX-2 pathway indeed.

On the other hand, PGE₂ production is a key indication of COX-2 activity in GI tract (66, 67). Our data showed that the concentration of stomach PGE₂ was decreased in DM rats and the effect was restored by insulin replacement. We suggested that the lost expression of COX-2 in stomach caused the decrease of PGE₂ production in DM. It meant that insulin reversed the expression of COX-2 and caused the stomach PGE₂ production relatively. In DM model, the PGE₂ concentration in stomach was decreased. It resulted in the decreased PGE₂ synthesis or production. Therefore, the increased PGE₂ caused by insulin is the reason of the contraction of smooth muscle in rat stomach in rats. However, the plasma PGE₂ levels were not changed in DM or insulin-pretreated rats (data not shown). It might be defined as the PGE₂ is a sort of paracrine and/or autocrine hormones so the plasma PGE₂ levels could not be used as an index for the contractility of GI smooth muscles. Finally, we found that PGE₂ stimulated the smooth muscle contraction, especially in insulin-pretreated group. Instead, the stimulatory effect was weak in DM rats. When combining with insulin and DM treatment, the restoration was almost to the normal range.

Although it has been known that blood glucose may affect the gastric emptying (36), our other data strongly suggested the
insulin can affect the gastric emptying and smooth muscle contraction in GI tract of DM rats.

Above all, our study has shown the down regulation of COX-2 / PGE2 production and the insulin receptor expression, in addition to an increase of plasma CCK levels in diabetic rats. These factors were all connected with the delay gastric emptying in diabetic rats. Of course, it might still exist other mechanism between diabetes and GI motility (68, 69). We believed that, in our studies, the more interesting point was focused on the COX-2 / PGE2 pathway because many mechanism up-regulated this COX pathway were unknown, including the newer subject cannabinoid (CB) receptors. On the other hand, NSAIDs were used extensively in clinical and had a variety of side effects.

We conclude that insulin changed the expression of IRs in stomach in DM rats. The delayed gastric emptying in diabetes was at least in part due to the COX-2 and PGE2 pathway (decreased COX-2 and diminished PGE2 production in stomach). The change of COX-2 expression was employed for the index of the smooth muscle contraction in stomach in diabetes. Insulin not only stimulated the smooth muscle contraction through the COX-2 expression plus PGE2 production in stomach but also reversed the delayed gastric emptying via the nervous actions of muscarinic M1, and M3, receptors in DM rats.

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