The Effect of Glucose and Glucagon-Like Peptide-1 Stimulation on Insulin Release in the Perfused Pancreas in a Non–Insulin-Dependent Diabetes Mellitus Animal Model

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This study was designed to investigate the effect of glucogon-like peptide-1 (GLP-1) on pancreatic β-cell function in normal, Zucker diabetic fatty (ZDF) rats, a model for non–insulin-dependent diabetes mellitus (NIDDM or type II diabetes) and their heterozygous siblings. Pancreas perfusion and enzyme-linked immunosorbent assay (ELISA) were used to detect the changes in insulin release under fasting and hyperglycemic conditions and following stimulation with GLP-1. Animals from the ZDF/Gmi-*fa* rats (ZDF) were grouped according to age, sex, and phenotype (obese or lean), and compared with LA lean rats. Glucose stimulation (10 mmol/L) in obese rats showed repressed response in insulin release. Glucose plus GLP-1 stimulation caused increased insulin release in all groups. The degree of this response differed between groups: lean > obese; young > adult; female > male. The LA lean control group was most sensitive, while the ZDF overtly diabetic group had the lowest response. In addition, the pulsatile pattern of insulin secretion was suppressed in ZDF rats, especially in obese groups. These results support the hypothesis that GLP-1 can effectively stimulate insulin secretion. Insulin release was defective in ZDF obese rats and could be partially restored with GLP-1. ZDF lean rats also showed suppression of β-cell function and there was a difference in β-cell function related to sex in ZDF strain. This study documents the efficacy of GLP-1 to stimulate insulin release and contributes to our understanding of the pathophysiological mechanisms underlying NIDDM.

NON–INSULIN DEPENDENT diabetes mellitus (NIDDM or type II diabetes) is a heterogeneous disease characterized by insulin resistance, impaired insulin secretion, and/or a change in the insulin secretion pattern. According to Creutzfeldt and Nauck,[1] a functional defect of β cells, rather than failure, occurs in NIDDM. Currently, considerable interest is focused on peptides ("excitants"), that may help improve the secretory function of pancreatic islet cells in NIDDM. A group of hormonal peptides secreted by the intestinal tract called "incretins" are thought to play an important role in the regulation of insulin secretion by the pancreas.[1] Among these incretins is glucagon-like peptide-1 (GLP-1), a recently defined peptide derived from proglucagon.[1] It is secreted from L cells of the distal small intestine and enhances insulin release in a glucose-dependent manner. Recent studies on normal pancreatic cells showed that only a subgroup of pancreatic β cells was sensitive to glucose and that pretreatment of cells with GLP-1 increased the number of glucose-competent β cells.[2] Many data showed potent insulin-releasing activities of GLP-1 infused in patients with type II diabetes.[3-9] However, few experiments have been performed to test directly its effect on pancreatic β-cell function in NIDDM subjects. It is not yet clear if GLP-1 can induce glucose competence in the endocrine pancreas of NIDDM.

It has also been observed that overall insulin secretion in type II diabetes is almost normal[10,11]; nevertheless, the secretion pattern, and the insulin response during glucose infusion, are reduced. In a study with ZDF rats reported by Johnson et al,[12] it was observed that glucose-stimulated insulin secretion was impaired and that this impairment was related to a defect in glucose transporter. Other clinical studies have suggested that the insulin secretion pattern was also defective in nondiabetic first-degree relatives of diabetics.[13] It is not clear whether the defect in insulin release could be altered by short-term or long-term GLP-1 stimulation. In addition, it is not known whether insulin secretion patterns in response to GLP-1 differ significantly between overtly diabetic, prediabetic, and nondiabetic subjects, or whether gender influences pancreatic β-cell function.

