The evolution of diabetes in the male leptin receptor–deficient (fa/fa) Zucker diabetic fatty (ZDF) rat is associated with disruption of normal islet architecture, β-cell degranulation, and increased β-cell death. It is unknown whether these changes precede or develop as a result of the increasing plasma glucose, or whether the increased β-cell death can be prevented. Early intervention with thiazolidinediones prevents disruption of the islet architecture. To determine the specific effects of rosiglitazone (RSG) on β-cell mass dynamics, male fa/fa (obese) and +/fa or +/+ (lean) rats age 6 weeks were fed either chow (control group [CN]) or chow mixed with rosiglitazone (RSG group) at a dosage of 10 μmol·kg⁻¹·body wt·day⁻¹. Rats were killed after 0, 2, 4, 6, or 10 weeks of treatment (at age 6, 8, 10, 12, or 16 weeks).

Plasma glucose increased from 8.9 ± 0.5 mmol/l at 0 weeks to 34.2 ± 1.8 mmol/l (P = 0.0001) at 6 weeks of treatment in obese CN rats and fell from 8.0 ± 0.3 to 6.3 ± 0.4 mmol/l in obese RSG rats (P = 0.02). β-cell mass fell by 51% from 2 to 6 weeks of treatment (ages 8–12 weeks) in obese CN rats (6.9 ± 0.9 to 3.4 ± 0.5 mg; P < 0.05), whereas β-cell mass was unchanged in obese RSG rats. At 10 weeks of treatment (age 16 weeks), β-cell mass in obese CN rats was only 56% of that of obese RSG rats (4.4 ± 0.4 vs. 7.8 ± 0.3 mg, respectively; P = 0.0001). The β-cell replication rate fell from a baseline value of 0.95 ± 0.12% in lean rats and 0.94 ± 0.07% in obese rats (at 0 weeks) to ~0.3–0.5% in all groups by 6 weeks of treatment (age 12 weeks). After 10 weeks of treatment, β-cell replication was higher in obese RSG rats than in CN rats (0.59 ± 0.14 vs. 0.28 ± 0.05%, respectively; P < 0.02). Application of our mass balance model of β-cell turnover indicated that net β-cell death was fivefold higher in obese CN rats as compared with RSG rats after 6 weeks of treatment (age 12 weeks). The increase in β-cell death in obese CN rats during the 6-week observation period was well correlated with the increase in plasma glucose (r² = 0.90, P < 0.0001). These results suggest that the development of hyperglycemia in ZDF rats is concomitant with increasing net β-cell death. β-cell proliferation compensates for the increased β-cell loss at a time when plasma glucose is moderately elevated, but compensation ultimately fails and the plasma glucose levels increase beyond ~20 mmol/l. Treatment with rosiglitazone, previously shown to reduce insulin resistance, prevents the loss of β-cell mass in obese ZDF rats by maintaining β-cell proliferation and preventing increased net β-cell death. Diabetes 50:1021–1029, 2001

In people with type 2 diabetes, insulin resistance and defects in β-cell function contribute to the development of hyperglycemia and glucose intolerance (1). Reduced β-cell function has been quantified in many ways, including loss of first-phase insulin release in response to glucose, loss of pulsatile insulin secretion, and increased secretion of proinsulin and the split products resulting from incomplete proinsulin processing (1,2). The total mass of β-cells in pancreas from people with type 2 diabetes also appears to be reduced compared with non-diabetic subjects matched for body weight (3). It is not known whether reduced β-cell mass is a cause or consequence of diabetes, and whether it results from increased β-cell death, decreased β-cell proliferation, or both.

It has been suggested that the continuing loss of β-cell function is the underlying cause of deteriorating metabolic control in people with type 2 diabetes (1,4). The results from the U.K. Prospective Diabetes Study suggest that deterioration of β-cell function may continue after the onset of hyperglycemia, despite intensive treatment (1,4). Although intensive treatment did reduce blood levels of HbA1c relative to the results in conventionally treated subjects, in both groups HbA1c increased over time (5). The finding that the rate at which HbA1c rises is similar in conventionally and intensively treated patients suggests that poor glycemic control does not drive the continuing loss of β-cell function. Whether the loss of β-cell function is attributable to loss of insulin secretion per se or includes a reduction in the number of β-cells remains to be determined.

The mass of β-cells in the pancreas is determined by the rate of β-cell formation, including self-replication and neofor-mation from stem cells (neogenesis), and by the loss of β-cells through necrosis or apoptosis (6). Thus changes in β-cell mass occur through changes in the rate of β-cell formation, changes in β-cell death, or both. Small increases in the plasma glucose level may lead to an
increase in β-cell replication (7) and a decrease in β-cell death (8), whereas further increases in glucose may lead to a reduction in β-cell proliferation and an increase in β-cell death (7–9). In type 2 diabetes, the effects of evolving hyperglycemia and/or increasing free fatty acids (FFAs) on β-cell mass dynamics (mass, replication, and death) remain to be clarified.

