The combination of colesevelam with sitagliptin enhances glycemic control in diabetic ZDF rat model

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Shang Q, Liu MK, Saumoy M, Holst JJ, Salen G, Xu G. The combination of colesevelam with sitagliptin enhances glycemic control in diabetic ZDF rat model. *Am J Physiol Gastrointest Liver Physiol* 302: G815–G823, 2012. First published January 26, 2011; doi:10.1152/ajpgi.00295.2011.—Bile acid sequestrants have been shown to reduce glucose levels in patients with type 2 diabetes. We previously reported that the bile acid sequestrant colesevelam HCl (Welchol) (COL) induced the release of glucagon-like peptide (GLP)-1 and improved glycemic control in insulin-resistant rats. In the present study, we tested whether adding sitagliptin (Januvia) (SIT), which prolongs bioactive GLP-1 half life, to COL would further enhance glycemic control. Male Zucker diabetic fatty (ZDF) rats were assigned to four groups: diabetic model without treatment (the model), the model treated with 2% COL or 0.4% (120 mg/day) SIT alone, or with the combination (COL+SIT). After 4 wk of treatment, the glucose area under the curve (AUC) was reduced more in the COL+SIT than the COL although both groups showed decreased glucose AUC with increased AUC of bioactive GLP-1 (GLP-1A) compared with the model group. The above changes were not observed after 8 wk. Increasing the SIT dose by 50% (180 mg SIT/day) in the diet reduced the glucose AUC in the COL+SIT group even after 8 wk but still not in the SIT alone group compared with the model. It was noteworthy that, after 8 wk, insulin levels in the SIT group declined to levels similar to the model. Histological examination of the pancreatic β-cell islets showed that islet sizes were larger, proliferation enhanced, and cell apoptosis reduced in the COL+SIT but not the SIT alone group compared with the model. We hypothesize that the combination of COL with SIT extends the half life of COL-induced GLP-1A and benefits preservation of the islets that delay the development of diabetes and improve glycemic control. This study suggests that the combined therapy (COL+SIT) is more effective than either drug alone for reducing glucose levels in diabetes.

hyperglycemia; plasma glucose; insulin; bile acid; apoptosis; glucagon-like peptide-1

RECENT STUDIES INDICATE that bile acid sequestrants, which block bile acid absorption in the intestine, improve hyperglycemia in diabetic animal models (22) as well as in patients with type 2 diabetes (2). Although various hypotheses have been proposed, the specific mechanism(s) by which bile acid sequestrants improve glycemic control remain unclear. Colesevelam HCl (Welchol) (COL) is an FDA-approved bile acid sequestrant, and, recently, several clinical studies have demonstrated that COL improves glycemic control in patients with type 2 diabetes (3, 10, 13–14).

We recently found that COL induced the release of glucagon-like peptide (GLP)-1 and suggested that the enhanced GLP-1 levels were responsible for improved glycemic control in insulin-resistant diet-induced obese rats (25). This hypothesis was supported by a study from another group (6) that cholestyramine, a classical older bile acid sequestrant, enhanced GLP-1 levels and improved glycemic control in male Zucker diabetic fatty (ZDF) rats, a type 2 diabetic rat model. However, the induced active GLP-1 has a very short half-life, as it is rapidly degraded by dipeptidyl peptidase IV (DPP4), which cleaves the NH_2-terminal dipeptides from GLP-1 in the intestine (15, 20). It is well known that sitagliptin (SIT) is a specific inhibitor of DPP4 that effectively prolongs the half-life of bioactive GLP-1 (GLP-1A) (4, 16, 21, 24).

As SIT might prolong the half-life of COL-induced bioactive GLP-1, we reasoned that the combination of SIT with COL might strengthen the beneficial effect of COL on glycemic control. In the present study, we tested whether adding SIT with COL would produce better glycemic control than each drug alone in type 2 diabetic rat models.

MATERIALS AND METHODS

Animal studies. Studies were conducted in type 2 diabetic rat model, male ZDF rats. To produce the type 2 diabetic model, fat male ZDF rats were fed Purina special diet no. 5008 (WF Fisher and Son, Bound Brook, NJ). Studies were carried out in male, 5-wk-old (250–325 g) ZDF rats that were purchased from Charles River, Kingston, NY. Two major studies were conducted in the animals, and the subgroups in these two studies were similar except that the dose of SIT (Januvia; Merck Sharp & Dohme, Rome, Italy) mixed in the diet was increased 50% in the second study.

Part 1 of the study consisted of four treatment groups: *1*) fat male ZDF rats fed Purina special diet 5008 as untreated positive controls or the model group (5008, $n = 8$); *2*) fat male ZDF rats fed Purina 5008 containing 0.4% SIT, (120 mg/day, ~200 mg of sitagliptin/kg body wt per day) as the type 2 diabetic model treated with SIT alone (SIT, $n = 8$); *3*) fat male ZDF rats fed Purina 5008 containing 2% COL as the type 2 diabetic model treated with colesevelam alone (COL, $n = 8$); and *4*) fat male ZDF rats fed Purina 5008 containing 2% COL + 0.4% SIT (COL+SIT, $n = 8$) as the type 2 diabetic model treated with the combination of COL+SIT.

In *part 2* of the study, the dose of SIT (Januvia) in the diet was increased 50% to 0.6% (180 mg/day, ~300 mg/kg body wt per day). *Part 2* also consisted of four treatment groups: *1*) fat male ZDF rats fed special diet Purina 5008 (5008, $n = 8$); *2*) fat male ZDF rats fed Purina special diet 5008 containing 0.6% SIT (0.6% SIT, $n = 8$); *3*) fat male ZDF rats fed Purina special diet 5008 containing 2% COL (COL, $n = 8$); and *4*) fat male ZDF rats fed Purina special diet 5008 containing 2% COL + 0.6% SIT (COL+0.6%SIT, $n = 8$). The special rat diets for different treatments (SIT, COL, and COL+SIT) were made by Purina, TestDiet, Richmond, IN. The studies started when the ZDF rats were 6 wk of age. All rats were treated for at least 8 wk, and oral glucose tolerance tests (OGTT) were performed after 4 and 8 wk of treatment, respectively, to evaluate and

compare the effectiveness of treatments on glycemic control. The animals then were killed after 2 days of further treatment. Under anesthesia, pancreas tissues were collected for evaluation of changes in β-cell islets. The contents/soft feces within the lumen of the ileum and ascending colon were collected and weighed, respectively. The mucosa of ileum and ascending colon were collected and frozen immediately in liquid nitrogen for measurement of mRNA expression of TGR5 in the intestine. The animal protocol was approved by the Institutional Animal Care and Use Committee at the Veterans Affairs Medical Center, East Orange, New Jersey.