If GLP-1 treatment could be shown to improve or normalize pancreatic response to glucose, it would provide a major advantage in the treatment of NIDDM over conventional regimens. If this could be achieved, effective treatment with GLP-1 would reduce complications, eg, hypoglycemia, caused by present medications. Further studies of GLP-1 regulation and function in the pancreas are necessary to determine its effectiveness as a clinical treatment for diabetes, which currently affects more than 4% of the US population.[14]

The purpose of this study was to investigate the effects of GLP-1 on pancreatic β-cell function in normal and NIDDM rats. We used two strains of rats to perform these studies, ZDF/Gmi-*fa* strain (ZDF) and LA/N (LA). We hypothesized that (1) pancreata from ZDF prediabetic (young obese male) rats, perfused with glucose, secrete more insulin in response to GLP-1 than severely diabetic rats; (2) the response of insulin secretion by β cells following GLP-1 stimulation in ZDF rats is sex-related; and (3) ZDF lean rats secrete more insulin in response to GLP-1 stimulation than ZDF obese rats, but less than other strains of control rats, such as LA lean rats, which is the background strain for the nondiabetic obese LA/N-*cp* strain.[15]

ZDF adult obese male rats served as our model of type II diabetes. This model has many of the characteristics of human type II diabetes.[16] The insulin secretion pattern during fasting, hyperglycemic conditions, and GLP-1 (7-36 amide) stimulation were studied. We also used obese female, lean female, lean male ZDF, and lean male LA rats to compare the β-cell function of

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these groups. The data document the pancreatic function under GLP-1 stimulation in amodel of type II diabetes.

MATERIALS AND METHODS

Animals

The ZDF rat strain, a model developed in our laboratory, is widely used to investigate type II diabetes. All of the animals in this inbred strain have the same genotype, except for the fatty locus, where they may be +/+, +/*fa*, or *fa*/*fa*. Animals with the *fa*/*fa* genotype become obese, whereas those +/+ or +/*fa* maintain a lean body phenotype. Obese adult male rats become diabetic between 7 and 10 weeks of age. Female rats, including adult obese females, do not become diabetic when fed normal rat chow. The characteristics of the diabetes seen in male rats correlate closely with those seen in human type II diabetes.

In the following experiments, ZDF rats were grouped into obese and lean. Each group was then subdivided according to age and sex (32 ZDF rats in total were equally divided between groups). Eight normal LA lean male rats served as controls. Average body weights, ages, and glucose levels obtained before surgery are listed in Table 1. Animals were kept under standard conditions with water and Purina 5008 chow (St Louis, MO) available ad libitum.

Isolated Pancreas Perfusion

Surgery. The surgical procedure for preparation of the pancreas for perfusion was a modification of previously described methods.[17,18] Blood was obtained by tail vein before surgery for plasma glucose measurement. Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital solution (50 mg/kg; Nembutal; Abbott, North Chicago, IL). A midline incision of the abdomen was made from approximately 1 cm below the xiphoid to the pelvis. The entire intestine was resected except for about 3 cm of duodenum surrounding the pancreas. The spleen and stomach were then removed following ligation of the esophagus. Vessels were double-ligated in the following order: iliac, iliolumbar, renal, adrenal, and lumbar. Heparin solution, 0.25 mL (1,000 U/mL; Elkins-Sinn, Cherry Hill, NJ),[17] was injected into the jugular vein, and within 2 minutes the inferior vena cava was tied above the renal veins. Perfusate solution was introduced via a polyethylene cannula entering the lower abdominal aorta and the aorta was then ligated just below the diaphragm. The portal vein was cannulated close to the liver for collecting the perfusate. The carcass, with the isolated pancreas, was kept over a water bath and the pancreas temperature was maintained at 38°C.

Pancreatic perfusion. The perfusate was a modified Krebs-Ringer bicarbonate buffered solution (KRB).[18] Polyvinylpyrrolidone (5.8%; Sigma Chemical, St Louis, MO)[19] was dissolved in KRB containing 2.8 mmol/L glucose or 10 mmol/L glucose with or without 100 pmol/L GLP-1 (7-36) amide (GLP-1) (human synthetic; Sigma Chemical).

