Original Article

Inhibiting Glycosphingolipid Synthesis Improves Glycemic Control and Insulin Sensitivity in Animal Models of Type 2 Diabetes

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Previous reports have shown that glycosphingolipids can modulate the activity of the insulin receptor, and studies in transgenic mice suggest a link between altered levels of various gangliosides and the development of insulin resistance. Here, we show that an inhibitor of glycosphingolipid synthesis can improve glucose control and increase insulin sensitivity in two different diabetic animal models. In the Zucker diabetic fatty rat, the glucosylceramide synthase inhibitor (1R,2R)-nonanoic acid[2-(2′,3′-dihydro-benzo [1, 4] dioxin-6′-yl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide-L-tartaric acid salt (Genz-123346) lowered glucose and A1C levels and improved glucose tolerance. Drug treatment also prevented the loss of pancreatic β-cell function normally observed in the Zucker diabetic fatty rat and preserved the ability of the animals to secrete insulin. In the diet-induced obese mouse, treatment with Genz-123346 normalized A1C levels and improved glucose tolerance. Analysis of the phosphorylation state of the insulin receptor and downstream effectors showed increased insulin signaling in the muscles of the treated Zucker diabetic fatty rats and diet-induced obese mice. These results suggest that inhibiting glycosphingolipid synthesis can significantly improve insulin sensitivity and glucose homeostasis and may therefore represent a novel therapeutic approach for the treatment of type 2 diabetes. *Diabetes* 56:1210–1218, 2007

The inability of tissues to respond normally to insulin is a defining characteristic of type 2 diabetes. Insulin signaling is composed of a cascade of phosphorylation events involving the activation of insulin receptor substrate 1 (IRS-1), phosphatidylinositol 3-kinase (PI 3-kinase), Akt, and several other kinases and effector molecules (1). Inflammation, obesity, and abnormal carbohydrate and lipid metabolism have all been associated with the disruption of insulin signaling (2–4). However, the initiating event of the pathway is the binding of insulin to the insulin receptor that activates its tyrosine kinase activity, and impairment of insulin receptor activation can thus also result in insulin resistance.

Gangliosides are sialic acid–containing glycosphingolipids that are known to modulate the activity of a number of receptor tyrosine kinases, including the insulin receptor (5). For example, the tyrosine kinase activity of the epidermal growth factor receptor can be enhanced or repressed by gangliosides GD1a or GM3, respectively (6,7). In addition, the activities of the platelet-derived growth factor (PDGF) receptor and the nerve growth factor receptor TrkA are negatively regulated by overexpression of GM1 (8,9). In both cases GM1 appears to act by displacing the PDGF receptor or TrkA from cholesterol and sphingolipid-enriched subdomains of the cell membrane, which are called lipid rafts or detergent-resistant microdomains (DRMs) (5,10,11).

The critical involvement of gangliosides in insulin resistance was demonstrated by Tagami et al. (12), who observed elevated levels of ganglioside GM3 in adipocytes made insulin resistant by chronic exposure to low levels of tumor necrosis factor-α (TNF-α). Inhibiting GM3 synthesis using D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) reversed the TNF-α–induced impairment of insulin signaling, whereas addition of exogenous GM3 could also mimic the effects of TNF-α. In addition, GM3 synthase mRNA was shown to be upregulated in adipose tissue of Zucker *fa/fa* rats and *ob/ob* mice (12). Furthermore, mutant mice lacking GM3 synthase were found to have increased sensitivity to insulin and were protected from high-fat diet–induced insulin resistance (13). Based on these observations, we hypothesized that a small-molecule inhibitor of glycosphingolipid synthesis could mimic the effects observed in the GM3 synthase knockout mice and increase insulin sensitivity in a diabetic animal model.

RESEARCH DESIGN AND METHODS

Male Zucker diabetic fatty rats (ZDF/GMiCrl-*fa/fa*), 4 weeks old, were purchased from Charles River Laboratories (Wilmington, MA). Male C57BL/6 mice, 4 weeks old, were purchased from The Jackson Laboratories (Bar Harbor, ME). Animals were cared for in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited facility in accordance with the guidelines established by the National Research Council. Studies were conducted using protocols approved by Genzyme's Institutional Animal Care and Use Committee. The animals were allowed to acclimate for 1 week in the facility before the initiation of treatment.

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D-PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; DRM, detergent-resistant microdomain; Genz-123346, (1R,2R)-nonanoic acid[2-(2′,3′-dihydro-benzo [1,4] dioxin-6′-yl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide-L-tartaric acid salt; IRS-1, insulin receptor substrate 1; NB-DNJ, *N*-butyldeoxynojirimycin; PI 3-kinase, phosphatidylinositol 3-kinase; TLC, thin-layer chromatography; TNF-α, tumor necrosis factor-α.

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Generation of diet-induced obese mice. Male C57BL/6 mice, 5 weeks old, were placed on a high-fat (45% of calories) diet (D12451; Research Diets, New Brunswick, NJ) for 8 weeks. The top ~20% in body weight gain were selected for use in the studies and placed in groups such that the treated and control groups had similar starting glucose and insulin levels.