OGTT. The rats that underwent OGTT were fasted overnight (from 4 PM to 8 AM). Immediately before the feeding of glucose, blood sample was taken via the tail and used as fasting baseline glucose levels (at 0 min). Glucose was then fed to rats at 200 mg/100 g body wt by gavage. Blood specimens were collected from the tail at 15, 30, 60, 90, and 120 min after the initiation of glucose feeding. Changes in plasma levels of glucose, insulin, and bioactive GLP-1 (GLP-1A) were measured.

Postprandial test. To determine the changes in the postprandial response in plasma glucose, insulin, and GLP-1A in these rats after different treatments, postprandial tests were performed 1 wk after the second OGTT (after 9 wk of the treatment). The tested ZDF rats were fasted overnight (4 PM to 8 AM). At 8 AM the rats were fed relevant special diet, respectively, for 2 h and then the diets were removed. The special diets were prepared as such that the powder of daily dose of drug mixed into 1 g of peanut butter (SKIPPY, Alameda, CA), respectively. The daily dose for COL was 0.6 g, and that for SIT was 120 mg. The untreated models were only fed 1 g of peanut butter. Blood samples were collected from the tail immediately prior (at 0) and at 1, 2, 3, and 4 h after the initiation of diet feeding. Thus blood samples at 3 and 4 h reflected the responses/changes in plasma glucose/insulin/GLP-1A 1 or 2 h, respectively, after the meal.

Immunofluorescence staining of β-cell islets. To show and compare the size of β-cell islets in the pancreas, immunofluorescence staining was performed using anti-insulin antibody. The collected rat pancreas tissues were placed in 10% formalin at 4°C overnight. The tissues were then immersed in 30% sucrose in PBS at 4°C for at least 24 h. Serial 16-μm sections were cut on a cryostat. All the pancreatic sections were blocked for 2 h in 0.4% Triton X-100 + 4% goat serum in PBS. Then the sections were incubated with guinea pig polyclonal to insulin antibody (Abcam, Cambridge, MA) diluted (1: 100) in PBS with 0.4% Triton X-100 + 4% goat serum. The incubation was performed at 4°C overnight. After several washes with PBS, the sections were incubated with the secondary antibody Alexa Fluor488-conjugated Donkey Anti-Guinea pig IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted with 1% goat serum (1: 250). The slides were examined under ×10 magnification using a digital camera (model CF2 camera) attached to the ECLIPSE Ti Series microscope (Nikon) and processed with an NIS-Elements BR3.1 Image software program.

TUNEL assay for β-cell apoptosis. To evaluate the degree of cell apoptosis in β-cell islets, the above insulin antibody-stained pancreatic sections (slides before mounting) were further subjected to terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (11). ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, Temecula, CA) was used to detect cell apoptosis (DNA fragmentation) by labeling the terminal end of nucleic acids. The sections were fixed in ethanol: acetic acid (2: 1) at −20°C for 10 min, and, after being washed twice with PBS, they were incubated with equilibration buffer for 10 min at room temperature. The sections were then incubated in a buffer solution with TUNEL at 37°C for 1 h. After the reaction was stopped by PBS, the sections were further incubated with CY-3 Mouse Anti-Digoxin (Jackson ImmunoResearch Laboratories) in PBS solution (1: 200) at room temperature for 1 h. The stained apoptotic cells within the insulin antibody-stained β-cell islet were examined under ×20 magnification using a digital camera (model CF2 camera) attached to the ECLIPSE Ti Series microscope

(Nikon) and processed with an NIS-Elements BR3.1 Image computer program.

Double immunofluorescence staining for insulin and Ki-67. The procedure of this double staining was similar to that described above in *Immunofluorescence staining of β-cell islets* except that another antibody (for staining KI-67) Rabbit mAb to Ki-67 (Abcam) was added simultaneously with the guinea pig insulin antibody, and Dylight 549-Conjugated Donkey Anti-Rabbit IgG (Jackson) was added with the Anti-Guinea Pig IgG in the later procedure.

Measurement of bile acid concentrations. Bile acid concentrations (mM) in portal blood were determined by gas-liquid chromatography (Hewlett-Packard, Palo Alto, CA), which was equipped with a fused silica CP-Sil 5-CB capillary column as described previously (25).

A measured proportion of the weighed total dried intestinal content was used to determine the intestinal bile acid concentrations (mM) using the routine autoclave, gas-liquid chromatography method available at our laboratory, as described previously (1).

Measurement of glucose, insulin, and GLP-1A. Plasma glucose concentrations were determined by Glucose Analyzer (Analox, London, UK). Plasma insulin concentrations were measured by Rat Insulin RIA kit (RI-13K; Millipore).

The concentration of GLP-1A, the biologically active form of GLP-1 (7–36 and 7–37 amide) was determined using GLP-1 RIA kit (GLP1A-35HK; Millipore) according to the instruction of the manufacturer. Briefly, the blood specimens were collected in ice-cooled EDTA-tubes with DPP4 inhibitor. Sample plasma specimens were extracted with 95% ethyl alcohol. After the alcohol was evaporated, the samples were rehydrated. GLP-1A antibody was then added and incubated overnight at 4°C. ^{125}I-GLP-1 was added to label the bound GLP-1A, which was then precipitated with carrier Ig and precipitating reagent. After centrifuging the samples, the ^{125}I-GLP-1A in the pellet was counted using the COBRA II Auto-Gamma.

Measurement of TGR5 mRNA expression. TGR5 expression in the mucosa of ileum and ascending colon was quantitatively determined with qRT-PCR described previously (25). TaqMan Expression Assays (Gpbar 1-Rn 01400316_S1) designed by Applied Biosystems (Foster City, CA) specifically for rat TGR5 was used, and reactions were performed in triplicate in a 7300 Real-Time PCR Systems (Applied Biosystems). The data were calculated by the comparative threshold cycle method using GAPDH as the internal standard. The relative mRNA expression shown in the text is presented as the ratio between the untreated diabetic model (5008) and treated groups.

Statistical analysis. The data reported in figures and text are presented as means ± SE except those listed in tables, which are presented as means ± SD. The statistical significance for multiple comparisons was analyzed with the Bonferroni method. When only two groups were compared, Student's *t*-test was used and would be stated specifically. GraphPad InStat V.3 (GraphPad Software, San Diego, CA) software was used for all statistical comparisons.