Physiological concentrations of GLP-1 range from 2 to 50 pmol/L.[20] In preliminary work, several concentrations of GLP-1 were tested (10 pmol/L, 100 pmol/L, and 1,000 pmol/L) and 100 pmol/L was chosen because it demonstrated an optimal insulin secretory response by β cells. This GLP-1 concentration is in the range used by other investigators.[21,22] The solution was bubbled with 95% O_2 and 5% CO_2 and a final pH of 7.3 was reached after oxygen saturation. A three-way valve was used to switch the solutions as needed during the procedure. The flow rate ranged from 1 to 1.5 mL/min and was adjusted by the height of the solution bottles for the entire perfusion period; this was monitored frequently. Samples from the portal vein catheter were collected into plastic tubes kept on ice. The first 40 minutes of each perfusion represented an equilibration period with KRB containing 2.8 mmol/L glucose, and samples were collected at 5-minute intervals after 20 minutes. Following equilibration, the perfusion was switched to KRB containing 10 mmol/L glucose and samples were collected every minute for 10 minutes followed by another 20 minutes of basal perfusion. The perfusate with 10 mmol/L glucose and 100 pmol/L GLP-1 was then administered for 10 minutes and the samples were again collected every minute. Finally, the pancreas was perfused with basal perfusate for 20 minutes. The viability of the preparation was confirmed by the peristaltic movement of the duodenum after the completion of perfusion. Collected samples were frozen and stored at −90°C for later insulin analysis.

Measurement of Rat Insulin

A modification of an enzyme-linked immunosorbent assay (ELISA) was used for insulin measurement.[23,24] In this assay, rat insulin was measured by the direct binding to antibovine insulin antibody (Sigma Chemical) adsorbed to the surface of 96-well plates (Nunc, Roskilde, Denmark). Rat insulin was used as standards (gift from Eli Lilly, Indianapolis, IN). The substrate solution was made by dissolving tetramethylbenzidine (TMB) in 1:9 acetone-methanol to make a TMB concentration of 20 mmol/L (5 mg/mL), then mixing 1 vol of 20 mmol/L TMB with 19 vol substrate buffer and H_2O_2 (5 μL/10 mL) (Sigma Chemical). The optical density was read at 630 nm with a spectrophotometric plate reader (Cambridge Technology, Watertown, MA). The data were analyzed using the Assayzap program (Biosoft, Ferguson, MO). Based on the principle of competitive saturation, standard ELISA curves were obtained from each plate. A typical dose-response curve started at 9.75 fmol/mL rat insulin and was clearly concentration-dependent in the range of standard insulin applied.

Measurement of Glucose

Plasma collected before surgery was measured for glucose using a Beckman Glucose Analyzer II (Fullerton, CA).

Statistics

Since the data were not normally distributed, logarithmic transformation was performed on all data before statistical analysis. ANOVA plus least-significant difference (LSD) tests and two-sample *t* tests were applied for between-group comparisons; $P \leq .05$ was considered significant. Both ratio increase of insulin over the basal levels (mean/base) and net increase of insulin over the basal levels (mean − base) were used for the statistical analyses. Basal level of insulin was determined by averaging the insulin concentrations of the four samples collected in the first 20 minutes (equilibration time). The mean was

Table 1. Age, Body Weights, and Glucose Levels of Rats Used for Pancreas Perfusion Studies

	ZDF Rats								
	Adult Male		Adult Female		Young Male		Young Female		Adult
Variable	Obese (n = 4)	Lean (n = 4)	Obese (n = 4)	Lean (n = 4)	Obese (n = 4)	Lean (n = 4)	Obese (n = 4)	Lean (n = 4)	LA Rats (n = 8)
Age (wk)	22 ± 1	22 ± 1	22 ± 1	22 ± 1	7 ± 1	7 ± 1	7 ± 1	7 ± 1	20 ± 5
Body weight (g)	480 ± 27	361 ± 10	447 ± 33	263 ± 18	232 ± 13	195 ± 1	240 ± 16	181 ± 13	350 ± 85
Glucose (mg/dL)	505 ± 15	114 ± 11	155 ± 8	115 ± 14	121 ± 17	116 ± 9	101 ± 6	100 ± 6	114 ± 7

NOTE. Values are means ± SD.

determined by averaging the insulin concentrations of the 10 samples collected during the 10-minute stimulation with either 10 mmol/L glucose or 10 mmol/L glucose plus GLP-1.

