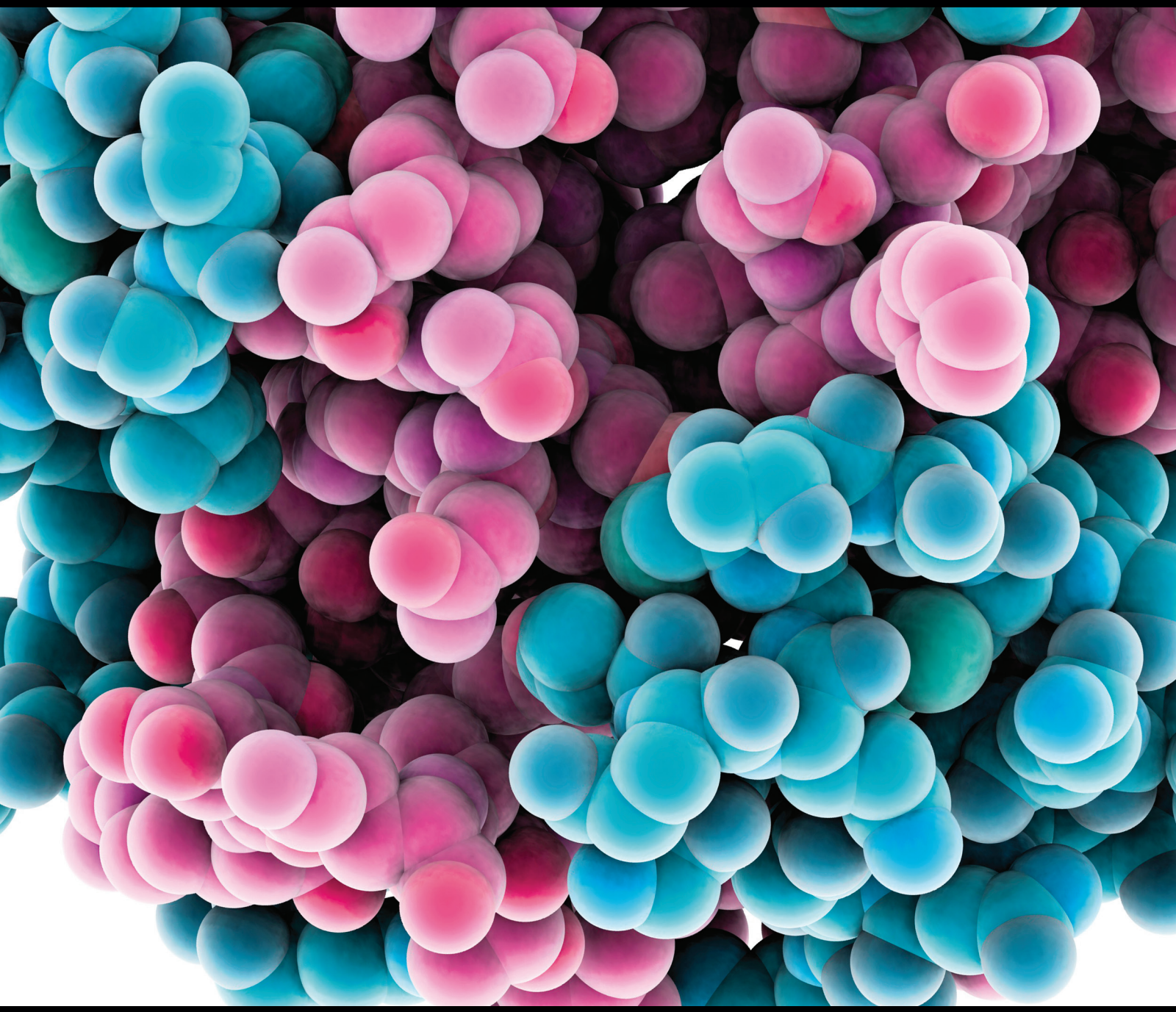
Journal of Diabetes Research

Animal Models of Diabetes and Metabolic Disease 2014

Guest Editors: Tomohiko Sasase, Norihide Yokoi, Marcus G. Pezzolesi, and Masami Shinohara



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Editorial Board

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Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2015, Article ID 571809, 2 pages
http://dx.doi.org/10.1155/2015/571809

Editorial

Animal Models of Diabetes and Metabolic Disease 2014

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Received 17 March 2015; Accepted 17 March 2015

Metabolic diseases include various disorders of metabolism. Diabetes is one of the major types of metabolic diseases and the number of diabetic patients is still increasing worldwide. Complications associated with diabetes, such as retinopathy, peripheral neuropathy, and nephropathy, threaten quality of life (QOL). Diabetes is frequently associated with dyslipidemia, a risk factor for premature atherosclerosis and cardiovascular disease. Hypertension also causes cardiovascular diseases and stroke, and obesity is a known major risk factor for diabetes, cardiovascular diseases, and cancer. Furthermore, these diseases often coincide and closely affect each other.

To study these diseases, numerous hereditary animal models have been developed. Although molecular biological techniques have dramatically improved and have become more important to clarify underlying mechanisms of diseases, the importance of hereditary animal models has not changed. In this special issue we introduce recent beneficial experimental animal models in this field and present up-to-date information on the pathophysiology of and therapeutic drugs for metabolic diseases using valuable animal models. We hope that the 12 articles included in this special issue provide valuable information.

Similar to the previous special issue "Animal Models of Diabetes and Metabolic Disease" published in 2013, several new animal models will be introduced in this special issue. Four papers will present new diabetic animal models, the Spontaneously Diabetic Torii (SDT) rat and its derivative, the SDT fatty rat. The SDT rat, a nonobese type 2 diabetes (T2D) model, exhibits severe hyperglycemia associated with hypoinsulinemia and severe diabetic microvascular complications. F. Toyoda et al. evaluated the effect of administration of ranirestat, a novel aldose reductase inhibitor currently in clinical trials, on diabetic retinopathy and diabetic peripheral neuropathy and reported their findings in "Effect of Ranirestat, a New Aldose Reductase Inhibitor, on Diabetic Retinopathy in SDT Rats." The SDT fatty rat is an obese T2D model established by introducing the *fa* allele in Zucker fatty rats into the SDT rat genome. Several articles introducing this new animal model were presented in the previous special issue. The discoverers of this model contribute additional information in this special issue. T. Ohta et al. mention gender differences in SDT fatty rats in their review article "Gender Differences in Metabolic Disorders and Related Diseases in Spontaneously Diabetic Torii-*Lepr*^{fa} Rats." Pathological perceptions regarding ocular inflammation in SDT fatty rats are reported in "Ocular Inflammation in Uveal Tract in Aged Obese Type 2 Diabetic Rats (Spontaneously Diabetic Torii Fatty Rats)" by Y. Kemmochi et al. The authors point out that this animal model has the potential for spontaneous uveitis. In the article entitled "Effects of Unilateral Nephrectomy on Renal Function in Male Spontaneously Diabetic Torii Fatty Rats: A Novel Obese Type 2 Diabetic Model," Y. Katsuda et al. report findings from their investigation of the effect of nephrectomy on renal function and morphology in SDT fatty rats. These papers clearly show that the SDT fatty rat

is useful in investigations to elucidate the pathogenesis of human diabetic microvascular complications.

In "Characterization of the Prediabetic State in a Novel Rat Model of Type 2 Diabetes, the ZFDM Rat," G. Gheni et al. investigate the phenotypic characterization of a new obese T2D model, the Zucker fatty diabetes mellitus (ZFDM) rat. The authors also characterize insulin secretory responses to both glucose and GLP-1 stimulation in the isolated pancreatic islets. In addition to severe insulin resistance and diminished insulin response to incretin, the fragility of islets is related to the development of T2D in this animal model.

Animal models that show obesity, metabolic syndromes, and diabetes (e.g., the ZF rat, ZDF rat, SDT fatty rat, *ob/ob* mice, and *db/db* mice) exhibit mutations in the leptin pathway. However, leptin pathway mutations are uncommon in the human population. The ZDSD/Pco (ZDSD) rat exhibits polygenic obesity and diabetes without defects observed in the leptin pathway. R. G. Peterson et al. report the characteristics of the ZDSD rat in "Characterization of the ZDSD Rat: A Translational Model for the Study of Metabolic Syndrome and Type 2 Diabetes." This new animal model may become a novel translational animal model for the study of human metabolic diseases and T2D.

Two pharmacological compounds effective against T2D and obesity were evaluated using diet-induced obesity (DIO)/diabetes or hereditary diabetic models in three articles. JTT-130 is a new intestine-specific microsomal triglyceride transfer protein (MTP) inhibitor expected to become a treatment for dyslipidemia, obesity, and diabetes. In the article "JTT-130, a Novel Intestine-Specific Inhibitor of Microsomal Triglyceride Transfer Protein, Reduces Food Preference for Fat," Y. Mera et al. showed that JTT-130 specifically decreases total caloric intake by reducing the preference for fat and reducing body weight. S. Sakata et al. administered JTT-130 and pioglitazone to ZDF rats to investigate the effects of these drugs on glucose and lipid metabolism in the article "Combination Therapy of an Intestine-Specific Inhibitor of Microsomal Triglyceride Transfer Protein and Peroxisome Proliferator-Activated Receptor γ Agonist in Diabetic Rat." Combination treatment with JTT-130 and pioglitazone resulted in intense glycemic control, strong hypolipidemic action, and an improvement in insulin sensitivity. These two articles suggest the possibility of using MTP inhibitors for the treatment of T2D associated with obesity and insulin resistance. JTT-551 is a new protein tyrosine phosphatase 1B (PTP1B) inhibitor and is a negative regulator of leptin signaling as well as insulin signaling that improves glucose metabolism by enhancing insulin signaling. In the article "Pharmacological Effects of JTT-551, a Novel Protein Tyrosine Phosphatase 1B Inhibitor, in Diet-Induced Obesity Mice," M. Ito et al. showed that the antiobesity effects of JTT-551 may be due to the enhancement of leptin signaling and that the compound may be useful in the treatment of T2D and obesity.

Gestational diabetes and macrosomia that cause adulthood obesity are associated with several metabolic disorders. Beneficial effects of ω-3 fatty acids on cardiovascular diseases, as well as diabetes and obesity, have been proposed. In the review article "Beneficial Effects of Omega-3 Polyunsaturated Fatty Acids in Gestational Diabetes: Consequences in Macrosomia and Adulthood Obesity," A. Yessoufou et al. summarized the effects of ω-3 PUFA, such as lowering high rates of macrosomia induced by diabetic pregnancy, reducing blood triglyceride levels, and protecting against oxidative stress. Based on these pharmacological effects, ω-3 PUFA is expected to become a treatment for gestational diabetes and inflammatory and immune diseases.

C1q/TNF-related protein-3 (CTRP3) is an adipokine that suppresses hepatic gluconeogenesis, thereby lowering blood glucose levels. X. Li et al. used high-fat diets plus a low-dose STZ-induced T2D model to investigate the expression of CTRP3 in a T2D model and to investigate the effects of GLP-1 receptor agonists in "Expression of CTRP3, a Novel Adipokine, in Rats at Different Pathogenic Stages of Type 2 Diabetes Mellitus and the Impacts of GLP-1 Receptor Agonist on It." As highlighted in this article, decreased CTRP3 levels in diabetic visceral adipose tissue and insulin sensitivity improved with GLP-1 receptor agonist exendin-4 administration.

Roux-en-Y gastric bypass (RYGB) is a common bariatric operation to reduce body weight and treat T2D in obese patients. To evaluate the potential mechanisms of bariatric surgery, a large animal model with anatomical similarities that mimics human metabolic diseases is helpful. In the article "Evaluating the Mechanisms of Improved Glucose Homeostasis after Bariatric Surgery in Ossabaw Miniature Swine" by J. G. Sham et al., Ossabaw miniature swine are introduced as an animal model that mimics human metabolic syndromes to elucidate RYGB's influence on glucose homeostasis.

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Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2015, Article ID 487816, 10 pages
http://dx.doi.org/10.1155/2015/487816

Research Article

Characterization of the ZDSD Rat: A Translational Model for the Study of Metabolic Syndrome and Type 2 Diabetes

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Received 30 June 2014; Revised 5 September 2014; Accepted 16 September 2014

Academic Editor: Norihide Yokoi

Metabolic syndrome and T2D produce significant health and economic issues. Many available animal models have monogenic leptin pathway mutations that are absent in the human population. Development of the ZDSD rat model was undertaken to produce a model that expresses polygenic obesity and diabetes with an intact leptin pathway. A lean ZDF rat with the propensity for beta-cell failure was crossed with a polygenetically obese Crl:CD (SD) rat. Offspring were selectively inbred for obesity and diabetes for >30 generations. In the current study, ZDSD rats were followed for 6 months; routine clinical metabolic endpoints were included throughout the study. In the prediabetic metabolic syndrome phase, ZDSD rats exhibited obesity with increased body fat, hyperglycemia, insulin resistance, dyslipidemia, glucose intolerance, and elevated HbA1c. As disease progressed to overt diabetes, ZDSD rats demonstrated elevated glucose levels, abnormal oral glucose tolerance, increases in HbA1c levels, reductions in body weight, increased insulin resistance with decreasing insulin levels, and dyslipidemia. The ZDSD rat develops prediabetic metabolic syndrome and T2D in a manner that mirrors the development of metabolic syndrome and T2D in humans. ZDSD rats will provide a novel, translational animal model for the study of human metabolic diseases and for the development of new therapies.

1. Introduction

Metabolic syndrome affects a significant proportion of the population and is becoming increasingly more prevalent in adolescents [1–4]. The syndrome embodies many components including central obesity, insulin resistance, dyslipidemia, and hypertension [3, 5]. In addition, the syndrome features a chronic low grade inflammatory state, vascular endothelial dysfunction, and a prothrombotic environment [3]. Long standing metabolic syndrome predisposes an individual to type 2 diabetes (T2D), atherosclerosis, microvasculature disease (of retina), stroke, renal injury, and neuropathy [5]. Due to the complicated mechanisms involved in the syndrome and its sequelae, current clinical standard of care embodies polypharmacological therapeutics aimed at controlling atherogenic dyslipidemia, hyperglycemia, and hypertension as well as intervening in secondary conditions such as renal dysfunction, stroke, and microvascular disease related to retinopathy. Development of new chemical entities with the potential to control more than one risk factor is hampered by the paucity of highly translational animal models.

The most frequently used rat and mouse models for obesity, metabolic syndrome, and T2D have defects in the leptin pathway. The Zucker diabetic fatty (ZDF) has been a gold standard for this disease complex and as such has many of the characteristics of the human condition but it becomes obese due a leptin receptor defect and becomes diabetic before the animals are mature. To circumvent these ZDF complications, the ZDSD/Pco (ZDSD) rat has been developed to be a more translational model of obesity, metabolic syndrome, and diabetes and to aid the study of these conditions and the development of drugs that could assist in control and treatment. The model was developed by crossing a homozygous lean Zucker diabetic fatty (ZDF) male rat with a substrain of

the Crl:CD (SD) rats that had been selectively bred for high fat diet induced obesity [6, 7]. The standard Crl:CD (SD) rat is a substrain of SD rats that is significantly heavier and more obese than other lines of SD rats; a percentage of these rats is very susceptible to develop obesity, fed high fat diets [6, 7]. The original design was to combine the defect in β-cell gene transcription that is found in lean and obese ZDF rats [8] with the obesity of the Crl:CD (SD) model to produce an obese diabetic model that preserves the critical leptin pathway. The animals were fed regular rodent chow (Purina 5008) during the 12 years of the model development process. The offspring from the initial crosses were screened and selected for obesity, the propensity to become diabetic and the expression of the other characteristics of metabolic syndrome. This model has been selectively inbred for >30 generations.

In the current study, ZDSD rats were followed for 6 months; clinically relevant metabolic endpoints were included throughout the study to extensively characterize the phenotype and development of metabolic disease. The authors believe that the ZDSD rat displays a phenotype with close similarity to metabolic syndrome/T2D in humans further representing a "one rat, many models" tool that will enable the study of early metabolic dysregulation, overt diabetes, and late stage complications of T2D in one rat. It is the sum of the presence of all the characteristics of the human prediabetic state, T2D, and the growing number of associated comorbidities that creates the belief that the ZDSD rat is highly translational to the human condition. It is our belief that this model will reduce development and clinical costs and facilitate discovery of new agents with potential to impact multiple components of the disease. This study was designed to follow a cohort of male ZDSD rats from early in life and as they progress through obesity/metabolic syndrome into full T2D with beta cell failure. The study objectives included comparison of the model's disease progression to the development of diabetes in the human condition. This paper will be followed by a publication that will use these pieces of data to compare this progression in the ZDSD rat to what occurs in the human condition using a model similar to a published paper [9].

2. Research Design and Methods

2.1. Animals. Twenty-four male ZDSD rats (ZDSD/Pco) of comparable body weight were sourced at 6 weeks of age for the study (PreClinOmics Inc., Indianapolis, IN, USA). Animals were housed 2 per cage, fed Purina 5008 chow, and given water *ad libitum* for the duration of the study unless otherwise noted.

2.2. Definitions of Diabetic State. The definition of the permanent onset of diabetes in this model is quite simple, two subsequent weekly morning glucose readings of over 250 mg/dL. When this occurs, the animals will consistently continue to remain hyperglycemic and continue to get more overtly diabetic. For the purposes of this publication, three metabolic states are defined and used to describe the progressive disease states of the model that are typically used in describing the human conditions: (1) metabolic syndrome/prediabetic hyperglycemia: fed/fasted glucose levels above 125 mg/dL, 50% increase in glucose AUC in the OGTT and a 25% increase in HbA1c; (2) diabetic: fed glucose levels above 250 mg/dL, >100% increase in HbA1c and a >200% increase in AUC in the OGTT; and (3) overt diabetes: equaling or exceeding values in (2) above decreases in insulin levels and weight loss. Depending on the methodology used for glucose measurements in an experiment, the actual glucose levels may vary.

2.3. Assays. Body weight was recorded and whole blood was collected every 2 weeks at 6 a.m. for purposes of obtaining fed analyte values. The whole blood (nonfasted samples) was collected by tail clip at 6 a.m. between 7–31 weeks of age for assessment of fed glucose levels using StatStrips (StatStrip Xpress Glucometer, Nova Biomedical, Waltham, MA, USA). Animals were then fasted for 6 hours and a sample of lithium heparin anticoagulated blood (100–150 μL) was taken from a second tail clip. HbA1c was measured in 20 μL of whole blood prior to processing to plasma (AU480; Beckman Coulter, Brea, CA, USA). Glucose, triglycerides, and cholesterol were assayed on fresh plasma samples using a clinical chemistry analyzer (AU480; Beckman Coulter, Brea, CA, USA). Oral glucose tolerance tests (OGTTs) were performed monthly after a 6-hour fast using a glucose load (2 g/kg, p.o.) and sampling from the tail at 0 (prior to glucose administration), 15, 30, 60, and 120 minutes postglucose load. The 7-week OGTT was done using blood glucose (StatStrip) methodology; plasma insulin levels were assayed at each time point of the OGTT. For subsequent OGTTs, glucose was determined from fresh plasma on the AU480 and insulin levels were determined from frozen plasma using a rat/mouse Meso Scale Discovery kit (K152BZC, Rockville, MD, USA). To insure that fasting did not negatively affect the phenotype, the fasts were limited to 6 hours (6:00 a.m.–12:00 p.m.) every two weeks.

2.4. Statistical Analysis. Data are represented as mean ± SEM. All data analysis was accomplished using JMP Statistical Software (SAS Institute). The effect of age on measured parameters (body weight, triglycerides, Cholesterol, HbA1c, NEFA, glucose, glucose AUC, insulin, and insulin AUC) was determined using one-way ANOVA with repeated measures ($P < 0.05$) and when necessary Tukey's multirange t-test was performed ($P < 0.05$). The effect of age on the 5-point oral glucose tolerance tests (OGTT) was determined using two-way ANOVA with repeated measures ($P < 0.05$). A Mahalanobis distance plot and T^2 statistic were used to identify outliers based on average fed glucose levels over the course of the study. Based on this analysis, one animal was retrospectively eliminated from analysis. A technical issue regarding the collection and processing of fasted blood samples was noted at the 17-week time point. This necessitated elimination of these samples (glucose, triglycerides, cholesterol, and insulin) from analysis. Fasting plasma glucose and insulin levels were used to calculate the homeostasis model assessment of β-cell function (HOMA-β) and insulin sensitivity index (ISI). HOMA-β was calculated as [20 × fasting

Table 1: Profile of spontaneous hyperglycemia in individual ZDSD rats (6:00 a.m. fed BG levels, mg/dL).

Animal ID	7 weeks	9 weeks	11 weeks	13 weeks	15 weeks	17 weeks	19 weeks	21 weeks	23 weeks	25 weeks	27 weeks	29 weeks	31 weeks
1	121	**130**	**133**	**149**	**140**	112	**163**	**230**	*270*	*375*	*472*	*487*	*527*
2	111	**130**	120	**133**	124	**129**	113	**129**	**172**	**234**	*407*	*487*	*505*
3	**139**	**146**	**128**	**158**	123	**138**	**136**	**237**	*296*	*341*	*406*	*443*	*494*
4	**133**	**125**	124	**137**	**133**	**160**	**133**	*313*	*367*	*405*	*482*	*537*	*531*
6	**134**	**127**	116	**151**	**144**	**140**	**140**	**172**	**180**	*324*	*456*	*463*	*483*
7	**140**	**131**	**133**	**146**	**158**	**197**	**153**	*438*	*472*	*502*	*535*	*523*	*531*
8	114	**131**	106	**131**	**125**	**147**	**198**	*301*	*378*	*408*	*489*	*495*	*545*
9	122	**144**	119	**152**	**160**	*292*	*395*	*394*	*514*	*541*	*535*	*593*	*564*
10	**131**	**154**	120	**162**	**169**	*265*	*322*	*404*	*490*	*451*	*496*	*592*	*524*
11	**133**	123	**140**	**168**	*326*	*405*	*442*	*511*	*555*	*544*	*545*	*536*	*592*
12	112	123	118	**141**	**147**	**207**	**247**	*411*	*446*	*461*	*501*	*566*	*621*
13	**129**	**138**	**134**	**146**	**148**	*265*	*366*	*453*	*438*	*507*	*600*	*502*	*572*
14	**141**	**143**	**128**	**157**	**171**	**199**	*325*	*386*	*447*	*493*	*509*	*540*	*514*
15	**133**	**126**	**128**	**134**	**141**	*278*	*365*	*436*	*507*	*544*	*578*	*526*	*584*
16	114	**135**	**129**	**148**	**148**	**178**	**142**	97	**210**	*322*	*473*	*427*	*465*
17	118	117	108	**138**	**135**	**149**	**132**	**169**	**144**	*371*	*472*	*457*	*483*
18	**140**	123	116	**133**	**153**	**176**	**219**	**140**	**235**	121	*393*	*391*	*515*
19	**129**	**131**	115	**141**	**139**	**158**	*287*	*363*	*423*	*451*	*525*	*511*	*549*
20	107	**142**	**126**	**188**	**162**	107	*284*	*379*	*416*	*463*	*487*	*541*	*531*
21	124	**141**	112	**143**	108	114	**138**	**189**	*410*	*424*	*458*	*472*	*511*
22	**136**	**129**	**140**	**133**	116	120	**128**	**225**	**231**	*295*	*427*	*454*	*457*
23	123	**129**	**125**	**138**	**159**	**132**	113	124	**221**	**180**	**233**	*303*	*407*
24	**146**	**131**	116	**140**	118	**207**	*286*	*397*	*508*	*494*	*525*	*575*	*635*

Values are represented as different fonts as each animal becomes progressively hyperglycemic. Normal represents relatively normoglycemic, bold represents hyperglycemic (125–249 mg/dL), and italic represents diabetic and overtly diabetic (>250 mg/dL).

insulin (μU/mL)/fasting glucose (mmol/L) − 3.5]. Insulin sensitivity was calculated from the OGTT using Matsuda index (1,000,000/square root fasting glucose × fasting insulin × glucose AUC × insulin AUC). Homeostatic assessment of insulin resistance (HOMA IR) was calculated using fasting glucose (mmol/L) × fasting insulin (μU/mL)/22.5 [10, 11].

3. Results

3.1. Body Weight. Male ZDSD rats weighed on average 221.9± 2.0 g at 7 weeks of age as study observations began. Body weights reached a plateau and peaked at 23 weeks of age (564.4 ± 8.2 g). Thereafter there was a significant ($P < 0.0001$) decline (8.2% from the peak) in body weight over the next 8 weeks (Figure 1).

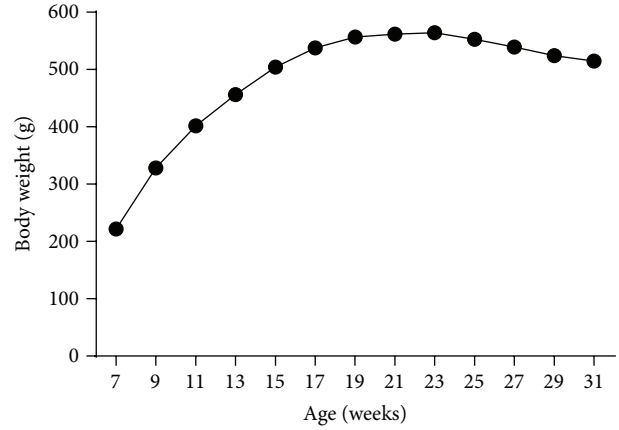

Figure 1: Body weight recorded biweekly throughout the study duration of 7- to 31-week-old ZDSD rats (mean ± SEM, $n = 23$, error bars included too small to see).

3.2. Glucose. Blood glucose (BG) values in the fed state were obtained from whole blood using StatStrips. BG in male ZDSD rats progressed significantly ($P < 0.0001$) from pre-diabetic hyperglycemia to diabetic levels with age (Table 1). Most animals (56.5%) were hyperglycemic as early as 7 weeks of age with a mean fed BG of 127.4 ± 2.3 mg/dL. BG levels rose steadily, reaching 227.3 ± 21.7 and 299.91 ± 26.4 mg/dL at 19 and 21 weeks, respectively, such that most animals (56.5%) could be classified as diabetic by 21 weeks. BG levels continued to increase such that 100% of animals were diabetic by 27 weeks of age (478.4 ± 15.6 mg/dL). BG levels rose to 527.8 ± 11.0 mg/dL at 31 weeks of age. Similarly,

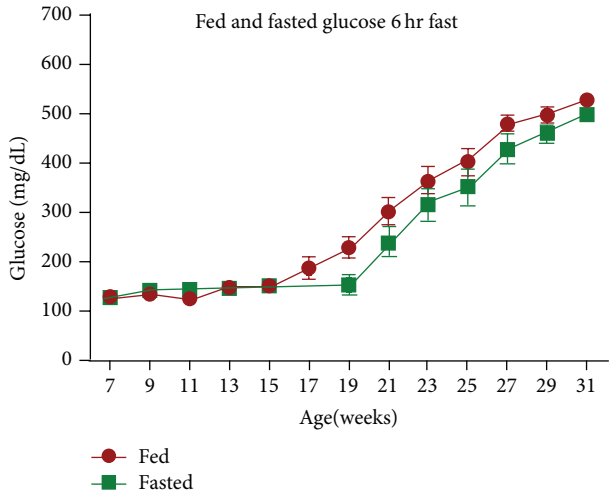

Figure 2: Glucose levels were taken biweekly in fed (●) (BG) or fasted (■) plasma glucose ZDSD rats throughout the study duration of rats in ages 7 to 31 weeks (mean ± SEM, $n = 23$).

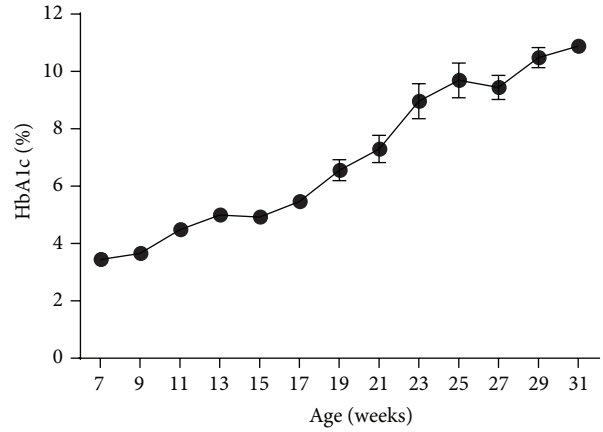

Figure 3: HbA1c levels in male ZDSD rats throughout the study duration of rats in ages from 7 to 31 weeks (mean ± SEM, $n = 23$).

mild hyperglycemia in the fasted state was also observed in animals as young as 7 weeks of age (125.7±2.1 mg/dL). Fasted plasma glucose levels remained relatively stable up to 19 weeks of age. Then significant ($P < 0.0036$) increases were noted (151.65 ± 15.4 versus 236.8 ± 27.6 mg/dL at 19 and 21 weeks, resp.). Fasted plasma glucose values progressed steadily to 498.1 ± 10.0 mg/dL in 31-week-old animals (Figure 2). Since fed blood glucose (BG) and fasted plasma glucose were taken using different methods, statistical comparison was not appropriate. Comparison of these two methods of glucose determination demonstrated that the BG levels run about 88% of the plasma glucose levels. This would effectively make the difference between fed BG and fasted plasma glucose significantly greater than what is shown in Figures 2 and 11. All animals that were analyzed in this study were overtly diabetic at the end of the study (as defined in methods above).

3.3. Glycated Hemoglobin. Chronic progressive hyperglycemia with an average fed BG level of 281.5 ± 11.1 mg/dL was confirmed as evidenced by a significant ($P < 0.0001$) increase in glycated hemoglobin (HbA1c) levels taken throughout the study period. HbA1c values in 7-week-old CRL:CD (SD) control rats ranged from 3.35 to 3.60 and remained relatively stable throughout their life span (data not shown). HbA1c levels in 7-week-old male ZDSD (3.45 ± 0.03) rats were the same as those of the control animals (3.44 ± 0.03%). Despite relatively steady morning glucose levels between 7 and 15 weeks of age (Figure 2), the HbA1c levels increased by 42% from baseline values during this period (3.45 ± 0.03 versus 4.92 ± 0.05%) suggesting that there likely were unseen hyperglycemic excursions during this period (see discussion on pm glucose levels). Thereafter, HbA1c levels rose rapidly, reaching 10.88 ± 0.23% (Figure 3).

3.4. Glucose Levels during Oral Glucose Tolerance Tests. Oral glucose tolerance tests (OGTTs) were performed every four weeks during the experiment. For clarity purposes, only data at eight-week intervals are presented (Figure 4(a)). Significant abnormalities in glucose disposal were observed in aging ZDSD rats during performance of oral glucose tolerance tests. Glucose values taken just prior to glucose load (time zero) increased significantly ($P < 0.0001$) over time (124.9 ± 2.1, 149.4 ± 2.4, 314.7 ± 34.0, and 498.1 ± 10.0 mg/dL for 7, 15, 23, and 31 weeks, resp.). Similarly, the peak glucose excursion and the glucose levels 2 hours after administration of glucose load rose dramatically as animals aged, creating a significant ($P < 0.0001$) age-dependent increase in the area under the curve ($\times 10^{-3}$ for glucose disposal, 18.4 ± 0.2, 33.5 ± 0.4, 58.5 ± 4.2, and 72.1 ± 2.4 for 7, 15, 23, and 31 weeks, resp.) (Figures 4(a) and 4(b)).

3.5. Insulin Levels during Oral Glucose Tolerance Tests. In contrast to glucose values obtained during OGTT, fasted insulin values obtained just prior to glucose load increased significantly ($P < 0.0001$) in animals 7 to 19 weeks of age, after which a precipitous drop in insulin level was noted. Insulin levels in 7-week-old animals were 446.44±38.9 pg/mL. Levels peaked at 19 weeks (1529.3 ± 153.3 pg/mL) and then fell significantly ($P < 0.0001$) from the peak to 189.4±27.4 pg/mL in 31-week-old animals (Figure 5). In 7-week-old animals, the insulin response to glucose loading was rapid, occurring at 15 minutes, and represents a dramatic 4.3-fold increase over values prior to glucose loading (448.8 ± 37.4 versus 1935.3±106.4 pg/mL). Further aging resulted in reduction of the magnitude of the insulin response in all age groups (1.7-, 1.35-, and 1.9-fold for 15, 23, and 31-week-old animals, resp.). The curve for the insulin response to glucose load revealed an increasingly flat profile as animals aged, such that insulin responses in 31-week-old animals were minimal throughout the 2-hour period. The area under the curve ($\times 10^{-3}$) for the insulin response increased significantly ($P < 0.0001$) and peaked at 19 weeks followed by a significant drop from peak in 23- ($P < 0.0016$) and 31- ($P < 0.0001$) week-old animals

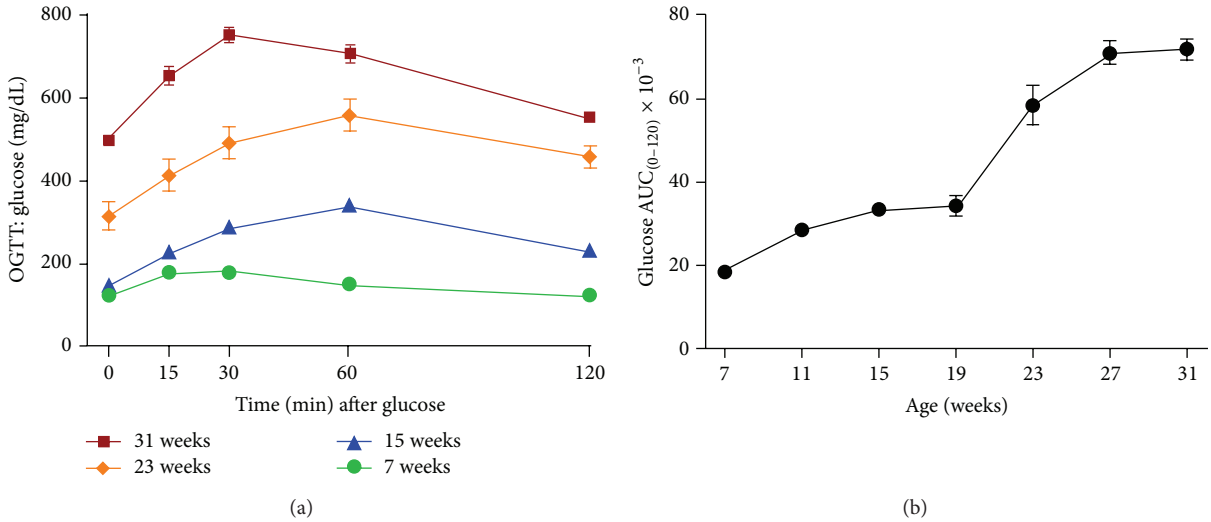

Figure 4: (a) Glucose levels from oral glucose tolerance tests in male ZDSD rats at 7 (●), 15 (▲), 23 (◆), and 31 (■) weeks of age and (b) glucose AUC calculations for all OGTTs (mean ± SEM, $n = 23$).

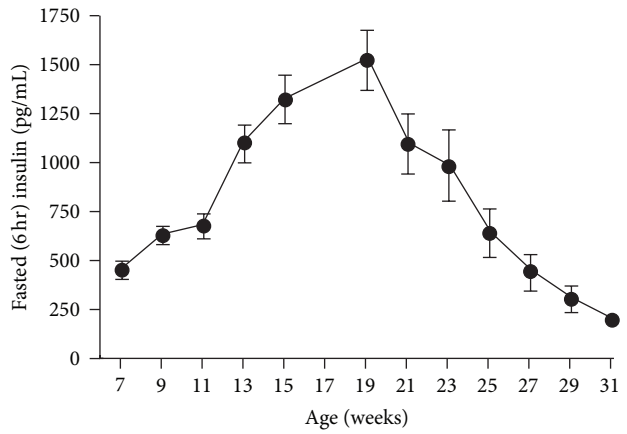

Figure 5: Fasted insulin levels in male ZDSD rats throughout the study duration of rats in ages from 7 to 31 weeks (mean ± SEM, $n = 23$).

(118.4 ± 5.3, 225.3 ± 17.5, 146.5 ± 21.7, and 32.1 ± 5.2 at 7, 19, 23, and 31 weeks, resp.) (Figures 6(a) and 6(b)).

3.6. Beta-Cell Function. Fasting plasma glucose and insulin levels obtained from OGTT's were used to calculate the homeostasis model assessment of β-cell secretory capacity. The values followed the same time course as did the fasted insulin levels presented in Figure 3, that is, a progressive increase 7–19 weeks of age, followed by a precipitous decline to values indicating very little insulin secretory capacity at 31 weeks. HOMA-β values were 3.92 ± 0.31 at 7 weeks of age, peaked at 19 weeks (12.6±1.22), and fell abruptly ($P < 0.0001$) to 0.44 ± 0.1 at 31 weeks, indicating only minimal secretion at 31 weeks (Figure 7).

3.7. Insulin Sensitivity Index (ISI). Insulin sensitivity in ZDSD animals reflects the progressive hyperglycemia and augmented insulin secretion before beta cell loss as animals age. ISI in 7-week-old animals (99.3 ± 5.8) decreased quickly to 61.9 ± 7.5 at 11 weeks ($P < 0.0002$) and plateaued at 19 weeks (33.01 ± 4.7). This decline represents a significant 67% decrease in sensitivity within the 7–19 week old animals ($P < 0.0001$) (Figure 8).

3.8. Lipid Levels. Fasted triglyceride levels increased significantly ($P < 0.0001$) compared to that of 7-week-old rats and peaked at 19 weeks of age (107.5 ± 3.7 and 292.5 ± 17.8 mg/dL, resp.). A slight but not significant decrease ($P < 0.1170$) to 251.1 ± 16.7 mg/dL was apparent at 21 weeks; however, levels remained relatively stable for the remainder of the study. Average triglyceride levels were 230.3 ± 8.8 mg/dL over the last 12 weeks (Figure 9). Fasted cholesterol levels increased significantly ($P < 0.0001$) over the course of data collection, ranging from 95.4 ± 1.1 to 127.9 ± 2.4 mg/dL at 7 and 31 weeks, respectively (Figure 10).

4. Discussion

Over the last decade, there has been a global increase in obesity and type 2 diabetes (T2D). In 2011, there was an estimated 375 million people worldwide with diabetes [12]. Ninety percent of individuals with diabetes were reported as having T2D, largely the result of obesity and lack of physical activity (WHO, 1999, 2003). T2D is thought to occur, in large part, as a consequence of the development of metabolic syndrome [13] which presents as a cluster of conditions defined by obesity, insulin resistance, hypertension, hyperlipidemia, and hyperglycemia [14]. In humans, the natural course of T2D progresses from insulin resistance to compensatory hyperinsulinemia resulting in beta cell failure and overt diabetes

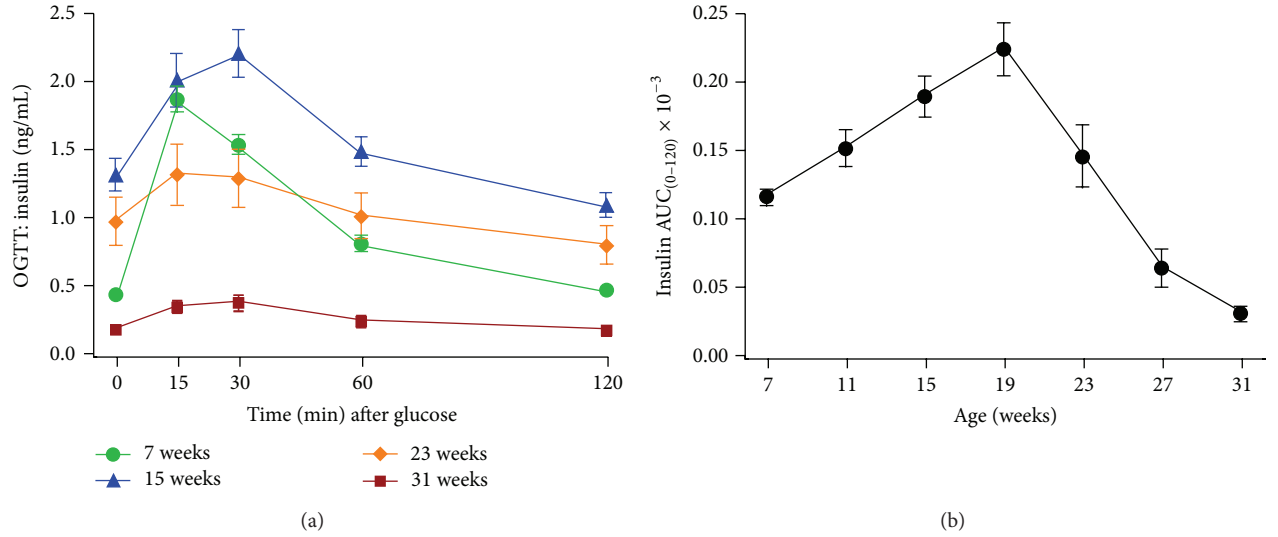

Figure 6: (a) Insulin levels from oral glucose tolerance tests in male ZDSD rats at 7 (●), 15 (▲), 23 (◆), and 31 (■) weeks of age and (b) insulin AUC for all OGTTs (mean ± SEM, $n = 23$).

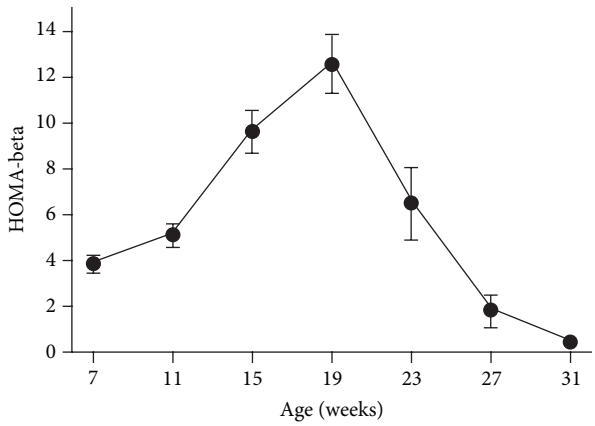

Figure 7: Calculated HOMA-β in male ZDSD rats throughout the study duration of rats in ages from 7 to 31 weeks (mean ± SEM, $n = 23$).

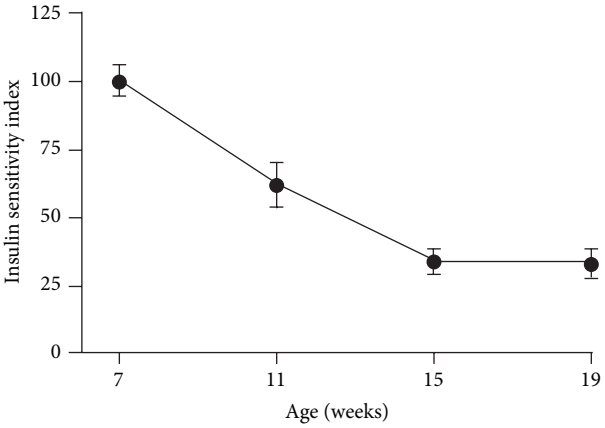

Figure 8: Calculated ISI in male ZDSD rats throughout the study duration of rats in ages from 7 to 19 weeks (mean ± SEM, $n = 23$).

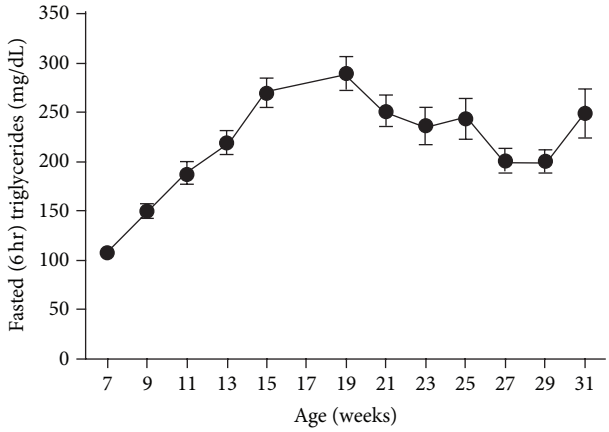

Figure 9: Fasted triglycerides in male ZDSD rats throughout the study duration of rats in ages from 7 to 31 weeks (mean ± SEM, $n = 23$).

[15]. Development can be initiated in humans by genetic and/or environmental factors and is thus considered a multifactorial disease [16]. Genetic polymorphisms linked to T2D have been identified in at least 3 dozen genes, most of which influence both hepatic and peripheral insulin resistance, and adipogenesis as well as beta cell mass and function [17]; however, no mutations in leptin signaling have been identified in human T2D [18, 19]. Since obesity is a major environmental factor predisposing humans to T2D [20], it becomes necessary for a translational animal model to become obese by a polygenetic mechanism.