RESULTS

OGTT. In *part 1* of the study, SIT was in the diet at 0.4% (120 mg/day). Figure 1*A1* summarizes the results of plasma glucose levels (shown as means ± SE) during the OGTT after different treatments for 4 wk in male fat ZDF rats. The results indicated that, at fasting baseline plasma, glucose levels were significantly ($P < 0.001$) reduced in the COL+SIT combination, and COL alone groups compared with the untreated diabetic rat models (5008). During OGTT, plasma glucose levels became significantly lower ($P < 0.01$) than in the diabetic model group after 60 min in the COL+SIT group and after 90 min in the COL group. Glucose area under the curve (AUC) during OGTT was reduced in the COL+SIT (-40%, $P < 0.001$) and COL (-27%, $P < 0.05$) groups compared with the untreated diabetic model group. The AUC of the COL+SIT
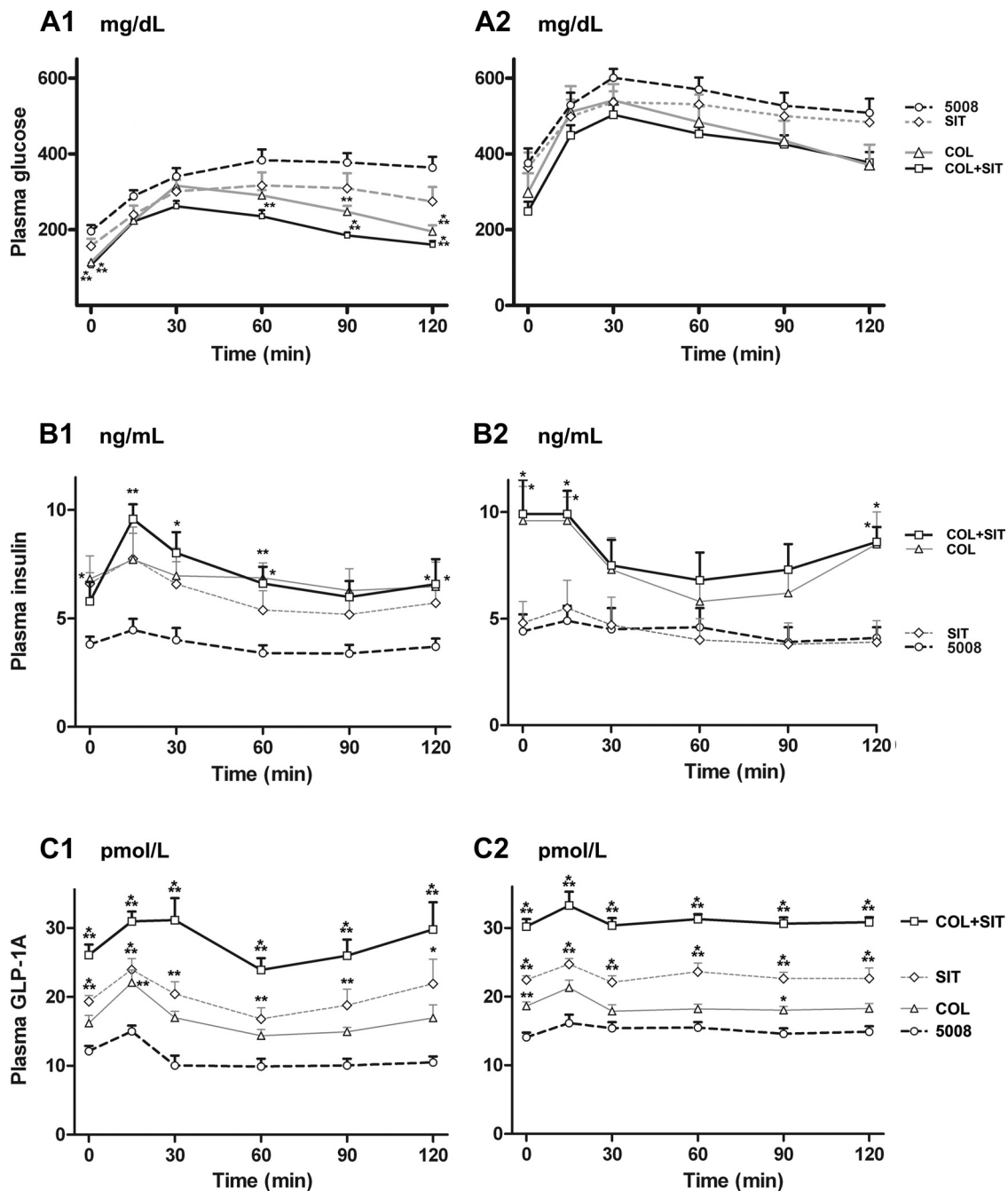
Fig. 1. Results from the blood collected during the oral glucose tolerance test in *part 1*, where sitagliptin (SIT) in the diet was 0.4% (120 mg/day). 5008, untreated diabetic rat model; SIT, treated with 0.4% SIT; COL, treated with 2% COL; COL+SIT, treated with 2% COL + 0.4% SIT. Data ($n = 8$) are represented as means ± SE. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with the untreated diabetic model (5008). *A*: plasma glucose levels. *A1*: after 4 wk; *A2*: after 8 wk of treatment. *B*: plasma insulin levels. *B1*: after 4 wk; *B2*: after 8 wk of treatment. *C*: plasma active form glucagon-like peptide (GLP)-1 (GLP-1A) levels. *C1*: after 4 wk; *C2*: after 8 wk of treatment.

group was significantly smaller ($P < 0.01$) than the COL alone group when only these two groups were compared using Student's *t*-test. There was no significant reduction in glucose levels/AUC during OGTT in the group treated with SIT alone.

After the above treatments were continued in the same animals for 4 additional wk (8 wk of total treatment), glucose levels were increased at each time point (Fig. 1*A2*) compared with 4 wk of treatment (Fig. 1*A1*). Although the mean values of plasma glucose levels were numerically lower in the group

treated with COL+SIT, the difference was not statistically significant between the COL+SIT-treated diabetic model and the untreated diabetic model (5008) as analyzed by multicomparison (Bonferroni method). The fasting glucose level before OGTT in the COL+SIT group was 34% lower (248 ± 72 vs. 375 ± 110 mg/dl) than the untreated diabetic model group.

Figure 1*B1* shows plasma insulin levels in different groups during OGTT after 4 wk of treatment. Insulin AUC increased 90% and 83%, respectively, in the COL+SIT ($P < 0.05$) and

COL ($P < 0.05$) groups compared with the untreated diabetic model group. Although insulin AUC in the SIT group also increased 61%, the increase was not significant compared with the untreated model group. Continuing the treatments for 8 wk, plasma insulin levels in the COL+SIT and COL groups were higher ($P < 0.05$) at 0, 15, and 120 min than the untreated model. Although insulin AUC in these two groups increased 79% and 65%, respectively, the increase was not statistically significant compared with the untreated model group. It should

be pointed out that, different from that after 4 wk of treatment, insulin levels of the SIT group after 8 wk declined to almost the same level of the untreated model group (5008) (Fig. 1*B2*).