Administration of drug. The synthesis of (1R,2R)-nonanoic acid[2-(2′,3′-dihydro-benzo [1,4] dioxin-6′-yl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide-L-tartaric acid salt (Genz-123346) is described in U.S. patent no. 6855830. Animals were gavaged once per day with a solution of Genz-123346 in water or water alone (placebo control group).

Glucose, insulin, and serum lipid measurements. Blood samples were collected by either tail-vein nick (glucose) or retroorbital plexus puncture (insulin and A1C). Nonfasting glucose levels were measured using an Accu-Chek Compact Meter (Roche Diagnostics, Indianapolis, IN). Insulin levels in plasma were measured using an ELISA kit (Crystal Chem, Downers Grove, IL). A1C levels were measured using A1cNow Monitors (Metrika, Sunnyvale, CA). For the glucose tolerance tests, the animals were fasted overnight and then injected intraperitoneally with glucose (1–2 g/kg body wt). Blood samples were taken at various time points (0–120 min), and glucose levels were measured as described above. Serum lipids (triglycerides, cholesterol, and HDL) were measured by IDEXX Laboratories (West Sacramento, CA).

Extraction and quantitation of glucosylceramide from liver. Levels of glucosylceramide (GL1) in the liver were measured using modifications of previously published protocols (14,15). Briefly, total lipid was extracted by homogenizing 0.1–0.5 g liver tissue in cold PBS using a TissueTearor (BioSpec Products, Bartlesville, OK). Chloroform (2.5 ml) and 1.25 ml methanol was added to 1 ml homogenate and then sonicated in a water bath sonicator for ~8 min. After 1 h incubation at room temperature, the samples were centrifuged at 2,190 × *g* for 30 min. The supernatant was saved, and then the pellets were re-extracted with 2 ml methanol, 1 ml chloroform, and 0.8 ml 0.9% (wt/vol) sodium chloride. After the pellet was sonicated and recentrifuged, the second supernatant was combined with the first supernatant. To the combined supernatants, 4.5 ml 1:1 (vol/vol) chloroform:0.9% NaCl mixture was added, vortexed, and centrifuged at 1,300 × *g* for 5 min. After discarding the upper aqueous layer, 2.25 ml methanol and 1.8 ml 0.9% NaCl were added to the bottom layer, vortexed, and recentrifuged. After again discarding the aqueous layer, 2.25 ml methanol, 1.5 ml water, and 0.3 ml 0.9% NaCl were added and centrifuged at 1,300 × *g* for 5 min. The organic phase was dried down under nitrogen. The samples were redissolved in 2:1 (vol/vol) chloroform:methanol using 1 ml/0.1g tissue and stored at −20°C. A portion of the lipid extract was used to measure total phosphate (16). The remainder was analyzed using high-performance thin-layer chromatography (TLC). Alkaline methanolysis was first performed on the samples to remove phosphatidylethanolamine and similar phospholipids that interfere with GL1 on the TLC plate. Aliquots of the extracts equivalent to the same amount of total phosphate were dried under nitrogen then redissolved in 2 ml chloroform and 1 ml 0.21 N NaOH in methanol. The samples were vortexed and allowed to sit at room temperature 2–16 h. Then 0.4 ml water and 0.4 ml 0.5 N HCl was added. After being vortexed, the samples were centrifuged at 1,300 × *g* for 5 min. The upper layer was discarded and the lower layer was extracted twice with 1 ml methanol and 0.8 ml 0.9% NaCl and then once with 1 ml methanol and 0.8 ml water. The lower layer was dried under nitrogen and stored at −20°C before analysis. The samples were then spotted on to an high-performance TLC plate (Silica 60 HPTLC plate; Merck, Darmstadt, Germany) along with known GL1 standards (Matreya, Pleasant Gap, PA). The lipids were resolved in chloroform:methanol (98:2, vol/vol) followed by chloroform:methanol:water (16:6:1). The plates were then dried, sprayed with 3% (wt/vol) cupric acetate monohydrate and 15% (vol/vol) phosphoric acid, and baked for 10 min at 150°C to visualize the separated lipid bands. The baked TLC plates were scanned on a densitometer (GS-700; Bio-Rad, Hercules, CA) and analyzed by Quantity One software (Bio-Rad).

Immunostaining of tissues. Frozen skeletal muscle sections were stained with an anti-GM3 mouse monoclonal IgM antibody (Seikagaku America, East Falmouth, MA) and an Alexa Fluor–conjugated goat anti-mouse IgM antibody (Molecular Probes, Eugene, OR). For insulin and glucagon staining, paraffin-embedded pancreatic tissue was deparaffinized, rehydrated, and incubated with either a guinea pig anti-swine insulin antibody or a rabbit anti-human glucagon antibody (DakoCytomation, Carpinteria, CA), followed by a peroxidase-labeled goat anti-guinea pig IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and detected using the DAKO Liquid DAB Substrate-Chromogen System (DakoCytomation).