The ZDSD rat was developed to address the disparity in disease development from the human condition that is apparent in commonly used rodent models. Previous models (Zucker fatty rat, ZDF rat, *ob*/*ob* mice, and *db*/*db* mice) have

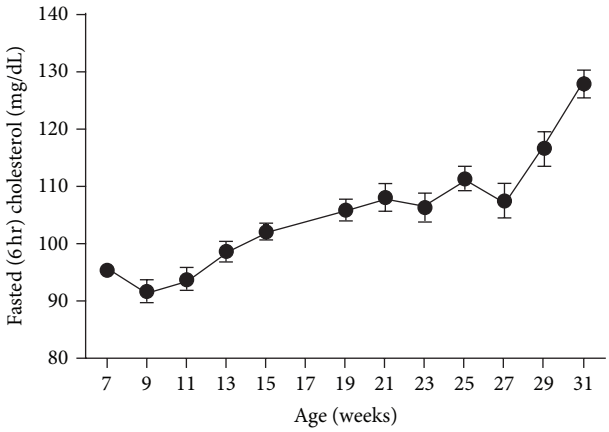

Figure 10: Fasted cholesterol in male ZDSD rats throughout the study duration of rats in ages from 7 to 31 weeks (mean ± SEM, $n = 23$).

proven useful in drug development and in research in the areas of obesity, metabolic syndrome, and diabetes. However, their mutations compromise the leptin pathway [21] which is essential for the normal function of CNS obesity and feeding behavior [22]. Since leptin pathway mutations are a very rare condition in the human population, the continued exclusive use of these models will impede the studies of CNS mechanisms that are central to combating the obesity conditions in the human population. The highly translational ZDSD model satisfies this important need.

The ZDSD rat model is similar to an animal model that was developed at the University of California at Davis [23, 24], in as much as it involved crossing homozygous lean ZDF rats with obese Crl:CD (SD) rats. Although direct comparisons have not been made, the model and the disease are similar to the ZDSD and thus it is probable that it will perform similarly in many experiments. The significant advantage of the ZDSD model is that it is commercially available.

The ZDF rat is the most commonly used leptin deficient rat model of metabolic syndrome, obesity, and diabetes. Obesity is severe and is apparent very early in life. Hyperglycemia with hyperinsulinemia and dyslipidemia are apparent as early as 8 weeks of age, before the animal reaches maturity, and are sustained throughout life [25, 26]. The time course for progression to beta cell failure is rapid in this model, leading to the absence of the extended metabolic syndrome (prediabetic) condition common to human T2D. Hypertension is a major contributor to the end-organ damage seen with long standing diabetes and as such is included in the cluster related to metabolic syndrome. Reports detailing blood pressure in ZDF rats vary greatly; when assessed by radiotelemetry, blood pressure is normal [27], but hypertension has been reported when pressure is assessed by tail cuff [28]. Although marked renal damage with albuminuria is also apparent with long standing diabetes [28], this model also has significant hydronephrosis which compromises its value for application. While this rat exhibits some components of metabolic syndrome, the rats progress rapidly to overt diabetes. The largest divergence from the human T2D for this model resides in the lack of a significant prediabetic period and the development of overt diabetes in the adolescent state.

In contrast to the ZDF male, the male ZDSD rat maintains a moderately long period (approximately eight weeks) of prediabetic metabolic syndrome with obesity, followed by progression to overt diabetes and its complications. This pattern is very similar to the development of T2D in humans. The female ZDSD rat does not become diabetic on ordinary rat chow such as 5008, but under the right conditions, feeding the females diabetogenic diet (5SCA or RD12468) can trip them into overt diabetes [29].

When compared to the rodent models extensively used over the past two decades, the ZDSD rat appears to be a better translational model of obesity, metabolic syndrome, and diabetes as well as its secondary complications associated with T2D. The ZDSD rat's polygenic phenotype, as it relates to metabolic syndrome, includes visceral obesity, insulin resistance, hyperglycemia, dyslipidemia, and mild hypertension [30]. In addition, ZDSD rats spontaneously develop diabetes independently of special diets or monogenic mutations making this a much more translational model. Moreover, the development of diabetes can be synchronized with the feeding of diabetogenic diets (Purina 5SCA or Research Diets 12468) beginning at 16 weeks of age; this has been described in previously published papers [29, 31–33]. As demonstrated here, the ZDSD males become spontaneously hyperglycemic when maintained on Purina 5008 and express a heterogeneous onset of overt diabetes which mimics that seen in T2D humans. When either spontaneous or synchronized diabetes is inadequately treated, this model develops the translational complications of a T2D profile with its sequelae. The resultant naturally occurring phenotype, in the presence of a functional leptin pathway, translates to a rodent model more characteristic of the disease conditions as expressed in the human population.

The characteristics of the ZDSD rat, as it goes through the prediabetic condition (metabolic syndrome) into overt diabetes, are described in this paper. From 7 to 19 weeks of age the ZDSD male rat demonstrated obesity, impaired glucose tolerance, dyslipidemia, and prediabetic hypertension [30]. Time dependent elevation in HbA1c, which occurs during the metabolic syndrome/prediabetic phase, was also noted. Although the morning fed glucose levels are quite stable in animals 7–15 weeks of age, they are higher than those in control rats; this study did not include blood glucose from control animals. More recent data using continuous blood glucose monitoring and StatStrip reading in the am and pm have reliably demonstrated that afternoon glucose levels are significantly higher than the morning levels in this model [11]. This is most likely a representation of the abnormal feeding pattern of ZDSD rats. They are not strictly nocturnal feeders and tend to eat a significant amount during the day [34]. It should also be noted that the late afternoon glucose elevations might be related to the human dawn effect since late afternoon just before lights go off is equivalent to the rat's morning since they are nocturnal animals.

As metabolic syndrome developed in the ZDSD rat, insulin levels increased in a compensatory fashion to combat increasing insulin resistance. Shortly after the peak in

insulin levels at 19 weeks of age, insulin values fell precipitously as beta cells began to fail. Progressive decreases in beta cell secretory capacity were demonstrated by the dramatic decrease in insulin and in HOMA-β calculated during this time. As the ZDSD model became more diabetic, further abnormalities in glucose disposal became apparent as the magnitude and duration of the glucose excursion following glucose load are increased with age, while the magnitude of the insulin response is minimized. Area under the glucose curve in the OGTT increased while area under the insulin curve was drastically diminished. In fact, in 31-week-old animals, the insulin response to glucose load was barely visible. The pattern of changes in glucose homeostasis in ZDSD is very much like that in humans as the disease progressed. Initially, obesity leads to insulin resistance due to many factors, including contributions from adipokines and cytokines from visceral fat. Glucose levels are temporarily maintained at normal level through hypersecretion of insulin from beta cells. Insulin resistance continues to progress until beta cells begin to cease functioning, insulin levels drop, and overt diabetes ensues.

Metabolic dysregulation in patients with metabolic syndrome includes hypertriglyceridemia. Similar to patients, progressive elevations in triglyceride levels were apparent in ZDSD rats. Cholesterol levels increased as animals age; interestingly, the rate of increase changed upward as ZDSD rats became overtly diabetic. Furthermore, although this study does not demonstrate the long-term complications of diabetes, we believe that this model will also develop complications that are seen in the human population with long-lasting, inadequately controlled diabetes. Indeed, in other studies, the ZDSD rat has been shown to develop diabetic osteoporosis [33], diabetic nephropathy [35], diabetic connective tissue changes [29, 32], and diabetic neuropathy [36] and hypertension prior to development of diabetes [30].

A review of the literature on the development of T2D in the human population demonstrated a high degree of translation with the ZDSD model. The review and analysis by many authors have led them to an understanding of the time course of events that occur in humans in the prediabetic and diabetic conditions [15, 37–39]. Figure 11(a) captures this in a single graphic that represents the progression of the "natural history of type 2 diabetes" in the human population. This figure produced by Kendall and Bergenstal represents the changes that occur during the prediabetic stages through the overt diabetic stages of diabetes. Similar to Figures 11(a) and 11(b) graphs from this paper have combined to compare the pattern which is observed in ZDSD rats where insulin resistance is represented by the progressive decrease in glucose disposal (increase glucose AUC) and insulin response is represented by the insulin response to a glucose load during an OGTT. Insulin AUC in the prediabetic state is exaggerated while a steady decline in insulin response is noted during the diabetic state.

The current study demonstrates similarities in fasting and fed glucose levels (Figure 2), insulin levels (Figure 5), the insulin AUC (Figure 6), and the HOMA-β (Figure 7) in the progression of the disease from the prediabetic state to the overt diabetic state. This comparison appears to support the case for translatability of the progression of T2D in ZDSD model to the human disease.

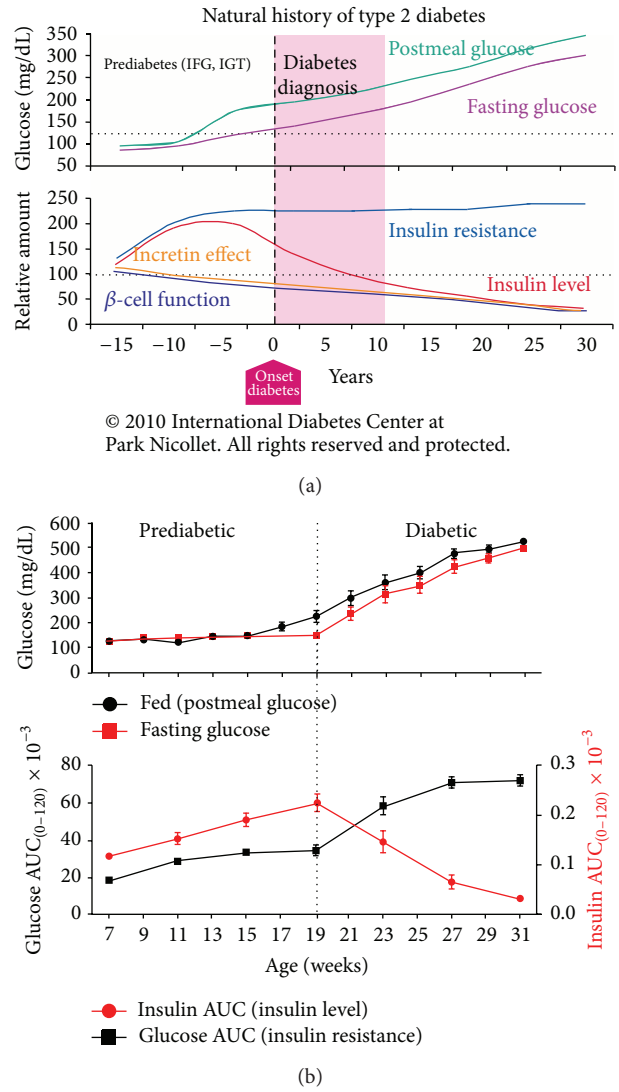

Figure 11: (a) The upper panel shows the graphic representation of the progression of both fasting and postmeal hyperglycemia. The lower panel demonstrates both insulin resistance and defective beta-cell function (Natural History of Type 2 Diabetes © 2010, International Diabetes Center, Minneapolis, MN, USA, used with permission). (b) demonstrates the similarity between the progression of diabetes between the human condition and ZDSD rat. The vertical dotted line represents the onset of diabetes.

5. Conclusions

The ZDSD rat model exhibits all stages and multiple components of metabolic syndrome/T2D and the complications of these conditions. Although further research is clearly needed, this study demonstrates that the ZDSD model may represent a truly translational model for the investigation of causative mechanisms, targeted interventions, and treatments for this

multifaceted disease. The expression of diabetic complications in ZDSD rats may strengthen the model's translatability. The authors believe that, in addition to the scientific merits of the ZDSD, the use of the ZDSD in the "one rat, many models" paradigm presents an opportunity for investigators to significantly reduce costs while evaluating compound effects on the multiple components of T2D and its sequelae.

Conflict of Interests

Richard G. Peterson is the developer of the ZDSD rat and could gain financially as a result of the marketing of this animal model. The other authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2015, Article ID 731434, 11 pages
http://dx.doi.org/10.1155/2015/731434

Review Article

Beneficial Effects of Omega-3 Polyunsaturated Fatty Acids in Gestational Diabetes: Consequences in Macrosomia and Adulthood Obesity

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Received 11 June 2014; Revised 25 August 2014; Accepted 4 September 2014

Academic Editor: Marcus G. Pezzolesi

Omega-3 polyunsaturated fatty acids (PUFAs) are increasingly being used to prevent cardiovascular diseases, including diabetes and obesity. In this paper, we report data on the observed effects of omega-3 PUFA on major metabolic disorders and immune system disruption during gestational diabetes and their consequences on macrosomia. While controversies still exist about omega-3 PUFA effects on antioxidant status regarding the level of omega-3 PUFA in diet supplementation, their lipid-lowering effects are unanimously recognized by researchers. Animal studies have shown that omega-3 PUFA contributes to the maintenance of the immune defense system by promoting the differentiation of T helper (Th) cell to a Th2 phenotype in diabetic pregnancy and by shifting the Th1/Th2 ratio from a deleterious proinflammatory Th1 phenotype to a protective anti-inflammatory Th2 phenotype in macrosomia and in adulthood obesity that results from macrosomia at birth. Based on the available evidence, international nutritional and food agencies recommend administration of omega-3 PUFA as triglyceride-lowering agents, for the prevention of cardiovascular disease risk and during human pregnancy and lactation. Furthermore, studies targeting humans are still required to explore application of the fatty acids as supplement in the management of gestational diabetes and inflammatory and immune diseases.

1. Introduction

Metabolic disorders as defined by the World Health Organization include disease conditions whose prevalence is reported to be on the increase more so in the developing countries. Based on the results of some epidemiological and clinical investigations in the past few decades, a number of studies have supported the beneficial effects of marine derived omega-3 polyunsaturated fatty acids (PUFAs) in cardiovascular diseases [1–3]. Indeed, low incidence of inflammatory diseases attributed to large consumption of cold water marine fish that contain omega-3 fatty acids has been observed in Greenland Eskimos and Japanese people [4–6]. Since evidence from experimental and clinical studies has proved the beneficial effects of omega-3 fatty acid consumption during diabetes, nutritional strategies have been proposed [7, 8]. Although the mechanism of action of omega-3 fatty acids remains unclear, many reports postulated that the beneficial effects on diabetes and diabetes outcomes may be due to the lipid-lowering action of the fats. However, controversies still exist regarding the beneficial effects of omega-3 PUFA in normal pregnancy or in the treatment and prevention of diabetes during pregnancy and its outcomes on the offspring. The results from most clinical trials performed in type 2 diabetes patients suggest that omega-3 PUFAs have no or marginal effects on metabolic control, while effectively reducing hypertriglyceridemia in these patients [9]. Some authors have recently demonstrated

that erythrocyte DHA enrichment with DHA+EPA treatment substantially decreases liver fat percentage in nonalcoholic fatty liver disease patients [10]. Similarly, consumption of lean fish (75–100 g/day) has exhibited beneficial effects by reducing the risk of type 2 diabetes mellitus compared to zero intake in Norwegian women [11]. In contrast, other results have shown that omega-3 PUFA did not provide any benefit on hepatic steatosis and insulin resistance in diabetic patients with nonalcoholic steatohepatitis [12].

Numerous studies have recommended the use of omega-3 PUFA supplementation during human pregnancy and lactation for the prevention of preterm birth, beneficial effects on fetal development, visual and cognitive development, and other functional outcomes of the infants [13–16]. While other authors have found that DHA supplementation (800 mg/day) during the second half of human pregnancy does not reduce the risk of gestational diabetes mellitus or preeclampsia in mothers, others have shown that DHA supplementation can reduce the risk of perinatal death and neonatal convulsions in newborns [17]. Other authors did not find any associations between maternal fatty acid intake or food consumption during human pregnancy and the development of type 1 diabetes in the offspring [18]. Despite these controversial reports on the effects of omega-3 PUFA, guidelines from the Polish Gynecological Association recommended the use of omega-3 PUFA either as supplements or through dietary counseling for women who are planning pregnancy and for patients with normal and/or gestational diabetes and during lactation [19, 20]. Koletzko et al. [21] have published the consensus statement and recommendations of several international research bodies on fatty acids. The adopted conclusions included dietary fat intake in human pregnancy and lactation and recommended that pregnant and lactating women should aim to achieve an average dietary intake of at least 200 mg DHA/day. In addition, since intakes of up to 1 g/day DHA or 2.7 g/day omega-3 long-chain PUFA have been used in randomized clinical trials without significant adverse effects, therefore, women of childbearing age should aim to consume one to two portions of sea fish per week, including oily fish [21]. Moreover, the American Pregnancy Association reports the recommendation of the International Society for the Study of Fatty Acids and Lipids (ISSFAL) that pregnant women should take 300 mg minimum to support themselves and the fetus for DHA requirements on a daily basis [22, 23]. The Institute of Medicine Food and Nutritional Board has developed what is considered as the recommended minimum adequate intake levels for the omega-3 PUFA group. The recommended adequate intakes for omega-3 PUFA are 1.3 g/day for nursing women, 1.1 g/day for adult women, 1.4 g/day for pregnant women, 1.3 g/day for girls ages 14 and above, 1.6 g/day for boys ages 14 and above and adult men, 0.5 g/day for infants, 0.7 g/day for children (1 to 3 years old), and a dosage of 0.9 g/day for children (4 to 8 years old) [22, 23].

The scientific evidence for cardioprotective effects of food sources of omega-3 PUFA, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), beyond the effect of changes in serum lipid profiles, has been recognized by the American Heart Association (AHA) Dietary Guidelines. The AHA recommended consumption of at least two servings of fish per week to confer cardioprotective effects [24]. In the same line the US Food and Drug Administration has approved administration of omega-3 fatty acids only as triglyceride-lowering agents in patients with hypertriglyceridemia [24]; and some European regulatory agencies have approved the use of omega-3 for the treatment of cardiovascular risk [9]. The aim of the present paper is to review data on the beneficial effects of omega-3 PUFAs on major metabolic disorders and immune system disruption observed during gestational diabetes and macrosomia. Details of outcomes of maternal diabetes in pregnancy on offspring have been reviewed elsewhere [25] and, therefore, will only be briefly discussed before focusing on beneficial effects of PUFA during gestational diabetes and evaluating the consequences on the macrosomia in newborns that become obese in adulthood.

2. Major Metabolic Complications during Gestational Diabetes and Macrosomia

Gestational diabetes mellitus (GDM, which refers to diabetes only during pregnancy) and obesity during pregnancy are both complications which significantly influence the development of offspring during fetal life and postnatal. Indeed, animal and human studies indicated that fetuses from mothers with gestational diabetes are at high risk of developing fetal macrosomia [26, 27], and they are prone to adverse side effects strongly associated with prematurity, birth trauma, respiratory distress syndrome, and fetal death [28]. Effectively, our observations are in agreement with previous epidemiological and clinical trials that have shown that either preexisting maternal diabetes (type 1 and type 2) or GDM appears to be important risk factor for fetal overnutrition and macrosomia [29–32].

Several modes exist for inducing experimental maternal diabetes with streptozotocin in animal models and the consequences on fetus and adult progeny are variable with each model [33, 34]. The streptozotocin, when administered at a high single dose, induces diabetes by the direct toxic effects on pancreatic β-islet cells [33]. The fetus is confronted with severe intrauterine hyperglycemia which induces fetal islet hypertrophy and β-cell hyperactivity and may result in early hyperinsulinemia [34]. The increased insulin secretion dramatically and rapidly decreases due to the overstimulation of fetal β cells which are depleted of insulin granules, resulting in fetal hypoinsulinemia [33, 34]. The growth of fetal protein mass is then suppressed, leading to fetal microsomia (small birth weight) [33]. Postnatal development is affected and retarded, and the offspring remain small at adulthood but develop insulin resistance [33, 35].

The animal model reported in this review concerns mild streptozotocin-induced type 1 diabetic pregnancy which also leads to macrosomia in newborns [36, 37]. Streptozotocin, administered at low doses during 5 consecutive days, induces mild type 1 diabetes, following a T-lymphocyte-dependent process, an autoimmune destruction of pancreatic β cells, mediated by both $CD4^+$ and $CD8^+$ T cells [38, 39] and this represents a good model of diabetes development for several

reasons [38, 40, 41]. When the streptozotocin is administrated at five low doses, starting on day 5 of gestation to preserve gestation in pregnant rats, [36] the infiltration of pancreatic islet β-cells by autoreactive T lymphocytes is observed two days after the last injection [38]; the hyperglycemia occurs one week (7 days) after the last injection. Diabetes (hyperglycemia) becomes maximal around 10-11 days after the last STZ injection (i.e., second trimester of gestation) [38–41] and it persists after delivery [25]. We have previously shown that the progenies of pregnant diabetic rats are prone to develop macrosomia at birth, obesity, type 2 diabetes, and impaired glucose tolerance in adulthood [27, 42].

Studies in humans with GDM revealed that diabetes determined by oral glucose tolerance test according to the criteria of the World Health Organization, as reviewed by the International Association of Diabetes and Pregnancy Study Groups (IADPSG) based on the Hyperglycemia and Adverse Pregnancy Outcomes (HAPO) Study [43], appeared at second or third trimester of pregnancy [28, 29], as we described elsewhere [25]. GDM patients are hyperglycemic and hyperinsulinemic at the diagnosis of the disease [28, 29], reflecting a decrease in insulin sensitivity in diabetic pregnant women [44]. Maternal diabetes is characterized by an increased placental transport of glucose and other nutrients from the mother to the fetus, resulting in macrosomia [33]. Convincing evidence from our studies and others has shown that either preexisting diabetes (type 1 and type 2 diabetes) or GDM (diabetes only during pregnancy) appears to be important risk factor for fetal overnutrition and macrosomia in newborns and for the development of diabetes and adulthood obesity that results from macrosomia [26, 29–32, 44–46]. Macrosomia, the most commonly reported effect of maternal diabetes in newborns [45], is usually defined in humans as birth weight above either 4 kg or birth weight above the 95th percentile of the gestational age. Babies from GDM patients whose birth weight was 2.0 SD greater than the mean birth weight of control infants were considered as macrosomic babies [26, 46, 47]. The risk of diabetes in the offspring of type 2 diabetes genitors is significantly higher when the mother rather than the father is diabetic [31]. Moreover, the risk of insulin resistance is higher in children of mothers with GDM (diabetes only during pregnancy) than in children from mothers developing diabetes after pregnancy [48]. Therefore, diabetic pregnancy appears to induce macrosomia that results in obesity in adulthood and these pathologies are associated with several metabolic disorders, implicating lipid metabolism, altered antioxidant status, and disrupted immune defense system.

2.1. Effects of Maternal Diabetes on the Lipid Metabolism: Implication in Macrosomia. Regarding metabolic processes, maternal diabetes induces alterations in the lipid metabolism which contribute to macrosomia in newborns. Indeed, we have previously shown in animal and human studies that diabetic pregnancy induces maternal hyperlipidemia which predisposed the fetus to macrosomia [26, 27, 42]. In fact, high levels of triglyceride in the maternal circulation of diabetic rats tend to create a steep concentration gradient across the placenta which accelerates the transport and deposition of the lipids in fetal tissues [49]. In addition, maternal hyperglycemia also leads to fetal hyperglycemia, which stimulates pancreatic islet cells and induces fetal hyperinsulinemia in animals and humans [26, 33–35, 37, 42, 46, 47]. Animal studies also showed that in macrosomic newborns hypertriglyceridemia exists and persists with age and is linked to the development of insulin resistance and hyperlipogenesis at adulthood [37]. Our observations are confirmed by several recent studies which have shown that maternal diabetes in human and rat is associated with increased risk of hyperlipidaemia [50–54] and metabolic syndrome and type 2 diabetes in the offspring [53, 54].

2.2. Effects of Maternal Diabetes on the Antioxidant Status: Implication in Macrosomia. In human studies as well as in experimental animal models, we have observed that maternal diabetes significantly alters the total antioxidant status as demonstrated by decreased antioxidant molecules (vitamins A and E), enzyme activities (superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase (GSSG-Red)), and increased serum thiobarbituric acid-reactive substances (TBARS) [27, 46]. The altered antioxidant system is also observed and persists with age in the macrosomic rat and human newborns that became obese adults [27, 46]. These observations are recently supported by several investigators who have observed increased oxidative stress in gestational diabetic women and animals [55–58] and their infants [59] and rat adult offspring [60]. In fact, our findings suggest that there is an increased oxidative stress in diabetic pregnant women and rats and their adult obese offspring that were macrosomic as newborns [27, 46], in agreement with the results of previous studies [61–64].

2.3. Effects of Maternal Diabetes on the Immune System: Implication in Macrosomia. In animal as well as in human studies, the immune system is also shown to be modulated during maternal diabetes which induces macrosomia in newborns. Several studies have implicated a pathological role of the immune system and inflammation in type 1 diabetes, type 2 diabetes, and GDM. Indeed, T cell-derived cytokines are involved in the autoimmune destruction of pancreatic islet cells leading to type 1 diabetes [38] while type 2 diabetes is associated with a generalized activation of the innate immune system, in which there is a chronic, cytokine-mediated state of low-grade inflammation [66–68]. Normal pregnancy or pregnancy complicated with diabetes is known to influence T helper cell differentiation. Evidence from our studies revealed that in normal pregnancy Th1 cytokines are downregulated whereas Th2 cytokines are upregulated in animals as well as in humans [65, 69] (Figure 1). Our observations were in agreement with the results of previous studies [70, 71]. Interestingly, we have observed that in diabetic pregnancy Th1 cytokines decrease and IL-10, a Th2 cytokine, increases [26, 65, 69] as presented in Figure 1. Therefore, evidence has

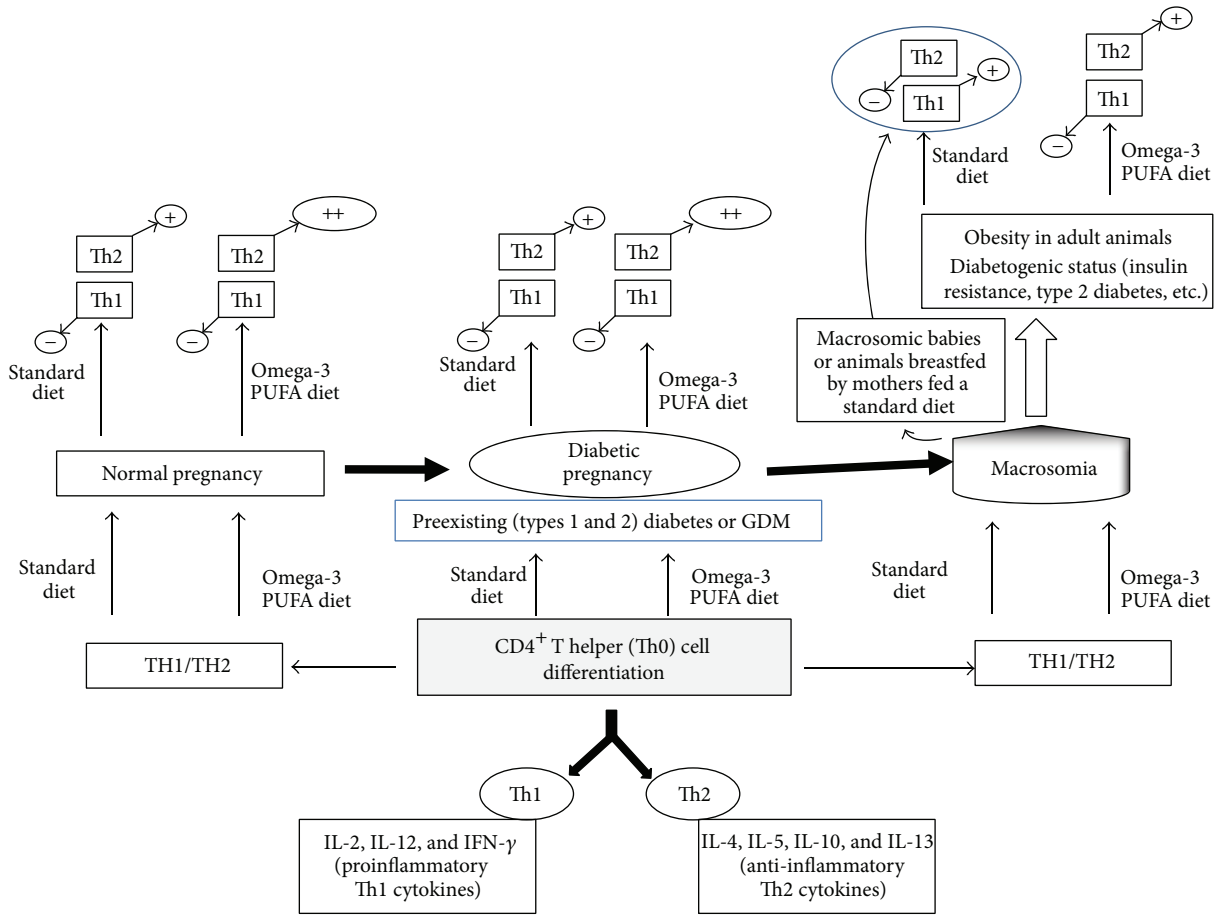

Figure 1: Effects of omega-3 PUFA diet on Th1 and Th2 dichotomy in animal diabetic pregnancy: implication in macrosomia. Naïve $CD4^+$ T helper (Th0) cells can be differentiated into either Th1 cells, producing proinflammatory cytokines (IL-2, IL-12, and IFN-γ), or Th2 cells, secreting anti-inflammatory cytokines (IL-4, IL-10, IL-5, and IL-13). Human and animal studies show that, in normal pregnancy as well as in diabetic pregnancy, the Th1/Th2 balance is shifted towards a protective Th2 phenotype. Feeding omega-3 PUFA diet to rats, in normal pregnancy as well as in diabetic pregnancy, enhances the increase of Th2 cytokines. In contrast, the Th1/Th2 balance is shifted towards a proinflammatory Th1 phenotype in macrosomic newborns as well as in adult obese animals that were macrosomic as newborns and the omega-3 PUFA diet shifts the ratio to an anti-inflammatory Th2 phenotype in adult obese animals. Th: T helper cells; GDM: gestational diabetes mellitus; PUFA: polyunsaturated fatty acid; (+): upregulation; (−): downregulation. Data are from the studies carried out by Khan et al., J Autoimmun, 2006 [65].

shown that Th2 cytokines may be beneficial for successful pregnancy in diabetic animals and GDM patients. Indeed, the shift of Th1/Th2 ratio to a protective Th2 phenotype during pregnancy has been shown to promote humoral immunity with high production of antibodies which contribute to the fight against infections during pregnancy and offer passive immunity to fetus [72]. However, animal and human studies have shown that, in macrosomic newborns and obese adult animals that were macrosomic as newborns, the Th1/Th2 balance is shifted to a proinflammatory Th1 phenotype [26, 65] (Figure 1). This upregulated-Th1 profile in obese adult animals that were macrosomic as newborns may confer to these animals a potential "diabetogenic status," as revealed by the hyperglycemia and hyperinsulinemia observed in these animals in the adulthood [42, 65].

3. Effects of Omega-3 PUFA in Gestational Diabetes: Incidence on Macrosomia and Lipid Metabolism

We have previously examined in animal model the effects of omega-3 PUFA on the incidence of macrosomia in diabetic pregnancy in rats [27, 42, 65]. The model of diabetic pregnancy was established through administration of five low doses of streptozotocin to pregnant Wistar rats starting on day 5 of gestation as described above [27, 37, 38, 42, 65]. Pups from diabetic pregnant rats whose birth weights were 1.7 SD greater than the mean birth weight of the control pups were considered as macrosomic newborns [27, 37, 38, 42, 65]. We observed that 62% to 75% of pups of diabetic pregnant rats were macrosomic at birth [27, 42, 65]. These macrosomic

newborns were hyperglycemic at birth and, when compared to offspring of control rats, they maintained an accelerated weight gain and become obese at adulthood (3 months of age) [27, 42, 65]. Interestingly, we observed that omega-3 PUFA diet consumption significantly reduced the incidence of gestational diabetes on macrosomia by decreasing the rate of macrosomic newborns by 16–25% [27, 65]. However, omega-3 PUFA diet did not show any effect on the hyperglycemia of macrosomic newborns that become obese at adulthood [27].

In the animal model, while diabetic pregnancy associated with hyperlipidemia [27, 49, 73] has been reported to induce hypercholesterolemia and hypertriglyceridemia in adult obese offspring from macrosomic newborns born to diabetic animals [27, 37, 74, 75], our studies have demonstrated that omega-3 PUFA diet significantly reduced the levels of cholesterol and triglyceride in diabetic pregnant animals and attenuated hyperlipidemia in their adult obese offspring from macrosomic newborns [27, 42, 65]. The hypolipidemic effects of omega-3 PUFA diet have also been demonstrated in animals [27, 42, 65] as the same findings have previously been reported in human studies by other researchers [4, 7, 8]. The hypocholesterolemic effects of omega-3 PUFA diet have been observed not only in the serum but also in the liver of diabetic pregnant animals and their adult obese offspring from macrosomic newborns. The findings suggest a decrease in cholesterol synthesis or increased cholesterol excretion into bile. It has been reported that fish oil induces changes in cholesterol metabolism in rat liver leading to an increase in the biliary excretion of cholesterol [76]. In our study, it also has been established in rat dams and their breastfed macrosomic pups that become obese in the adulthood that dietary intake of omega-3 PUFA induces a large increase in plasma omega-3 PUFA levels followed by a large decrease in omega-6 PUFA (LA and AA in particular) [77], in agreement with previous results [78]. Thus, we concluded that omega-3 PUFA exerts its beneficial effects on lipid metabolism observed in diabetic pregnant animals and their macrosomic newborns that become obese adults by attenuating the hyperlipidemia associated with these pathologies.

4. Antioxidant Effects of Omega-3 PUFAs during Gestational Diabetes and Macrosomia

In human studies as well as in animal models, we and several authors have previously reported that diabetes, diabetic pregnancy, macrosomia, and adulthood obesity that results from macrosomia are associated with increased oxidative stress related to decreased antioxidant molecules (vitamins A and E), decreased antioxidant enzyme activities (superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase (GSSG-Red)), and increased serum thiobarbituric acid-reactive substances (TBARS) [25, 25, 27, 61–64, 79, 80]. High blood glucose has been shown to induce an oxidative stress which in turn induces the production of highly reactive oxygen species toxic to cells particularly the plasma membranes where the radicals interact with the lipid bilayer [61]. Under normal conditions, endogenous antioxidant enzymes and vitamins are responsible for detoxification of the deleterious oxygen radicals. Thus, treatment with antioxidants may prevent or reverse the abnormalities associated with diabetes and its complications. Some studies have reported that dietary supplements with vitamins and minerals prevent or at least attenuate the organic deterioration caused by an excessive oxidative stress associated with diabetes in humans and animals [81, 82]. There is a general notion that omega-3 PUFA might deteriorate antioxidant capacity. Nonetheless, no consensus has been reached on this subject as shown in Table 1.

It has been argued that excessive intake of omega-3 PUFA may affect antioxidant status [8, 83–86] and enhance the susceptibility to oxidative damage. While some investigators [87–89] could not find any changes in the antioxidant status in humans and rats treated with omega-3 fatty acid-rich diet, we and other researchers have demonstrated that treating diabetic patients [90] or gestational diabetic rats and their adult obese offspring that were macrosomic as newborns [27] with omega-3 fatty acids significantly improves their antioxidant status. The details of fatty acid compositions of control and omega-3 PUFA diets used in our previous studies [27, 65, 91] are presented in Table 2. From the results presented in Table 1, we concluded that a moderate level of omega-3 PUFA dietary intake could be beneficial for improving the antioxidant status. This argument is supported by the findings that dietary fish oil modulates the composition of plasma membrane phospholipids by increasing omega-3 PUFA contents (EPA and DHA in particular) at the expense of arachidonic acid (AA, an omega-6 PUFA) levels [88]. Similarly, we have previously reported that feeding an omega-3-enriched diet to animals leads to an increased incorporation of EPA and DHA into the plasma membrane phospholipids of T lymphocytes and a decrease in arachidonic acid level (Table 3) [77, 91]. Our findings have been supported by other researchers [92–94]. Hence, we have concluded that omega-3 fatty acids influence T cell activity by being incorporated into their plasma membranes (see Table 3). The incorporation of omega-3 PUFA into the cell plasma membranes may diminish or counterbalance the negative effects of AA (n-6 PUFA) on antioxidant status and consequently modulate cell activation [92].

The balance between omega-3 and omega-6 fatty acids may also markedly affect cell metabolism. Evidence in the literature shows that essential fatty acids influence the physical properties of cell membranes in terms of fluidity and permeability, activity of membrane receptors, enzymes and ion channels, and cell response to various stimuli through the production of secondary messengers [92]. Therefore, the beneficial effect of omega-3-diet on antioxidant status likely involves DHA and EPA because EPA is known to give rise to eicosanoids of omega-3 fatty acid series which exert opposite effects to those of omega-6 series derived from linoleic acid (LA) and arachidonic acid (AA). In addition, EPA may also be converted into DHA which, along with dietary DHA, may further contribute to the beneficial effects. It has also been shown that DHA may give rise to some discovered derivatives like docosatrienes or resolvins which exert beneficial effects

Table 1: Effects of omega-3 fatty acids on antioxidant status as reported by various investigators. This table is adapted from our previous study, Yessoufou et al., Int. J Obesity, 2006 [27].

Antioxidant status	Species	Omega-3 PUFA level in the diet	References
Decreased	Diabetic rats	10% of diet (considered as excessive)	Cho and Coi, 1994 [83]
Decreased	Healthy humans	EPA: 2.5 g/day; DHA: 1.8 g/day	Wander and Du, 2000 [84]
Decreased	Healthy humans	6.26 g/day for 6 weeks	Allard et al., 1997 [85]
Decreased	Patients with myocardial infarction	850–882 mg/day (EPA + DHA) for 1 year	Grundt et al., 2003 [86]
Decreased	Diabetic rats	Fish oil	Yilmaz et al., 2002 [8]
Unchanged	Healthy humans	4 g/day (n-3) PUFA for 5 weeks	Hansen et al., 1998 [87]
Unchanged	Rats	n-3 fatty acid-rich diet (fish oil)	Ando et al., 1998 [88]
Unchanged	Hyperlipidemic patients	4 g/day (DHA or EPA)	Nordøy et al., 1998 [89]
Improvement	Diabetic humans	EPA: 1.08 g/day; DHA: 0.72 g/day	Kesavulu et al., 2002 [90]
Improvement	Diabetic rats	2.1% of diet	Yessoufou et al., 2006 [27]

Table 2: Fatty acid composition of control and omega-3 PUFA diets.

Fatty acids	Control diet (mg/g)	EPAX diet (mg/g)
C14:0	0.4	0.4
C16:0	5.1	2.1
C18:0	3.9	1.7
C18:1	18.5	9.1
C18:2n-6	21.3	11.2
C18:3n-3	0.83	0.5
C20:4n-6 (AA)	ND	0.9
C20:5n-3 (EPA)	ND	22.2
C22:6n-3 (DHA)	ND	2.0
Total fatty acids	**50.0**	**50.0**
∑n-6 PUFA	21.30	12.06
∑n-3 PUFA	0.83	24.59
(n-6)/(n-3)	25.80	0.49
(n-3)/(n-6)	0.04	2.04
∑SFA	9.40	4.26
∑PUFA	22.13	36.65
∑MUFA	18.50	9.07
PUFA/SFA	2.35	8.60

ND = not detectable. This table is adapted from our previous studies [27, 65, 91].

The chemical composition of control diet was as follows (g/kg dry diet): starch, 587; casein, 200; cellulose, 50; sucrose, 50; mineral mix, 40; vitamin mix, 20; DL-methionine, 3; vegetable oil-Isio-4 (Lesieur, Neuilly-sur-Seine, France), 50. Total oil represented 5% of the diet. In the omega-3 PUFA diet, half of the vegetable oil-Isio-4 was replaced by EPAX-7010 (the omega-3 PUFA oil). The vegetable Isio-4 oil contained the following: 47.2 mg/g 18:2 (n-6); 1.7 mg/g total (n-3); and 40.2 mg/g monounsaturated fatty acids (largely 18:1). EPAX-7010 oil, in the form of ethyl ester, contained approximately 85% (n-3) PUFA, that is, EPA, 70%, DHA, 12%, and α-tocopherol, 2.1 to 3.2%. It means that EPAX oil represented 2.5% of the diet. Since the omega-3 PUFA consisted of 85% of the 2.5% EPAX oil, the total n-3 PUFA represented only 2.1% of the total diet. After diets' preparation, the lipids from diets were extracted according to the method described in Yessoufou et al., 2006 [27], and then transmethylated by BF3/methanol after saponification, and fatty acids were analysed by gas liquid chromatography.

Table 3: Fatty acid composition of plasma membrane phospholipids of T lymphocytes purified from the spleen of mice fed on standard diet or omega-3-enriched diet, adapted from our previous study, Yessoufou et al., J Lipid Res., 2009 [91].

Fatty acids (% of total)	Cells from mice fed standard diet	Cells from mice fed omega-3 PUFA diet
C16:0	1.99 ± 0.31	2.16 ± 0.39
C16:1	27.01 ± 0.37	27.37 ± 0.20
C18:0	18.79 ± 0.25	18.31 ± 0.67
C18:1	13.15 ± 0.56	12.78 ± 0.75
C18:2n-6	11.12 ± 0.75	10.19 ± 0.11
C20:4n-6 (AA)	25.67 ± 0.98	13.12 ± 0.28*
C20:5n-3 (EPA)	0.41 ± 0.05	6.09 ± 0.15*
C22:6n-3 (DHA)	1.77 ± 0.06	9.99 ± 0.41*

Cells were purified from the spleen of mice fed the standard diet or omega-3-diet for 6 weeks. Values are mean ± SEM, $n = 10$ mice per group of diet. *$P < 0.01$: significant differences between omega-3-diet group and standard diet group. The lipids from cells were extracted according to the method described in the following reference: Yessoufou et al., J Lipid Res., 2009 [91]. Phospholipids were separated from silica gel by thin layer chromatography, using the following solvent: chloroform/methanol/acetic acid at 35 : 14 : 2.7 (v/v/v). After scraping off, the phospholipid fractions were transmethylated by BF3/methanol after saponification, and fatty acids were extracted and further analyzed by gas liquid chromatography. Analysis of fatty acid peaks was achieved with reference to the internal standard by using DELSI ENICA 21 integrator (Delsi Nermag, Rungis, France).

on the antioxidant status [95]. Similarly, the fatty acids have been shown to modulate cell signaling mechanisms via their incorporation in the plasma membrane phospholipids [96]. Although the exact mechanism by which EPA/DHA exert antioxidant action is not well understood, Das et al. [97] have suggested that EPA/DHA supplementation inhibits free radical generation and suppresses lipid peroxidation and NO synthesis in patients with nephritic syndrome. This finding suggests that EPA or DHA may be involved in scavenging of free radicals and NO. In support of the finding, Yazu et al. [93] reported that in aqueous micellar dispersions composed of methyl esters of EPA or linoleate (LA), the oxidizability of the methyl ester of EPA (omega-3) was lower than that of methyl linoleate (omega-6). The EPA micelle had ≥2 molecules of oxygen in the peroxyl radical while the linoleate micelle had only one molecule suggesting that EPA is more polar than linoleate and the oxygen species polar radicals may migrate from the lipophilic core of the micelle to the polar

surface. Due to this migration, an environment was created that favoured the termination and reduced the propagation of oxidation reactions [84]. This ability of EPA to behave as peroxyl and free radical scavenger is one of the mechanisms which may be used to explain the antioxidant properties of the fatty acid. With regard to the antioxidant properties of EPA/DHA, it has recently been proposed that DHA inhibited more efficiently than EPA the protein degradation by regulating NFκB (Nuclear Factor kappa B) signaling pathway in mouse C2C12 myotubes through activating PPARγ gene expression [98]. In addition, other authors have found that DHA and genistein exert complementary actions whilst genistein is antagonistic to arachidonic acid (an omega-6 PUFA) for controlling prostaglandin E_2 production as well as invasiveness of MDA-MB-231 human breast cancer cells in culture by modulating the level of NFκB expression [99].

5. Effects of Omega-3 PUFA on the Immune System in Gestational Diabetes: Implication in Macrosomia

As stated above, the immune system plays a preponderant role in the pathogenesis of maternal diabetes in pregnancy and macrosomia and both pathologies involve T cell activation. Many investigators have shown interest in the effects of omega-3 fatty acids on several diseases including diabetic pregnancy and obesity. It has been established that omega-3 PUFAs exert immunosuppressive effects [92, 94]. Being immune modulator agents, omega-3 PUFAs are thought to play an important role in the modulation of immune cell activation by exerting action through Th1/Th2 dichotomy in diabetic pregnancy and macrosomia. Consequently, the fatty acids are being used in the management of diabetes mellitus in human beings [100] and experimental models [94] and also in several inflammatory and autoimmune diseases including rheumatoid arthritis and multiple sclerosis [101]. Physiologically, n-3 PUFA suppresses mitogen-stimulated proliferation of lymphocytes isolated from lymph nodes [94]. It has also been shown that dietary EPA and DHA are equipotent in inhibiting IL-2 production in rodents [102, 103]. The production of IFN-γ is also decreased by the fatty acids [104]. Hence, it can be argued that the exhibited potential action of the fatty acids on cytokine secretion [102] is attributed to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which appear as the most potent immunomodulators of omega-3 PUFA family.

With regard to the effects of omega-3 PUFA on diabetic pregnancy, previous studies have established that feeding omega-3-enriched diet to pregnant healthy animals potentiates the increase of the Th2 phenotype in the lymphoid organ (spleen) and peripheral blood of the animals on standard diet [65, 105]. In diabetic pregnancy however, omega-3-enriched diet increases Th2 cytokines and decreases Th1 cytokines at both expressing and circulating levels [65].