Figure 1*C1* shows that, after 4 wk of treatment, plasma levels of the active form of GLP-1 (GLP-1A) increased during OGTT in all three treatment groups compared with the untreated diabetic model group although the increase in the COL group was not as high as in the COL+SIT or SIT groups. The AUC of GLP-1A increased in the COL+SIT (2.5-fold, $P <$
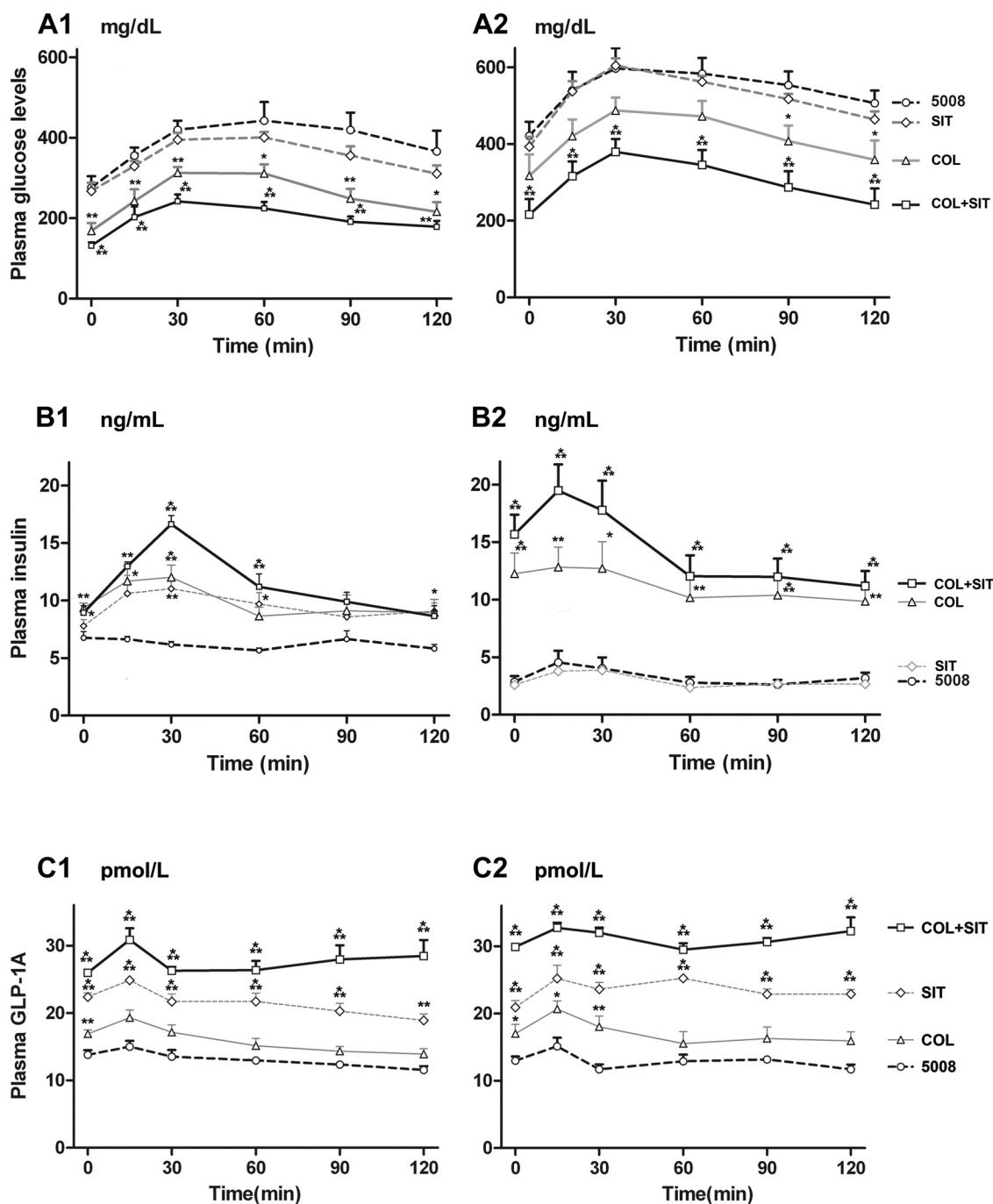

Fig. 2. Results from the blood collected during the oral glucose tolerance test in *part 2*, where SIT in the diet was increased to 0.6% (180 mg/day). SIT, treated with 0.6% SIT; COL, treated with 2% COL; COL+SIT, treated with 2% COL + 0.6% SIT. Data ($n = 8$) are represented as means ± SE. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with the untreated diabetic model (5008). *A*: plasma glucose levels. *A1*: after 4 wk; *A2*: after 8 wk of treatment. *B*: plasma insulin levels. *B1*: after 4 wk; *B2*: after 8 wk of treatment. *C*: plasma active form GLP-1 (GLP-1A) levels. *C1*: after 4 wk; *C2*: after 8 wk of treatment.

0.001), SIT (82%, $P < 0.01$), and COL (53%, $P < 0.05$) groups compared with the untreated diabetic models. Furthermore, the AUC of the combination group (COL+SIT) was significantly larger than the SIT ($P < 0.01$) and COL ($P < 0.001$) alone groups. After 8 wk of treatment, plasma GLP-1A levels remained higher in the treatment groups compared with the untreated model during OGTT. The AUC of GLP-1A was increased twofold in the COL+SIT ($P < 0.001$), 52% in the SIT ($P < 0.001$), and 22% in the COL ($P < 0.01$) group, respectively, compared with the untreated diabetic model. The AUC of GLP-1A in the combination group was also larger than the SIT ($P < 0.001$) and COL ($P < 0.001$) group at this stage.

In *part 2*, SIT in the diet was increased 50% from 0.4% (120 mg/day) to 0.6% (180 mg/day). Similar to that observed in *part 1*, 4 wk of treatment with increased SIT alone did not improve glycemic control (Fig. 2*A1*). Again, glucose levels during OGTT after 4 wk of treatment significantly decreased in the COL+SIT group as well as the COL group (Fig. 2*A1*). Glucose AUC decreased 48% and 33% in the COL+SIT ($P < 0.001$) and COL ($P < 0.01$) groups, respectively, compared with the untreated diabetic model group (5008). The AUC of the COL+SIT group was smaller ($P < 0.05$) than the AUC of the COL group when analyzed by Student's *t*-test. Continuing the treatment for 8 wk, different from that seen in *part 1*, glucose levels during OGTT were reduced significantly in the COL+0.6% SIT group. Glucose AUC in the COL+0.6% SIT was 43% ($P < 0.001$) less than the untreated diabetic model group (5008). However, the glucose levels in the 0.6% SIT group were still not improved (Fig. 2*A2*) compared with that in the 0.4% SIT or the model group although SIT dose increased from 120 mg to 180 mg/day.