Immunoprecipitation and Western blot analysis. Zucker diabetic fatty rats treated with Genz-123346 for 6 weeks were fasted overnight. The following morning, the fasted rats were anesthetized and injected with 5 units human insulin (Humulin; Eli Lilly, Lafayette, IN) into the hepatic portal vein. Quadriceps muscle and liver were harvested 2 min after injection and immediately frozen in liquid nitrogen. Insulin receptor was immunoprecipitated from muscle homogenates using an anti-IRβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and then the immunoprecipitates were analyzed by immunoblotting using an anti-phosphotyrosine antibody (Clone PY20; BD BioSciences, San Jose, CA) to measure phosphorylation levels or using the anti-IRβ antibody to measure insulin receptor protein levels. Akt was analyzed from liver homogenates by immunoblotting using an anti–phospho-Akt (Thr308) antibody (Cell Signaling Technology, Beverly, MA).

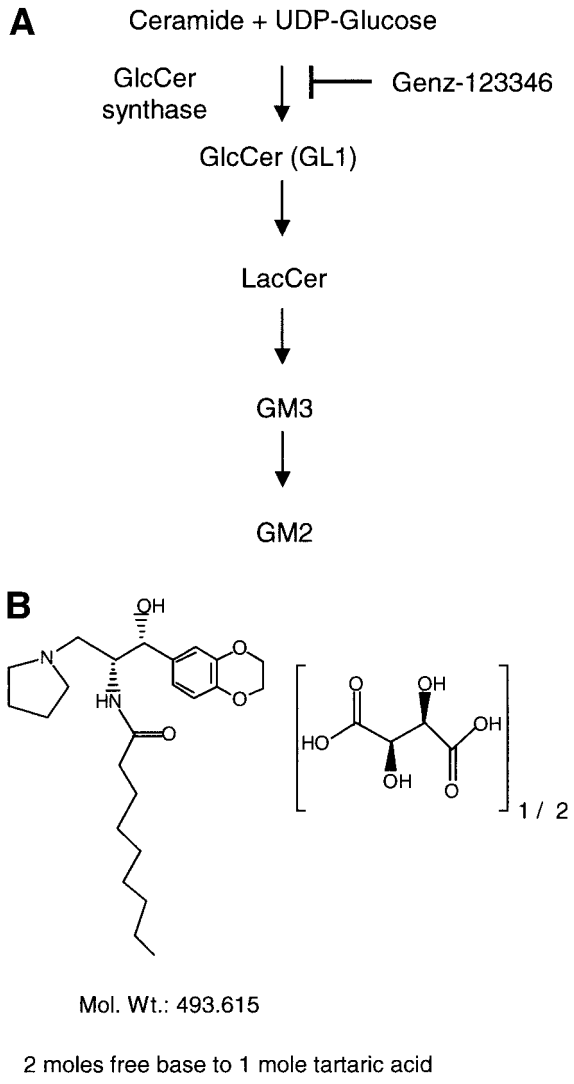

FIG. 1. *A*: Step in the synthesis of glycolipids inhibited by the GL1 synthase inhibitor Genz-123346. *B*: Structure of Genz-123346. The compound contains 2 mol free base/mol tartaric acid.

Statistical analysis. Data were evaluated using the two-tailed unpaired Student's *t* test (when two groups were analyzed) or one-way ANOVA followed by Bonferroni's post hoc test (for three or more groups) using the StatView software program (SAS Institute, Cary, NC). Data were considered significant if $P < 0.05$.

RESULTS

Characterization of Genz-123346. Genz-123346 (Fig. 1) is an inhibitor of GL1 synthase that blocks the conversion of ceramide to GL1, the first step in the biosynthesis of gangliosides and other glycosphingolipids (17). This compound was derived from one of the series of inhibitors of GL1 synthase described by Lee et al. (18). Unlike the original parent compound D-PDMP (19), the improved inhibitors and Genz-123346 have little effect on 1-*O*-acylceramide synthase activity at concentrations that effectively inhibit GL1 synthase activity and therefore do not