RESULTS

As indicated in Table 1; ZDF adult rats were about 22 weeks in age, while ZDF young rats were 7 weeks of age; LA rats were 20 ± 5 weeks of age. The average body weight listed in Table 1 demonstrates that all of the obese animals were heavier than their matched lean brothers or sisters ($P < .05$ or $P < .0001$, t test). Plasma glucose concentrations obtained before surgery showed that ZDF adult obese male rats were the only hyperglycemic (505 ± 15 mg/dL) group, and the values were significantly higher than in the other groups ($P < .0001$, ANOVA with LSD). This group was considered to be overtly diabetic.

In ZDF nondiabetic lean rat groups, the amount of basal insulin secretion under hypoglycemic conditions (2.8 mmol/L glucose) was similar, despite differences in age and sex. In contrast, basal level insulin secretion in the obese groups (adult, young, male, and female together) was much higher than ZDF lean rats (adult, young, male, and female together) ($P = .003$, t test) (Fig 1).

The degree of insulin release with 10 mmol/L glucose or glucose plus GLP-1 was calculated by (1) net increase, subtracting basal insulin from the averaged insulin during the stimulation (Figs 1 and 2); and (2) ratio increase, dividing the averaged insulin amount by basal insulin (Fig 3). The LA lean control group had a higher ratio increase of insulin in response to 10 mmol/L glucose stimulation than ZDF rats as a whole ($P = .004$, t test)(Fig 3). In the ZDF lean animals, pancreata were sensitive to glucose stimulation; 10 mmol/L glucose caused a three-fold

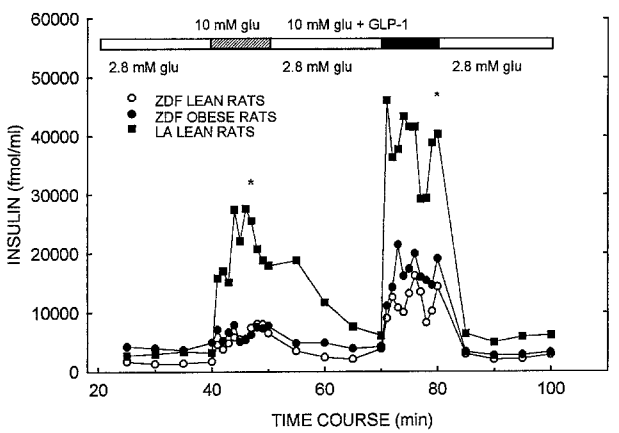

Fig 1. Comparison of insulin release during pancreatic perfusion between ZDF obese, lean, and LA male control rat groups. Each line represents the mean value of insulin release of 4 groups (ZDF obese or lean, including adult male, young male, adult female, and young female). Pancreata were perfused with 2.8 mmol/L glucose before (20 to 40 minutes) and between (51 to 70 minutes and 81 to 100 minutes) stimulations. During stimulations, perfusions were delivered with 10 mmol/L glucose (41 to 50 minutes) and 10 mmol/L glucose + 100 pmol/L GLP-1 (71 to 80 minutes).* (1) Significantly different in insulin release with 10 mmol/L glucose stimulation between LA rats and ZDF rats with obese and lean groups combined ($P = .004$, t test); (2) significantly different in insulin release with 10 mmol/L glucose + 100 pmol/L GLP-1 stimulation between LA rats and ZDF obese rats ($P = .04$, ANOVA with LSD test).