As shown in Fig. 2*B1*, after 4 wk of treatment, insulin levels in all three treatment groups were higher than the untreated model group during OGTT. Insulin AUC was significantly larger in the COL+SIT (83%, $P < 0.001$), COL (58%, $P < 0.01$), and SIT (54%, $P < 0.01$) groups, respectively, compared with the untreated model group. After 8 wk of treatment, similar to the 0.4%SIT in *part 1*, insulin levels in the 0.6%SIT group declined to the same level as the untreated diabetic model group during OGTT (Fig. 2*B2*). However, insulin levels in the COL+SIT and COL groups remained higher than the model group. Insulin AUC in these two groups was fourfold (COL+0.6% SIT, $P < 0.001$) and threefold (COL, $P < 0.001$) of that in the untreated model group.

GLP-1A levels were elevated in all three treatment groups (Fig. 2*C1*) after 4 wk of treatment during OGTT although the increase in the COL group was not as much as that in the COL+SIT or SIT groups. AUC of GLP-1A increased twofold ($P < 0.001$) in the COL+ SIT, 64% ($P < 0.001$) in the SIT, and 20% ($P < 0.05$) in the COL group compared with the diabetic model group. After the treatment was continued for 8 wk, GLP-1A levels during OGTT remained elevated in all the three groups (Fig. 2*C2*). The AUC of GLP-1A was increased 2.4-fold ($P < 0.001$) in the combination group, 85% ($P < 0.001$) in the SIT, and 32% ($P < 0.01$) in the COL group, respectively, compared with the untreated diabetic model group.

Postprandial test. Figure 3 summarizes changes in the postprandial plasma glucose, insulin, and GLP-1A levels in the untreated and treated rat diabetic models. During the test, the special diet was provided to the rats only during the first 2 h.
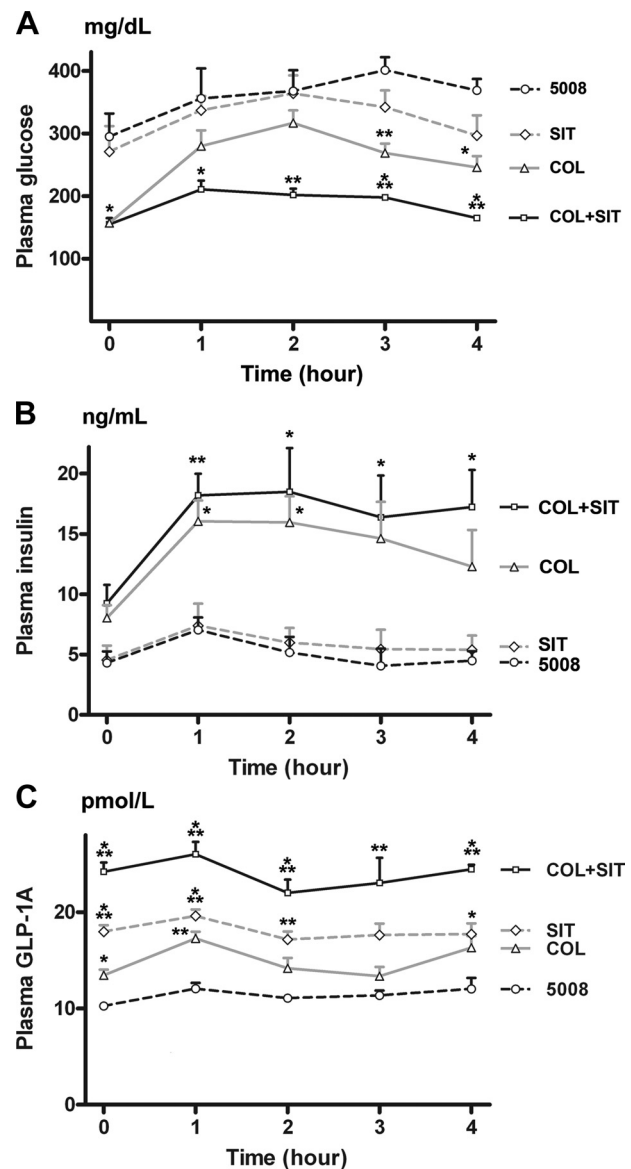

Fig. 3. Postprandial changes in glucose (*A*), insulin (*B*) and GLP-1A (*C*). SIT, treated with 0.4% SIT; COL, treated with 2% COL; COL+SIT, treated with 2% COL + 0.4% SIT. Data ($n = 6$) are represented as means ± SE. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with the untreated diabetic model (5008). The test was conducted in the Zucker diabetic fatty (ZDF) rats after 8 wk of treatment. After overnight fasting, the rats were fed the special diet respectively for only the first 2 h, and then the diets were removed.