The leptin receptor deficient (fa/fa) Zucker diabetic fatty (ZDF) rat has become a popular model for the investigation of type 2 diabetes (10,11). Diabetes develops spontaneously in male rats at ages 8–10 weeks, and is associated with the usual clinical symptoms of hyperphagia, polyuria, polydipsia, and impaired weight gain. The islets of ZDF rats appear normal at age 6 weeks, but by age 12 weeks are highly disorganized with significant fibrosis (12). β-cell degranulation occurs and the mass of β-cells fails to increase to the extent seen in the nondiabetic Zucker fatty rat. Pick et al. (13) attributed this failure of the β-cell mass to expand to an increased β-cell death at ages 6–12 weeks. Although these studies demonstrated that the change from normoglycemia to hyperglycemia in ZDF rats is associated with more β-cell death than appears to occur in nondiabetic Zucker fatty rats, the dynamics of β-cell mass, replication, and death, and their association with the prevailing plasma glucose, were not investigated. It is also unknown whether the increased β-cell death can be prevented.

Thiazolidinediones, agonists of the nuclear peroxisome proliferator–activated receptor-γ, enhance insulin sensitivity and improve metabolic control in patients with type 2 diabetes (14). These agents have been shown to prevent the development of diabetes in animal models of type 2 diabetes, including the ZDF rat (12,15,16). Treatment with rosiglitazone prevents the onset of hyperglycemia and, after treatment, islets retain a relatively normal architecture with well-granulated β-cells (12). It is unknown whether the total mass of β-cells remains normal and whether rosiglitazone treatment affects the dynamics of β-cell mass, replication, or death. Thus, the present studies were undertaken to characterize the evolution of the β-cell mass defect in the ZDF rat model of type 2 diabetes and to determine whether changes in β-cell mass, replication, or death are affected by treatment with the thiazolidinedione, rosiglitazone.

**RESEARCH DESIGN AND METHODS**

**Animals.** Experiments were carried out in obese male ZDF rats (ZDF/Gmi, fa/fa) and their lean male littermates (fa/+) obtained from Genetic Models (Indianapolis, IN). All procedures were performed in accordance with the standards set forth by the Canadian Council on Animal Care and were approved by the Animal Care Committee at Simon Fraser University. Rats were obtained at age 5 weeks and were allowed to adapt to the local environment for 1 week before study. Rats were housed in separate cages, under a 12:12-h light:dark cycle in a pathogen-free environment, and had free access to water. During the acclimatization period each animal was fed Purina 5008 Chow (Purina Mills, Richmond, IN) ad libitum. Food and water intake were monitored daily.

**Treatment.** At age 6 weeks, lean and obese rats were randomly divided into treatment (rosiglitazone [RSG]) and control (CN) groups. The CN rats continued to receive Purina 5008 chow ad libitum. The RSG rats were fed Purina 5008 Chow (Purina Mills) mixed with rosiglitazone maleate (Smith–Kline Beecham, Harrow, UK). The chow was obtained from the manufacturer in the powdered form and mixed with water and an appropriate amount of rosiglitazone maleate (range 0–100 mg/kg chow). The mixture was made into pellets and dried at <50°C on a food dehydrator (American Harvest, Chaska, MN). The concentration of RSG in the food was based on a target dosage of 10 μmol · kg⁻¹ · body mass · day⁻¹. RSG animals were pair-fed to their respective untreated control group (lean or obese). New formulations of food mixed with RSG were prepared one to two times per week. Drug-treated rats ingested an average daily dose of 0.8 ± 0.4 μmol/kg body mass of RSG.

**Experimental design.** Two separate studies were performed. In the first study, lean and obese CN and RSG rats were killed after 0, 2, 4, or 6 weeks of treatment (i.e., at age 6, 8, 10, or 12 weeks). Rats were obtained from the supplier in four groups of 28 rats (14 lean and 14 obese). From these groups, two animals were randomly assigned to each treatment/time cohort (i.e., 0 weeks, 2 weeks CN, 2 weeks RSG, 4 weeks CN, 4 weeks RSG, 6 weeks CN, and 6 weeks RSG). This approach ensured that animals from a given litter were represented throughout the experimental groups. Rats were studied for each treatment/time cohort. In the second study, 24 obese rats were obtained from the supplier and, at age 6 weeks, randomly assigned to one of three groups: CN, RSG, or CN + insulin (n = 8 each). All rats received chow for 10 weeks, either without (CN and CN + insulin groups) or with RSG (RSG group). Up to 96 h before killing, the CN + insulin group received subcutaneous implants that released insulin at 2–4 U/24 h (Labplant; LinShin, Scarborough, ON, Canada) to lower the blood glucose. All animals in the 10-week untreated, control group (lean or obese) were fasted overnight prior to killing. Blood samples were obtained at 0, 2, 4, or 6, or 10 weeks of treatment (ages 6, 8, 10, 12, or 16 weeks), rats were deeply anesthetized with sodium pentobarbital (35 mg/kg i.p.). A 2- to 4-ml blood sample with an average daily dose of 9.8 ± 0.4 mg/kg body mass of RSG.