In animal and human studies, a shift of Th1/Th2 balance to a proinflammatory Th1 phenotype has been observed in macrosomic newborns and adult obese animals that were macrosomic as newborns of diabetic dams [26, 65]. As compared to animals fed on a standard diet, feeding the omega-3 PUFA enriched diet to adult obese rats from macrosomic newborns significantly diminishes the mRNA expression of Th1 cytokines and increases that of IL-4 but not that of IL-10 [65] (Figure 1). While the circulating high concentrations of IFN-γ observed in adult obese rats from macrosomic newborns are lowered by omega-3 PUFA diet, the IL-4 level observed in the animals is increased by the omega-3 PUFA diet (Figure 1). These findings suggest that omega-3 PUFA diet exerts beneficial effects in obese rats from macrosomic newborns by significantly shifting the Th1/Th2 (IFN-γ/IL-4) ratio to a Th2 phenotype [65] (Figure 1). However, the omega-3 PUFA diet could not significantly influence glycaemia in macrosomic newborns and obese rats, suggesting that macrosomia may be a multifactorial pathology.

6. Conclusion

Gestational diabetes mellitus and macrosomia that results in adulthood obesity are pathologies associated with several metabolic disorders, implicating lipid metabolism, altered antioxidant status, and disrupted immune defense system. Based on the evidence available in animal studies, feeding omega-3 PUFA diet not only decreases the high rate of macrosomia induced by diabetic pregnancy but also exerts a lipid-lowering action in both pathologies. Omega-3 PUFA also contributes to the protection against oxidative stress during gestational diabetes and adulthood obesity that results from macrosomia and restores the immune defense system disrupted by diabetes. The protection is by enhancing Th2 phenotype in diabetic pregnancy and therefore shifting the Th1/Th2 phenotype from a deleterious proinflammatory Th1 phenotype to a protective anti-inflammatory Th2 phenotype in offspring that were macrosomic at birth and became obese in the adulthood. Further studies targeting humans are recommended to further explore application of the fatty acids as supplements in the management of gestational diabetes and inflammatory and immune disease conditions.

Abbreviations

GDM:	Gestational diabetes mellitus
PUFA:	Omega-3 polyunsaturated fatty acids
CD:	Cluster of differentiation
IL:	Interleukin
IFN:	Interferon
AA:	Arachidonic acid
LA:	Linoleic acid; eicosapentaenoic acid (EPA); and docosahexaenoic acid (DHA)
Th cells:	T helper cells
TBARS:	Thiobarbituric acid-reactive substances
TG:	Triglyceride
TC:	Total cholesterol
SOD:	Superoxide dismutase
GSH-Px:	Glutathione peroxidase
GSSG-Red:	Glutathione reductase.

Conflict of Interests

All of the authors have nothing to declare as far as the conflict of interests is concerned.

Acknowledgments

The work performed in the authors' laboratory was supported by the French Foreign Office and the Ministry of Higher Education and Research (France) which sanctioned the contingent grants for the work. The authors also express their sincere thanks to the Islamic Development Bank (IDB) and the Office of Scholarship Programme that granted a postdoctoral fellowship to A. Yessoufou at the Centre for Integrative Genomics (University of Lausanne, Switzerland).

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Journal of Diabetes Research
Volume 2015, Article ID 261418, 8 pages
http://dx.doi.org/10.1155/2015/261418

Research Article

Characterization of the Prediabetic State in a Novel Rat Model of Type 2 Diabetes, the ZFDM Rat

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Received 12 September 2014; Revised 16 February 2015; Accepted 23 February 2015

Academic Editor: Kazuya Yamagata

We recently established a novel animal model of obese type 2 diabetes (T2D), the Zucker fatty diabetes mellitus (ZFDM) rat strain harboring the *fatty* mutation (*fa*) in the leptin receptor gene. Here we performed a phenotypic characterization of the strain, focusing mainly on the prediabetic state. At 6–8 weeks of age, *fa/fa* male rats exhibited mild glucose intolerance and severe insulin resistance. Although basal insulin secretion was remarkably high in the isolated pancreatic islets, the responses to both glucose stimulation and the incretin GLP-1 were retained. At 10–12 weeks of age, *fa/fa* male rats exhibited marked glucose intolerance as well as severe insulin resistance similar to that at the earlier age. In the pancreatic islets, the insulin secretory response to glucose stimulation was maintained but the response to the incretin was diminished. In nondiabetic Zucker fatty (ZF) rats, the insulin secretory responses to both glucose stimulation and the incretin in the pancreatic islets were similar to those of ZFDM rats. As islet architecture was destroyed with age in ZFDM rats, a combination of severe insulin resistance, diminished insulin secretory response to incretin, and intrinsic fragility of the islets may cause the development of T2D in this strain.

1. Introduction

Type 2 diabetes (T2D) is the most common form of diabetes, afflicting more than 80% of all people with the disease. T2D is a metabolic disorder characterized by chronic hyperglycemia due to insulin resistance and/or impaired insulin secretion. Despite the increasing incidence and prevalence of T2D, little is known about effective treatment and prevention of the disease and its complications at early stages.

Spontaneous animal models have contributed greatly to the study of T2D. There are several useful rat models such as the Goto-Kakizaki (GK) rat [1], Wistar fatty rat [2], Zucker diabetic fatty (ZDF) rat [3], Otsuka Long-Evans Tokushima fatty (OLETF) rat [4], and Spontaneously Diabetic Torii (SDT) rat [5]. Spontaneous models enable comparison of normoglycemic, prediabetic, and diabetic states in a limited time period and are especially helpful in the study of mechanistic, pathophysiological, and prevention aspects of T2D. Only a few of these models are widely suitable for use in these studies, including the ZDF and the SDT rat.

We recently established a novel rat model of obese T2D, the Zucker fatty diabetes mellitus (ZFDM) rat harboring the *fatty* mutation (*fa*) in the leptin receptor gene. *fa/fa* male rats maintain the normoglycemic state until 7 weeks of age and then develop diabetes as early as 10 weeks of age, reaching 100% incidence at around 20 weeks of age [6]. In contrast to the original Zucker fatty (ZF) rat [7], the Wistar fatty rat, and the ZDF rat, *fa/fa* male rats in the ZFDM strain are

fertile and possess high reproductive efficiency. ZFDM rats therefore could serve as a useful *model of* T2D.

Here we performed a phenotypic characterization of the ZFDM strain, focusing mainly on the prediabetic state. We also characterized the insulin secretory responses to both glucose stimulation and the incretin GLP-1 in the isolated pancreatic islets. In addition, we compared these characteristics of ZFDM rats with those of original ZF rats.

2. Materials and Methods

2.1. Animals. Male ZFDM rats (Hos:ZFDM-*Lepr*^{fa}, *fa*/*fa* and *fa*/+) were provided by Hoshino Laboratory Animals, Inc. (Ibaraki, Japan). Male ZF rats (Slc:Zucker, *fa*/*fa* and +/+) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All animals were maintained under specific pathogen free conditions with a 12 h light-dark cycle and were provided with a commercial diet CE-2 (CLEA Japan, Inc., Tokyo, Japan) at the Animal Facility of Kobe Biotechnology Research and Human Resource Development Center of Kobe University. All animal experiments were approved by the Committee on Animal Experimentation of Kobe University and carried out in accordance with the Guidelines for Animal Experimentation at Kobe University.

2.2. Oral Glucose Tolerance Test (OGTT). Glucose (2.0 g/kg body weight) was administered orally to 6 h fasted rats. Blood samples were collected from the tail vein at indicated time points. Blood glucose levels were measured by a portable glucose meter (ANTSENSE III, HORIBA, Ltd., Kyoto, Japan) and plasma insulin levels were measured by insulin ELISA kit (Shibayagi Co., Ltd., Gunma, Japan).

2.3. Insulin Tolerance Test (ITT). Insulin (1.0 IU/kg body weight) (Humulin R, Eli Lilly Japan K.K., Kobe, Japan) was administered subcutaneously to 6 h fasted rats. Blood samples were collected from the tail vein at indicated time points. Blood glucose levels were measured by a portable glucose meter (ANTSENSE III).

2.4. Insulin Secretion from Isolated Pancreatic Islets. Pancreatic islets were isolated by the collagenase digestion and Ficoll gradient method [8, 9]. Isolated pancreatic islets were cultured for 3 days in RPMI1640, preincubated for 30 min in H-KRB with 2.8 mM glucose, and then incubated for 30 min in H-KRB with 11.1 mM glucose in the presence or absence of 10 nM GLP-1. Insulin released in the incubation buffer and cellular insulin content in the pancreatic islets were measured by insulin assay kits from CIS Bio international (Gif sur Yvette, France). The amounts of insulin secretion were normalized by the cellular insulin content determined by 0.1% Triton X-100 extraction.

2.5. Histological Analysis. Histological analysis of the pancreas was performed using procedures essentially as described previously [10]. Briefly, the pancreas was fixed in 10% neutral buffered formalin. The fixed specimens were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin for histopathological examination.

2.6. Statistical Analysis. Data are expressed as mean ± SEM. Differences in blood glucose level and plasma insulin level were assessed using Welch's t-tests. Differences in insulin secretion from the pancreatic islets were assessed using Tukey-Kramer method. Differences for which the P value was <0.05 were regarded as statistically significant.

3. Results

3.1. Characterization of Glucose Tolerance of the ZFDM Strain. To characterize glucose tolerance of ZFDM rats at the prediabetic state, we performed OGTT at 6–8 weeks of age and at 10–12 weeks of age. At 7 weeks of age, *fa*/*fa* male rats exhibited mild but significant glucose intolerance after oral glucose loading (Figure 1(a)). At 120 min after glucose loading, blood glucose levels of *fa*/*fa* rats were decreased to the fasting glucose levels. Plasma insulin levels of *fa*/*fa* rats were significantly higher at the fasting state (0 min) than those of *fa*/+ rats (Figure 1(b)). The differences in insulin levels were more evident after glucose loading.

At 11 weeks of age, *fa*/*fa* rats exhibited marked glucose intolerance at all time points examined after glucose loading (Figure 1(c)). Blood glucose levels of *fa*/*fa* rats were decreased but not to the fasting glucose levels at 120 min after glucose loading. Plasma insulin levels of *fa*/*fa* rats were markedly higher than those of *fa*/+ rats at the fasting state (0 min) and after glucose loading (Figure 1(d)). These results indicate mild glucose intolerance in *fa*/*fa* rats at 7 weeks of age and further deterioration with age.

3.2. Characterization of Insulin Sensitivity of the ZFDM Strain. To characterize insulin sensitivity of ZFDM rats at the prediabetic state, we performed ITT at 7 and 11 weeks of age. *fa*/*fa* rats at both ages exhibited severe insulin resistance (Figures 2(a) and 2(b)). There was no apparent difference in the degree of insulin resistance at 7 and 11 weeks of age. These results indicate severe insulin resistance in *fa*/*fa* rats already at 7 weeks, which remains with age.

3.3. Comparison of the Glucose Tolerance and Insulin Sensitivity of the ZFDM Strain with the Nondiabetic ZF Strain. To compare the features of glucose tolerance and insulin sensitivity of ZFDM rats with those of nondiabetic ZF rats, we performed OGTT and ITT on ZF rats at 12 weeks of age. ZF *fa*/*fa* rats exhibited mild but significant glucose intolerance after glucose loading (Figure 3(a)). In contrast to ZFDM *fa*/*fa* rats, blood glucose levels of ZF *fa*/*fa* rats were decreased to the fasting glucose levels at 120 min after glucose loading. Plasma insulin levels of ZF *fa*/*fa* rats were significantly higher at the fasting state (0 min) than those of +/+ rats (Figure 3(b)). After glucose loading, the insulin levels of ZF *fa*/*fa* rats were markedly increased and were significantly higher than those of ZFDM *fa*/*fa* rats (Figure 3(d)). These findings indicate that

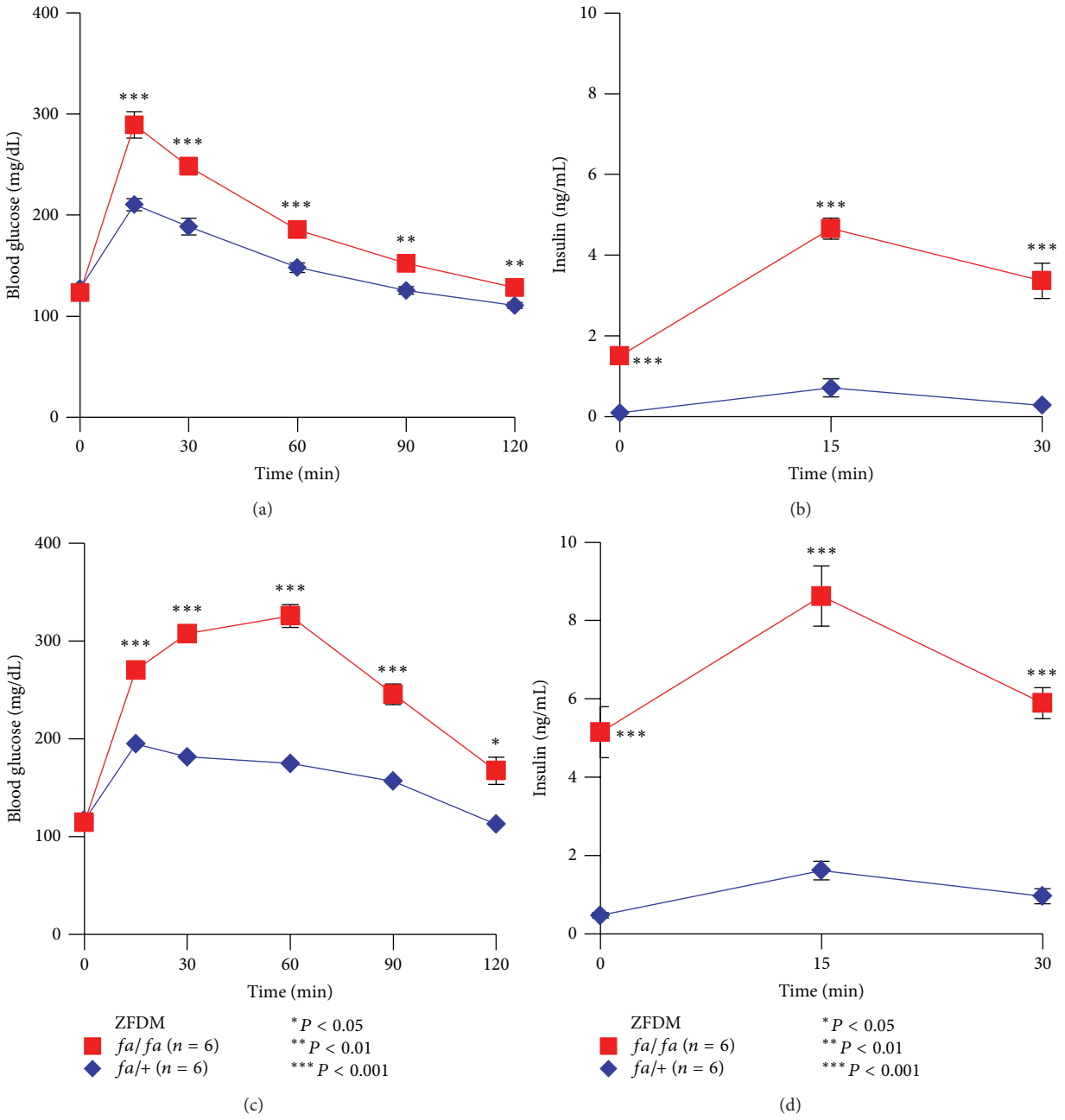

Figure 1: Characterization of glucose tolerance of the ZFDM strain. (a) Blood glucose levels and (b) plasma insulin levels during OGTT at 7 weeks of age. (c) Blood glucose levels and (d) plasma insulin levels during OGTT at 11 weeks of age. The data are expressed as mean ± SEM. Welch's *t*-test was used for comparisons between *fa/fa* (red, $n = 6$) and *fa/+* (blue, $n = 6$) rats.

fa/fa rats in the ZF strain can maintain the state of mild glucose intolerance by increasing insulin secretion, while *fa/fa* rats in the ZFDM strain cannot, due to defects in insulin secretion.

ZF *fa/fa* rats exhibited severe insulin resistance, as assessed by ITT (Figure 3(c)). There was no apparent difference in the degree of insulin resistance in *fa/fa* rats between the ZFDM and ZF strains. These results suggest a similar condition of severe insulin resistance in *fa/fa* rats in both strains.

3.4. Insulin Secretory Responses to Both Glucose and Incretin Stimulation in Isolated Pancreatic Islets. To clarify the insulin secretory responses to both glucose stimulation and the incretin GLP-1 in pancreatic islets of ZFDM rats at the prediabetic state, we performed batch incubation experiments using isolated pancreatic islets at 7 and 11 weeks of age. Although basal insulin secretion from the pancreatic islets of *fa/fa* rats was remarkably higher than that of *fa/+* rats at 7 weeks of age (Figures 4(a) and 4(b)), the insulin secretory responses to both glucose stimulation and incretin were retained in *fa/fa*

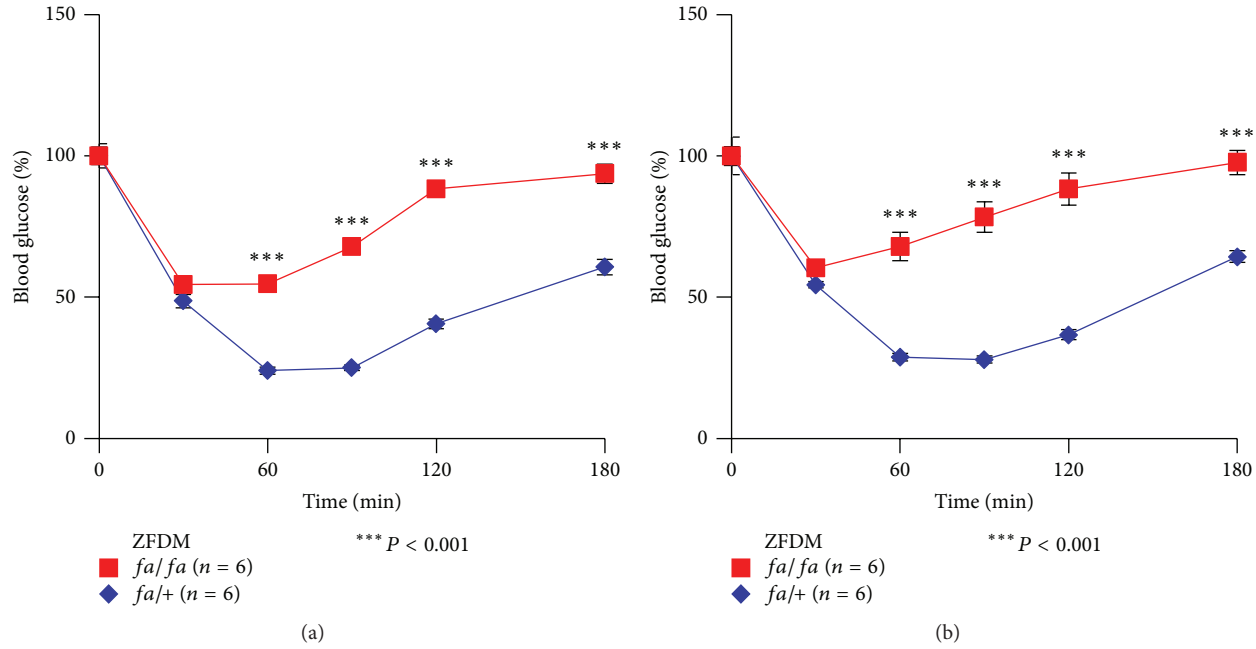

Figure 2: Characterization of insulin sensitivity of the ZFDM strain. Blood glucose levels during ITT at (a) 7 and (b) 11 weeks of age. The data are expressed as mean ± SEM. Welch's t-test was used for comparisons between *fa/fa* (red, $n = 6$) and *fa/+* (blue, $n = 6$) rats.

rats. At 11 weeks of age, the insulin secretory response to glucose stimulation was maintained but that to the incretin was extremely diminished in *fa/fa* rats (Figures 4(c) and 4(d)). Similar insulin secretory responses were observed in *fa/fa* rats in the nondiabetic ZF strain (Figures 4(e) and 4(f)). These findings indicate that defects in insulin secretion from the pancreatic islets are common in *fa/fa* rats of both strains.

3.5. Histological Characterization of the Pancreas of the ZFDM Strain. To clarify the histological changes in the pancreas of ZFDM rats at the prediabetic state, we performed histological characterization of the pancreas at 7, 12, and 20 weeks of age. There were no obvious pathological changes in endocrine and exocrine pancreas of *fa/+* rats (Figures 5(a), 5(b), 5(g), 5(h), 5(m), and 5(n)). In *fa/fa* rats, enlarged pancreatic islets were observed at 7 weeks of age (Figures 5(c) and 5(d)), and islet architecture was destroyed with age (Figures 5(i), 5(j), 5(o), and 5(p)). In contrast, islet architecture was substantially maintained with age in *fa/fa* rats in the ZF strain (Figures 5(e), 5(f), 5(k), 5(l), 5(q), and 5(r)).

4. Discussion

In this study, we characterized the prediabetic state of the ZFDM strain. Mild glucose intolerance exists in *fa/fa* rats at 7 weeks of age and deteriorates with age. In contrast, severe insulin resistance already exists in *fa/fa* rats at 7 weeks of age and remains severe with age. In the pancreatic islets, the insulin secretory response to glucose stimulation is retained but that to the incretin GLP-1 is diminished with age. In contrast, *fa/fa* rats in the nondiabetic ZF strain maintain mild glucose intolerance by increasing insulin secretion with age. However, the defect in the insulin secretory response to the incretin is common in *fa/fa* rats of both strains. These findings together indicate that, in addition to severe insulin resistance and diminished insulin response to the incretin, other defects are involved in the development of T2D in ZFDM rats.

In the ZFDM strain, *fa/fa* rats exhibit enlarged pancreatic islets at 7 weeks of age, in compensation for increased insulin demand due to severe insulin resistance. However, islet architecture is destroyed with age, resulting in relative insulin deficiency. In contrast, *fa/fa* rats in the ZF strain maintain enlarged pancreatic islets and architecture with age, which compensates for severe insulin resistance to maintain normoglycemia. These findings suggest that a high degree of fragility of islets is intrinsic to *fa/fa* rats in the ZFDM strain. ZFDM *fa/fa* rats fail to compensate for severe insulin resistance, resulting in the development of diabetes.

It has been reported that β-cell mass in 5- to 7-week-old prediabetic ZDF *fa/fa* rats was similar to that in age-matched ZF *fa/fa* rats and greater than that in Zucker lean (+/?) rats [11]. At 12 weeks of age (after diabetes onset), β-cell mass in ZDF *fa/fa* rats was lower than that that in ZF *fa/fa* rats. The failure of β-cell expansion was thought to be due to an increased rate of cell death [11]. In another report [12], β-cell mass was decreased by 51% from 8 to 12 weeks of age in ZDF *fa/fa* rats. The increase in β-cell death was well correlated with the increase in plasma glucose levels, suggesting that hyperglycemia in ZDF rats develops concomitantly with increasing net β-cell death. Despite the delay in onset of diabetes, a similar change in the β-cell mass might occur in ZFDM *fa/fa* rats.

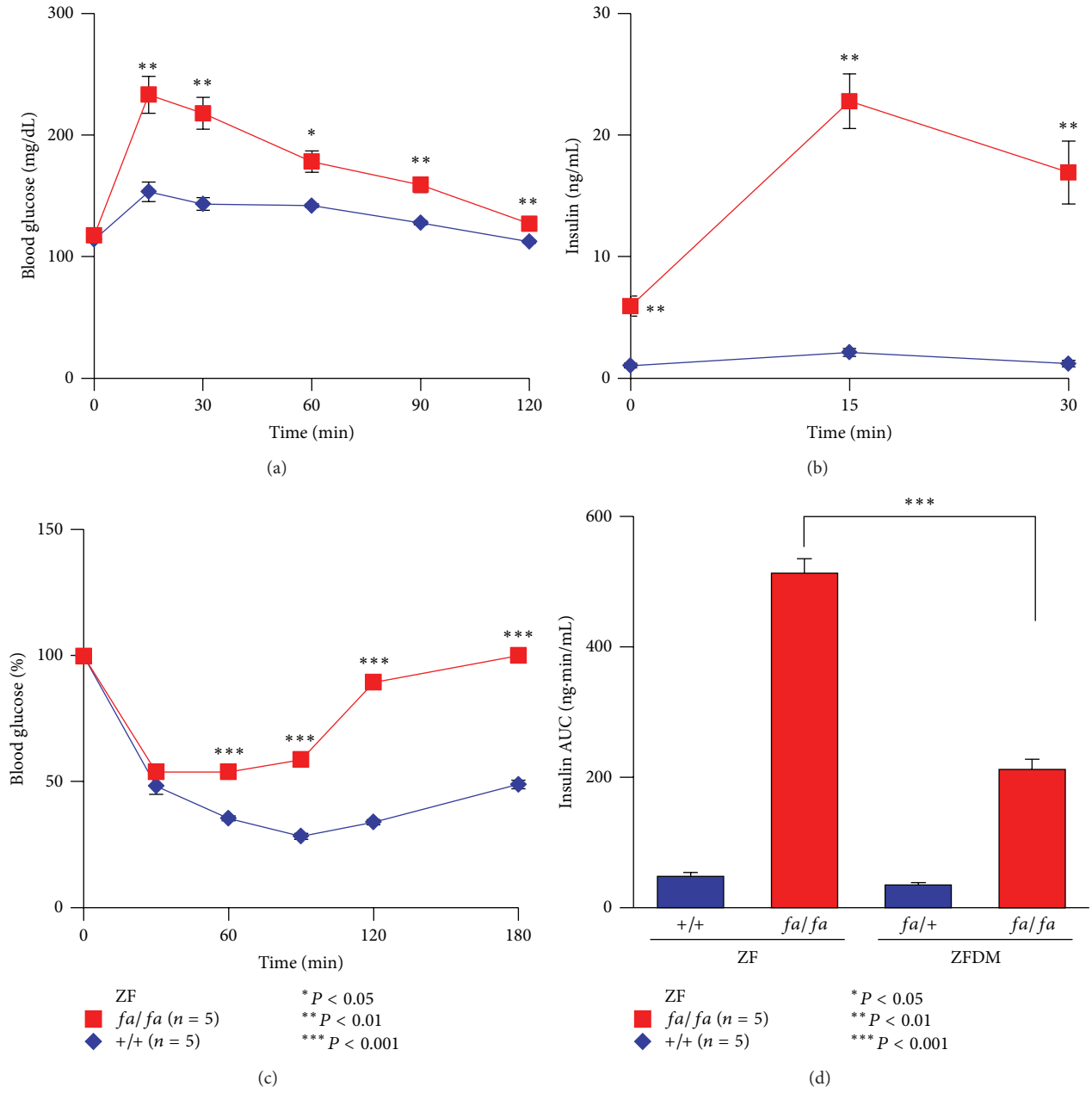

FIGURE 3: Characterization of glucose tolerance and insulin sensitivity of the ZF strain. (a) Blood glucose levels and (b) plasma insulin levels during OGTT at 12 weeks of age. (c) Blood glucose levels during ITT at 12 weeks of age. (d) AUC data on insulin secretion during OGTT in the ZF and ZFDM strains. The data are expressed as mean ± SEM. Welch's *t*-test was used for comparisons between *fa/fa* (red, $n = 5$) and +/+ (blue, $n = 5$) rats ((a), (b), and (c)) and between *fa/fa* rats in the both strains ($n = 5$-6) (d).

We found a diminished insulin response to the incretin GLP-1 not only in ZFDM *fa/fa* but in ZF *fa/fa* islets at 12 weeks of age, indicating a common defect in the pancreatic islets of both strains. In support of this finding, several pathological features have been reported in ZF *fa/fa* islets at 14 weeks of age, such as β-cell vacuolation, vascular congestion, haemorrhage, fibrosis, and minimal mononuclear cell infiltration [13]. These pathological changes could affect normal function of the islets, including that of incretin-induced insulin secretion. In addition to relatively large islets accounting for the majority of the islet population, there are relatively small and morphologically normal islets in 11-week-old *fa/fa* rats of both strains. Insulin secretory responses to both glucose stimulation and incretin were retained in these small islets (data not shown), which further supports the correlation between pathological changes in the pancreatic islets and the diminished insulin response to incretin.

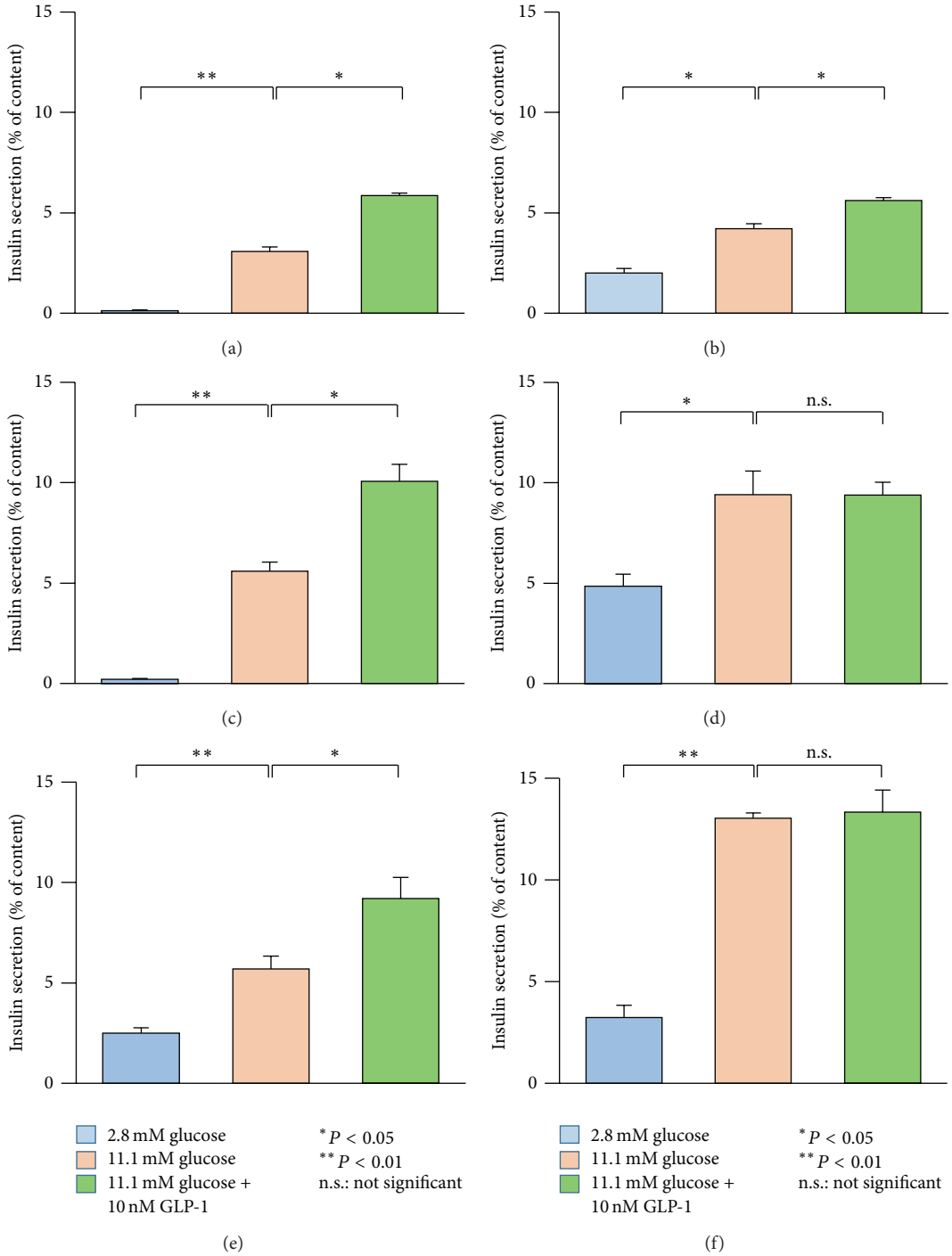

Figure 4: Insulin secretion from the pancreatic islets of ZFDM and ZF strains. Insulin secretory responses to glucose alone (11.1 mM) and glucose plus GLP-1 (10 nM) in (a) *fa*/+ ($n = 6$) and (b) *fa*/*fa* ($n = 6$) rats in ZFDM strain at 7 weeks of age. Insulin secretory responses in (c) *fa*/+ ($n = 6$) and (d) *fa*/*fa* ($n = 6$) rats in ZFDM strain at 11 weeks of age. Insulin secretory responses in (e) +/+ ($n = 6$) and (f) *fa*/*fa* ($n = 6$) rats in ZF strain at 11 weeks of age. The data are expressed as mean ± SEM. Differences in insulin secretion from the pancreatic islets were assessed using Tukey-Kramer method.

5. Conclusions

In this study, we characterized the prediabetic state of a novel animal model of obese T2D, the ZFDM strain. In addition to severe insulin resistance and diminished insulin response to incretin, intrinsic fragility of islets in ZFDM rats may contribute to the development of T2D in this strain. The ZFDM strain should be useful for studying the mechanisms

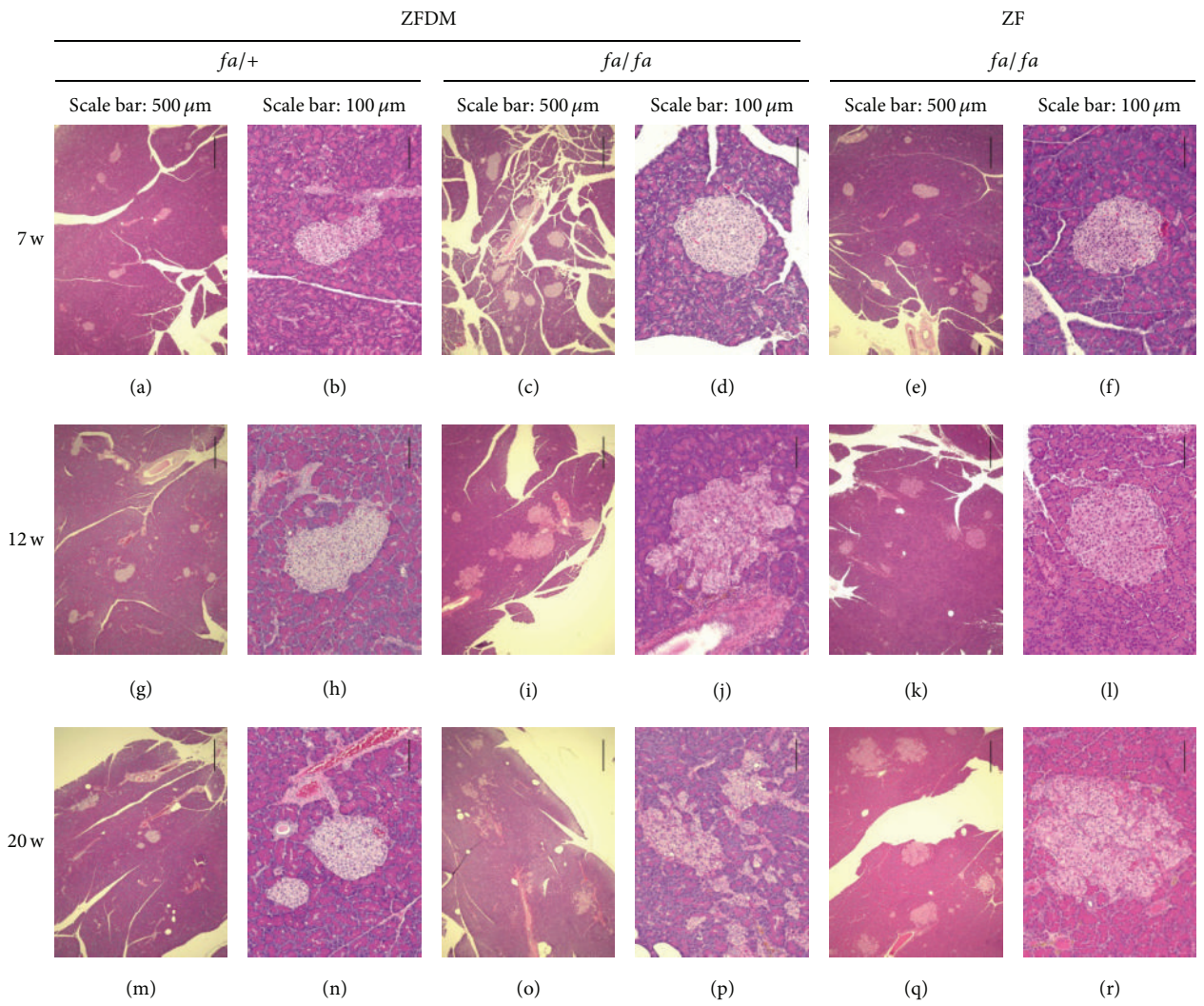

Figure 5: Histological characterization of the pancreas of the ZFDM and ZF strains. Representative pancreas histology of *fa*/+ ((a), (b), (g), (h), (m), and (n)) and *fa*/*fa* ((c), (d), (i), (j), (o), and (p)) male rats in the ZFDM strain and *fa*/*fa* ((e), (f), (k), (l), (q), and (r)) male rats in the ZF strain. Hematoxylin and eosin staining.

of incretin-induced insulin secretion and islet fragility in the pathogenesis of T2D.

Conflict of Interests

Yoshikazu Hoshino and Masayuki Hoshino are an employee and the president of Hoshino Laboratory Animals, Inc., respectively, the company providing the Hos:ZFDM-*Lepr*^{fa} rats.

Acknowledgments

The authors express appreciation and thanks to all of the colleagues in their laboratories. This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2014, Article ID 629016, 5 pages
http://dx.doi.org/10.1155/2014/629016

Research Article

Ocular Inflammation in Uveal Tract in Aged Obese Type 2 Diabetic Rats (Spontaneously Diabetic Torii Fatty Rats)

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Received 3 July 2014; Revised 2 September 2014; Accepted 4 September 2014; Published 14 September 2014

Academic Editor: Masami Shinohara

We report uveitis observed in an obese type 2 diabetes rat model, Spontaneously Diabetic Torii Lepr^{fa} (SDT fatty) rats aged over 50 weeks. The eyes of SDT fatty rats (16 animals: 7 males and 9 females with 50 or 60 weeks of age) were examined histopathologically. Infiltration of inflammatory cells in the uveal tract was observed in 13 of 16 animals. One female showed severe inflammation affecting the entire uveal tract including the iris, ciliary body, and choroid with a variety of inflammatory cells (neutrophils, lymphocytes, and macrophages). Those changes clinically mimic the findings of diabetic iridocyclitis in diabetic patients. Uveitis associated with diabetes can occur in diabetic patients but the pathogenesis still remains unknown. Since increased extramedullary hematopoiesis in the spleen and abscess in the genital and lower urinary tracts were observed in some SDT fatty rats, increased susceptibility to infection, prolongation of inflammatory states, and disorders of the immune system were considered to be possible factors of the uveitis in aged SDT fatty rats. There have been few reports on how diabetes has influence on the development of uveitis associated with bacterial infection. The SDT fatty rat can be an animal model to investigate diabetes-associated uveitis.

1. Introduction

Uveitis can be defined as inflammation in the uveal tract in the eye. Uveitis is the major cause of severe visual impairment in human and has been estimated to account for 5% to 15% of all cases of total blindness in the United States [1]. Diabetes has long been known to increase the chances of a variety of eye diseases, including retinopathy, cataracts, glaucoma, and uveitis [2]. However, the mechanism of cause of diabetes-associated uveitis still remains unknown.

The Spontaneously Diabetic Torii (SDT) fatty rat, established by introducing the fa allele of the Zucker fatty rat into the SDT rat genome, is a new model of obese type 2 diabetes [3]. The SDT-fa/fa (SDT fatty) rat shows overt obesity, hyperglycemia, and hyperlipidemia at a young age as compared with the other diabetes rat models [4, 5]. With an early incidence of diabetes mellitus, diabetes-associated complications in SDT fatty rats were observed at younger ages compared with those in the SDT rats [4–7]. Spontaneous ocular lesions of the SDT fatty rats up to 40 weeks of age have been reported but not in aged SDT fatty rats over 50 weeks of age [4–6]. We examined the eye of the SDT fatty rat over 50 weeks of age histopathologically in both sexes and report its characteristics.

2. Materials and Methods

Male and female SDT-fa/fa (fatty) rats and age-matched SDT-+/+ (SDT) rats were used from our colonies and age-matched Sprague-Dawley (SD) rats were purchased from CLEA Japan, Inc. (Tokyo, Japan). The rats were housed in a climate-controlled room with a temperature of 23 ± 3°C,

humidity of 55 ± 15%, and a 12 h lighting cycle. A basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and water were provided *ad libitum*. All the experiments received prior approval from the committee for the human care and use of animals of our laboratory, in accordance with the Standards Relating to the Care and Management of Experimental Animals. Necropsy was performed on the SDT fatty rats, SDT rats, and SD rat at 50 and 60 weeks of age. All animals were sacrificed by exsanguinations under light ether anesthesia. The eyes were sampled and fixed in formalin-glutaraldehyde mix fixative, then embedded in paraffin, sectioned, stained with hematoxylin and eosin (HE), and examined histopathologically (SDT fatty rats; 16 (7 males and 9 females), SDT rats; 17 (6 males and 11 females), and SD rat; 19 (9 males and 10 females)).

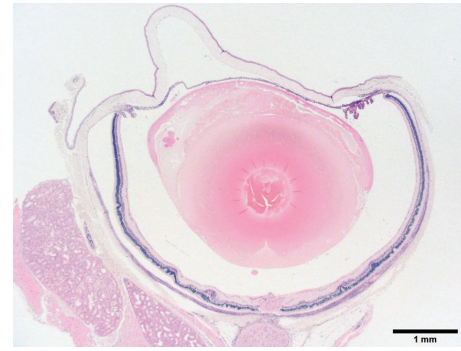

Figure 1: Photomicrograph of the eye of a 50-week-old female SDT fatty rat. Low power magnification of an eye showing deformation of lens, disarrangement of the retina (retinal fold), and infiltration of inflammatory cells in the uveal tract. Bar = 1 mm. Hematoxylin and eosin.

3. Results and Discussion

At necropsy, severe opacity of the lens was observed in all of the SDT fatty rats in both sexes (data not shown). A large-sized spleen and abscess surrounded in the genital and lower urinary tracts were also observed in SDT fatty rats in both sexes. Some SDT fatty rats also showed swelling and thickening of the foot pad, the so-called "bumble foot," possibly due to the increase in body weight and chronic stimulus from the wire-bottomed cages on the foot pad.

Histologically uveitis, including infiltration of inflammatory cells in the uveal tract in the eye, was observed in 13 of 16 SDT fatty rats of both sexes (Table 1). The inflammatory cells consisted of neutrophils, macrophages, and lymphocytes. The finding was observed mainly in the choroid, but one female showed severe inflammation throughout the iris and ciliary body (Figures 1, 2, 3, 4, and 5). The changes clinically mimic the findings in diabetic iridocyclitis in diabetic patients. Advanced cases with these findings result in posterior synechia and following this are iris bombe and acute glaucoma. In the SDT rats, very slight inflammatory cell infiltration was also observed in the iris and ciliary body but no findings were observed in the choroid. There were increases in the incidence and degree of thickened and disarranged retinal layers (retinal fold) and degenerative lens fiber (cataract) in the SDT fatty rats, compared with those in the SDT rats. The enlarged spleen in the SDT fatty rats was histologically characterized by severely increased extramedullary hematopoiesis (EMH). Myeloid and granulocyte components were predominant in the EMH in the spleen of the SDT fatty rats. A lesion in the foot pad showed histologically ulcer and acanthosis of the epidermis with inflammation (ulcerative pododermatitis).

Uveitis is an inflammatory ocular disease of the uveal tract which is composed of the iris, choroid, and ciliary body. Uveitis can be caused by various factors including infectious or noninfectious (autoimmune) processes, often associated with systemic disease [8]. Although retinopathy is a major diabetic complication in the eye, uveitis can also be observed in diabetic patients [8, 9]. Diabetic patients, 1% to 6%, had uveitis in the iris, the most common localization of uveitis in diabetic humans [8–10]. Destruction of the blood retina barrier, increased blood permeability, and increased susceptibility to infection are considered to be involved in diabetes-associated uveitis but the details of pathogenesis still remain unknown [11–13].

Diabetes has been identified as an important risk factor for infection [14]. Hyperglycemia can induce poor wound healing and increased susceptibility to infection [15]. Once infection occurs in diabetic conditions, inflammation tends to last because diabetes prolongs the inflammatory response to a bacterial stimulus through cytokine dysregulation [16]. Endotoxin induced uveitis (EIU) is known as an experimental animal model of uveitis initiated by injection of lipopolysaccharide (LPS) [17, 18]. Although inflammatory response in this model lasts only for 72 hours, EIU rats with diabetes induced by streptozotocin (STZ) tended to have a prolonged inflammatory response [16].

EMH is commonly seen in the normal spleen, especially in young rodents as compared to aged rodents [19, 20]. EMH consists mainly of erythroid and myeloid precursors, indicating physiopathological responses secondary to hemorrhagic and inflammatory conditions, respectively. Increased EMH in the spleen in aged SDT fatty rats could have resulted from infections elsewhere in the body because some SDT fatty rats had severe inflammation in the foot pad and in the lower urinary tract, which could have been caused by bacterial infection. Another hematologic analysis on young SDT fatty rats showed that the leukocyte count (WBC) in SDT fatty rats was significantly higher than that in SD rats [6]. Decreased resistance to infection and a dysregulated inflammatory response may be responsible for the systemic condition of the SDT fatty rats.

Type 2 diabetes has been redefined as an inflammatory disease. The state of the immune system can be altered in obesity and type 2 diabetes, with apparent changes occurring in adipose tissue, liver, pancreatic islets, vasculature, and circulating leukocytes [21]. Type 2 diabetes is associated with higher serum levels of inflammatory cytokines (tumor necrosis factor, TNF) [22–24]. This may be due to the production of TNF in adipose tissue [25], the activity of advanced glycation end products, or enhanced cytokine production caused by

Table 1: Histopathological findings of the eyes in SDT fatty rats at weeks 50 and 60 of age.