The fasting glucose levels in the COL and COL+SIT groups were lower ($P < 0.05$) than the untreated model group (Fig. 3*A*). In the combination group (COL+SIT), glucose levels were significantly lower than the untreated model group at each time point. The AUC of glucose was 47% smaller in the COL+SIT group ($P < 0.01$) compared with the untreated model group (5008). Although in the COL group the AUC of glucose was not significantly decreased, the postprandial glucose levels were 33% lower than the untreated model at 3 h ($P < 0.01$) and 4 h ($P < 0.05$), respectively. That means the glucose levels in the COL group had been significantly lower than the untreated models 1 h (at 3 h) after stopping the diet feeding to the rats. Glucose levels in the SIT group were not significantly reduced after the meal.
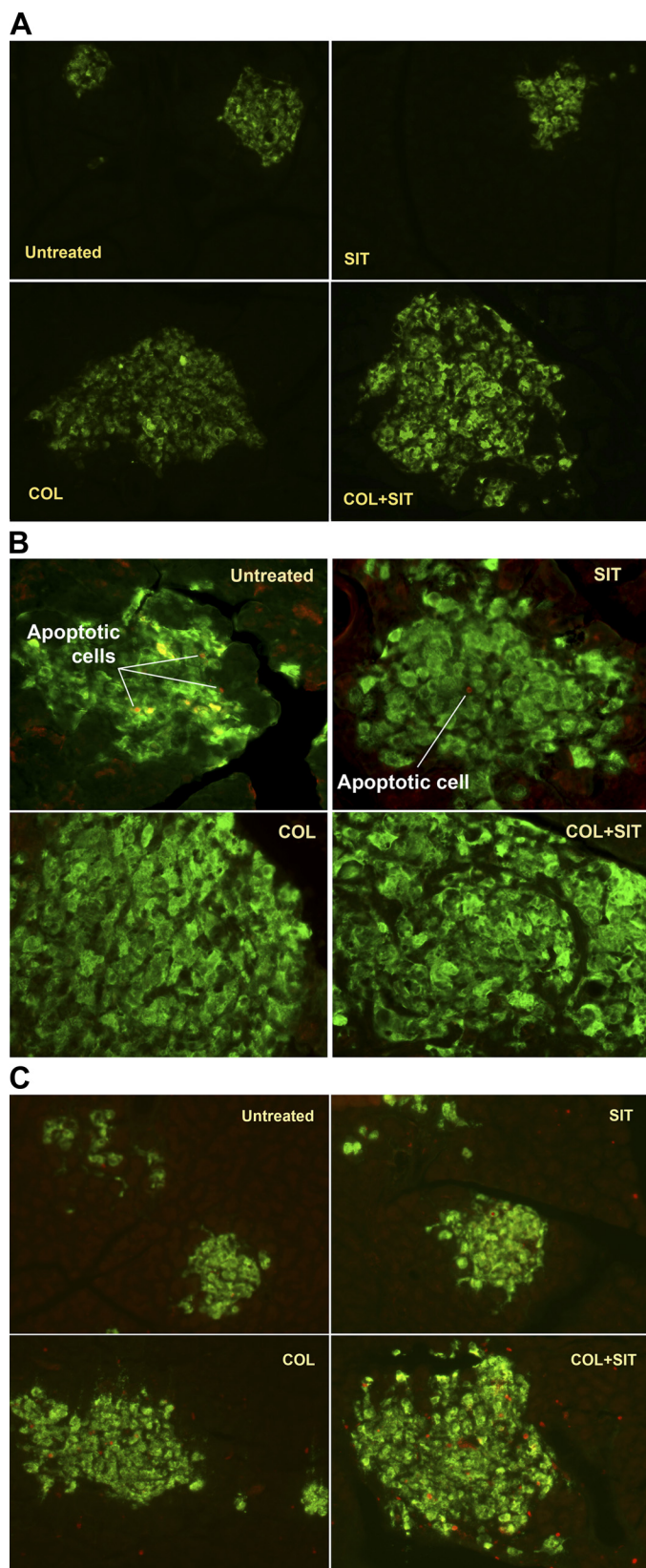
The plasma insulin levels were increased sharply after the meal was given in the COL+SIT and COL groups compared with the model (5008) and did not decline in the combination (COL+SIT) group (Fig. 3*B*). In contrast, insulin levels in the SIT group were similar to the model. The AUC of insulin during the postprandial test was larger in the COL+SIT (+3-fold, $P < 0.01$) and the COL (+2.6-fold, $P < 0.05$) group than the model group.

The fasting GLP-1A levels in the plasma (Fig. 3*C*) were significantly higher in the COL+SIT ($P < 0.001$), SIT ($P < 0.001$), and COL ($P < 0.05$) groups compared with the untreated model group. The AUC of GLP-1A during this test was larger in the COL+SIT (+2-fold, $P < 0.001$), SIT (+62%, $P < 0.001$), and COL (+31%, $P < 0.05$) groups than the untreated model. The AUC of GLP-1A in the combination group (COL+SIT) was also significantly larger than the SIT ($P < 0.01$) and COL ($P < 0.001$) groups.

Size of β-cell islets in pancreas. Figure 4*A* is a set of pancreatic tissue sections with immunofluorescence staining showing the β-cell islet from different treatment groups after 8 wk of treatment. Obviously, in the SIT (120 mg/day, 0.4%) group, β-cell islets labeled with the anti-insulin antibody shrank to the size similar to those in the untreated diabetic model. After 8 wk of treatment, the maximal size of islets in the (0.4%) SIT group was reduced 45% ($P < 0.05$ by *t*-test) as compared that after 4 wk (Table 1). The area size of the β-cell islet in the SIT group was similar to the untreated model group. In contrast, the size of the islet in the COL group and the COL+0.4%SIT group was 2.1-fold ($P < 0.01$) and 2.9-fold ($P < 0.001$) of that in the untreated models after 4 wk of treatment and was 2.6-fold ($P < 0.001$) and 3.4-fold ($P < 0.001$), respectively, after 8 wk. Furthermore, the size of the islet in the COL group and the COL+0.4%SIT group was also larger than the SIT alone group after 4 wk of treatment ($P < 0.05$ and $P < 0.001$) and after 8 wk ($P < 0.01$ and $P < 0.001$), respectively. After 8 wk of treatment, the islet size was not significantly changed in the COL or COL+SIT group as compared those after 4 wk of the same treatment (Table 1). There was no significant difference in the islet size between the combination of COL with 0.4% SIT and that with increased 0.6% SIT.

Evaluation of β-cell apoptosis and proliferation. To compare the status of cell apoptosis, TUNEL assay (11) was employed to stain the apoptotic cells in the islets. We noticed that more apoptotic cells were observed in the untreated model group than the COL+SIT and COL group. There were always several (1 to 3) spots (in red color) of stained apoptotic cells seen in the islet sections from the untreated model group and usually one stained spot in the sections from the SIT alone

Fig. 4. β-cell islets in different study groups after 8 wk of treatment under microscope (Nikon, ECLIPSE Ti Series). Untreated, diabetic ZDF models without treatment; SIT, diabetic ZDF models fed 0.4% SIT; COL, diabetic ZDF models fed 2% COL; COL+SIT, diabetic ZDF models fed 2% COL + 0.4% SIT. *A*: differences in the size of β-cell islet at ×10 (0.465 μm/pixel). β-cells (in green color) are labeled with guinea pig polyclonal to insulin antibody. *B*: evaluation of β-cell apoptosis using TUNEL assay at ×20 (0.232 μm/pixel). The apoptotic cells in the β-cell islet are labeled (red spots) with CY-3 mouse anti-Digoxin. *C*: status of β-cell proliferation in the islet by immunofluorescence staining Ki-67 at ×10 (0.465 μm/pixel). Antigen Ki-67 is labeled by its antibody as well as Dylight 549-Conjugated Donkey Anti-Rabbit IgG such that stained Ki-67 can be seen as red spots.