**Blood sampling and plasma assays.** Once per week, blood (≈500 μl) was withdrawn from the tail vein of each rat. A small cut was made at the tip of the tail using a scalpel and blood was drawn into 75-μl heparinized capillary tubes. The blood samples were placed on ice and centrifuged, and plasma was stored at −20°C until assayed. Plasma glucose concentrations were determined by an enzymatic assay method (Trinder; Sigma Diagnostics, St. Louis, MO). Plasma insulin concentrations were determined by an insulin radioimmunoassay kit (Linco Research, St. Louis, MO) that uses a specific anti-rat insulin antibody. Plasma nonesterified fatty acid (NEFA) concentrations were determined (second experiment only) by an enzymatic colorimetric method (ACS-ACOD; Wako Chemicals, Richmond, VA, Canada).

**Pancreas removal and tissue processing.** At 0, 2, 4, 6, or 10 weeks of treatment (ages 6, 8, 10, 12, or 16 weeks), rats were deeply anesthetized with sodium pentobarbital (35 mg/kg i.p.). A 2- to 4-ml blood sample with an average daily dose of 9.8 ± 0.4 mg/kg body mass of RSG. The blood samples were placed on ice and centrifuged, and plasma was stored at −20°C until assayed. Plasma glucose concentrations were determined by an enzymatic assay method (Trinder; Sigma Diagnostics, St. Louis, MO). Plasma insulin concentrations were determined by an insulin radioimmunoassay kit (Linco Research, St. Louis, MO) that uses a specific anti-rat insulin antibody. Plasma nonesterified fatty acid (NEFA) concentrations were determined (second experiment only) by an enzymatic colorimetric method (ACS-ACOD; Wako Chemicals, Richmond, VA, Canada).

**Measurement of β-cell mass.** Sections were dewaxed in xylene, dehydrated in petroleum ether, and incubated in 0.3% hydrogen peroxide in methanol for 30 min. Sections were washed in phosphate-buffered saline (PBS) and incubated in 10% lamb serum in PBS for an additional 30 min. Sections were then incubated in 10% sheep serum in PBS for 30 min, and subsequently in 0.2% bovine serum albumin in PBS for 30 min. Sections were incubated in guinea pig anti-insulin antibody overnight at 4°C (Dako Diagnostics, Mississauga, ON, Canada), biotinylated anti–guinea pig antibody for 1 h (Vector Laboratories, Burlington, ON, Canada), and avidin/biotin complex for 1 h (ABC; Vectastain Elite ABC Kit; Vector Laboratories). Samples were then incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma–Aldrich, Oakville, ON, Canada) for 10 min. The sections were washed several times with PBS between incubations. After incubation in DAB, sections were washed in tap water and counterstained with hematoxylin.

- β-cell mass was determined from the insulin antibody–stained sections using an image analysis system. This system consisted of an Olympus light microscope (Model BX40; Caron Group, Markham, ON, Canada) attached to a Sony color video camera (Model DXC-950; Sony, Japan) and Northern Eclipse software (Empix Imaging, Mississauga, ON, Canada). The relative cross-sectional area of β-cells was determined by quantification of the cross-sectional area occupied by β-cells and the cross-sectional area of all tissue in multiple fields per slide. Total β-cell mass was determined as the sum of the estimates from the two or three pieces of pancreas. Total β-cell mass and adipose content of the pancreas were determined in a similar manner. β-cell mass determinations were performed in a blinded fashion by two individuals. Equal numbers of samples from each treatment/time cohort were counted by each person, and
internal reference standards were used to ensure both observers were applying the methods in a comparable manner.

**Measurement of β-cell replication.** At 6 h before removal of the pancreas, the animals were injected intraperitoneally with 100 mg/kg 5-bromo-2′-deoxyuridine (BrdU; Amersham, Oakville, ON), a thymidine analogue incorporated into newly synthesized DNA. Sections were dewaxed and mounted in a glycerol gel (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.0) (21). The gel was dehydrated in a series of increasing concentrations of ethanol (70%, 90%, 100%) before it was embedded in historesin (Leica, Wetzlar, Germany). Sections 4 µm thick were cut, washed and counterstained with hematoxylin.