Organ	Sex	Male														Female													
	Strain	SD					SDT					SDT fatty					SD					SDT					SDT fatty				
Pathological finding	Number of animals	9					6					7					10					11					9				
	Grade	−	±	+	2+	3+	−	±	+	2+	3+	−	±	+	2+	3+	−	±	+	2+	3+	−	±	+	2+	3+	−	±	+	2+	3+
Choroid																															
Infiltration, inflammatory cell		9					6					1	1	3	2		10					11					2	1	5		1
Iris/ciliary body																															
Infiltration, inflammatory cell		9					4	2				5	2				10					6	5					3	5	1	
Deposit, hemosiderin, and trabecular meshwork		9					3	1	2			4	2	1			10					6	5				8	1			
Retina																															
Retinal fold		8	1				3	1	2					4	3		10					9	1	1				1	3	5	
Lens																															
Deformation		9							5	1					7		10					4	2	5			1		2	6	
Degeneration, lens fiber (vacuolation/morgani body)		9								6						7	10					3	4	4			1		2	5	1
Hypertrophy/proliferation, epithelium		9					1	2	3			3		3	1		10					11					7		2		

−: negative, ±: very slight, +: slight, 2+: moderate, and 3+: severe.

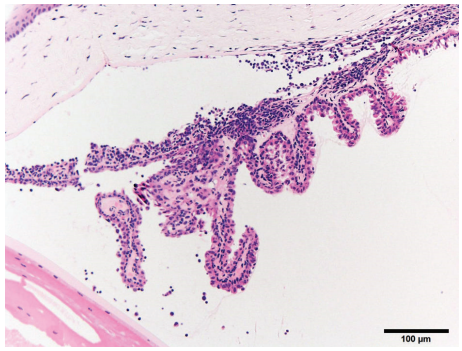

Figure 2: Photomicrograph of the ciliary body/iris of the eye from a 50-week-old female SDT fatty rat. There is infiltration of inflammatory cells in the ciliary body, iris, the anterior and posterior chambers, and the angle of anterior chamber. Bar = 100 μm. Hematoxylin and eosin.

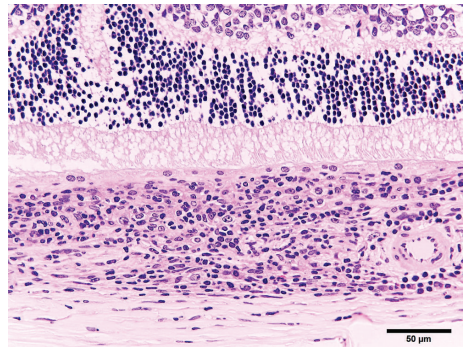

Figure 4: Photomicrograph of the choroid of the eye from a 50-week-old female SDT fatty rat. There is diffuse infiltration of inflammatory cells in the choroid. Inflammatory cells are partly spreading to the surrounding area in the sclera. Bar = 50 μm. Hematoxylin and eosin.

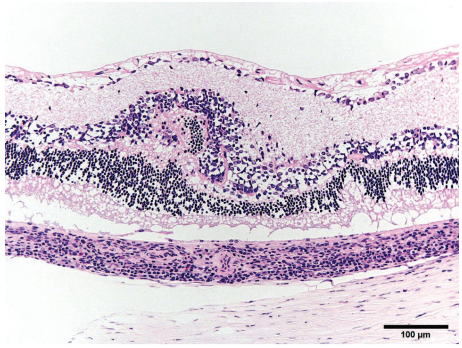

Figure 3: Photomicrograph of the retina and choroid of the eye from a 50-week-old female SDT fatty rat. There is infiltration of inflammatory cells in the choroid and the retinal fold characterized by disarrangement and thickness of the retinal layers. Bar = 100 μm. Hematoxylin and eosin.

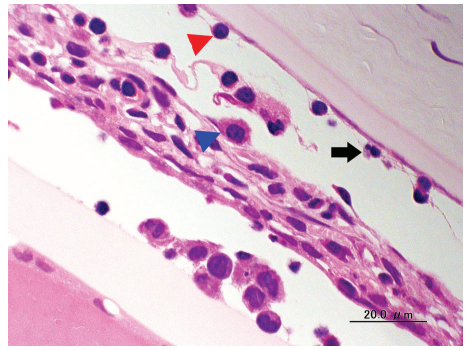

Figure 5: Photomicrograph of the iris of the eye from a 50-week-old female SDT fatty rat. There are inflammatory cells including a neutrophil (arrow), a macrophage (blue arrow head), and a lymphocyte (red arrow head) in the iris and the anterior and posterior chambers. Bar = 20 μm. Hematoxylin and eosin.

the indirect effects of hyperinsulinemia or hyperglycemia [26, 27]. It is reported that TNF mRNA levels in SDT fatty rats tended to be increased as compared with those in SDT rats [28].

Aldose reductase inhibitors, which are a class of drugs to prevent eye and nerve damage in diabetic patients, are associated with a decrease in ocular inflammatory complications such as uveitis [29]. When metformin was given to the experimental uveitis model rats induced by an endotoxin, which mimicked the inflammatory effects of bacterial infection, endotoxin induced uveitis was inhibited or was prevented in the metformin group [30]. These cases indicate that diabetes might be one of the contributing factors of uveitis because drugs for diabetes not only improved disorders of carbohydrate metabolism but also decreased ocular inflammation.

Hyperglycemia and dyslipidemia arise at a younger age in the SDT fatty rats compared with other diabetic rat models. Severe and prolonged metabolic disorders in the SDT fatty rats may result in uveitis possibly due to bacterial infection and dysregulated immune system. There are not many reports investigating the association between infection and uveitis by using diabetic animal models, possibly because the mechanism of uveitis is considered to be complicated and multifactorial. The SDT fatty rat is a possibly useful model for investigating the mechanisms of uveitis associated with obesity and diabetes mellitus, especially related to bacterial infection. Whether the SDT fatty rats have stronger susceptibility to infection followed by systemic inflammation leading to uveitis compared to other diabetic models still remains unknown. Further investigation to elucidate potential relationship between metabolic disorder and uveitis in the SDT fatty rats is required.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

T. Ohta and T. Sasase helped in the design of study. Y. Kemmochi, K. Toyoda, T. Ohta, and T. Sasase helped in the

data collection and conduct of the study. Y. Kemmochi, K. Miyajima, T. Ohta, K. Toyoda, Y. Yasui, K. Kakimoto, T. Shoda, and A. Kakehashi helped in the analysis of the study. Y. Kemmochi, K. Miyajima, T. Ohta, T. Sasase, K. Kakimoto, T. Shoda, and A. Kakehashi helped in writing the paper.

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Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2014, Article ID 672590, 7 pages
http://dx.doi.org/10.1155/2014/672590

Research Article

Effect of Ranirestat, a New Aldose Reductase Inhibitor, on Diabetic Retinopathy in SDT Rats

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Received 11 July 2014; Revised 1 August 2014; Accepted 4 August 2014; Published 25 August 2014

Academic Editor: Tomohiko Sasase

Purpose. To evaluate the effect of ranirestat, a new aldose reductase inhibitor (ARI), on diabetic retinopathy (DR) in Spontaneously Diabetic Torii (SDT) rats. *Methods*. The animals were divided into six groups, normal Sprague-Dawley rats ($n = 8$), untreated SDT rats ($n = 9$), ranirestat-treated SDT rats (0.1, 1.0, and 10 mg/kg/day, $n = 7, 8$, and 6, resp.), and epalrestat-treated SDT rats (100 mg/kg/day, $n = 7$). Treated rats received oral ranirestat or epalrestat once daily for 40 weeks after the onset of diabetes. After the eyes were enucleated, the retinal thickness and the area of stained glial fibrillary acidic protein (GFAP) were measured. *Results*. The retinas in the untreated group were significantly thicker than those in the normal and ranirestat-treated (0.1, 1.0, and 10 mg/kg/day) groups. The immunostained area of GFAP in the untreated group was significantly larger than that in the normal and ranirestat-treated (1.0 and 10 mg/kg/day) groups. There were no significant differences between the untreated group and epalrestat-treated group in the retinal thickness and the area of stained GFAP. *Conclusion*. Ranirestat reduced the retinal thickness and the area of stained GFAP in SDT rats and might suppress DR and have a neuroprotective effect on diabetic retinas.

1. Introduction

Diabetic retinopathy (DR) is a leading cause of visual loss and blindness in adults in most developed countries [1]. Appropriate and effective treatment against DR needs to be developed. Surgical treatments for DR, such as laser photocoagulation and vitrectomy, are well developed. The effect of laser photocoagulation for preventing and treating proliferative diabetic retinopathy (PDR) and diabetic macular edema (DME) has been proved [2, 3]. However, the effect of laser photocoagulation for treating PDR is somewhat limited and cannot be performed in patients with opaque media. The effect of grid laser photocoagulation for treating DME also is limited and advanced atrophic creep around the macula resulting in severe visual impairment has developed in some patients with DME treated with this therapy [4]. Vitrectomy, an established surgical treatment for PDR, is gaining in popularity for DME. However, the visual prognoses of vitrectomy for treating PDR and DME are unsatisfactory. Glycemic control is the primary medical treatment for DR. Several clinical trials have reported that intensive glycemic control reduces the incidence and progression of DR [5–7].

Although glycemic control seems to be the most important approach, achieving acceptable glucose homeostasis is difficult, even in patients who adhere strictly to treatment. Therefore, it is important to find medical options other than glycemic control to prevent DR. The metabolic changes that accompany hyperglycemia, such as activation of the polyol pathway [8], activation of protein kinase C (PKC) [9], increased oxidative stress [10], leukocyte adhesion to

the endothelial cells [11], and accumulation of advanced glycation end products (AGEs) [12], are related to the development and progression of diabetic ocular complications. In particular, the polyol pathway is correlated strongly with oxidative stress, activation of PKC, and accumulation of AGEs that lead to induction of vascular endothelial growth factor (VEGF). Intravitreous injections of triamcinolone [13, 14] and anti-VEGF agents [15–17] are recently developed major treatments for DME. However, they are associated with risk of infection due to multiple intravitreous injections and high cost; in addition, they are not indicated for PDR and only for limited cases without pathological changes such as vitreomacular traction.

Among the targeted metabolic factors, we focused on the polyol pathway. A key enzyme in the polyol pathway is aldose reductase (AR), which is found in the retina, lens, and Schwann cells of the peripheral nerves [18]. Our previous study showed an inhibitory effect of fidarestat (SNK-860, Sanwa Kagaku Kenkyusho, Nagoya, Japan), an AR inhibitor (ARI), on the development of DR in SDT rats [19]. Fidarestat suppressed the VEGF levels in the ocular fluid and prevented extensive fluorescein leakage around the optic disc in that study. We also confirmed the effect of a new ARI, ranirestat (AS3201, Sumitomo Dainippon Pharmaceutical Co., Osaka, Japan), on DR in SDT rats [20]. Ranirestat suppressed accumulation of VEGF and Nε-(carboxymethyl) lysine in the retina of SDT rats in that study. We confirmed that ranirestat also suppressed diabetic cataract and neuropathy in SDT rats [21]. However, we did not confirm whether ranirestat prevents retinal edema and neurodegeneration in diabetic retinas. In the current study, we evaluated the effect of ranirestat on the development of DR by preventing retinal edema and on the neurodegeneration in diabetic retinas by preventing glial fibrillary acidic protein (GFAP) accumulation within the retina in SDT rats.

2. Materials and Methods

2.1. Animals. The care and handling of animals were in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research and the Jichi Medical University Animal Care and Use Committee. Some procedures used in this study were the same as the methods we reported previously, and the animals used in this study were the same as those we used in a previous study [21]. We obtained male SDT rats and SD rats from CLEA, Inc. (Tokyo, Japan). All SDT rats were confirmed to be diabetic based on a nonfasting blood glucose concentration exceeding 350 mg/dL. The SDT rats were diagnosed with diabetes by 12 to 20 weeks after birth. All rats were fed standard rat chow (CRF-1, Oriental Yeast, Inc., Tokyo, Japan). Treated rats received oral ranirestat or epalrestat, an ARI obtained from Sumitomo Dainippon Pharmaceutical Co. (Osaka, Japan), which served as a positive control, once daily for 40 weeks after the onset of diabetes. Untreated rats and normal SD rats received no drug for 40 weeks. The animals were divided into six groups: normal SD rats ($n = 8$), untreated SDT rats ($n = 9$), ranirestat-treated (0.1 mg/kg/day for 40 weeks) SDT rats ($n = 7$), ranirestat-treated (1.0 mg/kg/day for 40 weeks) SDT rats ($n = 8$), ranirestat-treated (10 mg/kg/day for 40 weeks) SDT rats ($n = 6$), and epalrestat-treated (100 mg/kg/day for 40 weeks) SDT rats ($n = 7$). All rats were over 50 weeks old.

The untreated SDT rats that were 31 weeks old and normal SD rats that were 33 weeks old were dissected to examine the developmental process ($n = 4, 4$), and we compared these rats with over 50-week-old normal SD rats and untreated SDT rats.

2.2. Measurement of Body Weight, Blood Glucose, and Glycated Hemoglobin. Body weight, blood glucose, and glycated hemoglobin (HbA1c) were measured once monthly. Blood samples were collected from the tail vein of nonfasting rats to measure the blood glucose and HbA1c. Blood glucose was measured by the hexokinase-G-6-PDH method (L type Wako Glu2, Wako Pure Chemical Industries, Ltd., Osaka, Japan). HbA1c was measured using an automated glycohemoglobin analyzer (HLC-723GHb V, Tosoh Corporation, Tokyo, Japan) [21].

2.3. Ocular Histopathology. Some ocular histopathology procedures were the same as the methods we reported previously [21]. Under deep anesthesia induced by an intraperitoneal injection of pentobarbital sodium (25 mg/kg body weight, Nembutal, Sumitomo Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), the eyes were enucleated for conventional histopathologic studies and placed in a fixative (Super Fix KY-500, Kurabo, Japan). The fixed eyes were washed in 0.1% mol/L cacodylate buffer and embedded in paraffin. The paraffin block was sectioned to 4 μm and stained with hematoxylin and eosin for conventional histopathologic examination. The immunohistochemical procedures were based on the standard avidin-biotin horseradish peroxidase method using each antibody and developed with AEC Substrate-Chromogen (Dakocytomation, Carpinteria, CA, USA). GFAP mouse monoclonal antibody (Cell Signaling Technology, Inc., Danvers, MA, USA) was used at a dilution of 1 : 50. Bovine serum was used as a primary antibody for negative control of the immunostaining.

2.4. Measurement of Retinal Thickness and Area of Stained GFAP. The paraffin blocks sectioned to 4 μm were examined using a polarizing microscope (Olympus BX-51, Olympus Corporation, Tokyo, Japan), and the images were recorded and downloaded using the attached digital camera and software (Olympus DP 72, DP2-BSW, Olympus Corporation). Retinal tissue 300 to 600 μm from the optic disc was observed for retinal changes using the DP2-BSW. The retinal thickness and the area of stained GFAP 300 μm in width were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The retinal thickness was measured as the distance between the retinal internal limiting membrane (ILM) and the retinal pigment epithelium.

2.5. Statistical Analysis. All values were expressed as the mean ± standard deviation. The Mann-Whitney U test and

Table 1: The mean retinal thickness and mean area of stained GFAP in each group. 31 wDM: 31-week-old SDT rats; 33 wNDM: 33-week-old normal SD rats; NDM: normal SD rats older than 50 weeks; DM: untreated SD rats older than 50 weeks; rani (0.1): ranirestat-treated SDT rats (0.1 mg/kg/day); rani (1.0): ranirestat-treated SDT rats (1.0 mg/kg/day); rani (10): ranirestat-treated SDT rats (10 mg/kg/day); epa (100): epalrestat-treated SDT rats (100 mg/kg/day).

	31 wDM	33 wNDM	NDM	DM	rani (0.1)	rani (1.0)	rani (10)	epa (100)
Mean retinal thickness (μm)	152.6 ± 12.8	127.2 ± 11.1	90.3 ± 19.8	158.2 ± 23.0	114.7 ± 22.8	93.5 ± 18.2	102.9 ± 17.9	129.7 ± 21.8
P value	0.22 (compared with DM) 0.021 (compared with 33 wNDM)	0.023 (compared with NDM)	0.0076 (compared with DM)		0.017 (compared with DM)	0.0052 (compared with DM)	0.016 (compared with DM)	0.057 (compared with DM)
Mean area of stained GFAP (μm^2)	225.8±132.9	128.0 ± 60.1	1069.8±311.7	2512.5±971.9	1426.8±1131.7	669.0±524.2	789.0±799.0	1671.4±604.6
P value	0.0055 (compared with DM) 0.24 (compared with 33 wNDM)	0.0055 (compared with NDM)	0.0044 (compared with DM)		0.13 (compared with DM)	0.0052 (compared with DM)	0.028 (compared with DM)	0.11 (compared with DM)

Steel's test were used for comparisons between each group. Excel Tokei 2006 software (Social Survey Research Information Co., Ltd., Tokyo, Japan) was used for statistical analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Body Weight, Blood Glucose, and Glycated Hemoglobin. Figures 1, 2, and 3 show the changes in weight, blood glucose, and HbA1c, respectively, during the study. Compared with the SD rats, the SDT rats were significantly ($P < 0.01$) lighter with and without ARI treatment. The mean blood glucose levels and HbA1c levels of the SDT rats were significantly ($P < 0.01$) higher than those of the SD rats. However, there were no significant differences in the blood glucose levels and HbA1c levels in the treated and untreated rats. Because the ARIs did not affect glycemic control, we did not consider the glycemic effect in this study [21].

3.2. Retinal Thickness and Area of Stained GFAP. The values are shown in Table 1. The retinas in the untreated SDT rats were significantly ($P = 0.0076$, $P = 0.017$, $P = 0.0052$, and $P = 0.016$, resp., Steel's test) thicker than those in the normal SD rats and ranirestat-treated (0.1, 1.0, and 10 mg/kg/day) SDT rats. There was no significant ($P = 0.057$, Steel's test) difference between the untreated SDT rats and the epalrestat-treated SDT rats. The stained area in the untreated SDT rats was significantly ($P = 0.0044$, $P = 0.0052$, and $P = 0.028$, resp., Steel's test) larger than that in the normal SD rats and ranirestat-treated (1.0 and 10 mg/kg/day) SDT rats. There was no significant ($P = 0.11$, Steel's test) difference between the untreated SDT rats and the epalrestat-treated SDT rats.

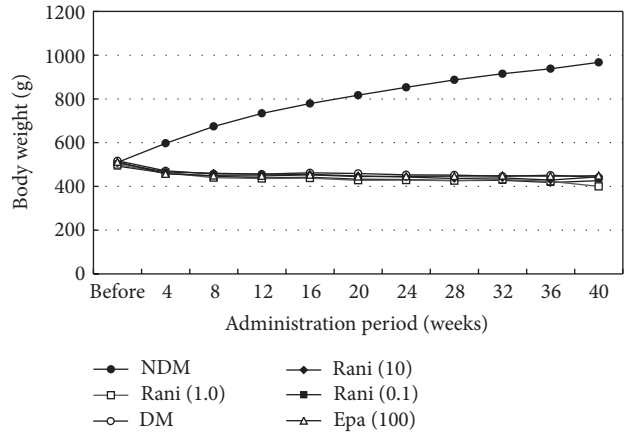

Figure 1: Body weight of the study animals. The SD rats are heavier than the SDT rats with or without treatment. NDM: normal SD rats; DM: untreated SDT rats; rani (0.1): ranirestat-treated SDT rats (0.1 mg/kg/day); rani (1.0): ranirestat-treated SDT rats (1.0 mg/kg/day); rani (10): ranirestat-treated SDT rats (10 mg/kg/day); epa (100): epalrestat-treated SDT rats (100 mg/kg/day) [21].

The retinas in the 33-week-old SD rats were significantly ($P = 0.023$, Mann-Whitney U test) thicker than those in the SD rats that were over 50 weeks old. The stained area in the SD rats over 50 weeks old was significantly ($P = 0.0055$, Mann-Whitney U test) larger than that in the 33-week-old SD rats. The stained area in the untreated SDT rats over 50 weeks old was significantly ($P = 0.0055$, Mann-Whitney U test) larger than that in the 31-week-old SDT rats, but there was

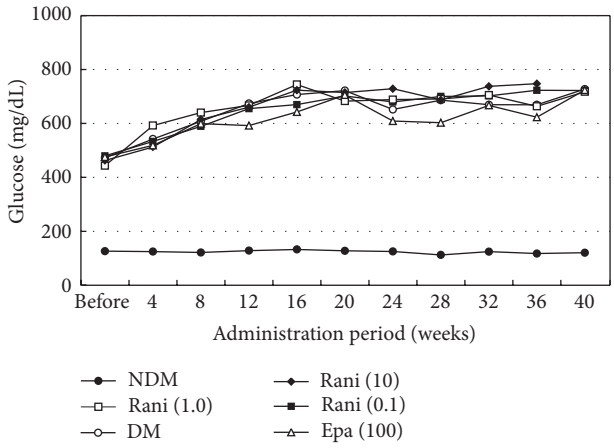

Figure 2: Blood glucose levels of the study animals. The mean blood glucose levels of the SD rats are significantly lower than those of the SDT rats with or without treatment. There is no significant difference in the blood levels among the SDT rats with or without treatment. NDM: normal SD rats; DM: untreated SDT rats; rani (0.1): ranirestat-treated SDT rats (0.1 mg/kg/day); rani (1.0): ranirestat-treated SDT rats (1.0 mg/kg/day); rani (10): ranirestat-treated SDT rats (10 mg/kg/day); epa (100): epalrestat-treated SDT rats (100 mg/kg/day) [21].

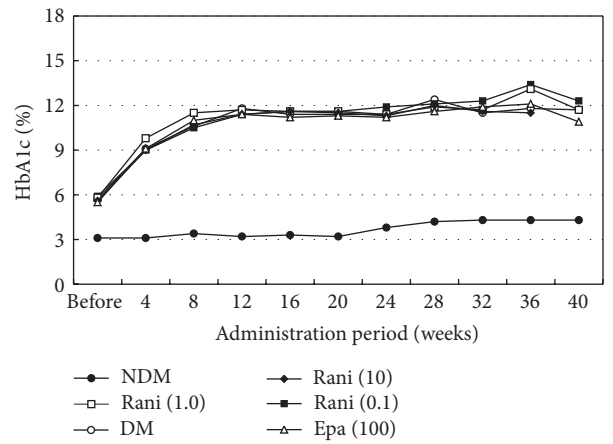

Figure 3: HbA1c levels of the study animals. The mean HbA1c levels of the SD rats are significantly lower than those of the SDT rats with or without treatment. There is no significant difference in the HbA1c levels among the SDT rats with or without treatment. NDM: normal SD rats; DM: untreated SDT rats; rani (0.1): ranirestat-treated SDT rats (0.1 mg/kg/day); rani (1.0): ranirestat-treated SDT rats (1.0 mg/kg/day); rani (10): ranirestat-treated SDT rats (10 mg/kg/day); epa (100): epalrestat-treated SDT rats (100 mg/kg/day) [21].

no significant ($P = 0.22$, Mann-Whitney U test) difference in the retinal thickness. The retinas in the 31-week-old SDT rats were significantly ($P = 0.021$, Mann-Whitney U test) thicker than those in the 33-week-old SD rats, but there was no significant ($P = 0.24$, Mann-Whitney U test) difference in the stained area.

Figures 4, 5, and 6 show the retinas in each group. In the normal SD rats, the region of stained GFAP was minimal near

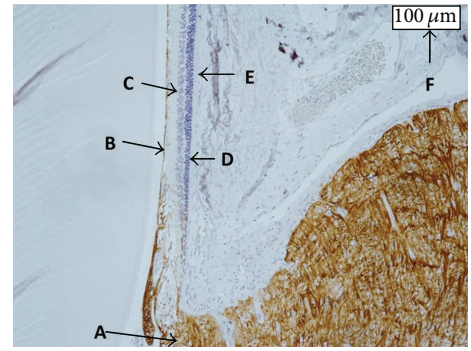

Figure 4: The retina in a normal SD rat older than 50 weeks. The brown region shows stained GFAP. A: optic nerve disc; B: ILM; C: INL; D: outer nuclear layer; E: retinal pigment epithelium; F: index of 100 μm. The area of stained GFAP is limited around the ILM and does not appear around the INL.

the ILM but became more extensive around the inner nuclear layer (INL) in the untreated SDT rats. The retinas in the SDT rats began to thicken at 33 weeks of age, but the stained area of GFAP had not begun to spread at that time point.

4. Discussion

The retinas and the stained area of GFAP in the 33-week-old SD rats were significantly thicker and smaller than those in the SD rats older than 50 weeks in this study. This suggested that the retinas may become thinner and the GFAP in the retina may increase in SD rats over time. The stained area of GFAP in the untreated SDT rats older than 50 weeks was significantly larger than that in the 31-week-old SDT rats, but there was no significant difference in the retinal thickness in this study. This suggested that the increased GFAP in diabetic retinas may begin after retinal edema develops.

We investigated whether ranirestat reduces the retinal thickness in SDT rats. We reported previously that ranirestat suppressed accumulation of VEGF and prevented extensive fluorescein leakage around the optic disc in the retinas of SDT rats [20]. Together with the current results, the results suggested that ranirestat may suppress vascular permeability and prevent DME.

GFAP is a specific component of the glial filaments present in astrocytes [22]. In the central nervous system of higher vertebrates, after injury from trauma, disease, genetic disorders, or chemical insult, astrocytes become reactive and respond in a typical manner, that is, astrogliosis, which is characterized by rapid synthesis of GFAP and increased protein content or immunostaining with the GFAP antibody [23]. It was reported that GFAP also increased locally in some ophthalmic diseases. Yang et al. reported that the immunoreactivity of GFAP was detected and increased in the optic nerve region of a glaucoma model rat [24]. Rungger-Brändle et al. reported that the density of Müller cells and microglia increased and GFAP expression in the Müller cells was prominent in the retinas of a diabetic rat model [25]. Thus, the increase in neuroglial cells and GFAP expression

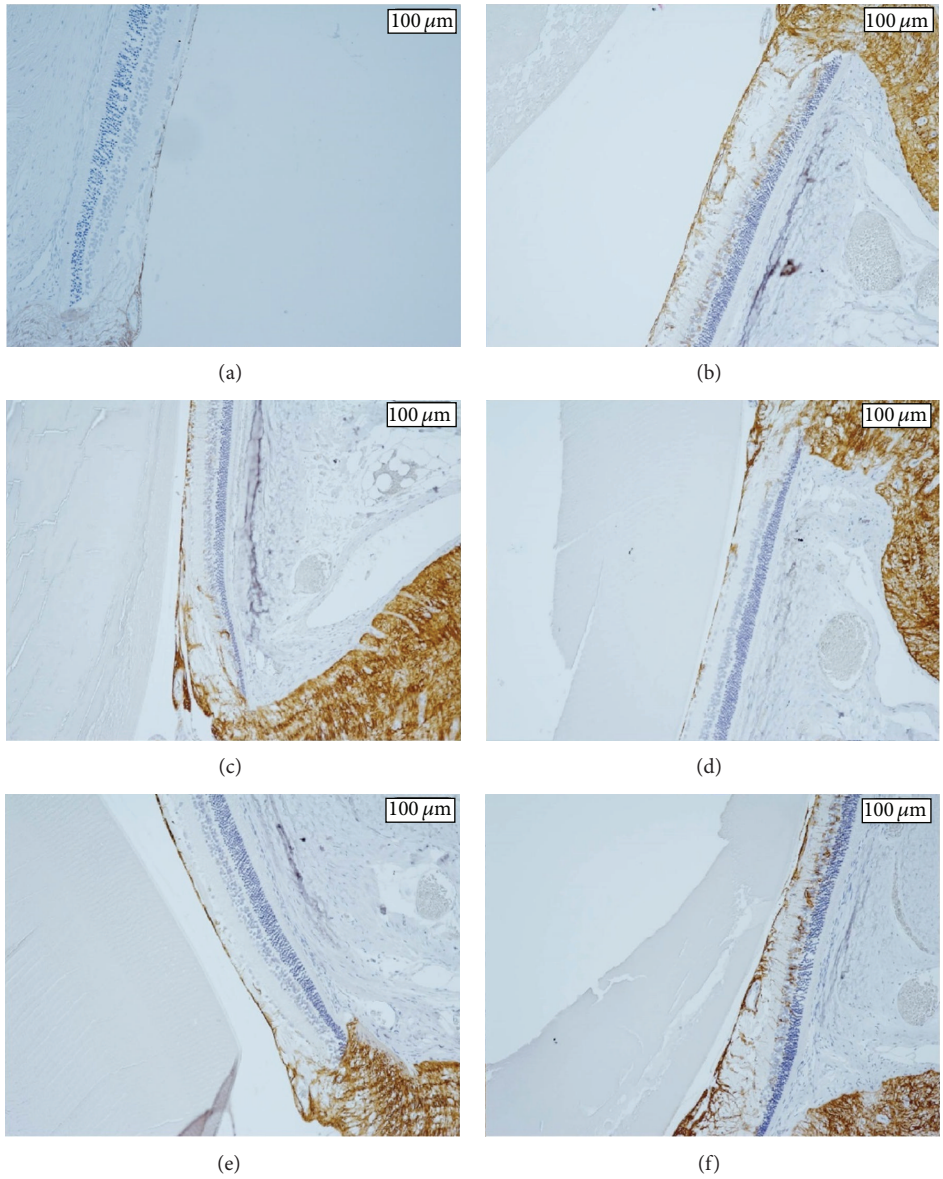

Figure 5: (a) The retina in a 31-week-old untreated SDT rat. The area of stained GFAP is significantly smaller in 31-week-old untreated SDT rats than in untreated SDT rats older than 50 weeks, but there is no significant difference in retinal thickness. (b) The retina in an untreated SDT rat older than 50 weeks. The retina is thicker and the area of stained GFAP is larger compared with the normal SD rat. GFAP is strongly stained from the ILM to around the INL in the untreated SDT rats. (c) The retina in a ranirestat-treated SDT rat (0.1 mg/kg/day). The retina is thinner and the area of stained GFAP is smaller compared with the untreated SDT rat. The stained area, which appears between the ILM and around the INL in the untreated SDT rats, is suppressed. (d) The retina in a ranirestat-treated SDT rat (1.0 mg/kg/day). The effect of ranirestat (1.0 mg/kg/day) is stronger than ranirestat (0.1 mg/kg/day) on the retinal thickness and the area of stained GFAP. (e) The retina in a ranirestat-treated SDT rat (10 mg/kg/day). Although the area of stained GFAP in the ranirestat-treated SDT rat (10 mg/kg/day) is suppressed compared with the ranirestat-treated SDT rat (0.1 mg/kg/day), the difference in retinal thickness is not clear. (f) The retina in an epalrestat-treated SDT rat. Epalrestat does not suppress the retinal thickness or the area of stained GFAP. The area of GFAP is intensely stained between the ILM and around the INL.

are thought to be associated with onset and development of neuropathy in the diabetic retina. Since neurodegeneration is an early event in the pathogenesis of DR [26], developing treatment for the early disease stage is important.

The effects of diabetes may appear in the retina before funduscopic or morphologic changes occur. Some studies have reported electroretinographic abnormalities. Shirao and Kawasaki [27] reported that the peak latency of the first oscillatory potential (OP) peak was prolonged in early diabetes with no funduscopic abnormality and increased as DR progressed and also that the summed amplitude of the OPs decreased as DR progressed. However, those investigators reported that visual functional disorders were unclear in patients with OP abnormalities who have no or minimal

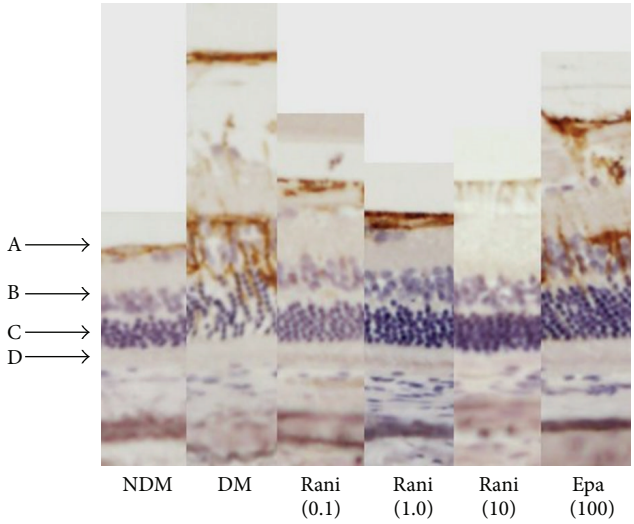

Figure 6: Comparison of retinas in each group. A: ILM; B: INL; C: outer nuclear layer; D: retinal pigment epithelium. NDM: normal SD rats; DM: untreated SDT rats; rani (0.1): ranirestat-treated SDT rats (0.1 mg/kg/day); rani (1.0): ranirestat-treated SDT rats (1.0 mg/kg/day); rani (10): ranirestat-treated SDT rats (10 mg/kg/day); epa (100): epalrestat-treated SDT rats (100 mg/kg/day).

fundus changes and presumed that amacrine cells were the most feasible candidates for OP generation [27]. Aung et al. reported abnormalities in visual acuity, and contrast sensitivity tested using the OptoMotry system (Cerebral-Mechanics, Lethbridge, AB, Canada), and delayed responses specifically in OP implicit times in streptozotocin-induced diabetes mellitus rats before expected onset of diabetes-associated retinal vascular lesions [28]. Previous studies have reported the depth profile of the OPs within the retina. Brindley reported that the maximal amplitude of the oscillations in the frog retina was in the INL [29], and Ogden and Wylie reported that the maximal amplitudes of the first three OPs in the pigeon, chicken, and monkey were at the level of the inner plexiform layer [30, 31]. In the current study, GFAP was strongly stained from the ILM to around the INL in the untreated SDT rats and ranirestat significantly suppressed the area of stained GFAP in the groups treated with 1.0 and 10 mg/kg/day. Considering that together with previous studies of OPs in diabetic retina, neural damage in a diabetic retina may begin in an inner retinal layer; however, GFAP expression in a diabetic retina may begin after the retinal edema develops. The current results suggested that ranirestat may have a neuroprotective effect on diabetic retina, but it may be affected by controlling retinal edema.

Although the mean retinal thickness and the mean area of stained GFAP in epalrestat-treated SDT rats were smaller than those in the untreated SDT rats, epalrestat did not significantly suppress them in the current study. Because the study included a small number of rats, we could not determine whether or not epalrestat was effective for treating DR. The values in the 1.0 mg/kg/day ranirestat group were smaller than those in the 10 mg/kg/day, and the dose-reaction relationship was unclear. The findings also may have resulted from the small number of rats; a larger study with more rats is needed to confirm that effectiveness of epalrestat and the dose-reaction relationship of ranirestat. However, ranirestat may prevent DME and have a neuroprotective effect on diabetic retinas. Further studies of ranirestat should be undertaken.

Conflict of Interests

The authors except Kakehashi declare that there is no conflict of interests regarding the publication of this paper. Kakehashi has received grant support from Sumitomo Dainippon Pharma Co., Ltd. Matsumoto and Tsuji are employees of Sumitomo Dainippon Pharma Co., Ltd.

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Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2014, Article ID 526972, 7 pages
http://dx.doi.org/10.1155/2014/526972

Research Article

Evaluating the Mechanisms of Improved Glucose Homeostasis after Bariatric Surgery in Ossabaw Miniature Swine

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Received 6 June 2014; Revised 29 July 2014; Accepted 7 August 2014; Published 24 August 2014

Academic Editor: Masami Shinohara

Background. Roux-en-Y gastric bypass (RYGB) is the most common bariatric operation; however, the mechanism underlying the profound weight-independent effects on glucose homeostasis remains unclear. Large animal models of naturally occurring insulin resistance (IR), which have been lacking, would provide opportunities to elucidate such mechanisms. Ossabaw miniature swine naturally exhibit many features that may be useful in evaluating the anti diabetic effects of bariatric surgery. *Methods*. Glucose homeostasis was studied in 53 Ossabaw swine. Thirty-two received an obesogenic diet and were randomized to RYGB, gastrojejunostomy (GJ), gastrojejunostomy with duodenal exclusion (GJD), or Sham operations. Intravenous glucose tolerance tests and standardized meal tolerance tests were performed prior to, 1, 2, and 8 weeks after surgery and at a single time-point for regular diet control pigs. *Results*. High-calorie-fed Ossabaws weighed more and had greater IR than regular diet controls, though only 70% developed IR. All operations caused weight-loss-independent improvement in IR, though only in pigs with high baseline IR. Only RYGB induced weight loss and decreased IR in the majority of pigs, as well as increasing $AUC_{insulin}/AUC_{glucose}$. *Conclusions*. Similar to humans, Ossabaw swine exhibit both obesity-dependent and obesity-independent IR. RYGB promoted weight loss, IR improvement, and increased $AUC_{insulin}/AUC_{glucose}$, compared to the smaller changes following GJ and GJD, suggesting a combination of upper and lower gut mechanisms in improving glucose homeostasis.

1. Introduction

Roux-en-Y gastric bypass (RYGB) has emerged as the most efficient and effective approach for weight loss and treatment of type 2 diabetes mellitus (T2DM) in obese patients [1, 2]. Postoperative effects on glycemic control and insulin resistance have been shown to precede significant weight reduction [3] and occur at least partially in a weight-independent fashion [4], generating controversy surrounding the mechanisms of RYGB's antidiabetic effects [5]. Hypotheses include the operation's impact on intestinal hormones [6, 7], bile acids [8, 9], gut flora [10, 11], and intestinal glucose sensing [12] and metabolism [13]; however, no definitive mechanism has emerged.

To evaluate potential mechanisms of bariatric surgery, an animal model that mimics human metabolic disease and possesses enough anatomic similarity to allow the evaluation of bariatric procedures used in humans would be helpful [14]. Ossabaw miniature swine appear to be a valuable model of acquired obesity and IR [15]. We hypothesized that these animals would facilitate the study of RYGB mechanisms in ways not feasible in toxin-induced, rodent, or other large animal models of diabetes or insulin resistance [16]. Due to harsh environmental pressures in their native habitat, Ossabaws evolved over time to gain large amounts of weight when exposed to abundant supplies of food, reflecting selection pressure from frequent periods of famine [15]. When exposed to a high-fat, high-calorie diet in a laboratory,

they develop marked obesity and many of the hallmarks of metabolic syndrome, including insulin resistance (IR) [15, 17–20], dyslipidemia [18, 20, 21], and hypertension [18, 20].

Our group has suggested several hypotheses pertaining to the antidiabetic mechanisms of various gastrointestinal (GI) rearrangements, particularly RYGB [22–31]. Among these is the "lower intestinal hypothesis," which postulates that enhanced delivery of ingested nutrients to the distal bowel increases secretion of the incretin glucagon-like peptide-1 (GLP-1). GLP-1 elevations would augment insulin secretion and improve glucose homeostasis. The "upper intestinal hypothesis" suggests that exclusion of nutrient flow from the proximal small bowel exerts direct antidiabetes effects on glucose homeostasis, most likely by promoting a factor that increases insulin sensitivity or antagonizing a factor that decreases sensitivity. Roux-en-Y gastric bypass, gastrojejunostomy (GJ), and gastrojejunostomy with duodenal exculsion (GJD) are GI operations that facilitate the study of these hypotheses. Gastrojejunostomy enables nutrients from the stomach to be delivered directly to the distal small bowel, GJD does the same while also preventing nutrient delivery to the duodenum and proximal jejunum, and RYGB provides both proximal exclusion and enhanced distal delivery while also largely eliminating gastric nutrient exposure.

This study aims to evaluate Ossabaw miniature swine as a large animal model for metabolic syndrome and to assess the effects of several GI rearrangements on body weight and IR, potentially elucidating RYGB's influence on glucose homeostasis.

2. Materials and Methods

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Washington (UW), with the recommendations outlined by the National Research Council and the American Veterinary Medical Association Panel on Euthanasia [32, 33].

2.1. Animals and Environment. 53 female Ossabaw swine were selected from the Indiana University School of Medicine (IUSM) Purdue Animal Facility and maintained on a standard diet of 2400 kcal/day (5L80; Purina Test Diet, Richmond, IN). A subset of 32 pigs, 12–18-month-old, were fed a high-calorie (4700 kcal/day), high-fat diet (KT324, Purina Test Diet, Richmond, IN) for ~180 days to promote weight gain and IR and were then shipped to UW. Pigs were housed in a temperature-controlled room on a 12-hour light/dark cycle with free access to drinking water and were removed daily for stall cleaning. While at the UW, pigs were maintained on a ~7500 kcal/day diet comprised of 16.1% proteins, 43.1% lipids, and 40.8% carbohydrates (5B4L, Purina Test Diet) as previously described in detail [20]. Food was provided twice daily, and intake was recorded daily. Body weight was monitored at least weekly using a digital scale (Waypig 15, Vittetoe Inc., Keota, IA).

2.2. Surgical Intervention. Approximately one week after pigs arrived at the UW facility, indwelling central venous access was obtained [15, 34] for repeated blood sampling as well as fluid/drug administration. After implantation of intravascular catheters, pigs were given 5–10 mg/kg of aspirin daily to reduce blood clotting around the internal catheter tip. Additionally, catheters were flushed daily with a heparin-saline solution containing 1 mg/mL vancomycin to prevent thrombosis and/or occlusion.

Approximately one month after arriving at UW, pigs were randomized to one of four GI operations, all performed in a standardized fashion: RYGB ($n = 13$), GJ ($n = 10$), GJD ($n = 7$), and Sham ($n = 2$). RYGB, which was developed in this animal model by our group [16], creates a small, functional gastric pouch measuring approximately 5 × 5 cm comprised of the proximal stomach and completely separated from the cardia, fundus, and body of the stomach. The Roux-en-Y small bowel reconstruction approximates a typical human RYGB in that ~1/3 of the small bowel is used for an alimentary Roux limb, and the biliary-pancreatic-duodenal (BPD) limb measures ~45 cm. GJ includes anastomosing the greater curvature of the stomach with the proximal jejunum, with full preservation of the stomach and pylorus. GJD is performed identically to GJ but with the additional detachment of the pylorus from the proximal duodenum using a surgical stapler, thereby excluding the duodenum from nutrient flow. In both GJ and GJD, the excluded length of small bowel is approximately the same length as the BPD limb in the RYGB procedure. The Sham operation includes an extended midline laparotomy with manual intestinal manipulation for ~130 minutes, the average time of the other procedures.

2.3. Glucose Tolerance and Insulin Sensitivity Testing. An intravenous glucose tolerance test (IVGTT) was performed on all pigs and within one week prior to surgery as a baseline. The IVGTT was then repeated in operated animals at 2 weeks and 8 weeks postoperatively. A standardized meal tolerance test (MTT) was performed on the day following each IVGTT in each pig. During IVGTT, after taking a baseline blood sample, 1 g/kg dextrose was administered intravenously, with subsequent venous blood sampling 5, 10, 20, 30, 40, 60, 90, and 120 min after injection. For MTT, pigs were given a test meal of 430 g of chow and allowed to eat for 15 minutes. Blood was sampled at –15, 0, 30, 60, 90, and 120 minutes after the completion of the test meal. Blood samples were evaluated at the UW's Northwest Lipid Metabolism and Diabetes Research Laboratory. Glucose levels were evaluated on a Hitachi Clinical Chemistry modular autoanalyzer (Hitachi Clinical, Tokyo), while insulin tests were performed using a Tosoh 1800 autoanalyzer (Tosoh Bioscience, San Francisco). Preoperative insulin resistance was evaluated from fasting blood samples using the homeostatic model assessment of insulin resistance (HOMA-IR) [35] using the formula HOMA-IR = (glucose × insulin)/405, where glucose and insulin are measured in mg/dL. Insulin resistance in this model was defined as a HOMA-IR >2 standard deviations from the mean for the 21 pigs that remained at the

IUSM/Purdue Animal Facility and were not exposed to the high-fat, high-calorie diet.

2.4. Statistical Analysis. All numeric data are expressed as the average value ± the standard deviation, unless otherwise indicated. Area-under-the-curve (AUC) values were calculated using the trapezoidal approximation formula $(h/2)\sum_{k=1}^{N}(f(x_{k+1}) + f(x_k))$, where h is serum insulin or glucose, x is time in minutes, and k and N are the lower and upper bounds of summation, respectively. Excel (version 12.3.6, Microsoft) was used for statistical analysis. Where appropriate, a paired, two-tailed, Student's t-test was used, with a P value of less than 0.05 considered statistically significant.

3. Results

3.1. Procedures. Twenty-one pigs were not fed the high-calorie diet and underwent IVGTT as regular diet controls. Thirty-two pigs were fed the obesogenic diet and subsequently randomized into one of the following groups: RYGB ($n = 13$), GJ ($n = 10$), GJD ($n = 7$), or Sham ($n = 2$). Of these, nine pigs were unable to complete the study due to perioperative complications, including intraoperative cardiac arrest ($n = 3$), postoperative infection ($n = 3$), anastomotic dehiscence ($n = 2$), and intraoperative hemorrhage ($n = 1$). Data were analyzed only for pigs that completed all three postoperative IVGTT and MTT ($n = 23$), resulting in the following cohort sizes: RYGB ($n = 7$), GJ ($n = 8$), GJD ($n = 6$), and Sham ($n = 2$).