Table 1. *Area (μm^2) of the β-cell islets in ZDF rats*

	Untreated	0.4%SIT	COL	COL +0.4%SIT	COL +0.6%SIT
4 wk	34320 ± 12854	45458 ± 13214	72734 ± 8774†	99487 ± 19074‡	93226 ± 24458‡
8 wk	25197 ± 11581	25028 ± 10244	66445 ± 11191*	87004 ± 25816‡	98739 ± 34267‡

Data ($n = 8$) are presented as means ± SD (μm^2). The size of islets was calculated using an NIS-Elements BR3.1 Image software program attached to the Nikon microscope at ×10 (0.465 μm/pixel). *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ compared with that in the untreated diabetic model group. ZDF, Zucker diabetic fatty mice; COL, colesevelam HCl; SIT, sitagliptin.

group. However, in the islet sections from the COL+SIT or COL groups, virtually no stained spot of apoptotic cell was observed (Fig. 4*B*).

In the present study, Ki-67 (12) was used as the marker for the β-cell proliferation, as Ki-67 protein expression is strictly correlated to cell proliferation and the active phases of the cell cycle. As shown in Fig. 4*C*, the stained Ki-67 spots (in red color) are much more numerous in the sections from the COL+SIT group than the untreated model or SIT groups. Furthermore, we used the ratio of red-colored Ki-67 intensity (the marker of active cell proliferation) vs. green-colored insulin intensity (the mass of β-cells) as an indicator of the activities of proliferation in the islets. The ratio in the COL+SIT group (0.0165 ± 0.0191, means ± SD) was 9.7-fold ($P < 0.05$) of that in the untreated model group (0.0017 ± 0.0010). Thus the activities of cell proliferation in the islets of the combination group were significantly more than the untreated model. Although the ratios in the COL (0.0104 ± 0.0073) and SIT (0.0034 ± 0024) group were sixfold and twofold of that in the model group, the differences were not statistically significant.

Bile acid concentrations in the intestine. Table 2 summarizes the total bile acid concentrations (mM) in the wet contents collected from the lumen of terminal ileum and ascending colon, respectively. The data indicate that bile acid concentrations in the ileum were not significantly increased in COL-treated diabetic ZDF rat models in either the COL or the COL+SIT groups compared with those in the untreated diabetic model group where the ZDF rats were only fed Purina diet 5008. However, bile acid concentrations in the ascending colon of the untreated diabetic models decreased almost 70% ($P < 0.001$) compared with that in the ileum, whereas the bile acid concentration did not decrease significantly in those treated with COL or COL+SIT. As a result, bile acid concentrations in the colon were higher in the COL ($P < 0.01$) or COL+SIT ($P < 0.001$) groups than the untreated diabetic model group.

TGR5 mRNA expression in the intestine. The mRNA expression of TGR5 in the mucosa of ileum and ascending colon in different treatment groups is summarized in Fig. 5. TGR5 mRNA expression in those treated with COL (the COL or COL+SIT groups) was not increased compared with the un-

treated diabetic model either in the ileum or in the ascending colon. However, in each group, the expression of TGR5 mRNA in the colon was significantly lower than that in the ileum when compared using Student's *t*-test (Fig. 5).

Changes in body weight and chow intake. Table 3 summarized the information on body weight and chow intake in different treatment groups. There was no significant difference observed between the treatment groups either in body weight or chew intake at 4 or 8 wk.

DISCUSSION

The present study demonstrated that COL combined with SIT produced better glycemic control than each single drug in the diabetic ZDF rat model. We believe that this combination enhanced the beneficial effects of COL. SIT extends the half-life of bioactive GLP-1, which is induced by COL. It is known that bioactive GLP-1 reduces insulin resistance and improves glycemic control (17, 7). Our study demonstrated that the combination treatment (COL+SIT) resulted in the highest levels of active GLP-1 and lowest levels of glucose among the four experimental groups. Furthermore, in the combination of COL+0.6%SIT, where the dose of SIT increased 50%, the degradation of active GLP-1 was more strongly inhibited, which resulted in even better glycemic control than the COL+0.4% SIT. The combination of COL with 0.6% SIT reduced plasma glucose levels during the OGTT not only after 4 wk but also after 8 wk (Fig. 2*A2*), whereas COL+0.4% SIT did not (Fig. 1*A2*) compared with the model group. These two findings together support the hypothesis that the combination improved glycemic control by in-

Table 2. *Total bile acid concentrations (mM) in the ileum and ascending colon*

	Untreated	COL	COL+SIT
Ileum	3.66 ± 0.34	3.24 ± 0.71†	4.93 ± 0.89†
Colon	1.14 ± 0.08	3.05 ± 0.30*	3.55 ± 0.50†

Data ($n = 8$) are presented as means ± SD (mM). *$P < 0.01$, †$P < 0.001$ compared with that in the colon of untreated diabetic model group.
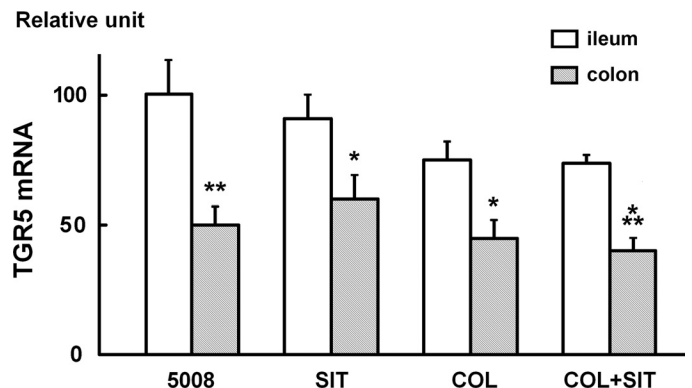

Fig. 5. TGR5 mRNA expression in the mucosa of ileum and ascending colon after 8 wk of treatment. The open bars represent the TGR5 expression in the ileum, whereas the shaded bars represent expression in the colon. The data (relative unit) are shown as means ± SE, the percentage change compared with the value of TGR5 mRNA in the untreated diabetic group in the ileum, which is regarded as 1. The statistical significance is shown as *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ when compared with the mRNA levels in the colon with that in the ileum in the same treatment group using Student's *t*-test.

Table 3. *Body weight and chow intake in different group of ZDF rats*

	Body Weight, g		Chow Intake, g/day	
Treatment	4 wk	8 wk	4 wk	8 wk
Untreated	313 ± 22	415 ± 36	26.8 ± 5.0	32.2 ± 5.7
0.4% SIT	326 ± 17	404 ± 40	27.3 ± 2.9	34.6 ± 4.9
COL	330 ± 21	440 ± 39	29.7 ± 3.3	34.4 ± 5.1
COL +0.4% SIT	313 ± 29	422 ± 30	26.8 ± 3.6	32.8 ± 5.4
COL +0.6% SIT	302 ± 30	407 ± 51	27.2 ± 3.3	30.7 ± 4.4

Data ($n = 8$) are presented as means ± SD.

creasing the bioactive form of GLP-1 in the plasma. We noticed that, in the present study, treatment with either COL alone or the combination of COL+SIT did not significantly reduce the chow intakes and body weights in the ZDF rat model. Therefore, the improvement of glycemic control mentioned above was not attributable to decrease in body fat but rather the increased active GLP-1.