The New Fuchsin Substrate System (Dako) for 10 min. Slides were then ABC (alkaline phosphatase (Standard Vectastain ABC Kit; Vector) for 1 h, and then cooled for 15 min at room temperature, washed with PBS, and incubated with biotinylated goat anti-mouse antibody (Vector) for 1 h at room temperature. Sections were then washed in PBS, incubated with ABC–horseradish peroxidase (1:1000; Vectastain Elite ABC Kit; Vector) for 1 h, and then developed for 5 min using DAB. Slides were then washed and incubated with guinea pig anti-insulin antibody (Dako) overnight at 4°C. Next they were serially incubated with goat anti-guinea pig antibody (Vector) for 1 h, ABC–alkaline phosphatase (Standard Vectastain ABC Kit; Vector) for 1 h, and the New Fuchsin Substrate System (Dako) for 10 min. Slides were then washed and counterstained with hematoxylin.

BrdU incorporation was determined by systematically sampling all of the β-cells in each section, using a light microscope under high magnification (×1000). β-cells incorporating BrdU (BrdU positive) had blue/black nuclei. β-cell replication was determined in a blinded fashion by one individual. An average of 1,956 ± 72 (± SE, range, 1,013–4,879) β-cells per animal were counted and classified as BrdU positive or negative.

**Calculation of β-cell mass dynamics.** We used our previously established mathematical model (6) to calculate net neogenesis or net β-cell loss over time in the four treatment groups (lean CN, lean RSG, obese CN, and obese RSG), as follows: neogenesis = death = d(β-cell number)/dt = replication. The mass balance equation estimates net β-cell neogenesis or net β-cell death based on the rate of change in β-cell number and the rate of β-cell replication. β-cell size was assumed to be constant, so d(β-cell number)/dt equals d(β-cell mass)/dt. d(β-cell mass)/dt was estimated by fitting a polynomial of the lowest significant order to the individual values of β-cell mass versus treatment time in days. The slope of the regression and the standard error of the estimate of the slope were used to estimate neogenesis minus death at 0, 2, 4, and 6 weeks of treatment (ages 6, 8, 10, and 12 weeks). β-cell replication in percentage per day was assumed to be four times the BrdU incorporation rate.

**Statistical analysis.** All statistical analyses were performed using SAS System for Windows (Release 6.12; SAS Institute, Cary, NC). Results are expressed as means ± SE unless otherwise stated. A two- or three-way analysis of variance (ANOVA) was carried out for the factors strain (lean and obese), treatment type (CN and RSG), and cohort (0, 2, 4, or 6 weeks of treatment) using the general linear models procedure. A repeated measures ANOVA was used for variables measured repeatedly (e.g., body weight, plasma concentrations) in animals studied over a 6-week period. These analyses did not include data obtained in the second study after 10 weeks of treatment. Comparison of data obtained in the second studies was not possible because the studies were carried out at different times and under somewhat different circumstances, and the quantitative morphometry was carried out by different people. Dunnett's post hoc test was used to test for differences from week 0. Plasma insulin levels were log transformed before performing the ANOVA. The 0 weeks (age 6 weeks) data were duplicated for use in both CN and RSG treatments. Differences were deemed statistically significant at P < 0.05.

**RESULTS**

**Body and pancreas weight.** As expected, body weight was significantly greater in obese as compared with lean rats (P = 0.0001) and increased with age (P = 0.0001) (Table 1). There was no effect of RSG treatment on body weight in the lean animals (P = 0.32); in contrast, by 4 weeks of RSG treatment (age 10 weeks), body weight was significantly greater in obese RSG as compared with obese CN rats (P = 0.0006). Pancreas weight was slightly lower in obese as compared with lean animals (P = 0.0001). Pancreas weight increased with time up to 4 weeks of treatment (P = 0.0001), but there was no significant effect of RSG treatment when analyzed by two-way ANOVA (P = 0.11) (Table 1). The ratio of pancreas to body weight was lower in obese as compared with lean rats (P = 0.0001) and decreased with time (P = 0.0001), but was not affected by RSG treatment (P = 0.19).

**Plasma glucose, insulin, and NEFA concentrations.** In the first experimental group, plasma glucose was significantly elevated in obese as compared with lean rats at age 6 weeks (0 weeks of treatment) (8.5 ± 0.3 vs. 6.6 ± 0.1 mmol/l, respectively; P = 0.0001). There were no effects of treatment (P = 0.59) or age (P = 0.53) on plasma glucose levels in lean animals studied for 6 weeks (ages 6–12 weeks). In contrast, both treatment (P = 0.0001) and age (P = 0.0001) had significant effects in obese animals. Plasma glucose increased from 8.9 ± 0.4 to 34.2 ± 1.8 mmol/l (P = 0.0001) in obese CN rats and fell from 8.0 ± 0.3 to 6.3 ± 0.4 mmol/l (P = 0.02) in obese RSG rats (Fig. 1).