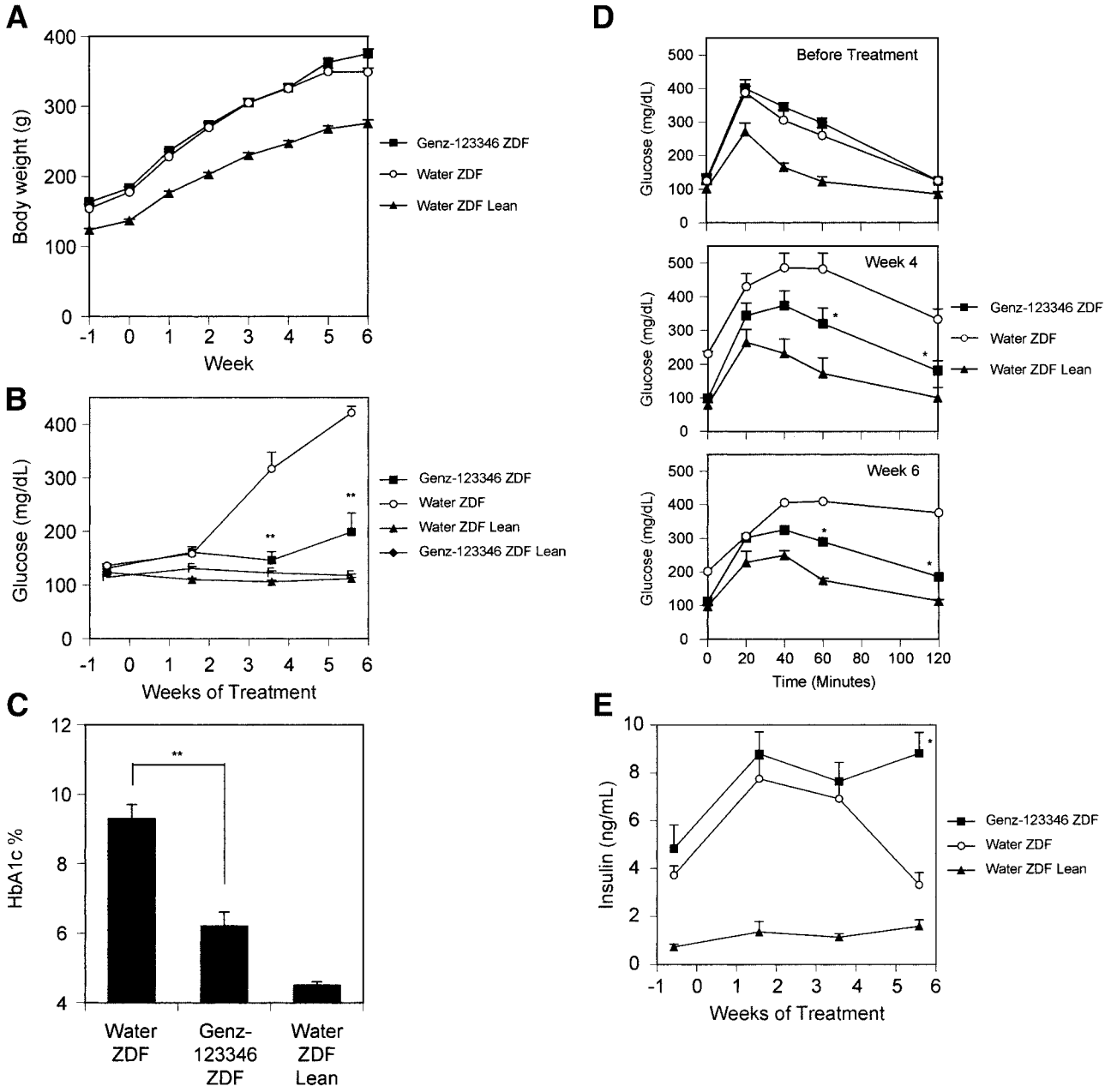

FIG. 2. Effect of Genz-123346 on glucose and insulin levels in Zucker diabetic fatty rats. Beginning at 6 weeks of age, rats were gavaged daily with Genz-123346 (75 mg/kg) or water for 6 weeks. *A*: Body weights. *B*: Nonfasting glucose levels. *C*: A1C levels after 6 weeks of treatment. *D*: Glucose tolerance tests before and after 4 and 6 weeks of treatment. Animals were fasted overnight and then injected intraperitoneally with glucose (2 g/kg body wt). *E*: Nonfasting insulin levels. Data are shown as means ± SE (n = 6 rats per group). *$P < 0.05$, **$P < 0.01$ (Genz-123346 Zucker diabetic fatty vs. Water Zucker diabetic fatty groups).

significantly elevate cell ceramide levels in vitro (18). Also, unlike *N*-butyldeoxynojirimycin (NB-DNJ), which is another inhibitor of GL1 synthase, Genz-123346 does not inhibit α-glucosidase or glucocerebrosidase (20,21). In addition to the improved specificity, Genz-123346 is a much more potent inhibitor than NB-DNJ when measured in in vitro assays of ganglioside inhibition (e.g., IC50 for GM1 inhibition = 14 nmol/l for Genz-123346 vs. >50 μmol/l for NB-DNJ).

Studies in normal mice dosed orally with Genz-123346 showed that the drug was well tolerated at doses up to 250 $mg \cdot kg^{-1} \cdot day^{-1}$. However, a loss of body weight of >5% was observed after 3 days of oral dosing at 500 $mg \cdot kg^{-1} \cdot day^{-1}$. The oral bioavailability of the drug was shown to be ~10 and 30% in mice and rats, respectively, with a half-life in plasma of 30–60 min. A dose-dependent reduction of GL1 was observed in the liver after 3 days of oral dosing normal mice with Genz-123346, with a 30% reduction of GL1 levels at the dose of 150 $mg \cdot kg^{-1} \cdot day^{-1}$ (data not shown).