3.2. Distribution of Insulin Resistance. The distribution of pig body weight and HOMA-IR (Figure 1) demonstrates high variability in the relationship between increasing body weight and IR. High-calorie fed Ossabaws weighed more (73.4 versus 62.3 kg, $P = 0.002$) and had higher average HOMA-IRs, with a much wider distribution (3.7 ± 1.9) than did their regular chow-fed counterparts (1.2 ± 0.7, $P < 0.001$). The wide distribution resulted in approximately 70% of high-calorie fed pigs developing insulin resistance, as we defined it (i.e., HOMA-IR >2 standard deviations above the mean for pigs on a regular diet or 2.61. Overall, there was a mild positive correlation between body weight and HOMA-IR (Figure 1 regression line, $R^2 = 0.08$, $P = 0.05$). However, there was significant heterogeneity, particularly in the high-calorie group, and the heaviest animals were not necessarily the most insulin resistant.

3.3. Postoperative Decreases in Body Weight and HOMA-IR. The percentage change in weight and HOMA-IR for each pig 8 weeks after their respective operations are displayed in Figure 2(a). In the GJ group, all pigs gained weight (+15 ± 5%, $P < 0.001$), with no net change in HOMA-IR. Pigs that underwent GJD demonstrated no change of either weight (−0.2 ± 10%, $P = 0.91$) or HOMA-IR (+3 ± 63%, $P = 0.53$) throughout the study and were distributed in all four quadrants of the scatter plot. In contrast with the other procedure groups, all RYGB pigs lost weight during the study

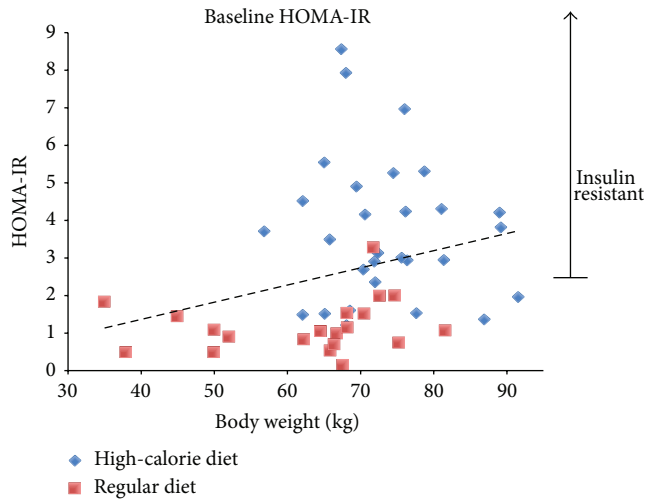

Figure 1: Preoperative body weight and HOMA-IR in high-calorie and regular diet Ossabaws. Scatter plot of preoperative weight and HOMA-IR in pigs exposed to the high-fat, high-calorie diet for >180 days and those maintained on a normal diet. The dashed regression line shows overall mild positive correlation between weight and HOMA-IR ($R^2 = 0.08$, $P = 0.05$). Insulin resistance in this model was defined as HOMA-IR > 2 standard deviations above the regular diet group mean (>2.61).

period (−14 ± 7%, $P = 0.002$) and the majority (66%) showed a trend towards improvement in insulin resistance.

When evaluating the more insulin-resistant pigs prior to surgery (above median baseline HOMA-IR), almost all pigs demonstrated a postoperative decrease in IR (Figure 2(b)). This improvement across GI procedures was not dependent on weight loss, as half of pigs actually gained weight despite experiencing a drop in HOMA-IR.

3.4. Insulin and Glucose AUCs. For IVGTT, there was no statistically significant difference in $AUC_{insulin}$, $AUC_{glucose}$, or $AUC_{insulin}/AUC_{glucose}$ ratio (Figure 3(a)) after 2 or 8 weeks, when compared with baseline values within each procedure, nor between procedures. During MTT (Figure 3(b)), $AUC_{insulin}/AUC_{glucose}$ in pigs undergoing RYGB was higher both at 2 weeks (0.7 ± 0.29, $P = 0.015$) and 8 weeks (0.46 ± 0.2, $P = 0.042$) compared with baseline values (0.28 ± 0.07). RYGB was the only procedure associated with a significant increase in $AUC_{insulin}/AUC_{glucose}$ during MTT.

4. Discussion

The availability of a large animal model to study the mechanisms of antidiabetes effects rendered by RYGB is critical. Identifying the mechanisms of improvement in IR and diabetes observed after bariatric surgery should lay the foundation for the development of targeted, less invasive therapies in the future. Evaluating the molecular pathways activated after RYGB requires evaluation of specific components of the procedure that cannot be easily or ethically performed in humans. Particularly given the limitations of

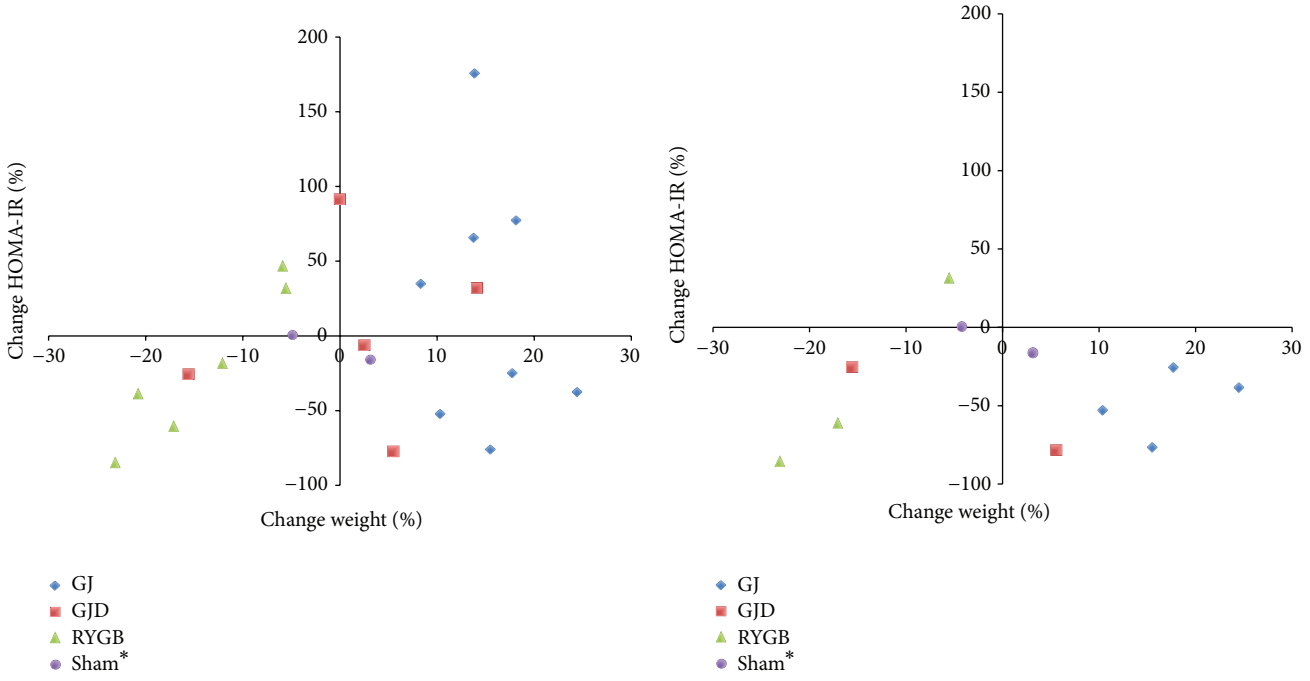

Figure 2: (a) Change in body weight and HOMA-IR by operation. Scatterplot shows percent change in weight and HOMA-IR 8 weeks postoperatively. *Sham pig values are at 2 weeks, as 8-week data was unavailable. (b) Change in body weight and HOMA-IR among high HOMA-IR pigs only. Percent change in weight and HOMA-IR 8 weeks postoperatively in pigs with above median baseline HOMA-IRs. *Sham pig values are at 2 weeks, as 8-week data was unavailable.

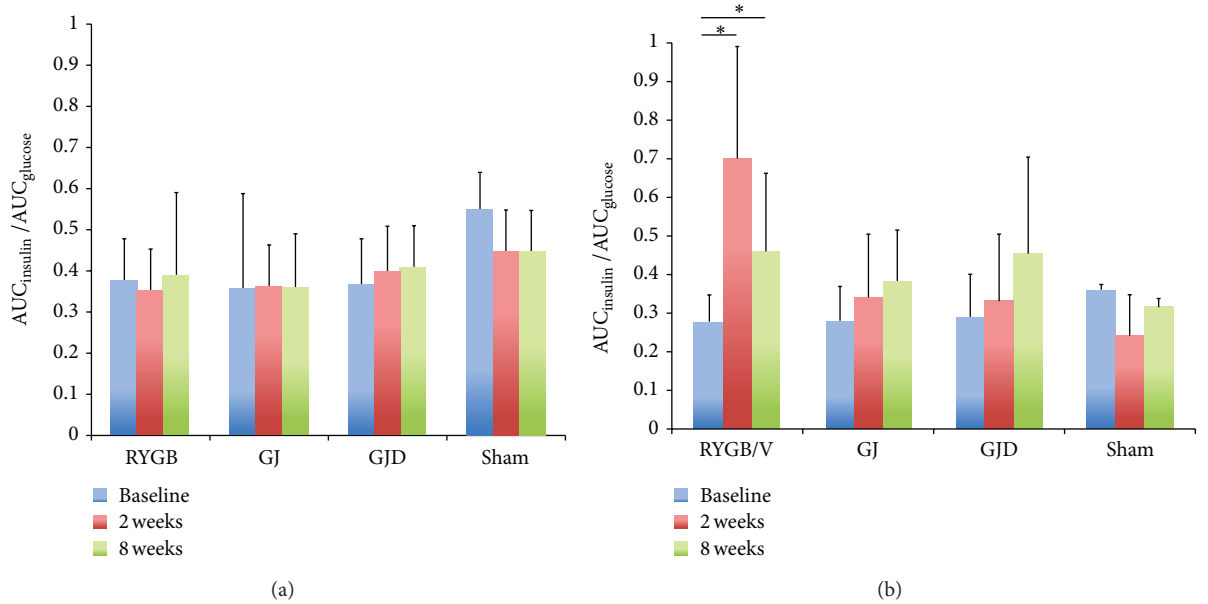

Figure 3: (a) IVGTT $AUC_{insulin}/AUC_{glucose}$. Pre- and postoperative IVGTT $AUC_{insulin}/AUC_{glucose}$ for RYGB, GJ, GJD, and Sham procedures. (b) MTT $AUC_{insulin}/AUC_{glucose}$. Pre- and postoperative MTT $AUC_{insulin}/AUC_{glucose}$ for RYGB, GJ, GJD, and Sham procedures. "* indicates" statistical significance ($P < 0.05$).

domestic pig and small animal models [36, 37], Ossabaw swine present a more human-like large animal model [15, 17–19, 21], in which long-term survival GI operations can be performed. The robust propensity to obesity in Ossabaw miniature swine clearly makes this breed superior to other laboratory animal swine, such as the Yucatan miniature pig, which show minimal obesity and complete absence of insulin resistance after long-term, high-calorie diets [19, 38]. The anatomy of the porcine upper GI tract, especially the stomach and its neural enervation, is far more similar to corresponding human anatomy than are mouse or rat models. In this report, we highlight not only the unique opportunity that this model affords, but also some important limitations for other investigators to consider.

The majority of Ossabaw swine clearly develop insulin resistance when exposed to a high-fat, high-calorie diet (Figure 1). However, although the overall difference between high-calorie-diet and regular diet pigs was statistically significant, these animals constitute a diverse population, with only ~70% developing insulin resistance, which marginally correlated to increased weight. This is consistent with human epidemiological data indicating that human obesity is itself a heterogeneous condition, with only about one-third of obese persons developing diabetes [39]. Because only a subgroup of obese Ossabaws exhibited deranged glucose homeostasis, future studies of insulin resistance should be performed in animals based on preoperative biochemical testing and validation of insulin resistance, not merely preoperative weight, as was done in this study.

Despite the fact that GI operations were performed in these pigs without consideration of preoperative HOMA-IR, important conclusions can still be gleaned from these data. All GJ pigs gained weight postoperatively (Figure 2(a)), indicating that exclusion of nutrient exposure to the duodenum and proximal jejunum does not, in isolation, promote weight loss in this animal model. Similarly, GJD pigs showed a mixed picture of weight loss/gain after surgery, with no significant change overall in the group. Perhaps for the same reason, when measured across all pigs, none of the operations improved HOMA-IR but pigs with higher baseline insulin resistance experienced a consistent decrease in HOMA-IR after GJ and GJD, regardless of their change in weight (Figure 2(b)). The fact that these pigs demonstrated improved IR despite gaining weight highlights the weight-independent nature of improved IR after GI surgery and is consistent with data from human studies [40]. This also implicates the role of distal nutrient delivery in IR improvement, as both operations deliver nutrients to the jejunum, but only GJD obligatorily bypasses the distal foregut.

The failure of these procedures to positively affect IR in below-median baseline HOMA-IR pigs is an important observation, particularly for investigators who choose to pursue this model in the future. This potentially reflects a "floor effect" of trying to improve HOMA-IR that is already low for that group. Alternatively, this finding could mirror data in some human studies suggesting that diabetes worsens in certain low-BMI or less-diabetic patients after bariatric surgery [41], although the true etiology is unclear and requires further study.

Across all pigs, RYGB was the only procedure to induce both weight loss and a reduction in HOMA-IR in the majority of pigs, regardless of baseline HOMA-IR (Figure 2(a)). Elimination of the gastric reservoir in RYGB is likely the main cause of sustained weight loss, as these pigs also exhibited the largest reduction in food intake of any operation, including GJD, which replicates a RYGB-like proximal intestinal bypass. Although there was a single high-baseline HOMA-IR pig whose insulin resistance did not benefit from the operation, it is important to note that two low-baseline HOMA-IR pigs also experienced an improvement in insulin resistance, an event observed in no other operation. This potentially underscores the ability of RYGB to normalize glucose homeostasis across a more diverse group of obese pigs, consistent with its substantial efficacy in humans.

The IVGTT and MTT data provide insight into the importance of altered nutrient delivery to the physiologic changes that occur after bariatric surgery. We observed a significant rise in $AUC_{insulin}/AUC_{glucose}$ in the RYGB cohort during MTT, despite no change during IVGTT in the same pigs. Although changes in $AUC_{insulin}/AUC_{glucose}$ have been reported during IVGTT after RYGB [42], it is reasonable to expect greater augmentation of insulin release after nutrient delivery directly to the distal intestine (as in MTT), rather than via a parenteral glucose load, particularly if the "lower intestinal hypothesis" holds true. This could be mediated through augmented secretion of nutrient-stimulated distal intestinal hormones, such as the incretin GLP-1, and/or via a yet undiscovered mechanism. The nonstatistically significant rise in $AUC_{insulin}/AUC_{glucose}$ in GJ and GJD pigs is important because these procedures also provide distal nutrient delivery. A key to further defining the interaction between proximal nutrient diversion and distal delivery could be the use of the vertical sleeve gastrectomy procedure, which removes 80% of the gastric mucosa but does not deliver nutrients directly to the jejunum, as in RYGB. This could isolate the effect of the stomach on glucose metabolism to determine if gastric exclusion is sufficient to obtain the observed insulin response, allowing us to further understand the underlying mechanisms of RYGB.

This study has several important limitations that must be considered. The relatively high mortality in the surgical intervention groups reduced the number of animals available for biochemical analysis. While the operative complication rate fell dramatically as the author's experience progressed, omitted animals reduced the statistical power of the remaining cohorts. Additionally, due to a technical error, the two control Sham animals were unable to contribute 8-week blood data, limiting their utility as negative controls. The unexpected finding that only two-thirds of high-calorie-fed animals developed insulin resistance further reduced available data, even in pigs that underwent a GI rearrangement. While this discovery is encouraging as it mimics the heterogeneous human response to obesity, it requires investigators who may use this model in the future to carefully identify insulin-resistant animals prior to randomization and treatment.

5. Conclusions

Ossabaw swine exhibit both obesity-dependent and obesity-independent IR and appear to be an effective model assisting in the evaluation of the effect of bariatric surgery on body weight and glucose homeostasis. RYGB, GJ, and GJD result in variable weight loss and improved insulin resistance, especially in pigs with baseline elevated IR. RYGB was associated with significant postoperative elevations in $AUC_{insulin}/AUC_{glucose}$ not observed after GJ and GJD, suggesting a combination of upper and lower gut mechanisms. Future studies isolating the effect of nutrient exposure to specific portions of the gastric mucosa and distal intestine should help to further elucidate these effects.

Disclosure

Financial support was received from NCI Grant T32CA138312 as well as NIDDK Grants T32DK070555 and R01DK084324. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NCI, NIDDK, or NIH.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jonathan G. Sham and Vlad V. Simianu contributed in analysis and interpretation, data collection, writing the paper, and critical revision of the paper. Skye D. Stewart contributed in data collection and conception and design. Mouhamad Alloosh and Michael Sturek contributed in conception and design, data collection, critical revision of the paper, and obtaining funding. Andrew S. Wright contributed in conception/design, data collection, writing the paper, and critical revision of the paper. David E. Cummings and David R. Flum contributed in conception/design, analysis and interpretation, data collection, writing the paper, critical revision of the paper, and obtaining funding.

Acknowledgments

The authors would like to thank Yuki Aoki for her assistance with animal husbandry and veterinary services. The authors received funding from the National Institute of Diabetes and Digestive and Kidney Diseases, National Cancer Institute.

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Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2014, Article ID 398518, 6 pages
http://dx.doi.org/10.1155/2014/398518

Research Article

Expression of CTRP3, a Novel Adipokine, in Rats at Different Pathogenic Stages of Type 2 Diabetes Mellitus and the Impacts of GLP-1 Receptor Agonist on It

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Received 17 June 2014; Revised 19 July 2014; Accepted 24 July 2014; Published 11 August 2014

Academic Editor: Tomohiko Sasase

This study aimed to investigate the expression of C1q/TNF-related protein-3 (CTRP3) in rats at different pathogenic stages of type 2 diabetes mellitus (T2DM) and the impacts of glucagon-like peptide-1 (GLP-1) receptor agonist on it. Male wistar rats were fed with high-fat diet for 10 weeks to induce insulin resistance (IR) and then were given low-dose streptozotocin (STZ) intraperitoneal injection to induce T2DM. Exendin-4 (Ex-4), a GLP-1 receptor agonist, was subcutaneous injected to the IR rats and T2DM rats for 4 weeks. The expression of CTRP3 mRNA and protein in epididymis adipose tissue of rats at the stage of IR was lower significantly than that of normal control (NC) rats and decreased more when they were at the stage of overt T2DM (all $P < 0.05$ or $P < 0.01$). After the treatment with Ex-4, the mRNA and protein expressions of CTRP3 were increased by 15.5% ($P < 0.01$) and 14.8% ($P < 0.05$), respectively, in IR rats and increased by 20.6% ($P < 0.01$) and 16.5% ($P < 0.05$), respectively, in T2DM rats. Overall, this study found that the expression of CTRP3 in visceral adipose tissue was progressively decreased in a T2DM rat model from the pathogenic stage of IR to overt diabetes, while Ex-4 treatment increased its expression in such animals.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic disease characterized by insulin resistance and pancreatic islet beta cells dysfunction. The T2DM pathogenesis represents the combined effects of genetics, nutrition, and lifestyle and involves both gene-gene and gene-environment interactions [1]. Over the past few decades, the correlation between the prevalence of T2DM and obesity has been well demonstrated, as more than 80% of T2DM patients are overweight or obese [2]. It is now well known that white adipose tissue is not only serving as a long-term energy store but also as an active endocrine organ that secretes a number of bioactive molecules called adipokines [3]. In addition to being important regulators of adipose tissue development and function, adipokines also have significant impacts on glucose metabolism in various tissues [4]. Abnormal expression and secretion of some adipokines in adiposity are strongly associated with the development and progression of insulin resistance and pancreatic islet beta cell dysfunction, which gradually and ultimately lead to the pathogenesis of T2DM.

C1q/TNF-related protein-3 (CTRP3) is a novel adipokine with multiple effects as lowering glucose levels, inhibiting the glyconeogenesis in liver, and increasing angiogenesis and anti-inflammation. Studies *in vivo* indicated that a modest 3-fold elevation of plasma CTRP3 levels by recombinant protein administration in normal and insulin resistant *ob*/*ob* mice is sufficient to lower glucose levels which may be mediated by up-regulating the protein kinase B (PKB) and inhibiting the expression of the gluconeogenic enzymes glucose-6-phosphatase and phosphoenolpyruvate carboxykinase in liver [5]. A recent study showed that CTRP3 attenuated diet-induced hepatic steatosis by regulating triglyceride metabolism [6], indicating that CTRP3 may be an important regulator of lipid metabolism as well as glucose metabolism.

CTRP-3 is expressed in subcutaneous and visceral adipocytes and is positively regulated by insulin and negatively regulated by chronic lipopolysaccharide (LPS) exposure [7]. One

clinical research showed that circulating CTRP-3 concentrations were elevated in patients with T2DM and metabolic syndrome (MS) [8], while another one reported that serum and omental adipose tissue CTRP3 were lower in women with polycystic ovary syndrome (PCOS) and metformin treatment increased serum CTRP3 levels in these women and in omental adipose tissue explants [9]. A study from Korea showed that a 3-month combined exercise program significantly decreased CTRP-3 levels in obese Korean women [10]. Although there has been some researches concerned the expression of CTRP3 and its modulation, little is known about its expression pattern at different stages of T2DM which is a chronic and progressive disease.

Glucagon-like peptide-1 (GLP-1) receptor agonists are a new class of pharmacological agents that improve glucose homeostasis in many ways, including potentiation of glucose-stimulated insulin secretion, glucose-dependent inhibition of glucagon secretion, and reduction in gastric emptying, appetite, food intake, and body weight [11]. Exendin-4 (Ex-4), a GLP-1 receptor agonist that has been used as a drug injected subcutaneously for treatment of T2DM, was shown to promote adiponectin secretion via the protein kinase A (PKA) pathway in 3T3-L1 adipocytes and ameliorate insulin resistance [12]. But it is unknown whether Ex-4 might modulate the expression of CTRP3 in rats with T2DM. So, in this report, we showed the gene and protein expression of CTRP3 in visceral adipose tissue of rats at different stages of T2DM pathogenesis and the effects of Ex-4 on it.

2. Research Design and Methods

2.1. Animal Feeding and Treatments. Seventy healthy male wistar rats, 8 weeks of age, were placed in a room with controlled lighting (12 hours light/dark cycle) and regulated temperature (18–25°C) and humidity. All rats were fed with regular chow (protein 21%, carbohydrate 55%, fat 6%, and total energy 15.36 kJ/g) for 2 weeks to be adapted for the environment. Twenty-four rats were randomly selected as normal control group (NC) and fed with regular chow throughout the study. The expression of CTRP3 in rats of NC group was detected at the beginning of the study (marked as week 0), week 10 and week 15, respectively, and each of the detection sacrificed 8 rats. The remaining 36 rats were fed with high-fat diet which consisted of regular feedstuff, sucrose, lard, fresh egg, and milk power (protein 16%, carbohydrate 38%, fat 46%, and total energy 20.54 KJ/g) according to one of our former studies [13]. After 10 weeks high-fat diet feeding, twenty-four rats were selected randomly as insulin resistance group (IR, $n = 16$) and IR + Ex-4 group ($n = 8$) which was given 10 μg/kg Ex-4 (ChemPep) administered by subcutaneous injection daily for 4 weeks. The expression of CTPR3 in rats of IR group was detected at week 10 and week 15, respectively, and each of the detection also sacrificed 8 rats. The other 22 high-fat feeding rats were given an intraperitoneal injection of streptozotocin (STZ, 25 mg/kg) in 0.1 mol/L citrate-buffered saline. One week later, random blood glucose of the rats were detected and rats with randomized blood glucose ≥16.7 mmol/L twice not in one day were considered as diabetic ones. There were 15 T2DM rats who were divided randomly into DM group (DM, $n = 7$) and DM + EX-4 intervention group (DM + Ex-4, $n = 8$) treated as IR + Ex-4 group. The control rats received daily saline injections. The expressions of CTRP3 in rats of IR + Ex-4 group, DM group and DM + Ex-4 group were also detected. The body weight of all rats was measured weekly during the study.

2.2. Hyperinsulinemic Euglycemic Clamp. Rats of NC group, IR group, DM group, DM + Ex-4 group, and IR + Ex-4 group at week 15 were anesthetized with pentobarbitone (80 mg/kg) after overnight fasting. Both sides of femoral veins were exposed and inserted by a catheter for infusion of glucose and insulin, respectively. Another catheter was inserted into the femoral artery for blood sampling. Rats were kept quiet for 30 minutes, then a 120 minute hyperinsulinemic euglycemic clamp was performed. Insulin was infused at a constant rate of 1.67 mU/kg per minute and the arterial blood glucose concentration was clamped at the basal fasting level by infusing glucose at variable rates. Under the hyperinsulinemic conditions, the steady glucose infusion rate (GIR) required to maintain euglycemia is a standard measure of the whole-body insulin sensitivity.

2.3. Real-Time Polymerase Chain Reaction (RT-PCR). Fresh epididymis adipose tissue specimens were homogenised in RLT lysis buffer (Rneasy Mini Kit, QIAGEN, Valencia, CA, USA) using a rotator-stator, followed by a chloroform delipidation step. The upper aqueous phase was processed for total RNA extraction using silica-based spin columns (Rneasy Mini Kit). The cDNAs were reverse-transcribed with SuperScript III (Invitrogen) from 1 μg of total RNA according to the manufacturer's protocol. PCR primers (Saibaisheng, Shanghai, China) used in the study were as follows: (1) CTRP3 sense 5-GCC CCC GTA TCA GGT GTG TAT TT-3; antisense: 5-TGA AGA CTG TGT TGC CGT TGT GC-3; (2) β-actin: sense 5-ACA CCC GCC ACC AGT TCG C-3; antisense 5-TCT CCC CCT CAT CAC CCA CAT-3. The β-actin mRNA level was quantified as an internal control. The $\Delta(\Delta Ct)$ method was used to calculate the results for each control and experimental group. The cycle conditions were 50°C for 2 min followed by 40 cycles at 95°C for 15 s and 65°C for 34 s.

2.4. Western-Blot. Rats epididymis adipose tissue protein homogenates were prepared in tissue protein extraction buffer (Thermo Scientific) supplemented with protease inhibitors (Roche Applied Science) and phosphatase inhibitors (Sigma). Samples were boiled for 5 min in SDS loading buffer and equal amounts (25–50 μg per sample) of protein extracts were then separated by 8–12% of SDS-PAGE and electrotransferred onto PVDF membrane (Bio-Rad). Membranes were blocked with 5% non-fat skim milk in Tris-buffered saline/0.1% Tween20 (TBS-T) for 1 h, and then incubated with affinity-purified goat polyclonal primary antibodies were used at the following working dilutions: CTRP3 (1 : 1000 dilution, Abcam) and beta-actin: (1 : 1000 dilution, Santa Cruz). Appropriate secondary antibodies conjugated to horseradish peroxidase were incubated with respective membranes for 1 h at room temperature. Following five times intermittent

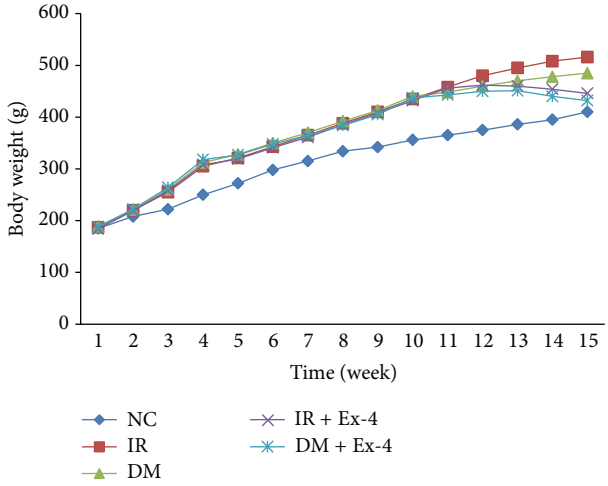

FIGURE 1: The curve of rats' body weight. NC: normal control group; IR: insulin resistance group; DM: diabetes mellitus group; IR + Ex-4: insulin resistance plus Exendin-4 treatment group; DM + Ex-4: diabetes mellitus plus Exendin-4 treatment group.

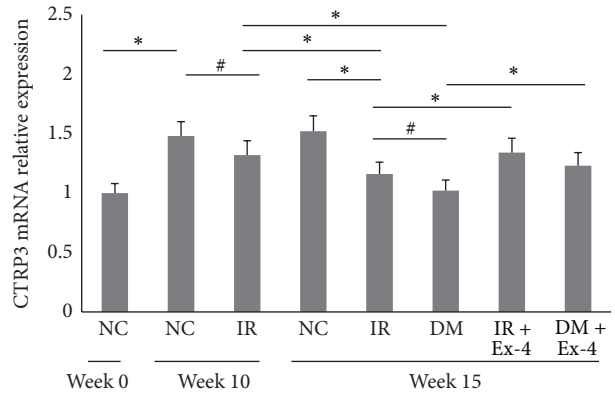

FIGURE 2: CTRP3 mRNA expression at the different stages of T2DM pathogenesis. NC: normal control group; IR: insulin resistance group; DM: diabetes mellitus group; IR + Ex-4: insulin resistance plus Exendin-4 treatment group; DM + Ex-4: diabetes mellitus plus Exendin-4 treatment group; $^{*}P < 0.01$, $^{\#}P < 0.05$.

washes with 1 × TBS-T, the membranes was processed for autoradiography using enhanced chemiluminescence (ECL, Pierce Chemical). The results were quantified by densitometric analysis using the Image-Quant software. All Western-blot experiments were performed in triplicate.

2.5. Statistical Analysis. Data were expressed as mean ± SE and were evaluated statistically using One-way ANOVA with SPSS (version 19.0) software. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Animal Model. The body weight of rats throughout study was shown in Figure 1. At the beginning of the study (week 0), there were no significant differences in body weight among the groups. After high-fat diet feeding, the body weight of rats in IR group, DM group, IR + Ex-4 group, and DM + Ex-4 group increased faster than NC group. There were no significant differences in body weight among IR group, DM group, IR + Ex-4 group, and DM + Ex-4 group before the treatment of Ex-4. At the end of the study (week 15) the body weight of rats in IR + Ex-4 group was lower than that of IR group ($P < 0.01$), and DM + Ex-4 group was lower than DM group statistically ($P < 0.01$). After the STZ intraperitoneal injection in 22 high-fat diet feeding rats, there were 15 rats whose random glucose ≥16.7 mmol/L twice not in one day, being considered as T2DM. Not surprisingly, the fasting blood glucose (FPG) in rats of DM group and DM + Ex-4 group was higher than that of NC group or IR group significantly (all $P < 0.01$), and the glucose level in DM + Ex-4 group was decreased obviously compared to that of DM group ($P < 0.01$) (Table 1).

3.2. Insulin Sensitivity. The whole-body insulin sensitivity was assessed by GIR. According to our previous study [13], high-fat diet feeding for 10 weeks may induce significant insulin resistance in wistar rats. So in this study the rats were given high-fat diet feeding for 10 weeks and then given STZ intraperitoneal injection to conduct T2DM model. At the end of study, the GIR of IR group and DM group was (4.9 ± 0.4) $mg*kg^{-1}*min^{-1}$ and 4.7±0.4 $mg*kg^{-1}*min^{-1}$, respectively, both being significantly lower than that of NC group [(7.2 ± 0.5) $mg*kg^{-1}*min^{-1}$] (all $P < 0.01$). After the intervention with Ex-4, the GIR in IR + Ex-4 group and DM + Ex-4 group were both increased significantly in comparison with that of IR and DM group accordingly (all $P < 0.01$) (Table 1).

3.3. CTRP3 mRNA Expression at the Different Stages of T2DM Pathogenesis. The relative expression of CTRP3 mRNA in NC group at week 10 was increased significantly than that of week 0 ($P < 0.01$), but there was no significant different between week 10 and week 15. CTRP3 mRNA relative expression of IR group at week 15 was decreased significantly by 12.1% ($P < 0.01$) than that of week 10 which was lower statistically than that of NC group at the same week ($P < 0.05$). Compared to IR group at week 10, the CTRP3 mRNA relative expression in DM group at week 15 was decreased by 22.7% ($P < 0.01$). At week 15, the CTRP3 mRNA relative expression in IR group and DM group was decreased by 25.0% and 32.9%, respectively, in comparison with that of NC group (all $P < 0.01$). The difference of CTRP3 mRNA expressions in DM group and IR group at week 15 was also significant ($P < 0.05$) (Figure 2).

3.4. CTRP3 Protein Expression at the Different Stages of T2DM Pathogenesis. Compared with NC group at week 0, the relative expression of CTRP3 protein in NC group at week 10 was increased significantly ($P < 0.01$). There was no significant difference in CTRP3 protein expression of NC group between week 10 and week 15. CTRP3 protein relative expression of IR group at week 15 was decreased significantly by 16.3% ($P < 0.01$) than that of week 10 which was lower statistically than that of NC group at the same time ($P < 0.01$). Compared to IR group at week 10, the CTRP3 protein

Table 1: Body weight, FBG, and insulin sensitivity of rats at the end of the study (mean ± SE).

Group	N	Body weight (g)	FBG (mmol/L)	GIR (mg $*$ kg^{-1} $*$ min^{-1})
NC	8	410.3 ± 23.6	5.1 ± 0.6	7.2 ± 0.5
IR	8	516.5 ± 30.7*	5.9 ± 1.1	4.9 ± 0.4*
DM	7	485.6 ± 26.8$^{*\Diamond}$	13.2 ± 2.1$^{*\Delta}$	4.7 ± 0.4*
IR + Ex-4	8	446.9 ± 25.6$^{\#\Delta\square}$	5.2 ± 0.8$^{\square}$	5.9 ± 0.5$^{*\Delta\square}$
DM + Ex-4	8	432.2 ± 24.7$^{\Delta\square}$	7.6 ± 0.9$^{*\Delta\square}$	5.8 ± 0.5$^{*\Delta\square}$

NC: normal control group; IR: insulin resistance group; DM: diabetes mellitus group; IR + Ex-4: insulin resistance plus Exendin-4 treatment group; DM + Ex-4: diabetes mellitus plus Exendin-4 treatment group; FBG: fasting blood glucose; GIR: glucose infusion rate; versus NC group $^{*}P < 0.01$, $^{\#}P < 0.05$; versus IR group $^{\Delta}P < 0.01$, $^{\Diamond}P < 0.05$; versus DM group $^{\square}P < 0.01$.

relative expression in DM group at week 15 was decreased by 24.8% ($P < 0.01$). Compared to NC group at week 15, the CTRP3 protein relative expression of IR group and DM group was decreased by 31.6% and 38.6%, respectively (all $P < 0.01$). The difference of CTRP3 protein expression in DM group and IR group at week 15 was also significant ($P < 0.05$) (Figure 3).

3.5. Effects of Ex-4 on the Expression of CTRP3 in T2DM and IR Groups. Compared to IR group at week 15, the relative expression of CTRP3 mRNA and protein of IR + Ex-4 group was increased by 15.5% ($P < 0.01$) and 14.8% ($P < 0.05$), respectively. The relative expression of CTRP3 mRNA and protein of DM + Ex-4 group was increased by 20.6% ($P < 0.01$) and 16.5% ($P < 0.05$), respectively, in comparison with that of DM group (Figures 2-3).

4. Discussion

Nowadays there are numerous animal models available for the study of T2DM, but the pattern of disease development and progress in most of them is not equal to the clinical situation in human beings [14]. Many studies have shown that rats fed with high-fat diet develop insulin resistance but not frank hyperglycemia or diabetes, indicating that high-fat diet might be a good way to initiate insulin resistance which is one of the important features and motivators of T2DM [15]. STZ is widely used to reproducibly induce diabetes mellitus by inducing pancreatic islet beta cells death through DNA alkylation. It is well known that high-dose STZ severely impairs insulin secretion, mimicking type 1 diabetes mellitus, and low-dose STZ after high-fat diet feeding induces a mild damage to beta cells on the background of insulin resistance, which is similar to the features of T2DM pathogenesis [16].

Our laboratory has used high-fat diet to induce insulin resistance model and high-fat diet plus low-dose STZ intraperitoneal injection to conduct T2DM model in wistar rats [13, 17]. In this study, male wistar rats were fed with high-fat diet for 10 weeks to induce insulin resistance and then 22 of them were given low-dose STZ (25 mg/Kg) intraperitoneal injection. One week later, the random glucose was detected and there were 15 rats whose glucose was ≥16.7 mmol/L twice not in one day. The diabetes model success rate was 68.2%, similar to other reports [18]. In addition to the detection of glucose, this study also measured GIR though hyperinsulinemic euglycemic clamp to reflect the insulin sensitivity, and found that the GIR of IR group and DM

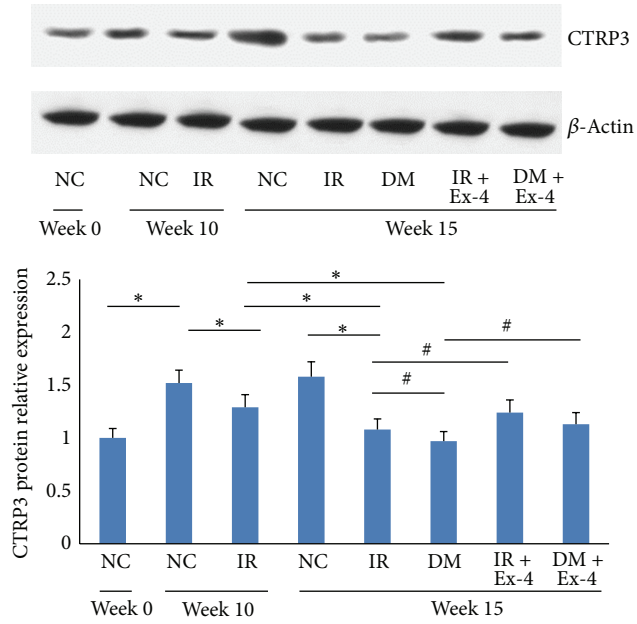

Figure 3: CTRP3 protein expression at the different stages of T2DM pathogenesis. NC: normal control group; IR: insulin resistance group; DM: diabetes mellitus group; IR + Ex-4: insulin resistance plus Exendin-4 treatment group; DM + Ex-4: diabetes mellitus plus Exendin-4 treatment group; $^{*}P < 0.01$, $^{\#}P < 0.05$.

group was lower significantly than that of NC group. Based on the results of glucose and GIR, week 10 (rats fed with high-fat diet for 10 weeks) and week 15 (rats fed with high-fat diet plus low-dose STZ intraperitoneal injection) were considered as different stages of T2DM pathogenesis, that is insulin resistance and overt diabetes.

Recently, CTRP3 was described as a novel adipokine with glucose-lowering effects achieved by suppression of hepatic gluconeogenesis [5]. Schmid et al. [7] used human subcutaneous and visceral adipocytes and murine 3T3-L1 adipocytes to analysis of CTRP-3 expression and function and found that CTRP-3 was expressed in subcutaneous and visceral adipocytes, being positively regulated by insulin and negatively by infection or inflammation-related factors. One study on women with PCOS which is associated with obesity, insulin resistance, and diabetes reported that the levels of serum and omental adipose tissue CTRP3 were lower in women with PCOS in comparison with control subjects [9].

However, another study found that CTRP-3 concentrations were significantly higher in patients with T2DM or prediabetes than the normal glucose tolerance group [8]. The possible explanations for the diverges between the two studies might be the effects of medications taken by T2DM patients, which may increase the expression of CTRP3, and the different ethnic groups. Although there have been some researches about the expression of CTRP3 under different status, the pattern of CTRP3 expression and its regulation at the different stages of T2DM is widely unknown.

This study detected the mRNA and protein expression levels of CTRP3 in T2DM rats at different pathogenic stages. In normal control rats, the expression of CTRP3 mRNA and protein at week 10 (aged 20 weeks) was increased significantly than that of week 0 (aged 10 weeks). Although the expressions of CTRP3 mRNA and protein were increased at week 15 (aged 25 weeks) in comparison with week 10, the differences were not significant, indicating that the expression of CTRP3 in visceral adipose tissues of normal rats increased with their growth and came to a relative stable state at the age of 20 weeks. As to T2DM rats, the expressions of CTRP3 mRNA and protein at week 10 when they were at the stage of insulin resistance were lower than that of normal control rats at the same week, and decreased more at week 15 when they were at the stage of overt diabetes. To our knowledge, this is the first time to report the expression of CTRP3 in rats at different stages of T2DM pathogenesis. These results resembled a report of CTRP3 expression in PCOS women as mentioned above but were opposite to one study showing that CTRP3 expression was decreased in T2DM patients in comparison with normal control subjects. The reasons may be the different species, the possible therapies in T2DM patients, and other unknown factors.

It is well known that inflammation is the common characteristic and mechanism of obesity, insulin resistance and T2DM [19]. Our former studies in insulin resistance rats induced by high-fat diet and T2DM rats induced by high-fat diet plus low-dose STZ intraperitoneal injection found that there were obvious inflammation in such animals which were the same as that used in this study [20, 21]. As some studies reported that CTRP3 expression in adipocytes were positively regulated by insulin and negatively by infection or inflammation-related factors, it is reasonable to infer that the relative low expression of CTRP3 in insulin resistance rats and T2DM rats was due to the status of inflammation and insulin resistance in them.

From recent decades, increasing the GLP-1 activity has emerged as a useful therapeutic tool for the treatment of T2DM [22]. The actions of GLP-1 on pancreatic islet beta cells and central nervous and digestive systems have been widely studied [23]. The action of this peptide in adipose tissue, however, is still poorly defined. Studies have shown the presence of GLP-1 receptor in adipose tissue, and that the expression of GLP-1 receptor mRNA and protein was increased in visceral adipose depots from morbidly obese patients with a high degree of insulin resistance [24]. Furthermore, prospective studies carried out with patients that underwent biliopancreatic diversion surgery showed that subjects with high levels of GLP-1 receptor expression in adipose tissue, which indicates a deficit of GLP-1 in this tissue, were those whose insulin sensitivity improved after surgery, suggesting the potential relationship between GLP-1 activity and insulin sensitivity [24]. This study found that after the treatment with Ex-4 for 4 weeks, GIR of IR rats and T2DM rats were increased significantly, indicating the sensitization of GLP-1 in adipose tissue, which may be due to its activities in inhibiting inflammatory and regulating adipogenesis and lipid metabolism [25, 26].

For the tight relationship between GLP-1 and adiposity and its related diseases, more and more studies focused on the effects and mechanisms of GLP-1 on adipose tissue which is the main source and target of adipokines that may be the key modulators in the pathogenesis of adiposity related diseases. GLP-1 receptor agonist has been shown to increase the expression of such adipokines as adiponectin [12] and visfatin [27] that may increase the insulin sensitivity in adipose tissue. CTRP3 is a novel multifunctional adipokine and its expression modulated by GLP-1 has never been studied before. This study, for the first time, reported that Ex-4 treatment increased the mRNA and protein expressions of CTRP3 in insulin resistance rats and T2DM rats. Kopp et al. [28] reported that the recombinant CTRP3 dose-dependently inhibited the release of chemokines in monocytes and adipocytes *in vitro* and *ex vivo*, and inhibited monocyte chemoattractant protein-1 (MCP-1) release in adipocytes, whereas small interfering RNA (Si-RNA)-mediated knockdown of CTRP-3 upregulated MCP-1 release, reduced lipid droplet size, and decreased intracellular triglyceride concentration in adipocytes, causing a dedifferentiation into a more proinflammatory and immature phenotype. These results indicated that CTRP3 has the effects of anti-inflammation, which may be one of the indirect mechanisms that GLP-1 increased insulin sensitivity in insulin resistance rats and T2DM rats as found in this study.

In whole, this study found that the mRNA and protein expressions of CTRP3 in visceral adipose tissue were progressively decreased at the pathogenic stages of insulin resistance and overt diabetes in a T2DM rats model induced by high-fat diet plus low-dose STZ intraperitoneal injection, while Ex-4, a GLP-1 receptor agonist, increased the expression of CTRP3 in such animals and improved their insulin sensitivity. But the expression of CTRP3 at different stages of T2DM pathogenesis in human beings, the factors that modulates the expression of CTRP3 in the pathogenesis of T2DM and the mechanisms that GLP-1 receptor agonists modulate the expression of CTRP3 are still largely unknown and need further research.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This research was supported by the Fundamental Research Funds for the Central University (4101017) and Natural Science Foundation of Zhongnan Hospital Wuhan University (2013A05).

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Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2014, Article ID 363126, 6 pages
http://dx.doi.org/10.1155/2014/363126

Research Article

Effects of Unilateral Nephrectomy on Renal Function in Male Spontaneously Diabetic Torii Fatty Rats: A Novel Obese Type 2 Diabetic Model

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Received 23 June 2014; Accepted 7 July 2014; Published 10 August 2014

Academic Editor: Masami Shinohara

The Spontaneously Diabetic Torii (SDT) fatty rat is a new model for obese type 2 diabetes. The aim of the present study was to investigate the effect of 1/2 nephrectomy (Nx) on renal function and morphology and on blood pressure in SDT fatty rats. Male SDT fatty rats underwent 1/2 Nx or a sham operation (Sham). Subsequently, animals were studied with respect to renal function and histological alterations. Induction of 1/2 Nx in SDT fatty rats led to functional and morphological damage to the remnant kidney and to hypertension, which are considered main characteristics of chronic kidney disease, at a younger age compared with the sham group. In conclusion, the SDT fatty rat is useful in investigations to elucidate the pathogenesis of human diabetic nephropathy and in new drug discovery.