In the second experimental group, plasma glucose levels were similarly elevated in obese CN and CN + insulin cohorts at 0–6 weeks of treatment (ages 6–12 weeks) (Fig. 2). The hyperglycemia persisted at 6–10 weeks of treatment, except in the CN + insulin cohort in which plasma glucose fell to 22.5 ± 2.5 mmol/l (P = 0.0001) at age 12 weeks (6 weeks of treatment) as a result of insulin treatment during the 4 days before killing (Fig. 2). Plasma glucose fell from baseline (7.8 ± 0.2 mmol/l) in RSG animals (P = 0.006), reaching a nadir of 5.9 ± 0.2 mmol/l at 3 weeks of treatment, but was not different from baseline by 4 weeks of treatment (age 10 weeks; 8.0 ± 0.2 mmol/l; P = 0.76).

In the first experimental group, plasma insulin was elevated 12-fold in obese as compared with lean rats...
There was no effect of age on plasma insulin levels in lean CN animals (P = 0.86) and a small decrease with age in lean RSG animals (P = 0.04) studied for 6 weeks. Plasma insulin levels varied with age in both CN (P = 0.0001) and RSG obese rats (P = 0.002). Plasma insulin levels increased significantly from 0 to 1 week of study (age 6–7 weeks; P = 0.003) in obese CN rats and decreased thereafter to a low at 6 weeks of treatment (age 12 weeks; 407 ± 64 pmol/l; P = 0.0001 vs. week 0 of treatment). The changes in plasma insulin levels were less dramatic in the obese RSG group. Plasma insulin was two- to fourfold higher in obese CN rats as compared with obese RSG rats at 5 and 6 weeks of treatment (ages 11 and 12 weeks) (P < 0.001). In the second experimental group, plasma insulin levels were significantly increased at 2, 3, and 4 weeks as compared with 0 weeks of treatment in CN and CN + insulin rats (P < 0.04) and were significantly reduced at 5–10 weeks as compared with 0 weeks of observation and as compared with RSG animals (P < 0.04) (Fig. 2).

In the second experimental group, plasma NEFA levels increased with age in obese CN and CN + insulin rats (P = 0.0001) and were significantly greater than levels at 0 weeks of treatment (age 6 weeks) and levels in obese RSG rats at 2–10 weeks of treatment (ages 8–16 weeks; P < 0.004) (Fig. 2). Overall, plasma NEFA levels did not vary significantly with age in obese RSG rats (P = 0.19), although at 2, 3, 6, 7, 8, and 9 weeks of observation, NEFA concentrations were significantly less than at 0 weeks (P < 0.02). In the CN + insulin group, plasma NEFA levels fell from ~1.0 mmol/l (sustained from 3 to 9 weeks of observation) to 0.6 ± 0.1 mmol/l at the end of the study.

**Islet morphology.** Before the development of hyperglycemia, islets of obese rats age 6 weeks appeared normal (Fig. 3A and B). The insulin content of the β-cells was normal, as demonstrated by dense immunohistochemical staining for insulin, and the islets maintained a normal rounded appearance. At age 12 weeks, islets of obese CN rats were enlarged and disorganized, with extensions into the surrounding exocrine tissue (Fig. 3C). By age 16 weeks (10 weeks of observation), many of the groupings of insulin-positive cells had lost any of the appearance of an islet structure and were seen as single cells and small groups of cells distributed throughout the exocrine tissue (Fig. 3C). After age 12 weeks, islets of obese CN rats were enlarged and disorganized, with extensions into the surrounding exocrine tissue (Fig. 3E). The islets of obese RSG rats had a normal appearance at ages 12 and 16 weeks (Fig. 3D and F).

**β-cell mass.** In the first experimental group, β-cell mass was significantly greater in obese as compared with lean animals at age 6 weeks (P = 0.0001) (Fig. 4). There was a significant effect of age (P = 0.001), but not of treatment (P = 0.10), on β-cell mass in lean animals as measured by two-way ANOVA. However, one-way ANOVA within treat-
ment groups demonstrated a significant increase in β-cell mass at 4 and 6 weeks of treatment (ages 10 and 12 weeks) as compared with 0 weeks of treatment (age 6 weeks) in lean CN (P < 0.05) but not in lean RSG rats (P = 0.17). In obese rats, there was no significant effect of treatment when considered by two-way ANOVA (P = 0.24), but with the obese CN rats, there was a significant effect of age (P = 0.046), with β-cell mass being decreased by 51% between 2 and 6 weeks of treatment (ages 8 and 12 weeks; P < 0.05). There was no effect of treatment time on β-cell mass in obese RSG rats (P = 0.56). In the second experimental group, there was no effect of insulin treatment on β-cell mass (P > 0.05) (Fig. 5). β-cell mass in CN and CN + insulin rats was only 50% of the β-cell mass in RSG rats after 10 weeks of treatment (age 16 weeks; P = 0.0001).