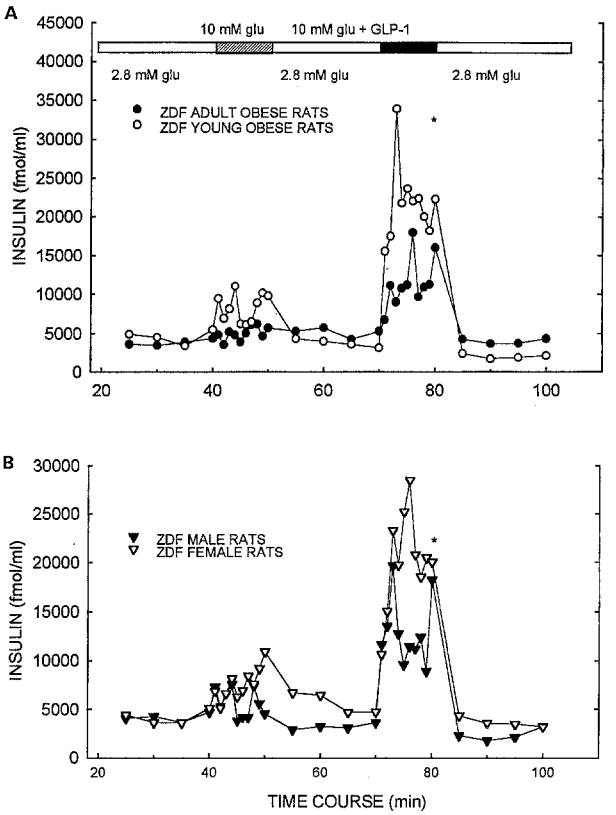

Fig 2. (A) Comparison of insulin release during pancreatic perfusion between ZDF adult obese and ZDF young obese rats. Each line represents the mean of insulin levels in two groups (obese male and female, young or adult) with 8 animals. The time course of the pancreas perfusion is the same as described in Fig 1. *Significantly different in insulin levels with 10 mmol/L glucose + 100 pmol/l GLP-1 stimulation between the 2 groups ($P = .05$, t test). (B) Comparison of insulin release during pancreatic perfusion between ZDF female and ZDF male rats. Each line represents the mean insulin levels in 4 female or male groups with 16 animals. The time course of the pancreas perfusion is the same as in Fig 1). *Significantly different in insulin levels with 10 mmol/L glucose + 100 pmol/L GLP-1 stimulation between the 2 groups ($P = .01$, t test).

to sixfold increase in insulin release over baseline. (Fig 3). On the other hand, the increase in insulin release over baseline in ZDF obese rats (young and old), was decreased compared with their lean siblings (both young and old) ($P = .004$, t test) (Fig 3) and LA lean rats ($P = .04$, ANOVA with LSD) (Figs 1 and 3). The overtly diabetic group (adult obese males) had the lowest ratio increase of insulin in response to 10 mmol/L glucose stimulation ($P = .04$, ANOVA with LSD) (Fig 3). The adult lean male rats (brothers of adult obese male group) had an intermediate ratio increase between the other lean groups and the adult obese male group ($P = .04$, ANOVA and LSD) (Fig 3).

Perfusion with 100 pmol/L GLP-1 in 10 mmol/L glucose caused a prominent net increase of insulin release in both ZDF rats and LA rat groups when compared with stimulation by glucose alone (Figs 1 and 3) ($P < .001$, paired t test). The increase between groups appeared different. In ZDF lean groups, the ratio increase in insulin release was more than the increase in their obese brothers and sisters ($P = .027$, t test) (Fig 3). The overtly diabetic ZDF male rats had only a twofold