Effect of Genz-123346 on glucose and insulin levels in Zucker diabetic fatty rats. To evaluate the effects of inhibiting glycosphingolipid synthesis on glucose homeostasis, Genz-123346 was administered to Zucker diabetic fatty rats by daily gavage (75 $mg \cdot kg^{-1} \cdot day^{-1}$) for 6 weeks. Zucker diabetic fatty obese and lean rats gavaged with water served as controls. Although a slight (~10%) decrease in food consumption was observed in the drug-

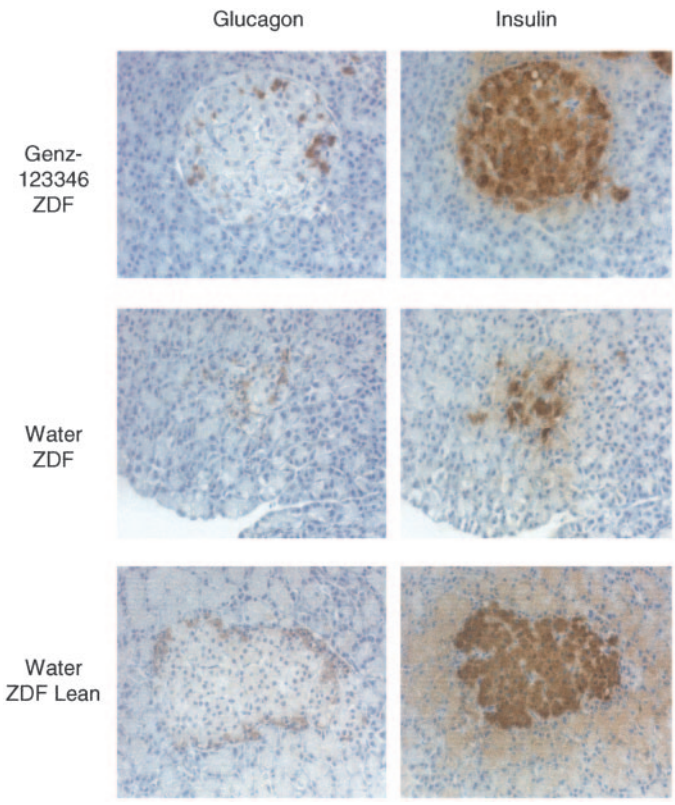

FIG. 3. Partial preservation of pancreatic islet architecture and function in Zucker diabetic fatty rats treated with Genz-123346. Rats were gavaged daily with Genz-123346 (85 mg/kg) for 10 weeks. The pancreas was sectioned and stained for glucagon or insulin. Shown are representative islets from each group.

treated animals, there was no effect on body weight gain (Fig. 2*A*). As expected for this severely hyperglycemic model (22), nonfasting glucose levels in the placebo-treated Zucker diabetic fatty rats increased markedly over the course of the study, exceeding 400 mg/dl (Fig. 2*B*). In contrast, glucose levels from the drug-treated rats increased to only ~200 mg/dl after 6 weeks of treatment. Treatment of lean rats with Genz-123346 had no effect on glucose levels (Fig. 2*B*). The levels of A1C in the serum were significantly lower in the drug-treated (6.2%) versus placebo-treated (9.3%) groups (Fig. 2*C*). Glucose tolerance tests performed after 4 and 6 weeks of treatment also showed an improved response to glucose challenge in the drug-treated group at both time points (Fig. 2*D*).

Nonfasting insulin levels in both the drug- and placebo-treated groups increased over the first 11 days of the study, likely in response to the rapid development of insulin resistance as the Zucker diabetic fatty rats aged (Fig. 2*E*). After 3.5 weeks, insulin levels in both the drug- and placebo-treated groups remained elevated. It should be noted that by this time point, glucose levels were markedly higher in the placebo-treated but not drug-treated animals (Fig. 2*B*), suggesting that drug treatment had improved insulin sensitivity. After 6 weeks, insulin levels in the placebo-treated group dropped precipitously, coinciding with the characteristic progressive loss of pancreatic β-cell function in these animals (22). In contrast, insulin levels in the drug-treated group remained elevated at the 6-week time point, indicating continued presence of β-cell function. The persistent hyperinsulinemia in the treated animals suggests that Genz-123346 is unable to completely correct the profound insulin resistance in this model.