1. Introduction

Chronic kidney disease (CKD) is a worldwide public health problem associated with significant morbidity and mortality [1]. Several risk factors contribute to the development and progression of CKD, including hypertension, diabetes, and dyslipidemia [2–4]. In particular the increase in number of patients with obesity-associated type 2 diabetes has resulted in a rapid increase in patients who have end stage renal disease (ESRD) and require dialytic life support [5, 6]. Despite efforts to develop means to prevent or arrest the progression of the disease, long-term prognosis of patients with established nephropathy remains poor [7]. Diabetic nephropathy has been recognized as a primary disease of CKD and the investigation of diabetic nephropathy is essential for the understanding of the pathogenesis of CKD.

Diabetic animal models have a critical role in the elucidation of mechanisms of diabetic complications and the development of novel drugs as treatments. Consequently, the understanding of the pathophysiology of renal lesions in diabetic models is beneficial in the design and development of therapies.

The Spontaneously Diabetic Torii (SDT) fatty rat, established by introducing the *fa* allele in Zucker fatty rats into the SDT rat genome, is a model for obese type 2 diabetes showing overt obesity, hyperglycemia, and hyperlipidemia from a younger age and resulting in early onset of diabetic complications [8–12]. Furthermore, SDT fatty rats showed elevated blood pressure, in addition to the aforementioned metabolic abnormalities [13, 14]. Therefore, the SDT fatty rat is considered to be a useful model for the analysis of diabetes and related complications such as diabetic nephropathy.

In this study, we investigated the effects of unilateral nephrectomy on renal function and morphology in SDT fatty rats.

2. Materials and Methods

2.1. Animals. This experiment was conducted in compliance with the Guidelines for Animal Experimentation established for our biological/pharmacological laboratories. Male SDT fatty rats from Japan Tobacco colonies were used in this study. Animals were divided into 2 groups: those undergoing 1/2 nephrectomy (1/2 Nx) or sham operation (sham). Animals at 8 weeks of age underwent 1/2 Nx or sham surgery under anesthesia. A small lumbar incision was made, and the left kidney was removed. In sham-operated animals, the left kidney was exposed and gently manipulated but left intact. Animals were housed in suspended bracket cages and given a standard laboratory diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) in a room with controlled temperature, humidity, and lighting.

2.2. Biological Parameters. Body weight, biochemical parameters, and renal parameters were assessed from 10 to 18 weeks of age, every 2 weeks. Blood samples were collected from the tail vein of nonfasted rats. Serum glucose, triglyceride (TG), and total cholesterol (TC) were measured as a biochemical parameter using commercial kits (F. Hoffmann-La Roche Ltd., Basel, Switzerland) and an automatic analyzer (Hitachi, Ltd., Tokyo, Japan).

Urine volume, urinary albumin, blood urea nitrogen (BUN), and creatinine clearance (Ccr) were evaluated as renal parameters. Urine samples were collected by placing the animals in metabolic cages with water for 6 h. Urinary albumin level was measured with a rat-albumin enzyme immunoassay (EIA) kit (Panapharm Laboratories Co., Ltd., Kumamoto, Japan). Urinary creatinine, serum creatinine, and BUN were measured using commercial kits (Roche Diagnostics, Basel, Switzerland) and an automatic analyzer (Hitachi, Ltd., Tokyo, Japan). Ccr was calculated by dividing the 6 h urinary excretion of creatinine by serum creatinine level and body weight.

Systolic blood pressure (SBP) and heart rate in a conscious nonfasted state were measured at 12 and 16 weeks of age by the indirect tail cuff method using a Softron BP-98A indirect blood pressure meter (Softron Co. Ltd., Tokyo, Japan). Blood pressure and heart rate were measured between 13:00 and 16:00 hours. Five measurements were taken for each rat and subsequently averaged.

2.3. Histopathological Examination. Necropsy was performed at 18 weeks of age and kidneys were collected from all animals. Kidneys were weighed and fixed with 4% paraformaldehyde. After resection, the tissues were paraffin-embedded using standard techniques and thin-sectioned (3–5 μm). Sections were stained with hematoxylin and eosin (HE) and periodic acid Schiff (PAS). Eight mice in the 1/2 Nx group and five mice in the sham group were examined histopathologically in a blinded manner.

2.4. Statistical Analysis. Results of biological parameters were expressed as means ± standard deviation (SD). Statistical analyses of differences between mean values in the sham group and the 1/2 Nx group were performed using the F-test, followed by the Student's t-test or Aspin-Welch's t-test. Differences were defined as significant when $P < 0.05$.

3. Results

3.1. Body Weight and Biochemical Parameters. Body weights in the 1/2 Nx group were comparable to those in the sham group from 10 to 18 weeks of age (1/2 Nx group; 479.7 ± 116.2 g at 18 weeks of age, sham group; 521.2 ± 40.3 g at 18 weeks of age). The 1/2 Nx group and sham group had similar levels of plasma glucose from 10 to 18 weeks of age (1/2 Nx group, 751.0 ± 63 mg/dL at 18 weeks of age; sham group, 799.9 ± 76.9 mg/dL at 18 weeks of age). Serum TG levels in the 1/2 Nx group at 18 weeks of age were significantly higher than those in the sham group (1/2 Nx group, 501.9 ± 211.4 mg/dL at 18 weeks of age; sham group, 309.6 ± 67.4 mg/dL at 18 weeks of age). Serum TC levels tended to increase in the 1/2 Nx group (1/2 Nx group, 154.4 ± 51.7 mg/dL at 18 weeks of age; sham group, 120.8 ± 18.7 mg/dL at 18 weeks of age).

3.2. Renal Parameters. Urinary albumin in the 1/2 Nx group increased from 14 weeks of age compared with those in the sham group (Figure 1(a)). Serum BUN levels in the 1/2 Nx group were significantly higher than those in the sham group during the experimental period (Figure 1(b)). Kidney weights in the 1/2 Nx group were higher compared with those in the sham group (Figure 1(c)). Ccr tended to decrease in the 1/2 Nx group (1/2 Nx group, 0.23 ± 0.12 mL/h∗g at 18 weeks of age; sham group, 0.31 ± 0.06 mL/h∗g at 18 weeks of age). Urine volumes in the 1/2 Nx group were comparable to those in the sham group from 10 to 18 weeks of age (1/2 Nx group, 13.17 ± 6.58 mL at 18 weeks of age, 0.23 ± 0.12 mL/h∗g; sham group, 15.90 ± 6.11 mL at 18 weeks of age).

3.3. Blood Pressure. SBP levels at 12 and 16 weeks of age in the 1/2 Nx group were significantly elevated compared with those in the sham group (Figure 2(a)). For heart rate, there were no differences among groups (Figure 2(b)).

3.4. Histopathological Examinations of the Kidney. The results of histopathological examinations of the kidney at 18 weeks of age are shown in Table 1 and Figure 3. The following findings in the glomerulus, tubule, and interstitium were observed in both the sham group and 1/2 Nx group. Glomerulosclerosis was characterized by an increase in size of the glomerulus and diffuse thickening of the glomerulocapillary wall and the mesangial expansion, showing partly segmental fibrosis in severe cases. Tubular lesions included tubular regeneration, dilatation, and hyaline casts, and interstitial lesions included fibrosis and inflammatory cell infiltration. The histological features of the kidney were not different between the 1/2 Nx group and the sham group; however, a more progressive pathological degree was observed in the 1/2 Nx group.

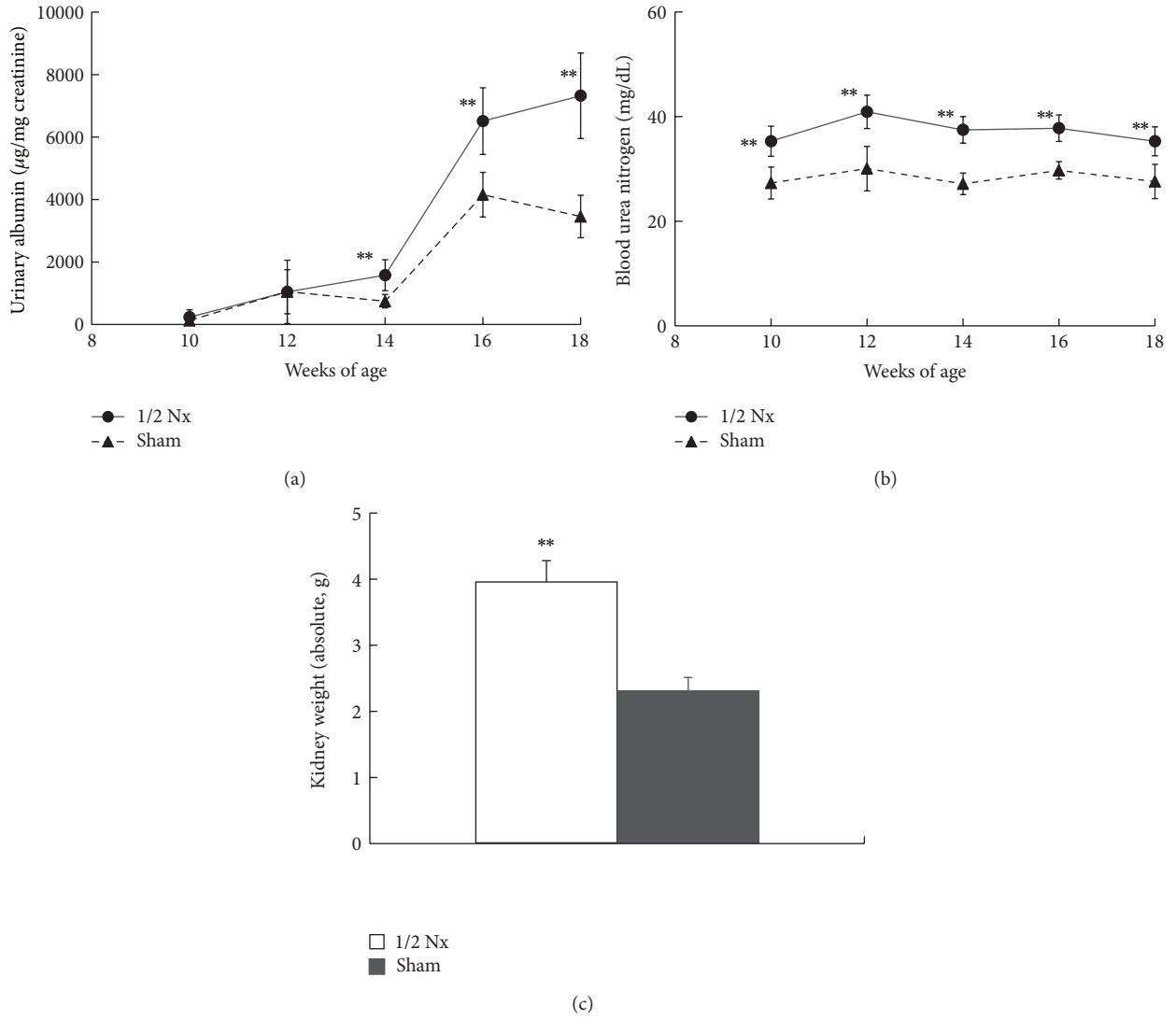

Figure 1: Changes in renal parameters ((a) urinary albumin, (b) blood urea nitrogen, and (c) kidney weight) in the 1/2 Nx group and sham group. Data shown as means ± SD ((a) and (b) n = 8–10 and (c) n = 8-9). $^{**}P < 0.01$; significantly different from the sham group.

4. Discussion

Glomerular lesions occurring due to renal mass reduction have been demonstrated [15, 16]. For example, kidney damage is exacerbated by nephrectomy in streptozotocin-diabetic rats [17] and Zucker fatty rats [18]. Therefore, unilateral nephrectomy is an effective method to accelerate the manifestation of renal alterations. In the present study, we evaluated the effects on renal function of SDT fatty rats subjected to unilateral nephrectomy, as well as the renal morphology of these animals.

Proteinuria, increased blood urea, hypertension, and glomerular sclerosis, which are considered main characteristics of CKD, were observed in the 1/2 Nx group at a younger age compared with the sham group.

Compensatory hypertrophy of the remnant kidney after unilateral nephrectomy is well recognized [19]. This phenomenon is accompanied by pathological changes that lead to reduced renal function, although the weight of kidneys has not been used as an indicator of renal dysfunction [19]. In agreement with these findings, the remnant kidney in the 1/2 Nx group was significantly heavier than those in the sham group, and histopathological examinations of remnant kidneys in the 1/2 Nx group showed degenerative changes such as glomerulosclerosis in the glomerulus and interstitial inflammation in the interstitium. These histopathological findings were not observed in normal Sprague Dawley rats.

Hyperglycemia is a known stimulus for renal hypertrophy and its association with reduction in renal mass affects this hypertrophy [15, 16, 20]. In the present study, levels of plasma glucose were similar among groups. This result suggests that the contribution of hyperglycemia to the exacerbation of renal function in the 1/2 Nx group is likely low.

Altered lipid metabolism influences the development and progression of glomerular injury [21, 22]. Lipid abnormalities

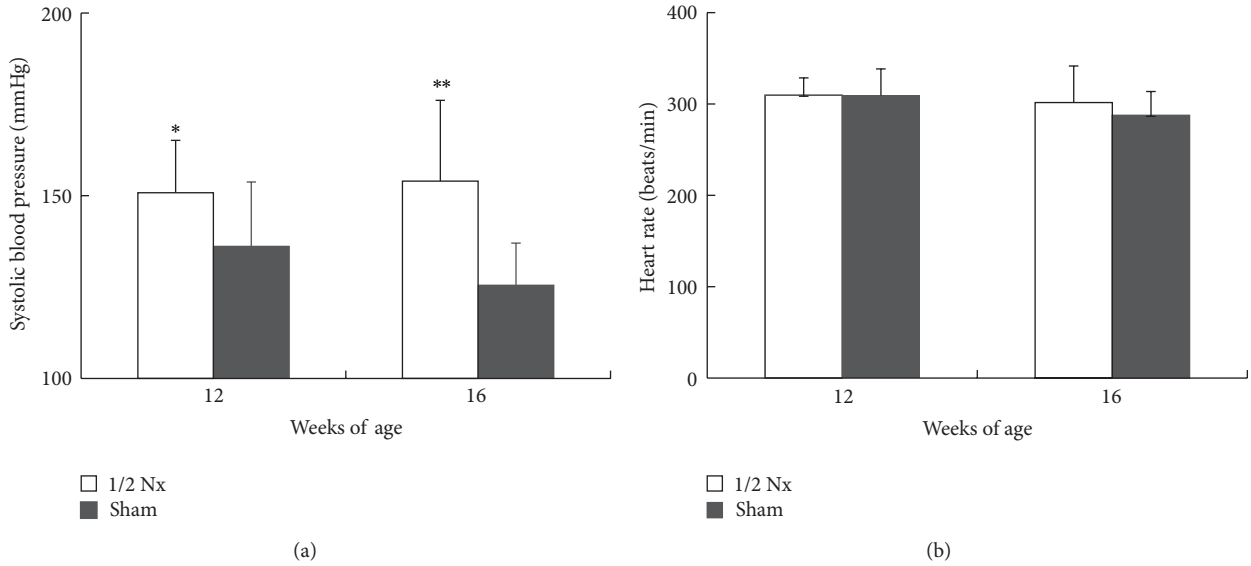

Figure 2: Systolic blood pressure (a) and heart rate (b) at 12 and 16 weeks of age in the 1/2 Nx group and sham group. Data shown as means ± SD (n = 8–10). $^{*}P < 0.05$, $^{**}P < 0.01$; significantly different from the sham group.

Table 1: Histopathological findings of kidney in sham group and 1/2 Nx group.

Findings	Group												
	1/2 Nx								Sham				
Animal number	1	2	3	4	5	6	7	8	9	10	11	12	13
Glomerulus													
Glomerulosclerosis	+	+	2+	+	+	2+	+	+	±	±	±	±	−
Tubule													
Regeneration	+	2+	2+	2+	2+	2+	2+	2+	+	+	+	+	+
Dilatation	2+	2+	+	2+	+	2+	2+	2+	+	+	±	+	+
Hyaline cast	+	+	±	±	+	±	+	+	+	+	±	+	±
Deposit, hyaline droplet	±	−	−	−	−	−	−	−	−	−	−	−	−
Armanni-Ebstein change	+	+	+	+	+	±	±	−	+	+	+	+	+
Mineralization	−	+	+	+	±	+	+	+	+	+	+	−	+
Interstitial													
Fibrosis, interstitial	−	+	−	−	−	+	−	+	−	+	±	−	±
Infiltration, inflammatory cell, interstitial	+	+	2+	2+	+	2+	2+	2+	±	−	+	±	+

−: negative; ±: very slight; +: slight; 2+: moderate; 3+: severe.

in the 1/2 Nx group, which accompany a reduction in renal mass, may contribute to progressive glomerular damage.

Hypertension is a hemodynamic characteristic of CKD, which could accelerate the progression of renal dysfunction by worsening glomerular injury and proteinuria [23]. It is possible that the increased blood pressure observed in the 1/2 Nx group in this study contributed to the accelerated glomerular injury and consequently led to marked proteinuria. Proteinuria has been considered a strong predictor of kidney disease outcome [24]. In addition, urinary biomarkers such as kidney injury molecule-1 (KIM-1) and N-acetyl-β-D-glucosaminidase (NAG) have been reported [25, 26]. In the further study, measuring these biomarkers may be useful to assess the severity of diabetic kidney damage.

In conclusion, induction of 1/2 Nx in SDT fatty rats led to functional and morphological damage of the remnant kidney and to hypertension, which are considered main characteristics of chronic kidney disease, at a younger age. The early onset of diabetic nephropathy in SDT fatty rats is an advantage for CKD research. The SDT fatty rat has promise in the further elucidation of the pathogenesis of human diabetic nephropathy and in new drug discovery.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

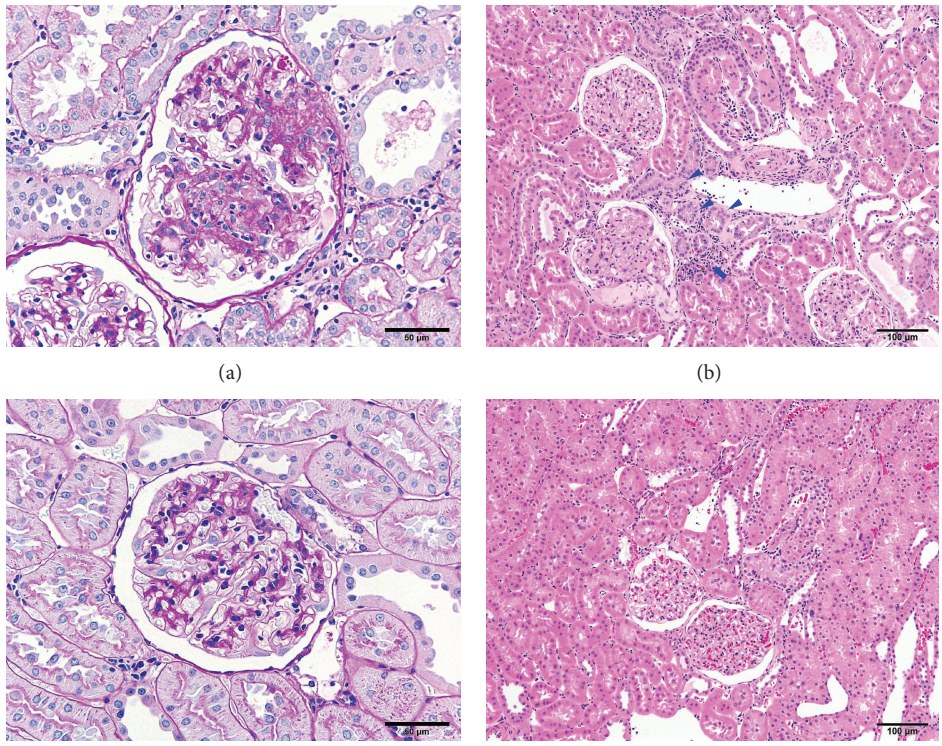

FIGURE 3: Photomicrograph of kidney tissues from the 1/2 Nx group (a) and (b) and sham group (c) and (d). Marked glomerulosclerosis (a) tubular regeneration (arrowhead in (b)) and inflammatory cell infiltration (arrow in (b)) observed in the 1/2 Nx group. Periodic acid Schiff ((a) and (c)), bar = 50 μm. Hematoxylin and eosin ((b) and (d)). Bar = 100 μm.

Authors' Contribution

Y. Katsuda, K. Kakimoto, and T. Ohta helped in the design of the study. Y. Katsuda, Y. Kemmochi, M. Maki, R. Sano, Y. Toriniwa, Y. Ishii, and K. Miyajima helped in the data collection and conduct of the study. Y. Katsuda, Y. Kemmochi, M. Maki, and T. Ohta helped in the analysis of study. Y. Katsuda and T. Ohta helped in writing the paper.

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Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2014, Article ID 680348, 7 pages
http://dx.doi.org/10.1155/2014/680348

Research Article

Pharmacological Effects of JTT-551, a Novel Protein Tyrosine Phosphatase 1B Inhibitor, in Diet-Induced Obesity Mice

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Received 24 April 2014; Revised 8 May 2014; Accepted 12 May 2014; Published 29 May 2014

Academic Editor: Norihide Yokoi

Protein tyrosine phosphatase 1B (PTP1B) is a negative regulator of leptin signaling as well as insulin signaling. JTT-551 is a new PTP1B inhibitor, which is reported to improve glucose metabolism by enhancement of insulin signaling. We have evaluated an antiobesity effect of JTT-551 using diet-induced obesity (DIO) mice. A single administration of JTT-551 was provided to DIO mice with or without leptin, and DIO mice were given food containing JTT-551 for six weeks. A single administration of JTT-551 with leptin treatment enhanced the food inhibition and the signal transducer and activator of transcription 3 (STAT3) phosphorylation in hypothalamus. Moreover, chronic administration of JTT-551 showed an antiobesity effect and an improvement of glucose and lipid metabolism in DIO mice. JTT-551 shows an antiobesity effect possibly by enhancement of leptin signaling and could be useful in the treatment of type 2 diabetes and obesity.

1. Introduction

The prevalence of obesity continues to increase rapidly worldwide. Body weight is normally maintained within a narrow range by a balance between energy intake and expenditure. When energy intake exceeds energy expenditure, excess energy is stored as triglyceride in adipose tissue, resulting in weight gain. Obesity is an important risk factor for type 2 diabetes, cardiovascular disease, and the metabolic syndrome. Effective antiobesity therapies are urgently needed [1–3].

Leptin, a hormone secreted by adipocytes, decreases body weight both by suppressing appetite and by increasing energy expenditure [4–6]. The brain, particularly the hypothalamus, integrates leptin and various other metabolic signals to regulate energy homeostasis and body weight by controlling both behavior and metabolic responses [7–9]. Genetic deficiency of leptin or functional leptin receptors also results in obesity and obesity-associated metabolic diseases in both animals and humans. Leptin administration decreases body weight and fat mass [10–12]; however, most obese individuals exhibit elevated circulating leptin levels and are less responsive to exogenously administrated leptin, consistent with a leptin resistance [13, 14].

Protein tyrosine phosphatase 1B (PTP1B) has been implicated in the negative regulation of the signaling pathway that phosphorylates the tyrosine residue. It is reported that PTP1B knockout mice exhibit increased insulin and leptin sensitivity and are resistant to high-fat diet-induced obesity (DIO) [15, 16]. DIO rats have a marked increase in PTP1B protein expression in the hypothalamus. It was recently reported that neuronal PTP1B knockout mice are hypersensitive to leptin and have reduced body weight and adiposity and increased energy expenditure [17, 18]. Treatment of DIO rats with PTP1B antisense oligonucleotide i.c.v. resulted in a decreased food intake, reduced body weight, and reduced adiposity [19]. These results suggest that PTP1B may play a pivotal role in the leptin resistance associated with obesity. Specific PTP1B inhibitors may thus be therapeutically beneficial in obesity as well as in type 2 diabetes.

JTT-551 is a novel PTP1B inhibitor, which is under development as an antidiabetic drug. JTT-551 increases the insulin-stimulated glucose uptake in L6 rat skeletal myoblasts (L6 cells), and single administration enhanced insulin receptor phosphorylation in liver. JTT-551 improves glucose metabolism by enhancement of insulin signaling [20].

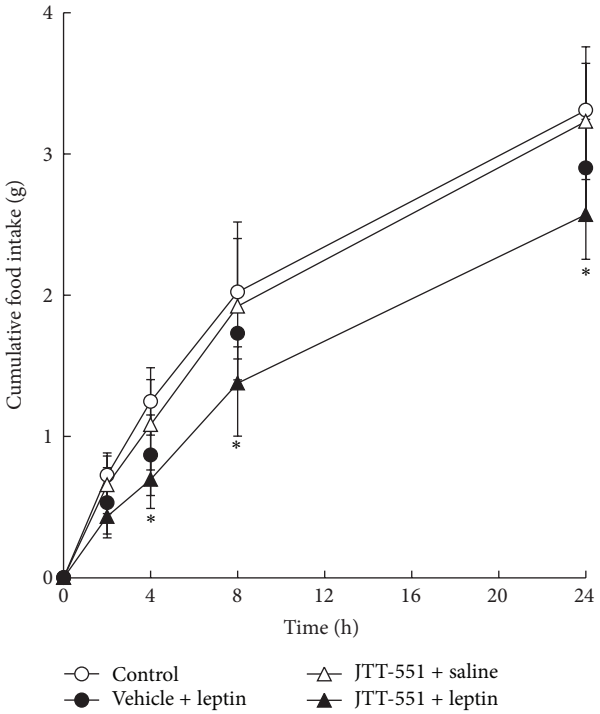

Figure 1: Enhancement effect of JTT-551 on leptin suppressed calorie intake in DIO mice. Data represent mean ± SD ($n = 5$). $^{*}P < 0.05$: significantly different from the control by Dunnett's test (two-tailed).

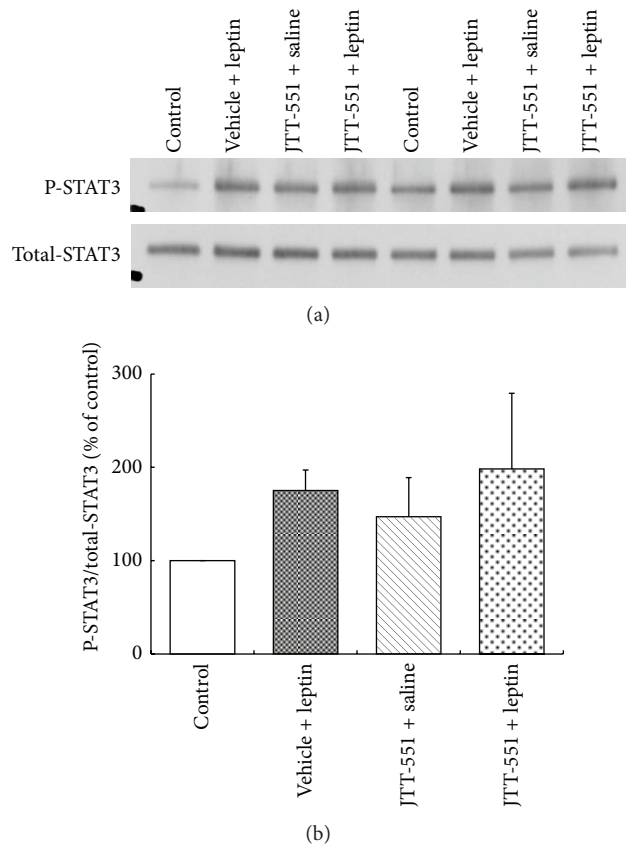

Figure 2: Enhancement effects of JTT-551 on STAT3 phosphorylation in the hypothalamus of DIO mice. The detected bands (typical bands) are shown in (a) and the phosphorylation-STAT3/STAT3 in each group in (b). The intensity of the STAT3 phosphorylation was calculated as the ratio of the density of phosphorylation-STAT3 to the density of STAT3.

In the present study, we evaluated the pharmacological profiles, especially the enhancement effect of leptin signaling, of JTT-551 *in vivo*, and examined whether the compound could be useful as an antiobesity agent.

2. Materials and Methods

2.1. Chemicals. JTT-551 was synthesized by Japan Tobacco Inc., Central Pharmaceutical Research Institute (Osaka, Japan).

2.2. Animals. All the experiments received prior approval from the Committee for the Humane Care and Use of Animals of Biological/Pharmacological Research Laboratories, Central Pharmaceutical Research Institute, Japan Tobacco Inc., in accordance with the Japanese Law on Humane Treatment and Management of Animals.

Male six-week-old C57BL/6J mice were purchased from Charles River Japan, Inc. (Yokohama, Japan). Animals were housed in a climate-controlled room (temperature 23 ± 3°C, humidity 55 ± 15%, and 12 h lighting cycle) and allowed free access to diet and water.

2.3. Acute Effect on DIO Mice. Seven-week-old C57BL/6J mice were provided with 35% fat diet (Oriental Yeast Co., Osaka, Japan) (Table 1) *ad libitum*. A single oral administration of JTT-551 100 mg/kg was provided to 12-week-old male DIO mice that had been fasting overnight and then leptin solution 10 mg/kg intraperitoneal administration 1 h before feeding. Feeding was resumed immediately after dosing and the food was weighed at 2, 4, 8, and 24 h. Cumulative food intake was calculated from difference in the weight from that before feeding. Calorie intake was determined under the following provisions: fat, 9 kcal/g; carbohydrate, 4 kcal/g; protein, 4 kcal/g.

Moreover, JTT-551 100 mg/kg was provided to 13-week-old male DIO mice that had been fasting overnight and then leptin solution 10 mg/kg intraperitoneal administration 1 h before feeding. The hypothalamus was removed at 2 h after feeding. The hypothalamus was homogenized and insoluble material was removed by centrifugation. Supernatants were separated using SDS polyacrylamide gel electrophoresis and immunoblotting as previously described [20]. Membranes were probed with antibodies for total and phosphorylated STAT3 (Santa Cruz Biotechnology, CA, USA). Protein phosphorylation was calculated as the ratio of phosphorylated-to-total protein expression.

2.4. Chronic Effect on DIO Mice. Eight-week-old DIO mice were given 10 or 100 mg/kg food containing JTT-551 for six weeks. Body weight and food consumption were measured

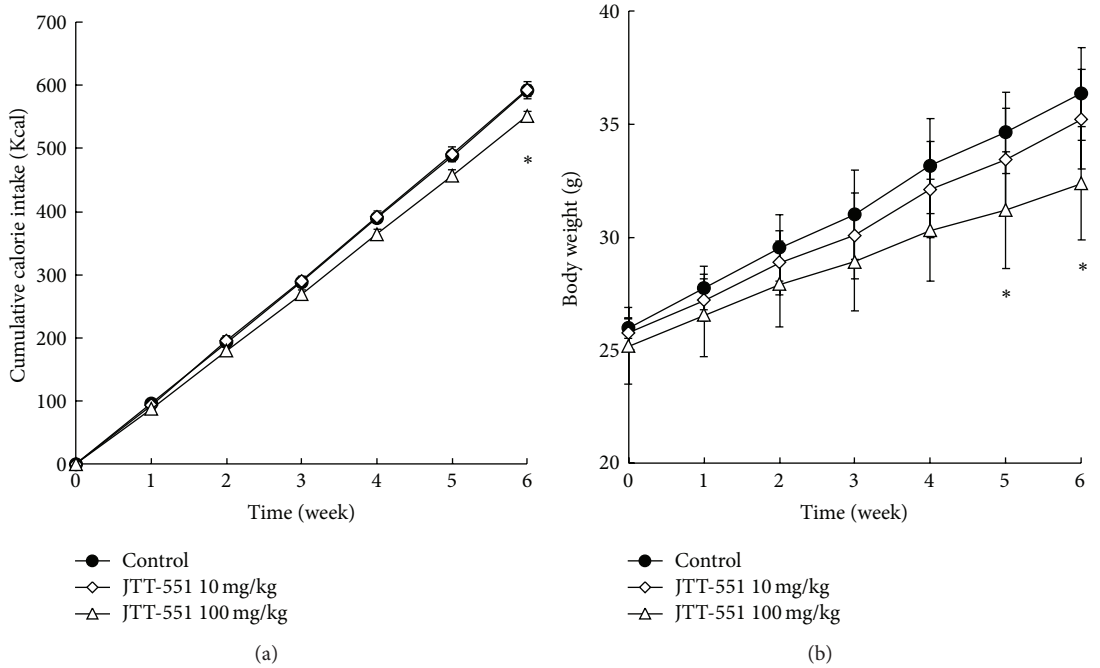

Figure 3: Effect of JTT-551 on cumulative calorie intake and body weight in DIO mice. DIO mice were given 10 or 100 mg/kg food containing JTT-551 for six weeks. Data represent mean ± SD ($n = 5$). $^*P < 0.05$: significantly different from the control by Dunnett's test (two-tailed).

Table 1: Composition of the experimental diet.

% (w/w)	35% fat diet
Lard	35.000
Cornstarch	7.308
Casein	28.810
Granulated sugar	14.410
Cellulose	7.200
AIN93M mineral mix	5.040
AIN93 vitamin mix	1.440
L-cystine	0.430
Choline bitartrate	0.360
Butylhydroquinone	0.002

every week. In fed or fasting DIO mice at six weeks after JTT-551 treatment, blood samples were collected from orbital venous plexus and the blood glucose, triglyceride (TG), and total cholesterol (TC) levels were measured using commercial kits (Roche Diagnostics, Basel, Switzerland) and an automatic analyzer (HITACHI 7170S; Hitachi, Tokyo, Japan). Blood insulin and leptin levels were measured with a rat enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Yokohama, Japan).

2.5. Statistical Analysis. Results of body weight, cumulative calorie intake, and blood chemistry values were expressed as the mean ± standard deviation (SD). Statistical analysis of mean values was performed using Dunnett's t-test (two-tailed). Differences were defined as significant at $P < 0.05$.

3. Results

3.1. Acute Effect on DIO Mice. The results for food intake in DIO mice are shown in Figure 1. In leptin administration group (leptin group), food intake was reduced compared with that in vehicle (0.5% MC) administration control group (control group) from 2 h after feeding. In JTT-551 administration group without leptin treatment (JTT-551 group), food intake was not reduced. In JTT-551 with leptin administration group (JTT-551 + leptin group), food intake was significantly reduced compared with leptin group from 4 h after feeding.

The results of western blot analysis are shown in Figure 2. The detected bands (typical bands) are shown in Figure 2(a) and the phosphorylated STAT3/STAT3 in each group in Figure 2(b). The STAT3 phosphorylation in the hypothalamus after administration of leptin and/or JTT-551 was increased compared with that in control group. In JTT-551 + leptin group, the STAT3 phosphorylation was more enhanced than in single administration groups.

3.2. Chronic Effect on DIO Mice. Effects of JTT-551 on the cumulative food intake and body weight are shown in Figure 3. In the JTT-551 100 mg/kg group, the cumulative calorie intake tended to decrease from two weeks after treatment (control: 193.3 ± 7.6 Kcal and JTT-551 100 mg/kg: 183.8 ± 14.2 Kcal) and was significantly decreased from six weeks after treatment (control: 591.8 ± 21.8 Kcal and JTT-551 100 mg/kg: 560.7 ± 28.6 Kcal) (Figure 3(a)). The body weight in JTT-551 treatment tended to decrease dose-dependently (control: 36.4 ± 2.1 g, JTT-551 10 mg/kg: 35.2 ± 2.2 g, and JTT-551 100 mg/kg: 32.5 ± 2.3 g, at six weeks after treatment); the decreases in JTT-551 100 mg/kg group were significant from five to six weeks after treatment (Figure 3(b)).

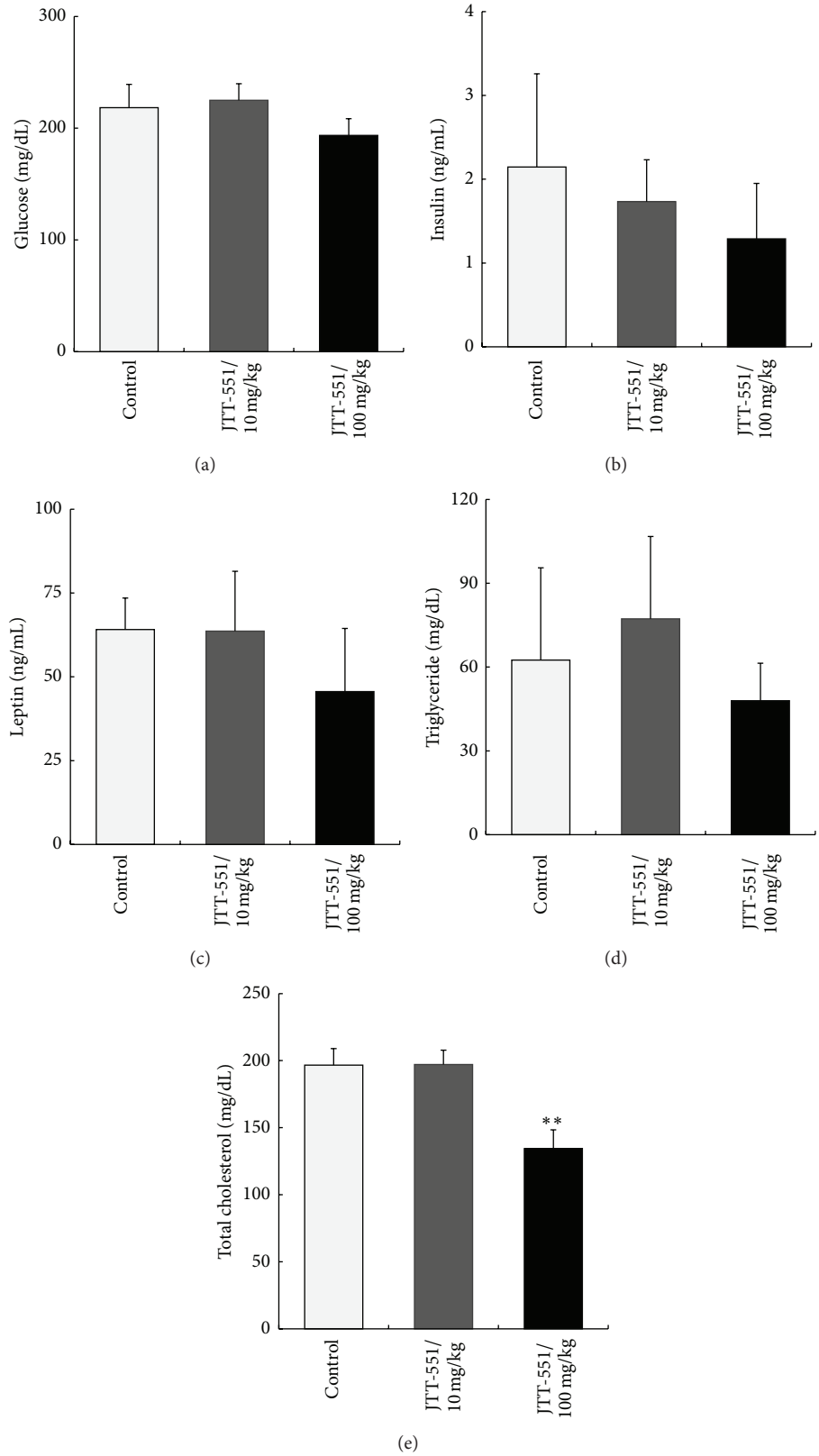

Figure 4: Effects of JTT-551 on blood glucose (a), insulin (b), leptin (c), triglyceride (d), and total cholesterol levels (e) in fed DIO mice. DIO mice were given 10 or 100 mg/kg food containing JTT-551 for six weeks. Data represent mean ± SD ($n = 6$). $^{**}P < 0.01$: significantly different from the control by Dunnett's test (two-tailed).

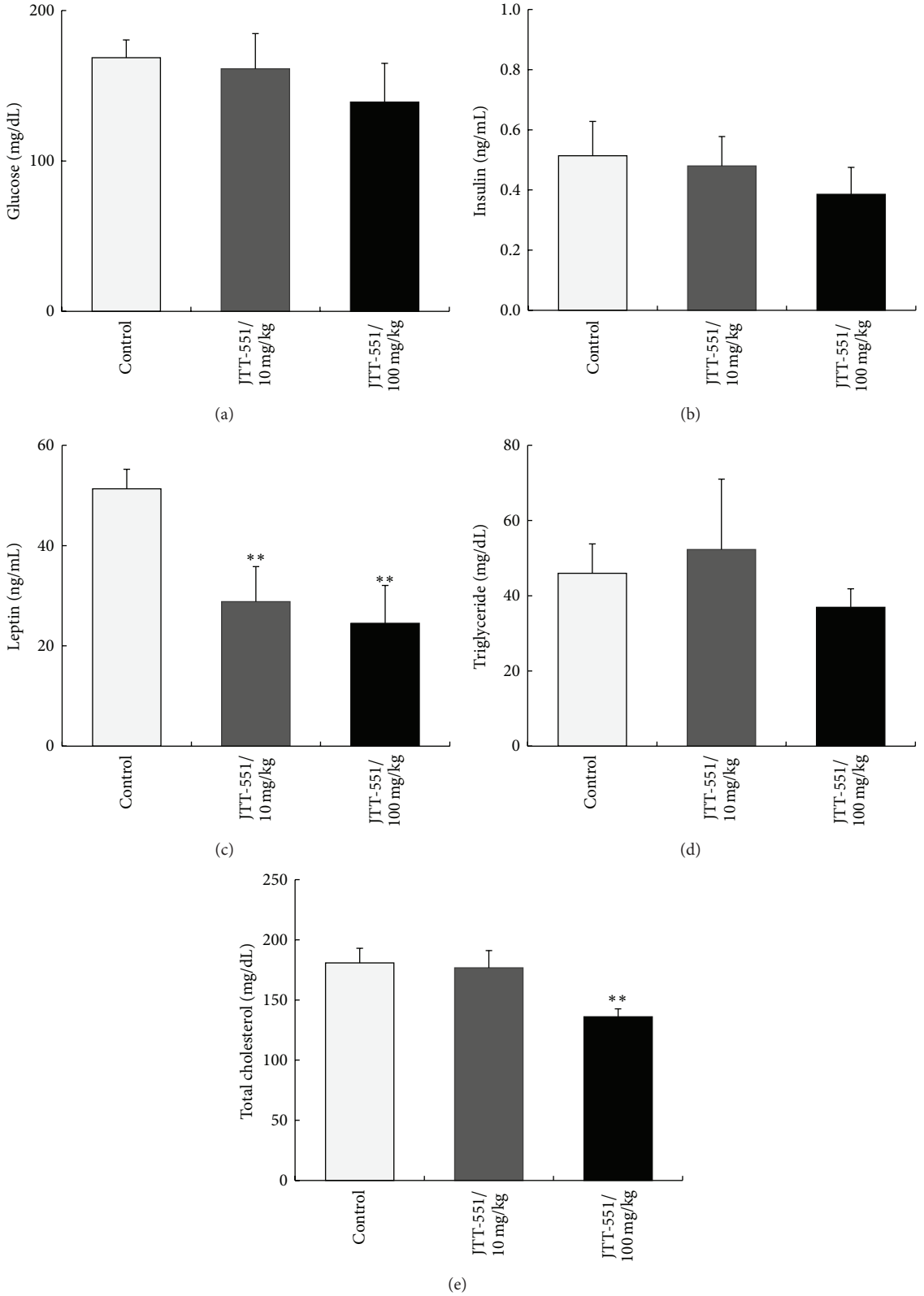

Figure 5: Effects of JTT-551 on blood glucose (a), insulin (b), leptin (c), triglyceride (d), and total cholesterol levels (e) in fasting DIO mice. DIO mice were given 10 or 100 mg/kg food containing JTT-551 for six weeks. Data represent mean ± SD ($n = 6$). $^{**}P < 0.01$: significantly different from the control by Dunnett's test (two-tailed).

Effects of JTT-551 on the blood chemistry values in six weeks after treatment are shown in Figures 4 and 5. The fed blood glucose level was not decreased (Figure 4(a)), but the fasting glucose level at JTT-551 100 mg/kg tended to decrease (control: 169 ± 12 mg/dL and JTT-551 100 mg/kg: 139 ± 26 mg/dL) (Figure 5(a)). The insulin levels in both fed and fasting mice tended to decrease, but not significantly (Figures 4(b) and 5(b)). The leptin levels in both fed and fasting mice tended to decrease dose-dependently, and those levels at JTT-551 treatment were significantly decreased (control: 51.3 ± 3.9 ng/mL, JTT-551 10 mg/kg: 28.8 ± 7.0 ng/mL, and JTT-551 100 mg/kg: 24.5 ± 7.5 ng/mL, in fasting mice) (Figures 4(c) and 5(c)). The TG levels in both fed and fasting mice tended to decrease, but not significantly (Figures 4(d) and 5(d)). The TC levels in both fed and fasting mice tended to decrease dose-dependently, and those levels at 100 mg/kg treatment were significantly decreased (control: 196.6 ± 12.4 mg/dL and JTT-551 100 mg/kg: 134.5 ± 13.9 mg/dL, in fed mice) (Figures 4(e) and 5(e)).