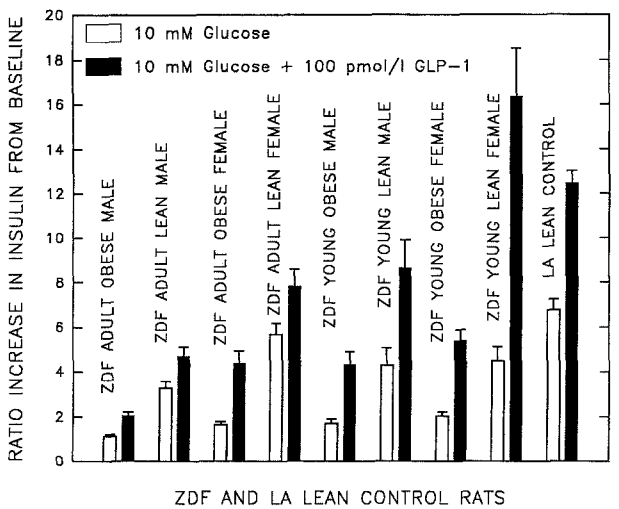

Fig 3. Comparison of the ratio increase in insulin release above baseline between perfusions of 10 mmol/L glucose alone and those containing 10 mmol/L glucose + 100 pmol/L GLP-1 in all experimental rat groups. Each bar represents the ratio increase of averaged insulin amount collected in 10 minutes within a group. Statistical significance is described in the Results.

increase in insulin release over basal levels (Fig 3); this increase was not statistically significant when compared with the increase in 10 mmol/L glucose stimulation alone; this may have reached a significant level with a larger sample size. In the younger male and female obese groups, the insulin increase was approximately fourfold and fivefold, respectively, whereas in the younger male and female lean groups, the increase reached 11- and 14-fold (Fig 3). Except for the latter two groups, the ZDF rats were significantly different from LA lean control rats in their GLP-1–stimulated insulin release over baseline ($P = .04$, ANOVA with LSD). Adult female obese rats had a fourfold increase over baseline, which was close to the increase in the adult male lean group (fivefold increase) (Fig 3). Female ZDF rats as a whole group showed a higher net increase of insulin release than their male brothers ($P = .01$, t test) (Fig 2B). Within the obese groups, the younger rats, male and female, had greater net increases of insulin release than those in the older groups ($P = .05$, t test) (Fig 2A).

DISCUSSION

The results of these experiments support our hypotheses described previously and further demonstrate that GLP-1 can effectively stimulate insulin secretion in the presence of elevated glucose. This was true not only in ZDF nondiabetic lean rats, but also in ZDF obese prediabetic and obese diabetic rats, although the degree of increase was different.

The insulin-stimulatory effect of GLP-1 has been studied in perfused pancreata of several species, including humans.[21,25-28] Until now, no studies have been performed using NIDDM rat models. In this study, we observed that GLP-1 retains its potency in enhancing glucose-stimulated insulin release in all of the experimental animals, including normal, prediabetic, and diabetic rats. As observed in a previous study,[1] the pancreas in NIDDM patients does not fail completely, rather a functional defect exists that prevents it from properly responding to glucose challenges. GLP-1 can work on the defective islet cells, making them more glucose-competent. This activity of GLP-1 contributes to the glucose-stimulated insulin release.[29,30] Increases in insulin release may also be accomplished by inducing proinsulin gene transcription with longer term stimulation with GLP-1.[31-32] In our experiment, diabetic rats (ZDF adult obese male) showed glucose resistance in the pancreas, but when GLP-1 was administered, insulin release increased about twofold. This occurred either because β cells became more glucose-competent and/or because more β cells recovered this function. This physiological change is consistent with the aforementioned molecular level consequence and gives further support to the hypothesis that the pancreatic dysfunction in NIDDM may be a functional defect. The modulatory effects of GLP-1 on β cells makes it a unique agent for stimulating insulin secretion, even in ill-controlled diabetic subjects after common drugs, such as sulfonylurea, have failed to work. Increasing β-cell sensitivity to glucose makes GLP-1 a better agent for regulating blood glucose levels than direct insulin or sulfonylurea administration, since hypoglycemia caused by exogenous insulin or sulfonylurea could be significantly reduced.