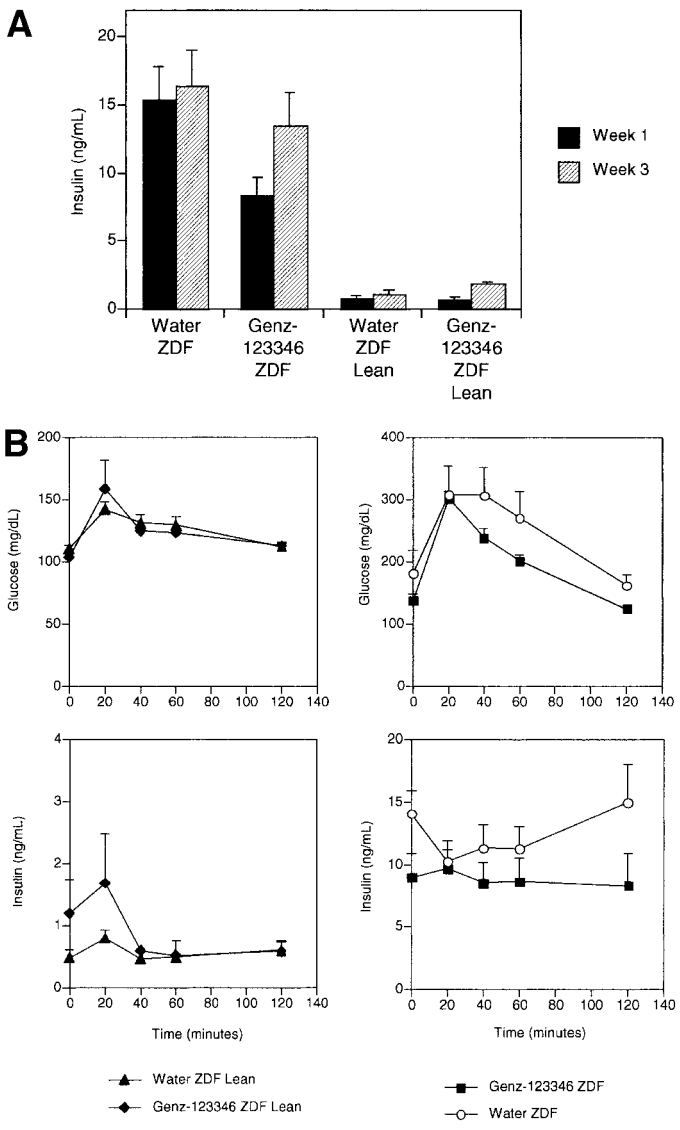

FIG. 4. Effect of Genz-123346 on insulin secretion from lean animals and during glucose challenge. Beginning at 6 weeks of age, Zucker diabetic fatty obese and heterozygous lean rats were gavaged daily with Genz-123346 (120 mg/kg) or water for 3 weeks. *A*: Nonfasting insulin levels after 1 and 3 weeks of treatment. *B*: Glucose tolerance tests and insulin levels after 1 week (Zucker diabetic fatty lean animals) or 3 weeks (Zucker diabetic fatty obese animals) of treatment. Animals were fasted overnight and then injected intraperitoneally with glucose (1 g/kg body wt). Data are shown as means ± SE (n = 4–6 rats per group).

The morphology of pancreatic islets after treatment with Genz-123346. The preservation of insulin production in the drug-treated rats was confirmed by immunohistological staining of the pancreas. Animals were treated for an additional 4 weeks, and at 16 weeks of age, the islets from the placebo-treated Zucker diabetic fatty rats were poorly defined and highly disorganized, with displacement of glucagon staining cells from the periphery and few cells staining positive for insulin (Fig. 3). In contrast, the islets from the drug-treated rats had significantly fewer structural alterations, greater number of insulin-positive cells, and more closely resembled the islets from the lean animals. Treatment of lean rats with Genz-123346 had no effect on islet morphology or the intensity of insulin staining (data not shown). The results suggest that drug treatment prolonged pancreatic β-cell function in the