4. Discussion

PTP1B is a 50-KD cytosolic tyrosine dephosphorylase consisting of 435 amino acids which is ubiquitously expressed in organs throughout the body. It is well known that PTP1B dephosphorylates both phosphorylated insulin receptor (IR) β subunit and phosphorylated IR substrate, to negatively regulate insulin signal transmission [21, 22]. On the other hand, it is reported that PTP1B is concerned with negative regulation of leptin signal transmission, to dephosphorylate phosphorylated STAT3 [17, 18]. In a recent study, mice lacking the PTP1B were protected from diet-induced obesity and were hypersensitive to leptin. Neuronal PTP1B KO mice especially showed increased leptin signaling in the hypothalamus and had reduced feeding, weight, and adiposity and increased energy expenditure [15, 16]. This suggests that PTP1B is a key regulator of the leptin signal transmission. PTP1B is a negative regulator of leptin signal, in which the PTP1B inhibits Janus kinase 2 (JAK2)/STAT3 phosphorylation. The inhibition of PTP1B might induce an enhancement of leptin sensitivity. In this study, we investigated an antiobesity effect of JTT-551, which has been developed as a novel PTP1B inhibitor.

Inhibition of food intake in DIO mice was observed in leptin group. In JTT-551 + leptin group, the food intake inhibition was more strongly observed than in leptin group (Figure 1). JTT-551 showed an enhancement of food intake inhibition with leptin treatment. Furthermore, analysis of leptin signal with JTT-551 treatment was examined in DIO mice. Leptin stimulated the phosphorylation of STAT3 in hypothalamus. Also, JTT-551 enhanced the phosphorylation of STAT3 in leptin treatment (Figure 2). The food intake inhibition with JTT-551 might be caused by an enhancement of leptin signal. Leptin signal in the hypothalamus by binding to Ob-Rb to activate the tyrosine kinase JAK2 and the activated JAK2 phosphorylates itself and residues Tyr985 and Tyr1138 within the Ob-Rb cytoplasmic tail [23, 24]. Phosphorylated Tyr985 recruits the tyrosine phosphatase Shp2, resulting in leptin-evoked activation of extracellular signal-regulated kinase (Erk). Moreover, Tyr1138 recruits and activates the transcription factor STAT3, and the phosphorylated STAT3 is translocated into the nucleus and transcribed to various leptin target genes. In examination of genetic models, it is reported that leptin injection activated STAT3 in the hypothalamus of ob/ob mice and the wild mice but not db/db mice [23]. Since PTP1B dephosphorylates the phosphorylated JAK2 with insulin stimulation and inhibits the phosphorylation of STAT3 [17, 18], it is considered that JTT-551 enhanced the leptin signal via an enhancement of phosphorylation of STAT3 in DIO mice.

Obese-related leptin resistance and hyperleptinemia induce promotion of obesity, glucose and lipid metabolic abnormality, and hypertension. Leptin therapy did not show an efficacy for those diseases, and one of the reasons is considered to be a deterioration of leptin signal. Since the blood leptin levels in DIO mice were decreased by JTT-551 treatment (Figures 4(c) and 5(c)), leptin resistance might be improved by an inhibition of PTP1B. Furthermore, chronic administration of JTT-551 showed an antiobesity effect (Figure 3). With an antiobesity effect, long-term treatment with JTT-551 improved lipid disorder and tended to improve glucose metabolic abnormality (Figures 4 and 5). Pharmacological effect of JTT-551 is considered to be induced by the enhancement of insulin and leptin signals. However, the cumulative calorie intake in JTT-551 100 mg/kg group was significantly decreased in the late chronic phase, at six weeks after treatment (Figure 3(a)). Since the chronic administration of JTT-551 100 mg/kg may act as a feeding deterrent and induce the reduction of body weight, it is necessary to examine carefully the mechanism of an antiobesity effect with JTT-551 treatment in further study.

JTT-551 showed a blood glucose reduction and an improvement of insulin resistance at 10 mg/kg in ob/ob mice and a decrease of hemoglobin A_{1c} (Hb A_{1c}) level at 30 mg/kg in db/db mice [20]. In our preliminary and present studies, an improvement of leptin signal in hypothalamus of DIO mice was observed at 100 mg/kg (Figures 1 and 2). An effective dose in leptin signal was higher than that in insulin signal. The reason for this might be a matter of brain penetration of JTT-551.

JTT-551, a novel developed PTP1B inhibitor, shows not only an improvement of glucose metabolism but also an antiobesity effect possibly by enhancement of leptin signaling and could be useful in the treatment of type 2 diabetes mellitus and obesity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2014, Article ID 583752, 7 pages
http://dx.doi.org/10.1155/2014/583752

Research Article

JTT-130, a Novel Intestine-Specific Inhibitor of Microsomal Triglyceride Transfer Protein, Reduces Food Preference for Fat

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Received 20 March 2014; Accepted 24 April 2014; Published 15 May 2014

Academic Editor: Norihide Yokoi

Microsomal triglyceride transfer protein (MTP) is involved in the assembly and secretion of triglyceride-rich lipoproteins from enterocytes and hepatocytes. JTT-130 is a novel intestine-specific MTP inhibitor, which has been shown to be useful in the prevention and treatment of dyslipidemia, obesity, and diabetes. JTT-130 has also been shown to suppress food intake in a dietary fat-dependent manner in rats. However, whether JTT-130 enables changes in food preference and nutrient consumption remains to be determined. Therefore, the aim of the present study was to investigate the effects of JTT-130 on food preference in rat under free access to two different diets containing 3.3% fat (low-fat diet, LF diet) and 35% fat (high-fat diet, HF diet). JTT-130 decreased HF diet intake and increased LF diet intake, resulting in a change in ratio of caloric intake from LF and HF diets to total caloric intake. In addition, macronutrient analysis revealed that JTT-130 did not affect carbohydrate consumption but significantly decreased fat consumption ($P < 0.01$). These findings suggest that JTT-130 not only inhibits fat absorption, but also suppresses food intake and specifically reduces food preference for fat. Therefore, JTT-130 is expected to provide a new option for the prevention and treatment of obesity and obesity-related metabolic disorders.

1. Introduction

Western diets that consist of high levels of fat have been closely related to dyslipidemia, obesity, and the induction of insulin resistance [1–3]. Fat contains more energy per unit weight than carbohydrates or proteins and, as a result, high-fat diets bring in more energy than low-fat diets at the same unit weight, leading to obesity. In addition, diets with a higher fat energy ratio are more likely to induce obesity even if the total daily energy is the same [4]. There have been reports that obese individuals have a higher preference for consuming fat than non-obese individuals [5–7]. Therefore, it may be important to decrease fat consumption and to correct the food preference for fat in the prevention and treatment of obesity and obesity-related metabolic disorders.

Microsomal triglyceride transfer protein (MTP) plays a pivotal role in the mobilization and secretion of triglyceride-rich chylomicrons in the enterocytes and very low-density lipoproteins (VLDL) in hepatocytes [8–10]. In particular, intestinal MTP plays a critical role in the absorption of dietary lipids, such as fat and cholesterol [11]. JTT-130, [diethyl-2-({3-dimethylcarbamoyl-4-[(4′-trifluoromethylbiphenyl-2-carbonyl)amino]phenyl} acetyloxymthyl)-2-phenylmalonate], a novel intestine-specific MTP inhibitor, was designed to be rapidly hydrolyzed and inactivated by the cleavage of the ester group in the structure immediately after intestinal absorption in order to avoid inhibition of hepatic MTP resulting in hepatic steatosis [12]. In our previous reports, we showed that JTT-130 may be useful in the prevention and treatment of dyslipidemia [12], obesity [13], and type II diabetes [14] in animals. In particular, JTT-130 showed food suppressive effect in a dietary fat-dependent manner in rats fed with diets differing in fat content [15]. As the mechanism of action, we already demonstrated that this effect may be attributed to free fatty acids that have accumulated in the gastrointestinal tract

as a result of inhibition of fat absorption. Recently, the gastrointestinal tract, which is the largest endocrine organ in the body, has been observed as playing important roles in the regulation of energy homeostasis, as well as maintaining its primary function in the digestion and absorption of nutrients [16–18]. Therefore, we postulated that JTT-130 may change food preference and nutrient consumption, in addition to reducing food intake. However, there has been no information to date about the effect of other MTP inhibitors on food preference. In this study, to test our hypothesis, we administered JTT-130 to rats with free access to two diets, one containing 3.3% and the other 35% fat, and determined each diet intake every day to analyze macronutrient consumption.

2. Materials and Methods

2.1. Chemicals. JTT-130, diethyl-2-({3-dimethylcarbamoyl-4-[(4′-trifluoromethylbiphenyl-2-carbonyl)amino]phenyl} acetyloxymethyl)-2-phenylmalonate, was synthesized by Japan Tobacco Inc. (Osaka, Japan). All other reagents used in this study were obtained commercially.

2.2. Animals and Diets. Male Sprague-Dawley rats (six weeks) were obtained from Charles River Japan Inc. (Yokohama, Japan) and maintained at a room temperature of 23 ± 3°C and an air humidity of 55 ± 15% in a 12-/12-hour light/dark cycle (lights on at 8:00 AM; lights off at 8:00 PM). Animals were given free access to water and experimental diets (Table 1). The diets were a 3.1% fat diet, a 3.3% fat diet (LF diet) and a 35% fat diet (HF diet) obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan) or Research Diets Inc. (New Brunswick, NJ). After acclimation under these conditions over 2 weeks, the rats were randomized into the control or JTT-130 treatment group by matching food intake, total caloric intake, ratio of HF diet to total caloric intake, body weight, and levels of body weight gain. All procedures were conducted according to the Japan Tobacco Animal Care Committee's guidelines.

2.3. Evaluation of Food Intake and Body Weight. Rats were individually housed and given free access to LF and HF diet, and the following experiment was conducted. The rats were given vehicle (0.5% methylcellulose solution) or JTT-130 at a dose of 10 mg/kg orally once daily (before the start of the dark cycle) for seven days, followed by a one-week recovery period. Before and during the treatment period and during the recovery period, LF and HF diet intake were determined daily before the start of the dark cycle. In addition, body weight was measured during the treatment period, and body weight gain was calculated.

2.4. Analysis of Caloric Intake and Macronutrient Consumption. Before and during the treatment period and during the recovery period, total caloric intake was calculated from the intake of two different diets and the respective caloric contents per unit weight (Table 1). The ratio of LF and HF diet intake was calculated as the ratio of caloric intake from each diet to total caloric intake. In addition, consumption of fat, carbohydrate, and protein was calculated from each diet and the content ratio of each component.

Table 1: Composition of experimental diets.

Components	3.3% fat diet (LF diet)		35% fat diet (HF diet)	
	% (w/w)	kcal/kg	% (w/w)	kcal/kg
Soybean oil	2.5	225	2.5	225
Lard	0.8	72	32.5	2925
Corn starch	35.148	1406	3.448	138
Maltodextrin 10	12.5	500	12.5	500
Sucrose	15.0	600	15.0	600
Casein	24.0	960	24.0	960
L-Cystine	0.30	12	0.30	12
Cellulose oil, BW200	5.0	0	5.0	0
t-Butylhydroquinone	0.002	0	0.002	0
Mineral mix S10022M	3.5	0	3.5	0
Vitamin mix V10037	1.0	40	1.0	40
Choline bitartrate	0.25	0	0.25	0
Total	100.0	3815	100.0	5400

2.5. Statistical Analysis. Data are presented as means ± S.E. Statistical analysis was performed using SAS systems, version 8.2 (SAS Institute, Cary, NC). If equality of variances was indicated by an F-test, statistical analysis was performed using Student's t-test. If equality of variances was not indicated by an F-test, statistical analysis was performed using Welch's t-test. A P value <0.05 was considered statistically significant.

3. Results and Discussion

3.1. Effects of JTT-130 on Food and Caloric Intake from LF and HF Diets. As shown in Figure 1, treatment with JTT-130, which is an intestine-specific MTP inhibitor, led to a reduction in food intake in rats fed a 35% (w/w) fat diet, but not in rats fed a 3.1% (w/w) fat diet. This effect was consistent with the previous finding in which JTT-130 suppressed food intake in a dietary fat-dependent manner [15]. In addition, the major metabolite of JTT-130 did not reduce the food intake at doses up to 30 mg/kg in rats fed a 35% (w/w) fat diet (data not shown).

To investigate the effect of JTT-130 on food preference, we administered JTT-130 to rats with free access to two diets containing 3.3% (w/w) fat and 35% (w/w) fat (LF and HF diet) (Table 1) and determined LF and HF diet intake every day to analyze macronutrient consumption. Before treatment with JTT-130, rats preferred the HF diet to the LF diet. A greater daily caloric intake from the HF diet was observed in both the control (90.4%) and JTT-130 (90.2%) groups, with no differences observed between the two groups (Table 2 and Figures 2 and 3(a)). This higher preference for the HF diet in our experiment was consistent with the previous report wherein rats were shown to have a higher preference for fatty diets [19, 20].

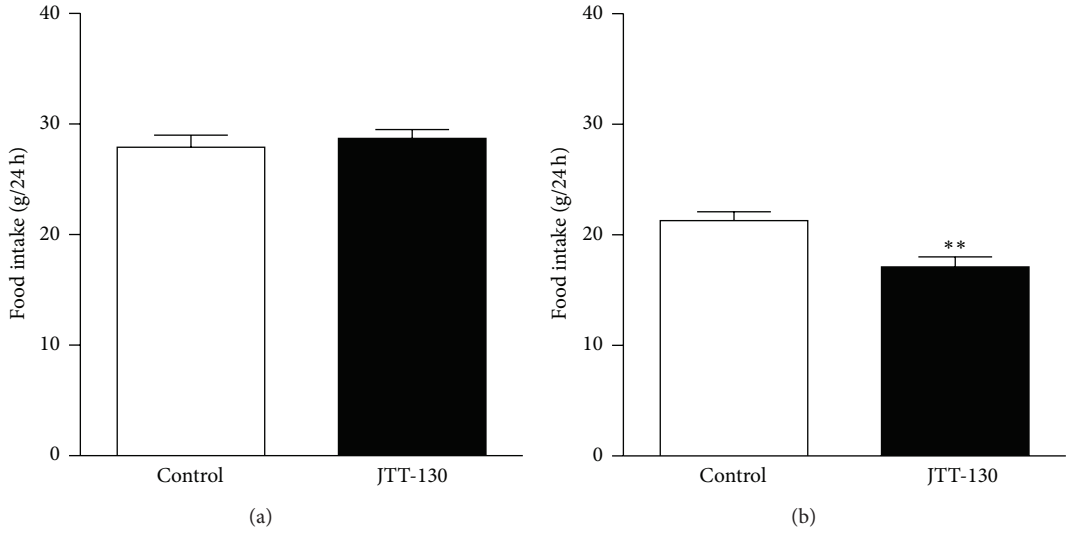

Figure 1: Effects of dietary fat on the suppression of food intake by JTT-130. Effect of JTT-130 on the suppression of food intake was assessed as described previously [15]. JTT-130 was administered orally to rats at a dosage of 10 mg/kg after 24 h food deprivation. Rats were allowed to have free access to 3.1% fat (a) or 35% fat (b) diets immediately after JTT-130 dosing. Cumulative food intake was monitored for up to 24 h after dosing JTT-130. Data are presented as means ± S.E. from six animals. $^{**}P < 0.01$ versus control group.

Table 2: Effect of JTT-130 on food intake from LF and HF diets during the experiment.

Study period	Baseline		Treatment		Recovery	
Groups	Control	JTT-130	Control	JTT-130	Control	JTT-130
			(g/day)			
LF diet	2.6 ± 0.5	2.4 ± 0.4	2.6 ± 0.6	6.9 ± 0.9	2.8 ± 0.5	6.3 ± 1.1
HF diet	16.5 ± 0.5	16.6 ± 0.6	16.7 ± 0.6	10.4 ± 0.6	17.6 ± 0.8	15.4 ± 0.8

Food intake from LF and HF diets was presented as average values during baseline, the treatment, and recovery period. Data are presented as means ± S.E. from ten animals.

During the seven-day treatment with JTT-130, there were no apparent changes from baseline in food intake in the control group, with an intake ratio of 10.0 ± 2.3% for the LF diet and 90.0±2.3% for the HF diet (Table 2 and Figures 2 and 3(b)). In the JTT-130 group, food intake was characterized by decreased HF diet intake and increased LF diet intake compared with the control group (Table 2 and Figures 2 and 3(b)), with an intake ratio of 32.9 ± 3.8% for the LF diet and 67.1 ± 3.8% for the HF diet, showing that JTT-130 decreased the HF diet intake ratio to total caloric intake (Figure 3(b)). In addition, total caloric intake during this period was 100.3± 3.4 kcal/day in the control group and 82.3 ± 2.1 kcal/day in the JTT-130 group, which significantly decreased after treatment with JTT-130 ($P < 0.01$) (Figure 4(b)).

3.2. Effects of JTT-130 on Macronutrient Consumption. Since the results showed that JTT-130 changed the intake ratio of LF and HF diets, nutrient consumption was analyzed according to the following nutrient components: carbohydrates, proteins, and fats (Table 1). Fat consumption was 5.9 ± 0.2 g/day in the control group and 3.9 ± 0.2 g/day in the JTT-130 group, showing that JTT-130 significantly decreased fat consumption (Table 3). On the other hand, JTT-130 had no effect on carbohydrate consumption and slightly decreased protein consumption (Table 3). Since protein content (w/w (%)) was the same in both LF and HF diets, the decrease in protein consumption was presumably caused by a decrease in total food intake after treatment with JTT-130 and not by direct alteration of the protein preference. These results demonstrated that JTT-130 specifically decreased the fat preference. In our previous report, we demonstrated that the food suppressive effect of JTT-130 may be attributed to free fatty acids that have accumulated in the gastrointestinal tract [15]. Recently, several receptors that are activated by free fatty acids in the gastrointestinal tract have been identified in other studies, and these receptors reportedly play important roles in nutrition regulation [21, 22]. Furthermore, infusion of fatty acids into the intestine has been shown to reduce the food preference for fat [23]. Although further studies are needed to clarify the mechanism of action, it is possible that free fatty acids which have accumulated in the gastrointestinal tract as a result of the inhibition of fat absorption may contribute to the decrease in fat preference after treatment with JTT-130.

3.3. Effects of JTT-130 on Body Weight Gain. To evaluate the effect of JTT-130 on body weight due to total caloric intake, body weight was measured during the treatment period. JTT-130 significantly inhibited increases in body weight, and body

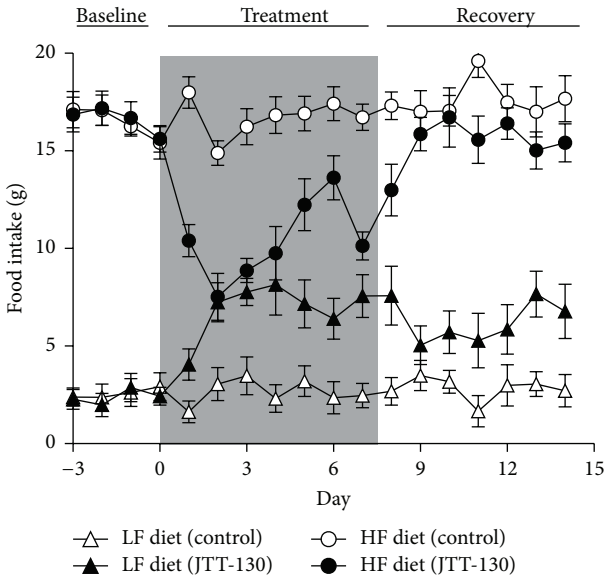

Figure 2: Effect of JTT-130 on LF and HF diet selection during the study period. Rats were allowed to have free access to LF and HF diet. JTT-130 was administered orally to rats at a dosage of 10 mg/kg for seven days. Data are presented as means ± S.E. from ten animals. Open circle: HF diet intake in the control group; closed circle: HF diet intake in the JTT-130 group; open triangle: LF diet intake in control group; closed triangle: LF diet intake in the JTT-130 group.

Table 3: Effects of JTT-130 on daily macronutrient consumption during the treatment period.

	g/day		kcal/day	
	Control	JTT-130	Control	JTT-130
Carbohydrate	7.0 ± 0.4	7.7 ± 0.4	28.1 ± 1.5	30.8 ± 1.7
Fat	5.9 ± 0.2	3.9 ± 0.2**	53.4 ± 2.0	34.7 ± 1.6**
Protein	4.7 ± 0.2	4.2 ± 0.1*	18.8 ± 0.7	16.8 ± 0.5*

Macronutrient analysis was performed as described in Section 2. The caloric intake from each component was calculated using calories per unit weight of 4 kcal/g of carbohydrates and protein and 9 kcal/g of fat. *$P < 0.05$; **$P < 0.01$ versus control group.

weight gain observed during the treatment period was 50.7 ± 2.4 g in the control group and 37.4 ± 2.0 g in the JTT-130 group ($P < 0.01$). Interestingly, food efficiency during the treatment period also significantly decreased in the JTT-130 group ($P < 0.05$) compared with that of the control group (control group, 7.16 ± 0.21 g/100 kcal; JTT-130 group, 6.30 ± 0.27 g/100 kcal). These results indicate that inhibition of body weight gain by JTT-130 may be due to the previously reported increase in fecal excretion of fatty acids resulting from the inhibition of triglyceride (TG) absorption [12, 15], in addition to the decrease in total caloric intake.

3.4. Post-treatment Changes after Treatment with JTT-130. To evaluate whether the change in LF and HF diet intake observed during the treatment period with JTT-130 was reversed by discontinuation of JTT-130 treatment, LF and HF diet intake were measured for seven days following the cessation of treatment. During the seven-day recovery period, LF and HF diet intake and the ratio of LF and HF diet intake in the JTT-130 group tended to recover to a rate similar to what was observed in the control group (Figures 2 and 3(c)). In addition, total caloric intake in the JTT-130 group recovered to a similar level to what was observed in the control group (Figure 4(c)), and no significant differences were seen between the groups. These results suggest that the JTT-130-induced decrease in fat preference may be due to a decreased attractiveness of consuming fat rather than an avoidance response associated with taste aversion.

There were no differences in total caloric intake in the recovery period between the control and JTT-130 groups, showing that total caloric intake did not increase after discontinuation of treatment with JTT-130 (Figure 3(c)). When compared with a central anti-obesity agent such as sibutramine, which is a serotonin-noradrenaline reuptake inhibitor (SNRI) that is associated with a "rebound phenomenon" or increased food intake after discontinuation of treatment [24, 25], the same phenomenon was not observed with discontinuation of JTT-130 treatment.

3.5. Differences between JTT-130 and Existing Anti-Obesity Agents. In the present study, we have shown that JTT-130 specifically reduced the food preference for fat accompanied with a decrease in total caloric intake. As compared with existing anti-obesity agents in terms of the effect on food preference, sibutramine decreases both carbohydrate and fat consumption [26], showing that this agent has a different profile from that of JTT-130. Postmarketing surveillance studies of sibutramine showed that treatment with this agent increased cardiovascular risk, leading to a decision from the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) to withdraw the product from the market. Therefore, development of safer anti-obesity agents is necessary. Orlistat is known to reduce fat absorption by lipase inhibition and is used as an anti-obesity agent. However, orlistat does not decrease body weight as effectively as what is expected for the degree of fat malabsorption. This phenomenon is partly attributed to the compensation of energy loss due to the inhibition of fat absorption with increased energy intake [27]. In fact, we showed that orlistat increased cumulative food intake in high-fat diet-fed rats with suppressed food intake due to JTT-130 treatment [15]. Another report has also demonstrated that orlistat decreased fat preference. The authors proposed that orlistat might reduce the attractiveness of consuming fat due to fat malabsorption, although the mechanism remains unknown [28]. In contrast, JTT-130 specifically decreased fat preference without increasing total caloric intake. Moreover, orlistat has been shown to increase carbohydrate and protein consumption in a compensatory manner and, thus, also increase total caloric intake [28]. These findings indicate that JTT-130 and orlistat have different effects on energy intake and food preference, although both inhibit fat absorption locally in the gastrointestinal tract. After administration of orlistat, ingested fats may be found primarily in the form

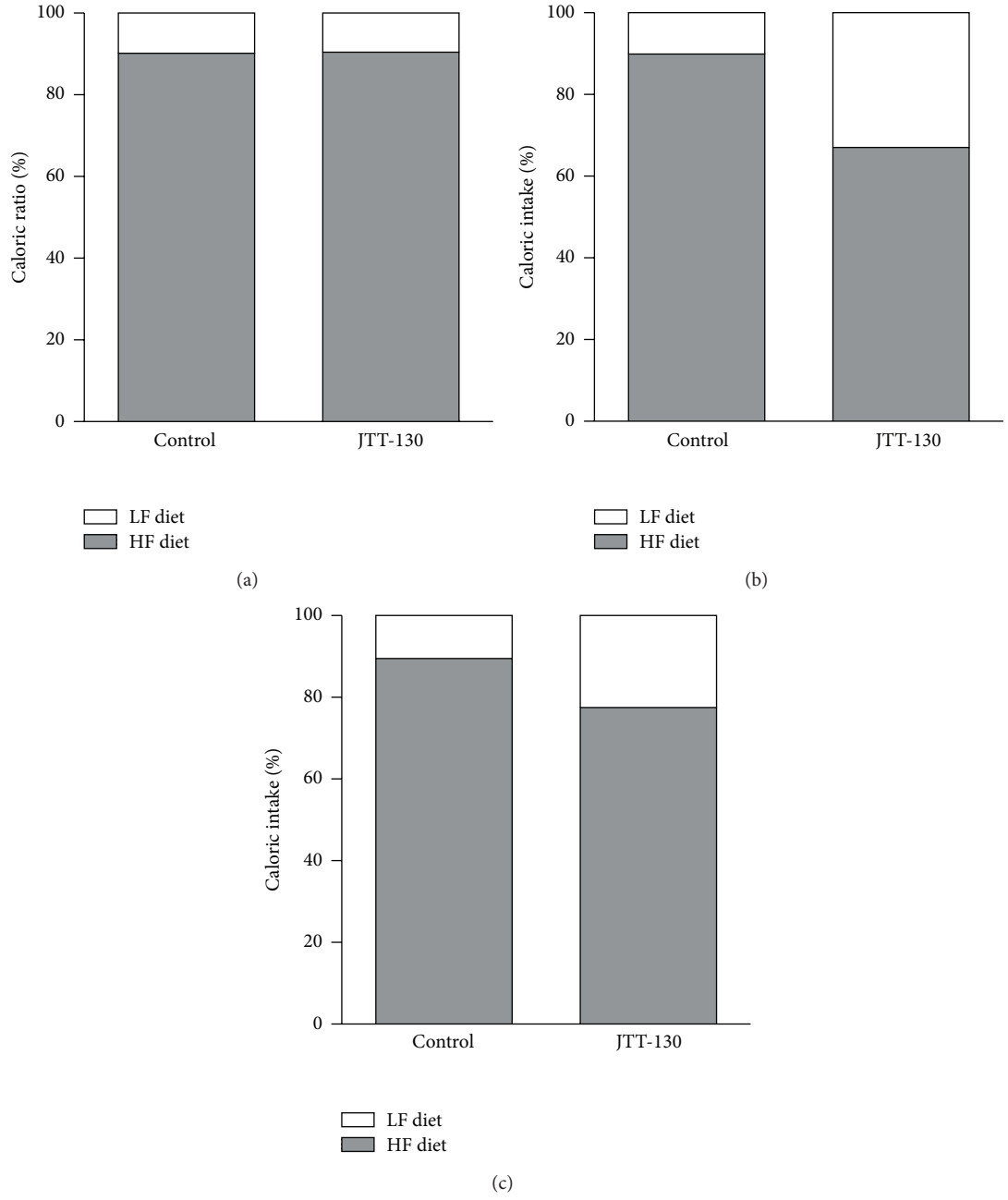

Figure 3: Effects of JTT-130 on caloric intake ratio from LF and HF diets. Caloric intake ratios were calculated from average daily intake from LF and HF fat dietsduring baseline (a), the treatment period (b), and recovery period (c). Data are presented as means ± S.E. from ten animals.

of TG in the intestinal lumen. After administration of JTT-130, in comparison, increases in TG and free fatty acids in the intestinal lumen and increases in TG in the small intestine tissue are observed [15]. These differences in types and amounts of ingested fats in the gastrointestinal tract may result in different effects of these compounds on food intake and food preference.

In conclusion, we have demonstrated that JTT-130, an intestine-specific MTP inhibitor, specifically decreases total caloric intake by reducing the preference for fat without changing the preference for carbohydrates, resulting in a reduction in body weight gain. After discontinuing JTT-130 treatment, total caloric intake returns to a similar level as the control and the reducing effect on the food preference

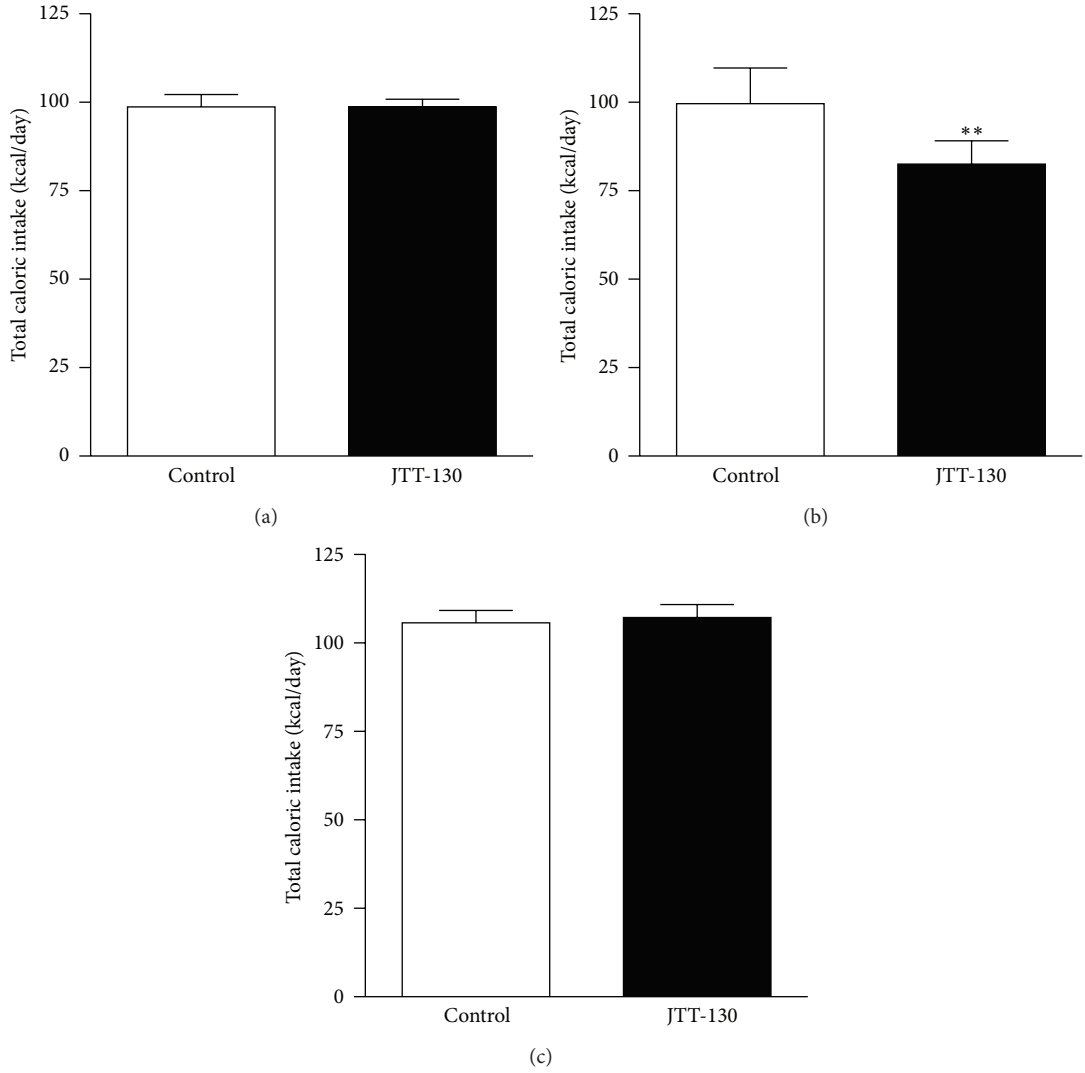

Figure 4: Effects of JTT-130 on total caloric intake. Total caloric intake was calculated from average daily intake of LF and HF diets during baseline (a), the treatment period (b), and recovery period (c). Data are presented as means ± S.E. from ten animals. $^{**}P < 0.01$ versus control group.

for fat disappears. Thus, JTT-130 suppresses food intake with reducing the fat preference in addition to inhibiting fat absorption, suggesting that JTT-130 might be expected to provide a new option for the prevention and treatment of obesity and obesity-related metabolic disorders.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

The authors express their gratitude to Dr. Tomohiko Sasase (Japan Tobacco Inc.) for his support regarding the writing of the paper.

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Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2014, Article ID 841957, 7 pages
http://dx.doi.org/10.1155/2014/841957

Review Article

Gender Differences in Metabolic Disorders and Related Diseases in Spontaneously Diabetic Torii-*Lepr*^{fa} Rats

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Received 17 March 2014; Accepted 18 April 2014; Published 8 May 2014

Academic Editor: Norihide Yokoi

The Spontaneously Diabetic Torii *Lepr*^{fa} (SDT fatty) rat is a novel type 2 diabetic model wherein both male and female rats develop glucose and lipid abnormalities from a young age. In this study, we investigated gender differences in abnormalities and related complications in SDT fatty rats. Food intake was higher in males compared to female rats; however, body weight was not different between genders. Progression of diabetes, including increases in blood glucose and declines in blood insulin, was observed earlier in male rats than in females, and diabetic grade was more critical in male rats. Blood lipids tended to increase in female rats. Gonadal dysfunction was observed in both male and female rats with aging. Microangiopathies, such as nephropathy, retinopathy, neuropathy, and osteoporosis, were seen in both genders, and pathological grade and progression were more significant in males. Qualitative and quantitative changes were observed for metabolic disease gender differences in SDT fatty rats. The SDT fatty rat is a useful model for researching gender differences in metabolic disorders and related diseases in diabetes with obesity.

1. Introduction

Recently, gender-specific medicine (GSM) has become an active field in current medical care. The field of GSM examines how normal human biology and physiology differ between males and females and how diagnosis and treatment of diseases differ as a function of gender. Males and females differ in their experience of diabetes mellitus. For optimal prevention and treatment of the disease, these differences must be acknowledged [1–3]. Some studies have shown that serum endogenous sex hormone levels are related to type 2 diabetes, insulin resistance, and other components of metabolic syndrome [4, 5].

The Spontaneously Diabetic Torii *Lepr*^{fa} (SDT fatty) rat, established by introducing the *fa* allele of the Zucker fatty rat into the SDT rat genome, is a new model of obese type 2 diabetes. Both male and female SDT fatty rats showed overt obesity, and hyperglycemia and hyperlipidemia were observed at a young age as compared with SDT rats [6, 7]. Female rats have the potential to become an animal model of type 2 diabetes with obesity for women, for which few models currently exist [8]. In this study, we investigated gender differences in glucose and lipid abnormalities and related diseases in SDT fatty rats. New findings of gonadal dysfunction in male and female SDT fatty rats were reported in Section 3.

2. Gender Differences in Glucose and Lipid Abnormalities

2.1. Materials. In male and female SDT fatty rats, body weight and blood biochemistry parameters, such as glucose, insulin, triglycerides (TG), and total cholesterol (TC), were periodically examined from 5 to 20 weeks of age. Blood samples were collected from the tail vein of nonfasted rats. Serum glucose, TG, and TC levels were measured using commercial kits (Roche Diagnostics, Basel, Switzerland) and an automated analyzer (Hitachi, Tokyo, Japan). Serum insulin level was measured with a rat-insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Yokohama, Japan).

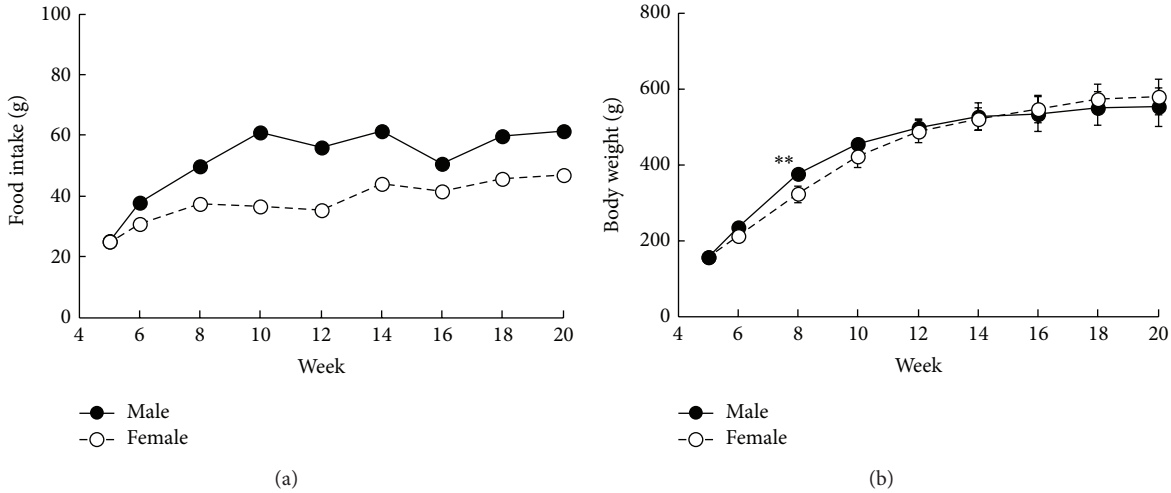

Figure 1: Changes in food intake (a) and body weight (b) in male and female SDT fatty rats. Data are shown as mean (a) or means ± standard deviation (b) ($n = 5$). Statistical analysis of differences between mean values was performed using the F-test, followed by Student's t-test or Aspin-Welch's t-test. $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different from the female SDT fatty rats.

2.2. Gender Differences in Diabetic Models. Food intake was higher in male SDT fatty rats after 6 weeks of age, as compared with intake observed in female rats (Figure 1(a)). Changes in body weight were almost comparable in both male and female rats; however, body weight in male rats only increased at 8 weeks of age (Figure 1(b)). In addition, visceral fat weight was determined in male and female rats at 16 weeks of age using computed tomography (CT) analysis (LATheta, ALOKA Co., Ltd., Osaka, Japan) (mean ± standard deviation: male rats, 69.5 ± 8.6 g and female rats, 75.1 ± 8.2 g), and results showed that levels were higher in fatty rats, regardless of gender, as compared with lean rats. Blood glucose levels in male rats were remarkably elevated after 6 weeks of age, and hyperglycemia was observed in female rats after 8 weeks of age (Figure 2(a)). Hyperglycemia observed in male rats was more significant than that in female rats. Due to the severe hyperglycemia observed in male rats, body weight in male and female rats was comparable despite differences in food intake. Blood insulin levels in male rats increased from 6 to 10 weeks of age as compared with those in female rats; however, insulin levels rapidly decreased after 10 weeks of age (Figure 2(b)). Since hyperglycemia observed in male rats was more severe than observed in female rats, the decline in insulin level was considered more pronounced compared to female rats. The severe decline in insulin levels in male rats was considered as leading to the stagnation of body weight gain despite hyperphagia (Figure 1). A gender difference was observed in the onset of diabetes in SDT rats [9], and the reason may be partly attributed to estrogen, which inhibits the development of diabetes. Female SDT fatty rats were considered as maintaining higher insulin levels, which suggests a possible pancreatic protection effect, as compared with male rats for similar reasons. Blood TG levels in male rats were temporarily elevated at 8 weeks of age as compared with those in female rats, whereas TG levels after 14 weeks of age were significantly higher in female rats (Figure 2(c)). Moreover, a tendency towards increases in blood TC levels in female rats was observed after 10 weeks of age, as compared with those in male rats (Figure 2(d)). Glucose levels were higher in males than in female rats, whereas lipid levels were higher in females than in male rats.

Gender differences in diabetes were reported in other diabetic models. Hyperglycemia was more often observed in younger male rats than in female rats in some diabetic rat models. In Zucker diabetic fatty rats, diabetes developed in male rats after 9 weeks of age, whereas hyperglycemia was not seen in female rats until 22 weeks of age [10]. Male Otsuka Long-Evans Tokushima fatty (OLETF) rats developed diabetes after 18 weeks of age and the incidence of diabetes was 100% at 25 weeks of age, whereas the incidence of diabetes in female rats was about 30% even at 60 weeks of age [11]. In both ZDF and OLETF rats, the incidence of diabetes in female rats was remarkably lower than the incidence in male rats. In Tsumura Suzuki obese diabetes (TSOD) mice, the incidence of diabetes was also 100% in male mice, whereas female mice did not develop diabetes [12]. In BALB/cA mice, males gained more body weight and body fat weight and had higher energy intake than females from high-fat diet feeding. BALB/cA female mice were resistant to HFD-induced obesity compared to males [13]. The reason for metabolic abnormalities in females being significantly smaller compared to those in males is considered due to female hormones, such as estrogen, that have a protective effect on metabolic dysfunction. It is well known that oophorectomy promotes the development of diabetes [9]. In female nonobese diabetic (NOD) mice, diabetes was observed at around 13 weeks of age and incidence gradually increased with age. In males, however, symptoms were only observed in a few animals [14]. Moreover, in Komeda diabetes-prone (KDP) rats, there were no gender differences in onset of diabetes [15].

3. Gender Differences in Gonadal Dysfunction

3.1. Materials. In male SDT fatty rats, serum testosterone level was measured with a Testosterone EIA kit (Cayman

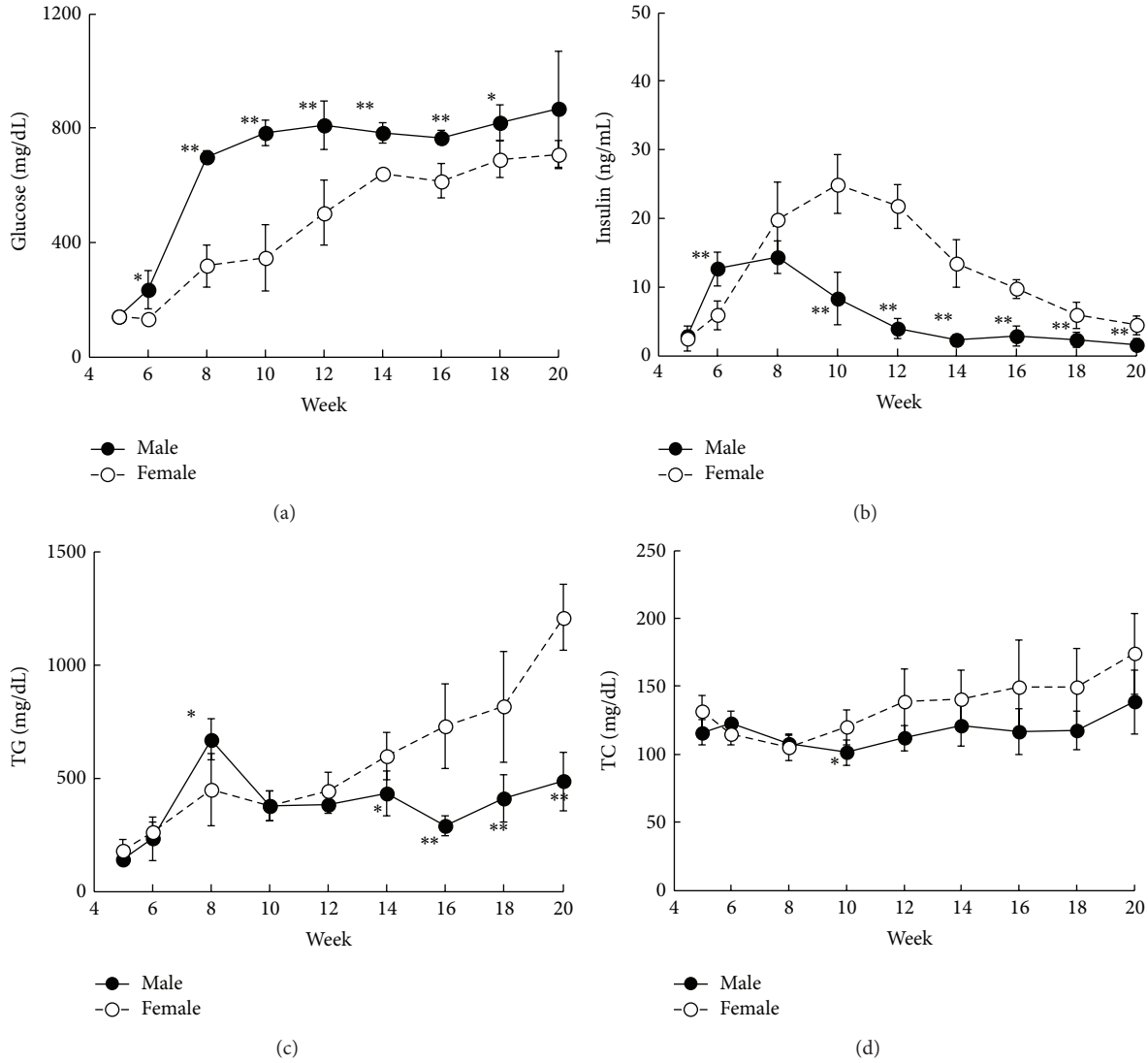

Figure 2: Changes in biological parameters ((a) glucose, (b) insulin, (c) triglyceride (TG), and (d) total cholesterol (TC)) in male and female SDT fatty rats. Data are shown as means ± standard deviation ($n = 5$). $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different from the female SDT fatty rats.