**BrdU incorporation.** There was no difference in the rate of BrdU incorporation between lean and obese animals at age 6 weeks (0 weeks of treatment) in the first experimental group (P = 0.95) (Table 2). There was a significant effect of age (P = 0.0001), but not of treatment (P = 0.89), on BrdU incorporation into β-cells in lean animals as measured by two-way ANOVA. In both CN and RSG lean rats, BrdU incorporation was significantly greater at 0 weeks as compared with 2, 4, or 6 weeks of treatment (P < 0.05). In obese rats, there was a significant effect of treatment (P = 0.0024) and age (P = 0.0001) when considered by two-way ANOVA. In obese CN rats, BrdU incorporation was significantly greater at 0 and 2 weeks of treatment (ages 6 and 8 weeks) as compared with at 4 and 6 weeks of treatment (ages 10 and 12 weeks) (P < 0.05). In obese RSG rats, BrdU incorporation was similar to that in lean CN and lean RSG rats, with only the value at 0 weeks of treatment being greater than the values at subsequent weeks. By 10 weeks of treatment (age 16 weeks), BrdU

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**FIG. 3.** Islets of Langerhans from lean and obese rats with and without RSG treatment. Staining with anti-insulin antibody visualized with DAB. A: Lean CN rats age 6 weeks; B: obese CN rats age 6 weeks; C: obese CN rats age 12 weeks (week 6 of observation); D: obese RSG rats age 12 weeks; E: obese CN rats age 16 weeks (week 10 of observation); F: obese RSG rats age 16 weeks. Black bar denotes 25 μm.
incorporation in RSG rats was twofold greater than in CN and CN + insulin rats \( (P = 0.02) \) (Fig. 5). β-cell replication rates were related to plasma glucose levels by a nonlinear relationship in which replication was lower at both low and very high glucose levels, but increased at moderate plasma glucose levels (Fig. 6).

Net cell death. To calculate net β-cell neogenesis or net cell death, we estimated the rate of change in β-cell mass by least squares regression of the β-cell mass over time for each treatment group. The order of the regression used was the lowest order in which all coefficients were significant. For lean CN rats, β-cell mass versus age in days was fit with a linear regression (intercept \( = 1.94 \pm 0.23, P < 0.0001 \); slope \( = 0.052 \pm 0.010, P < 0.0001 \)). In lean RSG rats, the slope of β-cell mass versus age was not significant \( (P = 0.12) \), making the rate of change of β-cell mass equal to zero. Obese CN rats had the most complex β-cell mass dynamics, as all coefficients in a second order polynomial were significant (intercept \( = 5.36 \pm 0.75, P < 0.0001 \); 1st order coefficient \( = 0.182 \pm 0.087, P = 0.046 \); 2nd order coefficient \( = -0.0054 \pm 0.0020, P = 0.013 \)). Obese RSG rats were similar to lean RSG rats, with a slope that was not significant \( (P = 0.49) \), making \( \frac{d(\text{β-cell mass})}{dt} = 0 \).

The net β-cell flux (neogenesis minus death) was calculated as the difference between \( \frac{d(\text{β-cell mass})}{dt} \) and the β-cell replication rate. When the net flux is positive (when the rate of increase in the β-cell mass is greater than can be accounted for by replication of β-cells), net neogenesis is deduced. When the net β-cell flux is negative (when the rate of increase in the β-cell mass is less than can be accounted for by replication of the β-cells), then net cell death must occur. In lean rats, there was net β-cell death at age 6 weeks (Fig. 7). In both CN and RSG lean rats, net β-cell death decreased from week 0 of treatment (age 6 weeks) to subsequent weeks. A similar decrease in net cell death occurred in obese RSG rats, whereas net β-cell death increased to \( \sim 10\% \) per day in obese CN rats at 6 weeks of treatment (age 12 weeks) (Fig. 7). The increase in net β-cell death observed in the obese CN rats was well correlated with the increase in plasma glucose during the period of observation \( (r^2 = 0.90, P < 0.0001) \) (Fig. 8).

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Data are means ± SE. *\( P = 0.0002 \) vs. lean; †\( P = 0.0005 \) vs. control; ‡\( P < 0.02 \) vs. control.
DISCUSSION

This study provides the first detailed examination of β-cell mass dynamics during the evolution of hyperglycemia in an animal model of type 2 diabetes. This detailed observation of β-cell mass, and the rates of β-cell replication and death, during the development of glucose intolerance provides novel insight into the sequence of events associated with the progressive deterioration of β-cell function.