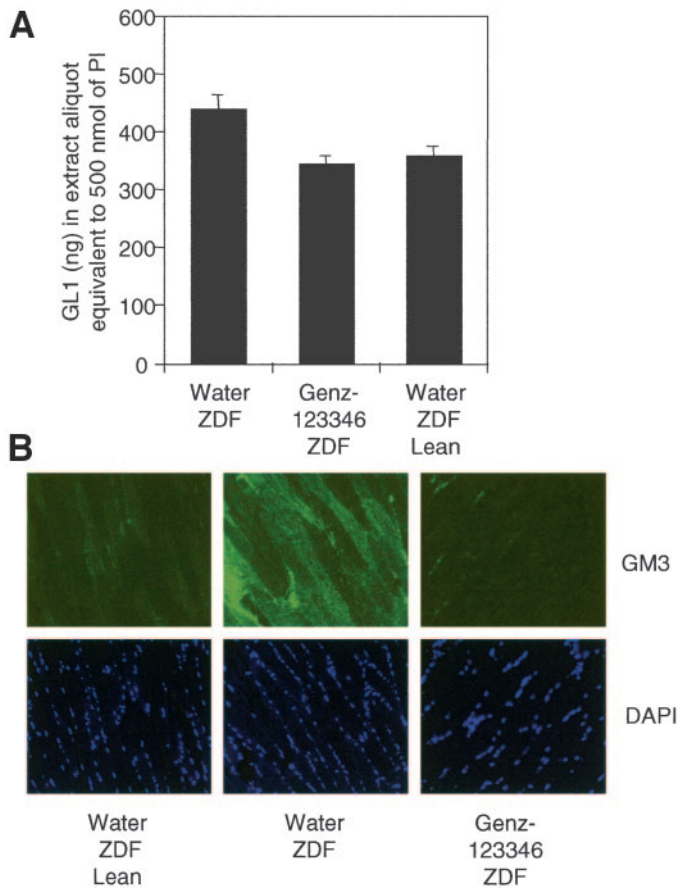

FIG. 5. Reduction of gangliosides GL1 and GM3 in Zucker diabetic fatty rats after treatment with Genz-123346. Rats were gavaged daily with Genz-123346 (85 mg/kg) or water for 10 weeks. *A*: GL1 was extracted from livers and analyzed by TLC. Shown are GL1 levels in aliquots of liver extract equivalent to 500 nmol total inorganic phosphate (PI). Data are shown as means ± SE (n = 6 rats per group). *B*: Quadriceps muscles were frozen, sectioned, and stained using an anti-GM3 antibody. Representative sections from each group are shown.

Zucker diabetic fatty rat, either via a direct effect on the pancreas or (perhaps more likely) indirectly by decreasing the glucotoxicity in the islets.

Effect of Genz-123346 on insulin secretion from lean animals and during glucose challenge. We next asked whether treatment with the compound lowered glucose levels by primarily affecting insulin secretion. Zucker diabetic fatty obese and heterozygous lean rats were gavaged daily with Genz-123346 (120 mg/kg) for 3 weeks. As observed before (Fig. 2*E*), the Zucker diabetic fatty obese animals became hyperinsulinemic, and drug treatment for 1 or 3 weeks had no significant effect on nonfasting insulin levels (Fig. 4*A*). Treatment of lean animals with Genz-123346 likewise had no effect on insulin levels at either time point.

Insulin levels were also measured during glucose challenge. In Zucker diabetic fatty lean rats after 1 week of treatment, a minimal rise in glucose levels was observed in the lean animals (Fig. 4*B*) in response to intraperitoneal injection of glucose. Insulin levels rose slightly higher in the drug-treated versus placebo-treated animals, but this was not statistically significant. In Zucker diabetic fatty obese rats after 3 weeks of treatment, an improved response to glucose challenge was observed in the drug-treated group (Fig. 4*B*). Insulin levels in the drug-treated animals did not increase, and the levels were comparable with the placebo-treated group during this time course. Thus, Genz-123346 did not significantly affect either nonfasting circulating levels of insulin or insulin levels in response to a bolus glucose injection, suggesting that the glucose-lowering effect of the drug was not primarily due to altering insulin secretion.

Effect of Genz-123346 on serum lipid levels. Serum triglyceride, cholesterol, and HDL levels were measured in the Zucker diabetic fatty rats after 5 weeks of drug treatment. As expected, elevated levels of triglycerides and cholesterol were present in the obese animals compared with the lean groups (Table 1). A slight decrease in serum triglycerides was observed in the Genz-123346–treated Zucker diabetic fatty rats compared with the placebo control group, as well as an increase in cholesterol and HDL levels. However, these changes were not statistically significant (P = 0.4–0.6). Thus, inhibiting glycosphingolipid synthesis had no effect on serum lipid levels at this time point.

GL1 and GM3 levels in control and treated Zucker diabetic fatty rats. Next, we examined the levels of GL1 and GM3 in tissues from control and treated Zucker diabetic fatty rats. GL1 was extracted from the livers of the rats that had received Genz-123346 for 6 weeks and analyzed by TLC. Slightly elevated levels of GL1 were observed in the livers from the obese animals compared with their lean counterparts (Fig. 5*A*), and treatment with Genz-123346 decreased GL1 to levels similar to that of the lean animals. As expected, drug treatment was found to have no effect on liver ceramide levels compared with the placebo-treated group (data not shown). We also analyzed the levels of GM3 in skeletal muscle sections by immunofluorescent staining using an anti-GM3 antibody. More intense fluorescence was observed in muscle sections from the obese Zucker diabetic fatty animals compared with their lean controls (Fig. 5*B*). Treatment with Genz-123346 decreased the level of fluorescent staining to that of the lean animals. These results show that treatment of Zucker diabetic fatty rats with Genz-123346 inhibited ganglioside synthesis and normalized tissue ganglioside levels in vivo.

Effects of Genz-123346 in diet-induced obese mice. We next examined the effects of Genz-123346 in C57BL/6 mice that had been placed on a high-fat (45% of kcal) diet. After feeding the diet for 8 weeks, obese mice with

TABLE 1
Effect of Genz-123346 on serum lipid levels in Zucker diabetic fatty rats after 5 weeks of treatment

Group	Triglycerides (mg/dl)	Cholesterol (mg/dl)	HDL (mg/dl)
Genz-123346 Zucker diabetic fatty	679 ± 96	163 ± 7	60 ± 2
Water Zucker diabetic fatty	797 ± 88	141 ± 1	51 ± 3
Water Zucker diabetic fatty lean	66 ± 5	83 ± 4	38 ± 3
Genz-123346 Zucker diabetic fatty lean	73 ± 12	107 ± 14	40 ± 4

Data are means ± SE.