Chemical Company, MI, USA) from 8 to 40 weeks of age. Semen analyses and pathological analyses of testes were performed at 32 and 40 weeks of age. Testes with epididymis were removed, and the cauda epididymis was separated from the testis to collect semen. Squeezed semen was incubated in buffer containing BSA at 37°C for 30 minutes. Ten μL of sperm suspension was dropped on a glass side, and 700–1000 sperm cells were observed for motility under a microscope using 100–400x magnification. The percentage of motile sperm cells (sperm motility) was calculated as the number of motile sperm cells divided by the total number of sperm cells. Sperm viability was evaluated with SYBR-14/propidium iodide (LIVE/DEAD Sperm Viability Kit, Life Technologies, Carlsbad, CA, USA). Live sperm ratio was defined as the number of viable sperm cells divided by the total number of sperm cells. Moreover, a total of 200–300 sperm cells per animal were randomly selected under a microscope using 400x magnification and were assessed to determine the presence or absence of morphological abnormalities. The ratio of normal sperm cells was expressed as the percentage of total sperm cell count. Necropsy was performed at 32 and 40 weeks of age. After weighing the testes and the epididymis, organs were fixed in 10% neutral buffered formalin. After resection, the tissue was paraffin-embedded by standard techniques and thin-sectioned (3 to 5 μm). The sections were stained with hematoxylin and eosin (HE).

3.2. Gender Differences in SDT Fatty Rats. Testosterone levels in male SDT fatty rats were decreased as compared with those in SD rats during the experimental period (Figure 3). Streptozotocin- (STZ-) induced diabetic rats showed a significant decrease in serum testosterone levels [16]; however, serum testosterone levels in Otsuka Long-Evans Tokushima fatty (OLETF) rats were comparable to those in control

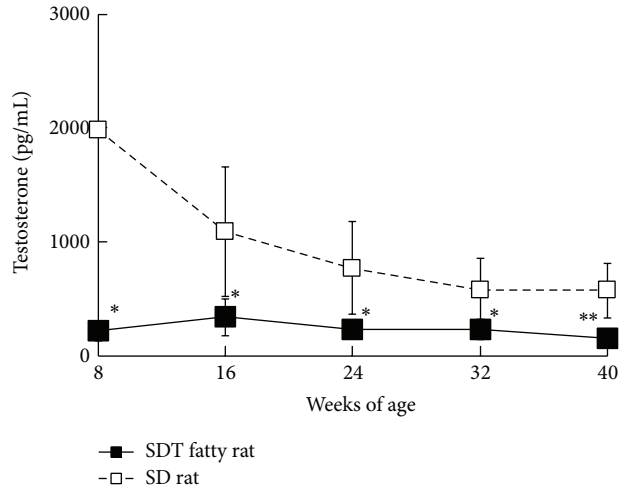

Figure 3: Changes in blood testosterone levels in male SDT fatty rats and SD rats. Data are shown as means ± standard deviation (n = 4–6). $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different from the SD rats.

rats [17]. In obese males, total testosterone, free testosterone, and sex hormone-binding globulin (SHBG) levels were all commonly decreased [18, 19]. Both serum testosterone and SHBG levels were significantly lower in males with type 2 diabetes, and increasing insulin resistance was associated with decreased testosterone secretion at the testicular level (Leydig cell) [20, 21]. The decrease in serum testosterone levels in SDT fatty rats is therefore considered to be caused by several metabolic disorders, such as obesity, hyperglycemia, and insulin resistance (Figures 1 and 2). The testosterone level in SDT fatty rats was significantly lower at the start of the experimental period, when rats were 8 weeks of age. The hypotestosteronemia may be related to the prominent increase in blood glucose levels from 6 to 8 weeks of age (Figure 2(a)).

Sperm characteristics in SDT fatty rats are shown in Table 1. Sperm motility in SDT fatty rats decreased significantly at 32 weeks of age, as compared with that in SD rats. Sperm motility in SD rats decreased with aging, and no significant differences were observed between SDT fatty and SD rats at 40 weeks of age. Sperm viability in SDT fatty rats also tended to decrease at 32 weeks of age, as compared with that in SD rats; however, there were no significant differences in sperm viability at 40 weeks of age between rats. In sperm morphological analyses, the percentage of normal spermatozoa in SDT fatty rats was significantly decreased at 40 weeks of age, as compared with that in SD rats. In OLETF rats, the sperm count decreased significantly at 64 weeks of age, in comparison to control rats [17]. In humans, total sperm count and total motile sperm are negatively correlated with weight, waist circumference, and hip circumference, suggesting a potential link between obesity, hypogonadism, and infertility as indicated by semen analyses [22].

Testis and epididymis weights in SDT fatty rats are shown in Table 2. Testis and epididymis weights were determined in different experiments. The absolute weights in SDT fatty rats

Table 1: Semen analysis in male SD and SDT fatty rats.

	32 weeks of age	40 weeks of age
Sperm motility (%)		
SD rat	79.9 ± 10.9	63.6 ± 14.7
SDT fatty rat	70.0 ± 8.2**	67.1 ± 5.0
Sperm viability (%)		
SD rat	70.9 ± 11.4	61.4 ± 12.0
SDT fatty rat	63.8 ± 5.9	67.4 ± 6.7
Sperm morphology (%)		
SD rat	84.5 ± 6.6	86.5 ± 5.3
SDT fatty rat	79.8 ± 2.5	75.3 ± 2.2**

Data represent the mean ± standard deviation (n = 4–6). $^{**}P < 0.01$ versus age-matched SD rats.

Table 2: Organ weights in male SD and SDT fatty rats.

	32 weeks of age	40 weeks of age
Testes		
Absolute weight (mg)		
SD rat	4174.9 ± 341.9	3885.3 ± 102.1
SDT fatty rat	3796.6 ± 183.1	3320.3 ± 438.2
Relative weight (mg/g)		
SD rat	4.813 ± 0.758	4.072 ± 0.525
SDT fatty rat	7.578 ± 1.039**	6.294 ± 0.895**
Epididymides		
Absolute weight (mg)		
SD rat	585.8 ± 82.9	551.4 ± 43.3
SDT fatty rat	502.3 ± 6.2	494.4 ± 3.0**
Relative weight (mg/g)		
SD rat	0.837 ± 0.121	0.615 ± 0.092
SDT fatty rat	0.919 ± 0.114	0.930 ± 0.053**

Data represent the mean ± standard deviation (n = 4–6).
$^{*}P < 0.05$ and $^{**}P < 0.01$ versus age-matched SD rats.

tended to decrease, as compared with those in SD rats, and the epididymis weight in SDT fatty rats decreased significantly at 40 weeks of age. On the other hand, a significant increase in the relative weights of testes and epididymis in SDT fatty rats compared to those in SD rats was seen, since the body weights of SDT fatty rats were lower at 32 and 40 weeks of age. In histological analyses of the testes and epididymis, no significant abnormalities were observed in SDT fatty rats at 32 and 40 weeks of age (data not shown). In the testes of STZ-induced diabetic mice, ultrastructural changes, such as an increase in lipid droplets and a decrease in smooth endoplasmic reticulum, were observed in Leydig cells [23]. A tendency toward seminiferous tubular atrophy has been reported in 64-week-old OLETF rats; however, structures for interstitial tissues including Leydig cells remained conserved [17]. No histological abnormalities were observed in testes or epididymis in SDT fatty rats, and the reason for hypotestosteronemia or sperm abnormalities should be elucidated in further studies.

In female SDT fatty rats, gonadal abnormalities in the estrus cycle and histopathology were reported by Inaba et al. [24]. The female rats showed an irregular estrus cycle with a

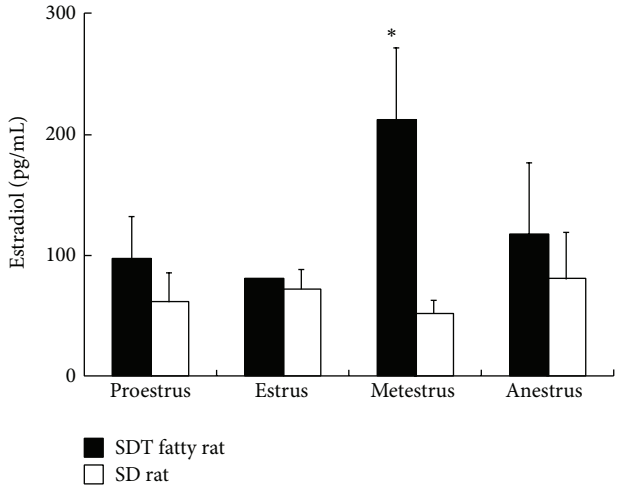

Figure 4: Changes in blood estradiol levels in female SDT fatty rats and SD rats at 12 weeks of age. Estradiol levels were measured in each estrus stage (proestrus, estrus, metestrus, and anestrus). Data are shown as means ± standard deviation ($n = 4$). $^{*}P < 0.05$, significantly different from the SD rats.

persistent estrus stage, an extension of metestrus, and a large number of leukocytes in smears from the metestrus stage. Decreases in relative weights of the ovary, uterus, and vagina and histopathological changes, such as atrophy of the uterus and inflammation of the vagina, were observed in female rats at 12 weeks of age. Furthermore, blood estradiol levels in the estrus cycle were determined using an Estradiol EIA kit (Cayman Chemical Company, MI, USA) at 12 weeks of age. Estradiol levels in metestrus were increased as compared with that in SD rats, and levels tended to increase in other estrus stages (Figure 4).

In both male and female rats, gonadal dysfunction was observed with progression of metabolic disorders, such as hyperglycemia, hyperlipidemia, and hyperinsulinemia. In blood biochemistry analyses, male rats showed hypotestosteronemia, whereas female rats showed hyperestrogenemia.

4. Gender Differences in Microangiopathy and Osteoporosis

In both male and female SDT fatty rats, increases in renal parameters such as urine volume and urine protein were observed after 4 weeks of age [7, 8, 25]. In the renal tubules, glycogen deposition in the tubular epithelium (Armanni-Ebstein lesions) and tubular dilation were noted from 8 weeks of age in male rats, and those changes were observed from 16 weeks of age in female rats [7]. In the glomeruli, glomerulosclerosis was observed from 16 weeks of age in male rats and from 32 weeks of ages in female rats [8]. Moreover, progression of tubular and interstitial lesions, including fibrosis and inflammatory cell filtration, was observed in both male and female rats at 60 weeks of age. Nodular lesions in the glomeruli were only observed in male rats after 40 weeks of age [7].

In male SDT fatty rats after 16 weeks of age, a prolongation of the peak latencies of oscillatory potentials was observed [7] and prolongation at 22 weeks of age was observed in female rats (peak latencies of oscillatory potentials ($\sum(OP_1 - OP_3)$) on electroretinogram, SDT fatty rats: 89.5 ± 3.4 ms versus SD rats: 82.5 ± 3.3 ms, $n = 5$). Histopathological findings in the lens, including hyperplasia of the epithelium, vacuolation of fibers, and formation of Morgagnian globules, were observed from 8 weeks of age in male rats, and similar changes were also observed in female rats from 16 weeks of age [7, 8]. Furthermore, retinal lesions, such as folding and thickening, were observed with aging in both male and female rats [25, 26].

Caudal motor nerve conduction velocity in both male and female SDT fatty rats was delayed at 24 weeks of age (female SDT fatty rats: 54.4 ± 8.4 m/s versus female SD rats: 66.3 ± 7.5 m/s, $n = 6$). Histopathologically, at 40 weeks of age, fiber number in male rats significantly decreased, and rats revealed significant atrophy in myelinated nerves [27]. There have been no reports in histological findings in female rats.

The effects of obese type 2 diabetes on bone metabolism were investigated in SDT fatty rats [28–30]. Both serum osteocalcin and urine deoxypyridinoline levels were lower in male rats as compared to normal rats from 8 to 40 weeks of age; however, urine deoxypyridinoline levels in female rats increased after 24 weeks of age as compared to normal rats [26]. Bone mineral density (BMD) of the whole tibia in male rats decreased as compared with that in female rats, whereas bone mineral content (BMC) in male rats increased as compared with that in female rats (Figure 5). The differences in BMD in both genders may be related to the degree of glucose and lipid metabolic disorders, and the differences in BMC may be associated with body weight.

SDT fatty rats of both genders showed metabolic abnormalities, such as hyperphagia, obesity, and increases in blood glucose and lipids, after weaning. Since hyperglycemia and hyperlipidemia were sustained for a long period afterwards, quantitatively equal changes were observed in diabetic complications in both male and female rats. On the other hand, there are gender differences in the development of diabetes in other obese diabetic models, such as ZDF rats, OLETF rats, and Wistar fatty rats, and critical findings in diabetic complications for these diabetic rats were mainly observed in male rats [25].

5. Conclusion

In both male and female SDT fatty rats, glucose and lipid abnormalities and related diseases were observed; however, the week of onset and severity were varied. Gender differences in pathophysiological changes seem to be based on gonadal hormones, such as testosterone and estrogen. Furthermore, pathophysiological changes in males may be associated with severe hyperglycemia, and changes in females may be related to insulin resistance as well as hyperglycemia. Gender differences in metabolic diseases in SDT fatty rats were accompanied with qualitative changes as well as quantitative changes. The SDT fatty rat is a useful model for

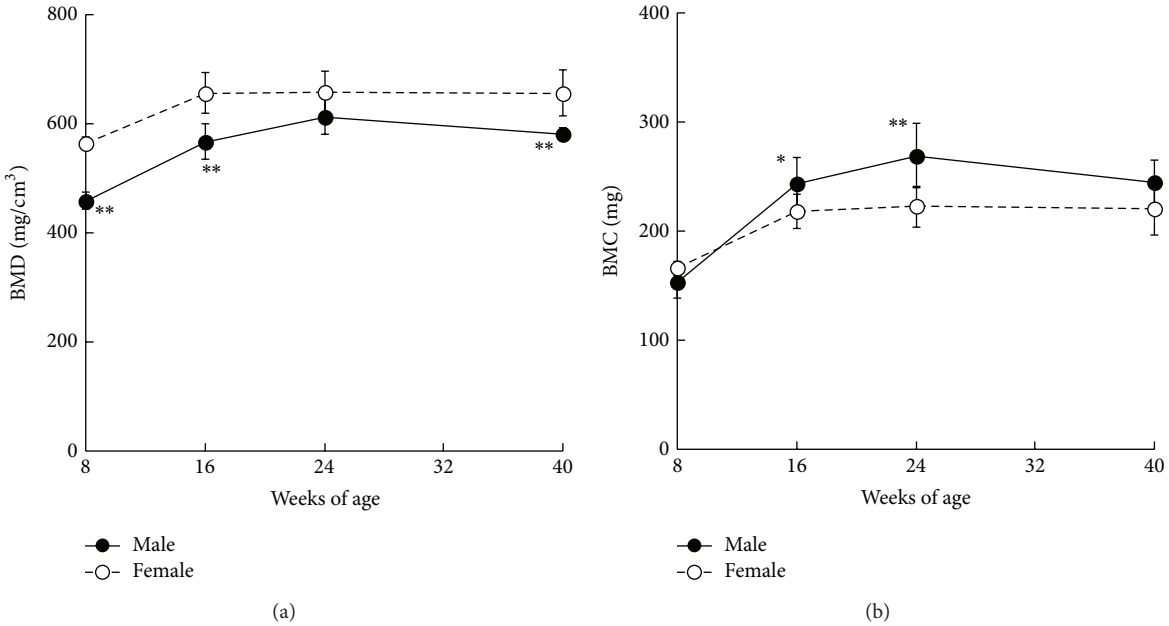

Figure 5: Changes in BMD (a) and BMC (b) in male and female SDT fatty rats. Data are shown as means ± standard deviation (male: $n = 5$ and female: $n = 10$). $^{*}P < 0.05$ and $^{**}P < 0.01$, significantly different from the female SDT fatty rats.

researching the gender differences in metabolic abnormalities in diabetes with obesity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Journal of Diabetes Research
Volume 2014, Article ID 890639, 7 pages
http://dx.doi.org/10.1155/2014/890639

Research Article

Combination Therapy of an Intestine-Specific Inhibitor of Microsomal Triglyceride Transfer Protein and Peroxisome Proliferator-Activated Receptor γ Agonist in Diabetic Rat

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Received 7 January 2014; Accepted 12 February 2014; Published 17 March 2014

Academic Editor: Masami Shinohara

We investigated effects on glucose and lipid metabolism in combination of JTT-130, a novel intestine-specific microsomal triglyceride transfer protein (MTP) inhibitor, and pioglitazone, peroxisome proliferator-activated receptor (PPAR) γ agonist. Male Zucker diabetic fatty rats were divided into 4 groups: control group, JTT-130 treatment group, pioglitazone treatment group, and combination group. The Zucker diabetic fatty rats were fed a regular powdered diet with JTT-130 and/or pioglitazone as a food admixture for 6 weeks. Effects on glucose and lipid metabolism were compared mainly between JTT-130 treatment group and combination group. JTT-130 treatment showed good glycemic control, while the plasma glucose and glycated hemoglobin levels in combination group were significantly decreased as compared with those JTT-130 treatment group. The reduction in the plasma triglyceride and free fatty acid levels in combination group was higher than that in JTT-130 treatment group, and glucose utilization was significantly elevated in adipose tissues. In Zucker diabetic fatty rats, combination treatment of JTT-130 and pioglitazone showed better glycemic control and a strong hypolipidemic action with an enhancement of insulin sensitivity. Combination therapy of MTP inhibitor and PPARγ agonist might be more useful in the treatment of type 2 diabetes accompanied with obesity and insulin resistance.

1. Introduction

Diabetes mellitus is not a single disease but a group of metabolic disorders affecting a huge number of populations worldwide [1, 2]. It is mainly characterized by chronic hyperglycemia, resulting from defects in insulin secretion and insulin action. With progress in elucidation of diabetic etiology, oral hypoglycemic drugs, such as sulfonylureas, α-glucosidase inhibitor, thiazolidinediones, and dipeptidyl-peptidase (DPP) IV inhibitor, have been successfully developed so far. Recently, moreover, combination therapies have become important in diabetic treatment [3, 4]. It seems useful to combine different antidiabetic agents based on specific needs and contraindications in an individual patient. Addressing not only glycemic control but also the underlying pathophysiological etiology might help to improve the prognosis in type 2 diabetic patients in the long run [5, 6]. Beyond lowering blood glucose levels, each combination of different antidiabetic drugs evolves specific pleiotropic effects, which might be considered on an individual basis in certain patients. Treatment with pioglitazone in type 2 diabetes was shown to improve insulin resistance and blood glucose levels without increasing the risk of hypoglycemia. Pioglitazone is approved in combination with several other antidiabetic drugs for treatment of type 2 diabetes [7, 8].

JTT-130, a novel intestine-specific inhibitor of microsomal triglyceride transfer protein (MTP), suppresses the absorption of dietary fat and cholesterol in the intestine and decreases plasma triglyceride and total cholesterol levels without accumulation of hepatic triglyceride [9, 10]. JTT-130 suppresses high-fat diet-induced obesity and improves

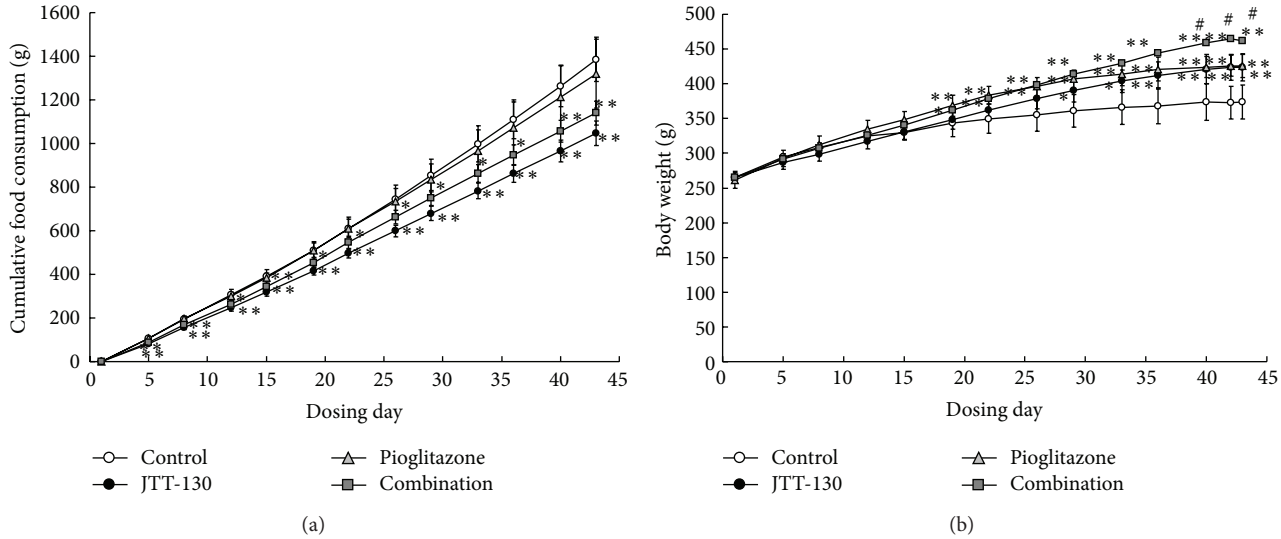

Figure 1: Changes in food intake (a) and body weight (b) in control group, JTT-130 treatment group, pioglitazone treatment group, and combination group. Data represent mean values ± standard deviation ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control group. $^{\#}P < 0.05$, significantly different from JTT-130 treatment group.

glucose and lipid metabolic abnormalities with the elevation of plasma glucagon-like peptide-1 (GLP-1) levels in SD rats and Zucker diabetic fatty rats [11–13]. In this study, we investigated the effects on glucose and lipid metabolism in combination treatment of JTT-130 and pioglitazone in Zucker diabetic fatty rats.

2. Materials and Methods

2.1. Materials. JTT-130, diethyl-2-({3-dimethylcarbamoyl-4-[(4′-trifluoromethylbiphenyl-2-carbonyl) amino] phenyl }acetyloxymethyl)-2-phenylmalonate, and pioglitazone were synthesized by Japan Tobacco Inc. (Osaka, Japan). All other reagents used in this study were obtained commercially.

2.2. Animals and Diets. Male Zucker diabetic fatty rats were obtained from Charles River Laboratories (Yokohama, Japan), individually housed with controlled temperature, humidity, and lighting (23 ± 3°C, 55 ± 15%, and a 12 h light/dark cycle with lights on at 8:00 AM), and provided with a powder diet (CRF-1; Oriental Yeast, Osaka, Japan) and water *ad libitum*. Zucker diabetic fatty rats were divided into 4 groups: a control group, JTT-130 treatment group, pioglitazone treatment group, and combination group. The assignment of rats was performed by body weight and nonfasting plasma parameters. Zucker diabetic fatty rats in the JTT-130 treatment group were fed a powder diet mixed with an appropriate amount of JTT-130 (0.01-0.02%) to achieve a daily dose of approximately 10 mg/kg for 42 days, from 7 to 13 weeks of age. The rats in the pioglitazone treatment group were fed a powder diet mixed with an appropriate amount of pioglitazone (0.001-0.002%) to achieve a daily dose of approximately 0.3 mg/kg. The rats in the combination group were fed a powder diet mixed with JTT-130 (0.01-0.02%) and pioglitazone (0.001-0.002%). Food intake and body weight were measured every 3 or 4 days during the experimental period. All procedures were conducted in accordance with the guidelines of the Japan Tobacco Animal Care Committee.

2.3. Measurement of Blood Chemical Parameters. Nonfasting plasma parameters, such as glucose, glycated hemoglobin, insulin, triglyceride, total cholesterol, and free fatty acid levels, were examined every 7 days. Blood samples were collected from the tail vein. Glucose, glycated hemoglobin, triglyceride, and total cholesterol levels were measured using commercial kits (Roche Diagnostics, Basel, Switzerland) and an automatic analyzer (Hitachi7170S, Tokyo, Japan). Plasma insulin level was measured with a rat-insulin enzyme-linked immunosorbent assay kit (Morinaga Institute of Biological Science, Yokohama, Japan). Plasma free fatty acid level was measured using NEFA *C*-test (Wako Pure Chemicals Industries Ltd., Osaka, Japan).

2.4. Glucose Utilization in Adipose Tissues. Small pieces (approximately 200 mg) of epididymal and mesenteric adipose tissues were incubated in Hank's balanced salt solution (pH 7.4) containing D-[U-^{14}C]-glucose (GE Healthcare UK, Little Chalfont, Buckinghamshire, England) in the absence or presence (100 nmol/L) of insulin at 37°C for 2 h. After stopping the reaction by the addition of 0.05 mol/L H_2SO_4, the produced $^{14}CO_2$ was trapped with filter paper. Radioactivity of filter paper was measured using a liquid scintillation counter (TRI-CARB 2500TR, Packard BioScience, Waltham, MA, USA). The protein contents of the adipose tissue pieces were determined using a BCA protein assay kit (Piece Biotechnology, Rockford, IL, USA), and the radioactivity of filter paper was normalized for comparison with the tissue protein content.

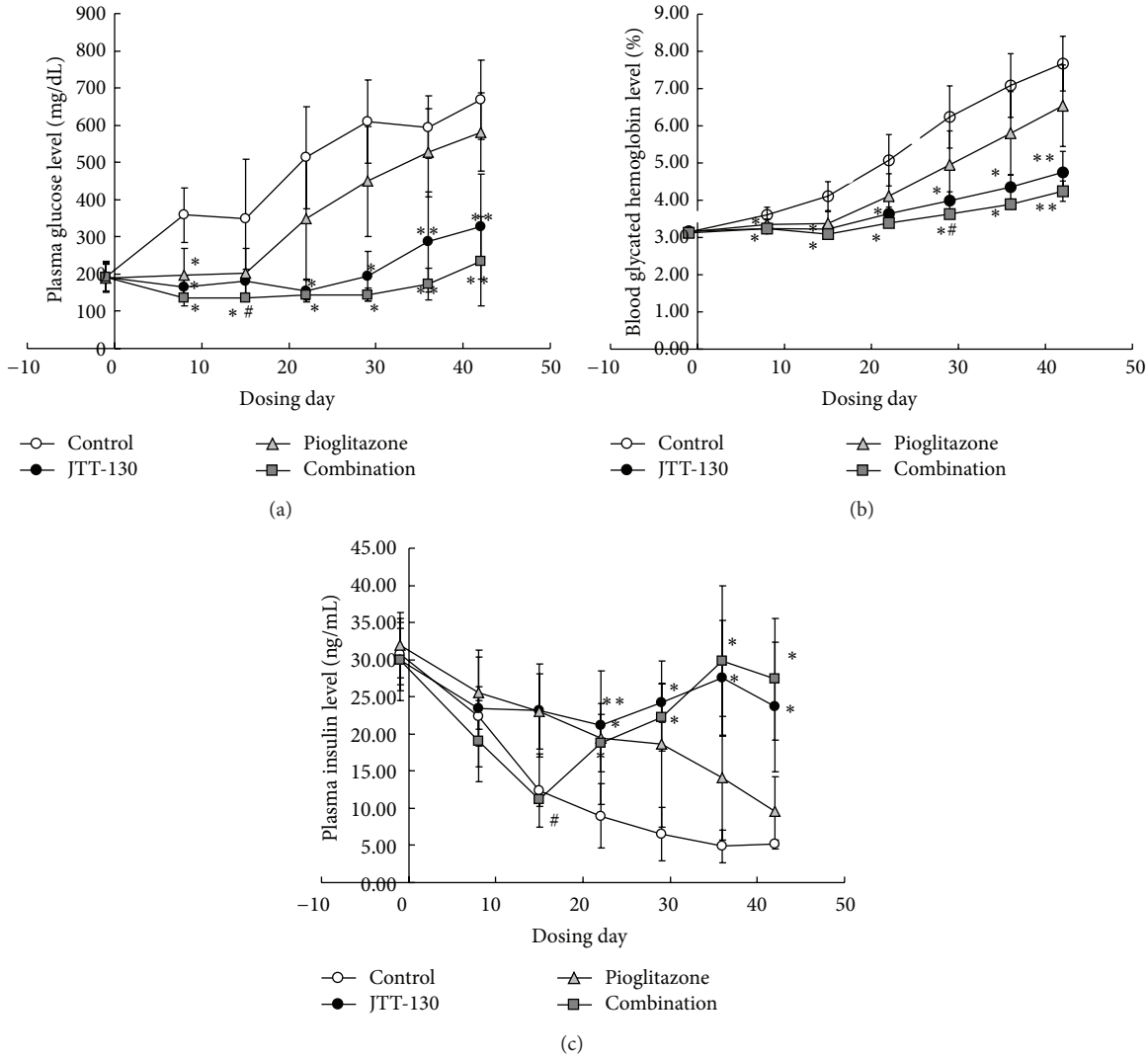

FIGURE 2: Changes in blood glucose (a), glycated hemoglobin (b), and insulin (c) levels in control group, JTT-130 treatment group, pioglitazone treatment group, and combination group. Data represent mean values ± standard deviation ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control group. $^{\#}P < 0.05$, significantly different from JTT-130 treatment group.

2.5. Statistical Analysis. Data were expressed as mean values ± standard deviation or + standard deviation. Tukey or Steel-Dwass test was used to determine statistical significance. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Food Intake and Body Weights. Changes in cumulative food intake and body weight are shown in Figure 1. The cumulative food intakes were decreased in JTT-130 treatment and combination groups after day 4 of treatment as compared with those in control group, but there were no significant differences in the food intake between the groups (Figure 1(a)). The cumulative food intakes in pioglitazone group were comparable to those in control group during the experimental period.

The body weights in JTT-130 treatment group were slightly lower in the early period of experiment and, inversely, increased after day 33 of treatment as compared with those in control group, and the body weights in pioglitazone treatment and combination groups were increased after day 19 (Figure 1(b)). The body weights in combination group were higher than those in JTT-130 treatment group after day 40 of treatment.

3.2. Blood Chemical Parameters. Changes in blood chemical parameters are shown in Figures 2 and 3. Zucker diabetic fatty rats in control group showed severe hyperglycemia with the significant elevation in the plasma glucose and glycated hemoglobin levels with time. The plasma glucose levels in JTT-130 treatment and combination groups were significantly decreased after day 7 of treatment as compared

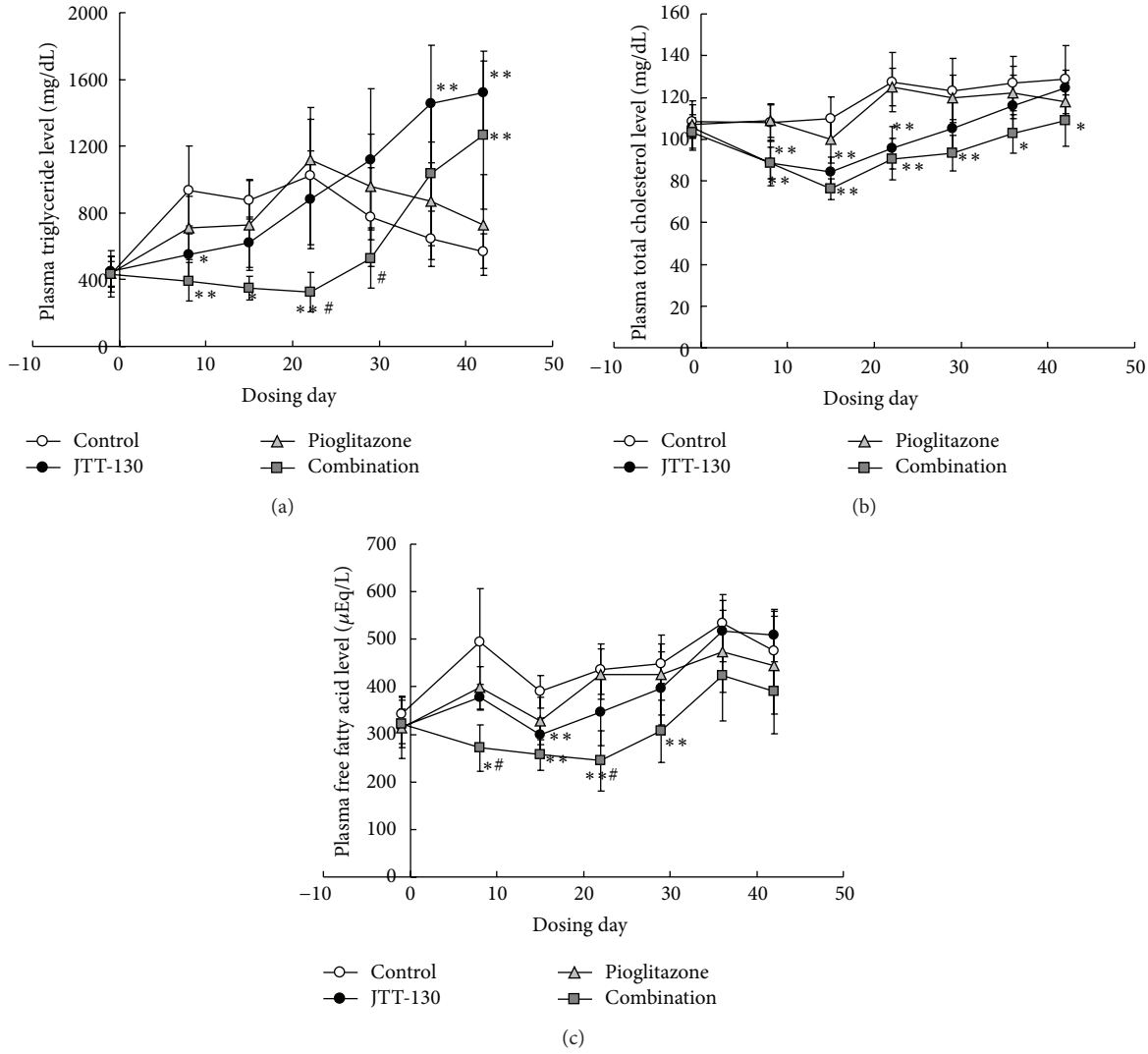

Figure 3: Changes in blood triglyceride (a), total cholesterol (b), and free fatty acid (c) levels in control group, JTT-130 treatment group, pioglitazone treatment group, and combination group. Data represent mean values ± standard deviation ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control group. $^{\#}P < 0.05$, significantly different from JTT-130 treatment group.

with control group. The plasma glucose levels in pioglitazone treatment group were also lower than those in treatment group, but the effects were significant only at day 7. Moreover, the plasma glucose levels in combination group were significantly decreased at day 14 of treatment, as compared with those in JTT-130 treatment group (control group, 359 ± 159 mg/dL; JTT-130 treatment group, 182 ± 32 mg/dL; combination group, 137 ± 23 mg/dL) (Figure 2(a)). Along with the plasma glucose levels, the glycated hemoglobin levels in JTT-130 treatment and combination groups were significantly decreased after day 7. Furthermore, the glycated hemoglobin levels in combination group were significantly decreased at day 28 of treatment, as compared with those in JTT-130 treatment group (control group, 4.11 ± 0.39%; JTT-130 treatment group, 3.22 ± 0.12%; combination group, 3.09 ± 0.10%) (Figure 2(b)). The glycated hemoglobin levels in pioglitazone treatment group tended to be decreased as compared with those in control group but not significantly. The plasma insulin levels in JTT-130 treatment and combination groups were significantly increased after day 21 of treatment as compared with those in control group, and the plasma insulin levels in pioglitazone group were significantly increased only at day 21. At day 14 of treatment, the plasma insulin levels in combination group showed a significant reduction as compared with those in JTT-130 treatment group (Figure 2(c)).

The plasma triglyceride levels in JTT-130 treatment group were lower in early period of experiment and significantly decreased on day 7 of treatment as compared with that in control group, but the levels were, inversely, elevated after day 35. The plasma triglyceride levels in combination group were also lower and significantly decreased from day 7 to 21 of treatment, as compared with those in control group, but the levels were also, inversely, increased at day 42 (Figure 3(a)).

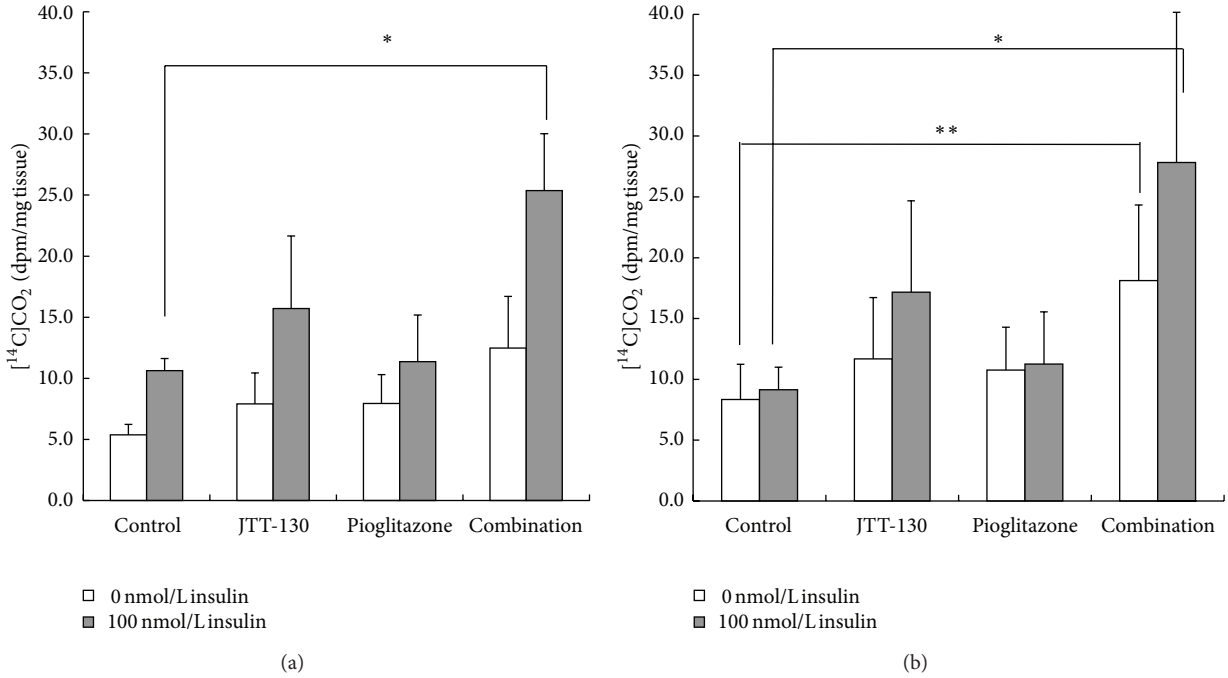

Figure 4: Effect on glucose utilization in the adipose tissues of epididymal fat (a) and mesenteric fat (b) in control group, JTT-130 treatment group, pioglitazone treatment group, and combination group. Data represent mean values + standard deviation ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different from control group.

The plasma triglyceride levels in combination group showed significant reduction as compared with those in JTT-130 treatment group at days 21 and 28 of treatment (control group, 1021 ± 412 mg/dL; JTT-130 treatment group, 881 ± 293 mg/dL; combination group, 327 ± 117 mg/dL, at day 21) (Figure 3(a)). The triglyceride levels in pioglitazone treatment group were comparable to those in control group during the experimental period. As compared with control group, the plasma total cholesterol levels in JTT-130 treatment group were significantly decreased from day 7 to 21 of treatment and the levels in combination group were significantly decreased after day 7 of treatment (Figure 3(b)). The total cholesterol levels in pioglitazone treatment group were comparable to those in control group. As compared with control group, the plasma free fatty acid levels in JTT-130 treatment group were significantly decreased at day 14, and the levels in combination group were decreased from day 7 to 28 of treatment. The plasma free fatty acid levels in combination group showed significant reduction as compared with those in JTT-130 treatment group (control group, 437 ± 53 μEq/L; JTT-130 treatment group, 347 ± 71 μEq/L; combination group, 244 ± 63 μEq/L, at day 21) (Figure 3(c)). There were no significant differences in the free fatty acid levels between pioglitazone group and control group.

3.3. Glucose Utilization in Adipose Tissues. To examine the effects of JTT-130 on glucose metabolism in the adipose tissues, we evaluated the abilities of epididymal and mesenteric adipose tissues to metabolize glucose to carbon dioxide using D-[U-^{14}C]-glucose. The production of carbon dioxide in the adipose tissues in combination group was significantly higher than that in control group in both the epididymal and mesenteric fat (Figure 4). The production of carbon dioxide in JTT-130 treatment and pioglitazone groups tended to be increased but not significantly.

4. Discussion

Type 2 diabetes mellitus is a progressive disease characterized by an impairment of insulin action and failure of pancreatic β-cells to compensate for the enhanced insulin demand. Several oral diabetic agents, with actions such as enhancement of insulin sensitivity in insulin target organ and insulin secretion from pancreas, have been approved for type 2 diabetes management so far. Combination therapies using these antidiabetic drugs have been employed in recent years.

JTT-130, a novel intestine-specific MTP inhibitor, improves the glucose and lipid metabolic abnormalities in obese diabetic models without any hepatotoxicity [11–13]. JTT-130 was shown to ameliorate impaired glucose metabolism in high-fat diet-induced obese rats and Zucker diabetic fatty rats with reduced food intake and body weight gain and thus is expected to be useful as a drug for the treatment of type 2 diabetes accompanied with obesity-related metabolic disorders. Pioglitazone, peroxisome proliferator-activated receptor (PPAR) γ agonist, is an insulin-sensitizing agent available for treatment of type 2 diabetes. The mechanism of action involves binding to PPAR γ, a transcription factor that regulates the expression of specific genes, especially in fat cells [14]. Pioglitazone

has been shown to interfere with expression and release of mediators of insulin resistance originating from adipose tissue. In this study, we investigated the combination effect of pioglitazone on JTT-130 treatment. Since significant pharmacological effects on 10 mg/kg of JTT-130 and 0.3 mg/kg of pioglitazone were confirmed in our preliminary and previous studies [12], those doses were established in the present study.

The glucose-lowering effect in combination group was more significant than that in JTT-130 treatment group (Figure 2(a)), and the glycated hemoglobin levels in combination group showed lower levels than in JTT-130 treatment group (Figure 2(b)). The value of glycated hemoglobin in ZDF rats (approximately 4% at 11 weeks of age) was lower than that of human, but similar values in diabetic rats were reported in other studies [12, 15]. In brief, better glucose control was achieved by combination of pioglitazone with JTT-130 treatment. The good glycemic control was considered to be caused by the interaction between an improvement of glucose metabolism by feeding restriction with JTT-130 and an enhancement of insulin sensitivity with pioglitazone. The plasma insulin levels at day 14 in combination group were significantly decreased as compared with those in JTT-130 treatment group (Figure 2(c)), suggesting that the combination treatment might enhance the insulin sensitivity more than JTT-130 alone. Moreover, the inhibition of progression of diabetes in JTT-130 and combination groups might induce the increases of body weight and blood insulin level after day 20 of treatment (Figures 1(b) and 2(c)).

Combination treatment significantly decreased the plasma triglyceride and free fatty acid levels (Figures 3(a) and 3(c)). These results suggested that the combination treatment ameliorated impaired lipid metabolism via the suppressed fat absorption by JTT-130 and the enhanced insulin sensitivity by pioglitazone, and therefore reduction of lipotoxicity in the combination treatment group was expected. Thus, the good glycemic control achieved in the combination treatment group might be caused not only by enhanced insulin sensitivity or decreased food intake but also the reduction in lipotoxicity. The current study using D-[U-^{14}C]-glucose showed that combination treatment of JTT-130 and pioglitazone significantly increased glucose utilization as compared with the monotherapy in adipose tissue, indicating that the combination therapy enhanced the insulin sensitivity (Figure 4).

We have previously reported that JTT-130 improved glucose intolerance with increase in the plasma GLP-1 levels in high-fat diet-induced obese rats [13], and thus the elevated GLP-1 might contribute the combination effects in the current study. In fact, there have been some reports on the combination therapies of incretin-related drugs and insulin sensitizers. In combination study using Zucker diabetic fatty rats with liraglutide, a human GLP-1 analogue, and ragaglitazar, a dual PPAR α/γ agonist, nonfasting blood glucose level was significantly decreased in combination therapy after treatment for 1-2 weeks, and there was a significant interaction between combination therapy and monotherapy for glycated hemoglobin level after 30-31 days of treatment [16]. Additionally, in combination study using ob/ob mice with alogliptin, a DPP-4 inhibitor, and pioglitazone, the combination therapy improved glycemic control and lipid profiles; moreover, increased pancreatic insulin contents were shown in the study [17]. Those combinations were shown to be useful also in a clinical trial [18].

In further study, the mechanism of synergistic effects between JTT-130 and pioglitazone such as protection of pancreas and preventive action of ectopic fat accumulation should be investigated, and merits of the combination therapy elucidated. Additionally, it is of importance for development of JTT-130 to elucidate the synergistic effect with pioglitazone treatment observed in this study, since pioglitazone has been widely approved as an antidiabetic agent.

In summary, combination treatment of JTT-130 and pioglitazone showed good glycemic control and a strong hypolipidemic action with an enhancement of insulin sensitivity in Zucker diabetic fatty rats. The combination therapy might be useful in the treatment of type 2 diabetes accompanied by insulin resistance.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

S. Sakata, Y. Mera, and Y. Kuroki helped in the design of study. Y. Mera, Y. Kuroki, and R. Nashida helped in the data collection and conduct of the study. S. Sakata, M. Kakutani, and T. Ohta helped in the analysis of study. S. Sakata and T. Ohta helped in writing the paper.

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