While the effect of GLP-1 is strongly supported, the acute GLP-1 effect may have only partially restored pancreatic function in our ZDF animals, including those with diabetes mellitus and their heterozygous siblings. This effect is relative to the severity of the disease, age of the animals, obesity, and sex, as is the defect in pancreatic function. GLP-1 can sensitize the glucose response of β cells, but may not be able to completely rescue defective cells to their former normal condition. Longer term treatment may stimulate and restore the cellular activity, including insulin production and receptor sensitization.

When ZDF and LA adult male lean rats were compared, the LA rat group had a significantly higher insulin response to GLP-1 stimulation. Comparing ZDF lean male rats with the ZDF obese male group, ZDF lean male rats showed greater insulin release from basal levels. ZDF obese animals are homozygous for the *fa*, and the ZDF lean rats used in the study were heterozygous for the *fa* gene, while the LA rats were homozygous for the lean trait. The ZDF rats as a group likely contain some other gene defect along with its *fa* gene, such as those causing leptin receptor defect, which are absent in other control animals like LA lean rats. These genetic abnormalities in ZDF lean rats could result in a partial defect in β-cell function, though not enough to produce hyperglycemia. This may explain our observation in ZDF adult male lean rats, which showed relatively low concentrations of insulin in response to glucose and glucose plus GLP-1 compared with LA control rats. This finding suggests that ZDF lean rats may not be a good lean control animal when ZDF obese male rats are used as models of NIDDM. Similar observations have also been made in humans. Nondiabetic relatives of patients with profound diabetes showed abnormal pulsatile patterns of insulin secretion.[13] The roles of the heterogeneous *fa* gene and other unknown background genes in the development of NIDDM and their influence on β-cell response to stimulation also need to be explored further.

In human NIDDM patients, peripheral insulin resistance and pancreatic defects usually occur together. ZDF obese male rats were originally characterized as having peripheral resistance to

insulin.[33] Our present observations in these animals indicate that the endocrine pancreas is also defective in insulin release in response to either glucose or glucose plus GLP-1 stimulation. Thus, ZDF obese male rats show similar pathophysiological changes to human NIDDM, further validating the use of these animals as a model of type II diabetes. However, there has been confusion about ZDF animals. In the ZDF strain, only ZDF adult obese male rats are overtly diabetic and are used as the model of NIDDM. Other ZDF rats, including lean and female, are not overtly diabetic in their life span and should not be considered as NIDDM animals. However, along with ZDF adult obese male rats, our study showed that they can be served as a whole family strain for the study of NIDDM.

In our ZDF animal model, obese female subjects did not become hyperglycemic even with age, while their obese brothers became very diabetic when fed normal chow.[34] From the present study, we observed that ZDF obese female rats responded to glucose or glucose plus GLP-1 better than ZDF obese male rats. Sex differences in insulin response to a glucose challenge were also reported in humans in the age group of 16 to 29 years.[35] In cases of human hyperinsulinemia, an association between hyperinsulinemia and the risk of cardiovascular disease exists in men, but not in women before menopause.[36] Other studies have shown that in nondiabetic first-degree relatives of NIDDM patients, adult males demonstrated greater intolerance to oral glucose tolerance tests than adult females.[35] These findings appeared to be related to the pattern of body fat distribution or differences in sex hormones and their binding proteins.[37] These factors may also contribute to the differences between ZDF male and female animals. We have also found that diets high in saturated animal fats induce hyperglycemia in ZDF obese female adult rats,[34] indicating that ZDF obese female rats are only less susceptible to NIDDM and that environmental factors could accelerate the pathological change. Further study is necessary to investigate the relationship of fat distribution or sex hormone function to NIDDM.

In conclusion, our experiments further support the hypothesis that GLP-1 can effectively stimulate insulin secretion in the presence of elevated glucose. This was true not only in LA control and ZDF nondiabetic lean rats, but also in ZDF prediabetic and diabetic rats, although the degree of increase was different. More long-term studies are necessary to understand the sustained effect of GLP-1 stimulation. Detailed analysis of the *fa* gene, other background genes, and sex differences would increase our understanding of the pathophysiology of type II diabetes and the mechanism by which GLP-1 affects β-cell function.

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