It was unexpected that SIT alone did not show a significant effect on improving glucose levels in this diabetic ZDF rat model. It suggested that SIT may be ineffective in some animal models. However, SIT alone in the present study served as a negative control and suggested that the significant improvement of glycemic control in the combination group is mainly attributable to the beneficial effects of COL.

We noticed that, after 8 wk of treatment in the SIT alone group, either 0.4% (120 mg/day) or 0.6% (180 mg/day) plasma insulin declined to the levels similar to the untreated diabetic model (Figs. 1*B2* and 2*B2*). The diminished insulin levels in the SIT group suggested that at this stage (8 wk) β-cell islets were as severely damaged as in the untreated model group and too exhausted to secrete sufficient insulin in response to glucose and GLP-1 challenges. The marked shrinking of β-cell islets observed in the SIT group after 8 wk (see Fig. 4*A* and Table 1) mirrored the diminished plasma insulin levels. The data explain why, during the OGTT performed after 8 wk, the insulin files (low insulin levels) did not reflect the profiles of GLP-1A (high GLP-1A levels) as seen in Figs. 1 and 2, *B2* and *C2*. It also suggested that diabetes developed in the SIT group as severely as the untreated diabetic model at this stage. It is clear that, with or without colesevelam, treatment makes significant difference in preservation of β-cell islets in this type 2 diabetic rat model. In either the COL alone or COL combined with SIT groups, the size of β-cell islets was always significantly larger than those without COL (the model and SIT alone groups). It seems that, unless the β-cell islets were severely damaged such that plasma insulin levels were significantly reduced, the significant shrinking in the islet size would not be observed. This finding, on the other hand, demonstrates that COL or COL combined with SIT preserves the β-cell islets in this diabetic rat model to delay the development of diabetes and that COL plays an essential role in this improvement. However, it must be emphasized that combination of COL with SIT produced better improvement in glycemic control than COL alone. Present study provided evidence that the reduction of glucose (AUC) either during the OGTT or in the postprandial test was greater in the combination group (COL+SIT) than the COL alone and that the AUC of GLP-1A in the combination group was larger than the COL group, and pro-

liferation in β-cell islets indicated by stained Ki-67 was significantly enhanced in the combination group but not the COL compared with the model group. More importantly, at 8 wk, glycemic control was significantly improved only in the combination group (COL + 180 mg/day SIT) but not in the COL alone group compared with the model group. Again, the above findings further support the hypothesis that the COL-induced GLP-1A is the essential cause responsible for the improvement of glycemic control.

Recent study suggested that infusing bioactive GLP-1 to diabetic ZDF rat models would increase the size (area) of β-cell islets, enhance proliferation of β-cells, and inhibit β-cell apoptosis (5). In the present study, we also found that β-cell apoptosis was reduced in the COL+SIT and COL groups and that proliferation was enhanced in the COL+SIT group compared with the model group. We propose that the preservation of β-cell islets is the result of reduced apoptosis and enhanced proliferation because COL enhances release of GLP-1 and combination of COL with SIT prolongs and increases the bioactivity of the COL induced GLP-1. Consequently, development of type 2 diabetes is slowed in this ZDF rat model, and that should be the major mechanism by which glycemic control is improved.

It is known that activation of TGR5, a bile acid receptor located superficially in the ileum and colon (19, 23), can stimulate L-cells to release GLP-1 (18, 26). In the present study, we carefully observed whether the elevated plasma GLP-1A levels in the COL or COL+SIT groups were caused via activation of TGR5 in the ileum and colon, as COL was expected to diminish intestinal bile acid absorption and increases bile acid concentrations in the intestine. It has been reported that infusing taurocholate solution at a concentration of 10 mM does not increase but 20 mM does increase secretion of GLP-1 from an isolated vascularly perfused rat ileum (8). However, our data show that bile acid concentrations in the ileum were not significantly increased in COL-treated diabetic rat models in either the COL or the COL+SIT groups compared with those in the untreated diabetic model group. In the untreated diabetic model group, bile acid concentrations in the contents collected from the ascending colon decreased 70% ($P < 0.001$) compared with that in the ileum. This decrease was due to the function of ileum where 95% of the bile acids are reabsorbed from the ileal lumen and returned to the liver via portal system. However, because of the effect of COL, which sequesters bile acids in the lumen of intestine to diminish bile acid reabsorption, the bile acid concentration in the colon of the COL-treated diabetic models (the COL or COL+SIT groups) did not decrease and remained at a level significantly higher than that in the untreated diabetic model group. However, total bile acid concentrations inside the lumen of either ileum or colon were all lower than 5 mM in both the untreated diabetic model or COL-treated models (the COL or COL+SIT groups). This finding suggests that the concentrations of bile acids within the lumen of ileum or colon in the ZDF models were all below the threshold required to activate TGR5. It remains to be clarified whether the relatively higher bile acid concentration in the colon of the COL-treated rats would cause activation of TGR5 and stimulate L-cells in the colon to release GLP-1. Thus, regarding the mechanism how COL induces GLP-1 release, we prefer to favor our earlier hypothesis (25) that COL diminishes uptake of fatty acids in the jejunum by

sequestering bile acids, which should have been available to form micelles. Consequently, increased amounts of fatty acids reach L-cells in the ileum to stimulate GLP-1 secretion.

In summary, this study demonstrated that the combination of COL with SIT resulted in better improvement of glycemic control than COL or SIT alone in this ZDF diabetic rat model. This improvement was mainly due to induction of GLP-1 secretion plus reduction of active GLP-1 degradation such that apoptosis was reduced and proliferation enhanced in β-cell islets. We postulate that the combination of COL with SIT or even COL alone delays the development of diabetes in the ZDF rat model by preserving β-cell islets.

AUTHOR CONTRIBUTIONS

Author contributions: Q.S. and G.X. conception and design of research; Q.S., M.K.L., and M.S. performed experiments; Q.S., M.K.L., M.S., and G.X. analyzed data; Q.S., J.J.H., G.E.S., and G.X. interpreted results of experiments; Q.S. and G.X. drafted manuscript; Q.S., J.J.H., G.E.S., and G.X. approved final version of manuscript; G.E.S. and G.X. edited and revised manuscript; G.X. prepared figures.

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