In untreated ZDF rats, the plasma glucose level increased linearly at ages 6–9 weeks (0–3 weeks of observation). During the early part of this rise, the β-cell mass continued to expand in response to the high demand for insulin necessitated by the developing obesity and associated insulin resistance. Thus, plasma insulin levels continued to rise, supported by a sustained β-cell mass, despite an increase in the rate of β-cell death. At the early, moderately increased plasma glucose levels, high β-cell replication rates were sustained in counterbalance to the increasing rate of β-cell death. By age 10 weeks (4 weeks of observation), the plasma glucose level had reached a plateau of ~30 mmol/l, and the plasma insulin level had fallen 70% from its peak value. Under these conditions, the β-cells continued to die at a rapid rate, the high β-cell replication rates could no longer be sustained, and the mass of β-cells began to decline. The nonlinear relationship between plasma glucose and β-cell replication suggests that the inability to maintain a relatively high replication rate may have been attributable to the exceptionally high plasma glucose level (7,9), although the direct causal effect of glucose remains to be confirmed.

By 6 weeks of observation (age 12 weeks), the decline in β-cell mass was significant because of further increases in β-cell death. This loss of β-cell mass presumably contributed to the further reduction in plasma insulin levels. The good correlation between increases in plasma glucose and increases in β-cell death suggests that the developing hyperglycemia of the diabetic state could be a driving force for loss of the β-cell mass (8). Likewise, increases in plasma fatty acids or intracellular triglycerides could also drive the loss of β-cells (17,18). Plasma NEFA levels were well correlated with plasma glucose levels in our obese ZDF rats, suggesting that increasing plasma NEFA could also drive the loss of the β-cell mass.

Treatment of obese ZDF rats with the thiazolidinedione, rosiglitazone, before the onset of significant hyperglycemia prevented the development of diabetes. Although the specific mechanism of action of rosiglitazone in preventing the development of diabetes in ZDF rats cannot be determined from the present study, the effects on plasma glucose and insulin and the effects on β-cell mass dynamics could all be the indirect result of rosiglitazone’s action.

FIG. 6. Nonlinear relationship between plasma glucose and the rate of β-cell replication. △, lean CN rats; ▲, lean RSG rats; ○, obese CN rats; ◆, obese RSG rats. Solid line, second order polynomial fit across all groups.

FIG. 7. Net β-cell death (negative β-cell flux) in first experimental group lean and obese ZDF rats with and without RSG treatment. □, CN rats; ■, RSG rats. Net β-cell death is the difference between the rate of β-cell mass change and the rate of replication. Differences in the estimate of net β-cell death at 0 weeks of treatment are attributable to the use of a polynomial regression of β-cell mass versus days of treatment to estimate d(β-cell mass)/dt.

FIG. 8. Increase in net cell death as a function of the increase in plasma glucose. △, lean CN rats; ▲, lean RSG rats; ○, obese CN rats; ◆, obese RSG rats. Solid line, first order linear regression across all groups. Correlation significant at P < 0.001.
in enhancing insulin sensitivity (19). An increase in insulin sensitivity would remove the demand for further adaptation of the β-cell mass to the developing obesity in these leptin receptor−deficient rats. As a result, plasma glucose, insulin, and the β-cell mass remained relatively constant throughout the period of observation in the obese RSG animals. Consistent with a decreased demand for adaptation of the β-cell mass was a reduction in the β-cell replication rate in obese RSG rats within 2 weeks of commencing treatment, as compared with the delayed fall in replication in obese CN rats.

In obese CN rats, insulin resistance in conjunction with an impaired ability to adapt to changes in the plasma glucose level could account for the initial rise in plasma glucose. That ZDF rats have an impaired adaptation of the β-cell mass is suggested by comparison with the nondiabetic Zucker fatty rat. These latter rats, the parent strain for the ZDF rat, have a fourfold increase in β-cell mass at ages 6–12 weeks (13), suggesting that a large increase in β-cell mass is required to maintain normoglycemia in this obese, insulin-resistant rodent. A similar conclusion was drawn by Pick et al. (13), as they found a limited increase in β-cell mass in the ZDF rat. In contrast to the present study, however, β-cell mass doubled in the male ZDF rats observed by Pick et al. (13) at ages 6–12 weeks. Differences in β-cell mass between the present report and that of Pick et al. (13) may be attributable to differences in the degree of hyperglycemia achieved, although this is difficult to evaluate given that Pick et al. reported only fasting plasma glucose levels (131 ± 2 mg/dl at age 12 weeks) and we have only fed-state plasma glucose levels (>500 mg/dl at age 12 weeks in untreated rats). Differences in the application of quantitative morphometric methods might also account for differences between the present study and that of Pick et al. (13).

In ZDF rats, the failure of the β-cell mass to adapt may be caused by an impaired ability of the β-cell mass to increase self-replication with increasing plasma glucose or FFAs (20). Glucose-infused SD rats also demonstrated a similar second order nonlinear relationship between β-cell proliferation and plasma glucose, but the peak rate of proliferation at ∼20 mmol/1 glucose was twofold greater in SD than in ZDF rats (21). This defect in β-cell mass adaptation with glucose remains to be confirmed in a time-independent manner with a more appropriate lean control strain, and the defect with increasing FFAs needs to be investigated in vivo. The failure of the β-cell mass to adapt could also be attributable to β-cells that more readily undergo apoptosis in response to increased plasma glucose or FFAs (18) or to an accumulation of triglycerides in the β-cells (22). It is also possible that thiazolidinediones have a direct effect on β-cell replication and death, but reports based on in vitro studies are inconsistent (16,23).

Intervention with a thiazolidinedione in the ZDF rat can reverse the diabetic state if it is recently developed (i.e., at age 11 weeks) (R.E.B., unpublished observations), but not after it has persisted for a considerable length of time (i.e., at age 21 weeks) (12). In the present study, the β-cell mass at age 12 weeks might have been at a level that could maintain normoglycemia if insulin resistance was reduced by treatment with rosiglitazone. This is inferred by the similarity of the β-cell mass in CN and RSG obese rats after 6 weeks of treatment (age 12 weeks). However, at age 16 weeks in the present study, the β-cell mass was only half that present in the normoglycemic RSG-treated rats, suggesting that after persistence of the diabetic state, the β-cell mass may be insufficient to permit correction of hyperglycemia, even if insulin sensitivity is restored or markedly improved.

The application of a mass balance equation to calculate net β-cell death provides a novel method for detailed investigation of β-cell mass dynamics in vivo (6). Apoptotic cells undergo a series of distinct changes—including condensation or margination of the chromatin, activation of caspases, and DNA fragmentation—that can be detected with light microscopy (24). Although apoptotic cells can be found in tissue samples, apoptosis occurs with low frequency, making it difficult to detect differences among treatment groups (25). In addition, the corpses of dying cells are rapidly engulfed by macrophages and other phagocytes (26), making it even more difficult to detect apoptosis in tissue samples and making the incidence of apoptotic cells dependent on both the rate of apoptosis and the clearance rate of apoptotic debris. Pick et al. (13) noted that the frequency of β-cell apoptosis in ZDF rats was too low to allow for meaningful direct quantitative analysis. Although assessment of many pancreas sections per animal may provide sufficient apoptotic events for detection of differences among groups, our model-based approach provides a novel means for quantitative investigation of β-cell death dynamics in vivo.

The rate of net cell death is dependent on estimates of the rate of β-cell replication and change in β-cell number. Cell number is proportional to the mass of β-cells and their average size (6), which was not determined in the present study because of limitations resulting from the deterioration of the islet structure. Instead, the rate of change in β-cell number was assumed to be equal to the rate of change in β-cell mass. If β-cell size increased in obese hyperglycemic animals (CN rats), then the rate of decline in cell number at 2–6 weeks of treatment (ages 8–12 weeks) would be greater than that used to estimate net cell death. A greater decline in cell number would indicate a bigger increase in net cell death, increasing the difference between treated and untreated rats.

Our methodology gives an estimate of the rate of cell death, but does not allow us to determine the specific mode of cell death. In vitro studies by Shimabukuro et al. (18) suggest that the β-cell loss we calculated in the present study was attributable to excessive apoptotic cell death. Those researchers observed significantly greater DNA laddering in islets isolated from fa/fa as compared with +/+ ZDF rats at age 5 weeks and found an increase in laddering with increasing age in the obese animals. Ceramide, a fatty acid−containing messenger in cytokine-induced apoptosis, was significantly increased in islets from obese animals ages 7 and 14 weeks because of increased ceramide synthesis from fatty acids (18). The triglyceride content of isolated islets increased with age in ZDF rats, apparently because of enhanced de novo lipogenesis (27,28). Islets cultured with FFAs demonstrated an increase in DNA laddering that could be blocked by culture with an inhibitor of ceramide synthetase, an inhibitor of fatty acyl-CoA synthetase, and by troglitazone,
another thiazolidinedione (18). Thus, the excessive β-cell apoptosis in untreated ZDF rats is likely to be associated with excessive accumulation of intracellular triglycerides. Whether the action of troglitazone observed in those studies was caused by enhancement of fatty acid oxidation, decreased nitric oxide formation, or protection from cytokine toxicity remains to be determined (18,29,30). Likewise, whether these mechanisms are important in the action of thiazolidinediones in preventing apoptotic β-cell loss in vivo remains to be clarified.

In summary, this study demonstrated that loss of β-cell mass resulting from an increase in β-cell death is an important contributor to the evolution of the diabetic state in ZDF rats. In addition, impaired glucose-induced stimulation of β-cell proliferation also may contribute to the development of hyperglycemia. Treatment with the thiazolidinedione, rosiglitazone, prevents the development of hyperglycemia and allows for maintenance of adequate β-cell mass and function. Application of a novel model-based method for estimation of net β-cell death allows for a detailed investigation of β-cell mass dynamics in vivo.

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