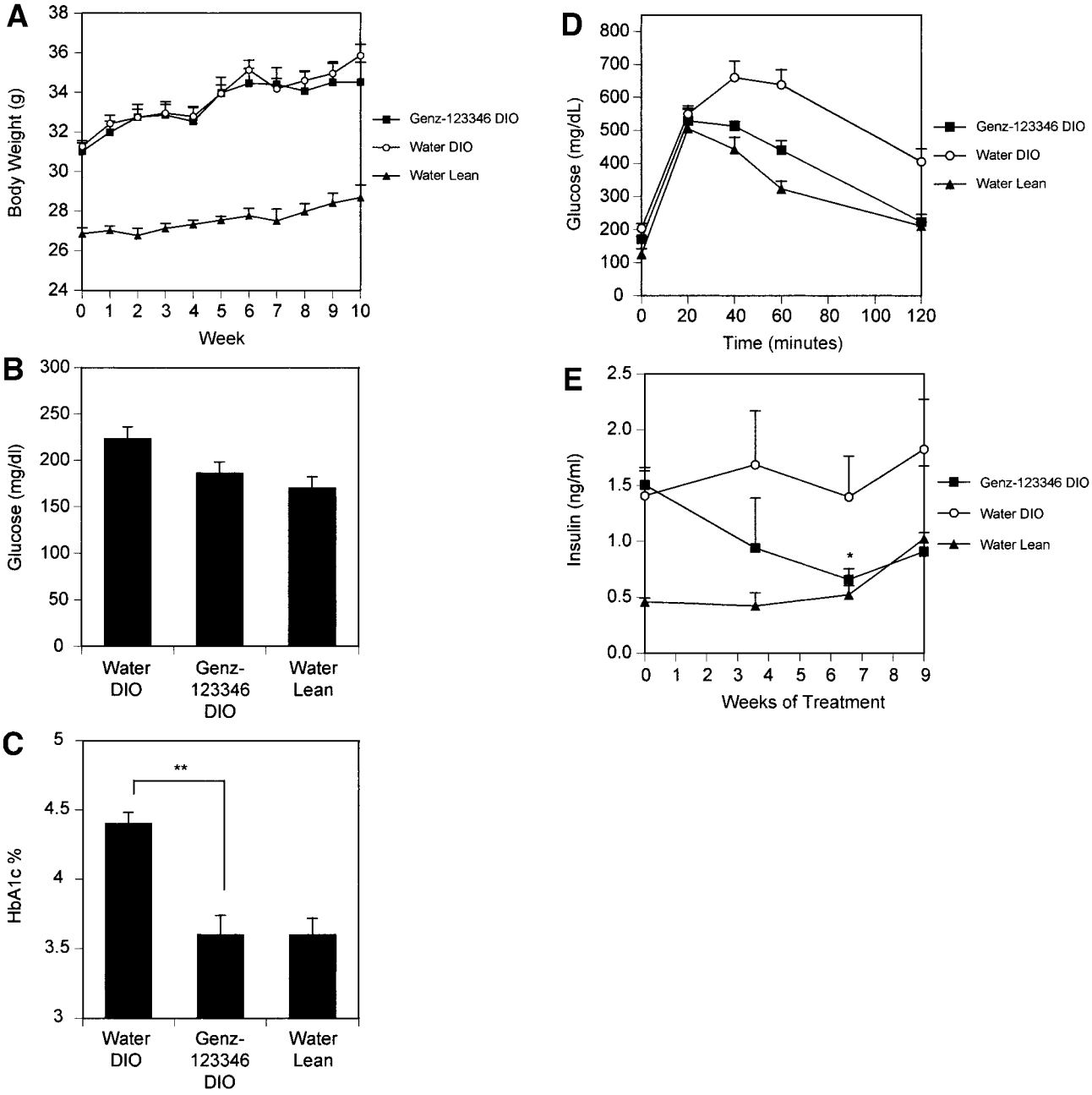

FIG. 6. Effect of Genz-123346 on glucose and insulin levels in diet-induced obese mice. After being placed on a high-fat diet for 8 weeks, obese mice were selected and then gavaged daily with Genz-123346 (125 mg/kg) or water for 10 weeks. *A*: Body weights during treatment with Genz-123346. Nonfasting glucose levels (*B*), A1C levels (*C*), and glucose tolerance test (*D*) after 10 weeks of treatment. *E*: Nonfasting insulin levels. Data are shown as means ± SE (n = 6–10 mice per group). *$P < 0.05$, **$P < 0.01$ (Genz-123346 diet-induced obese vs. water diet-induced obese groups).

comparable body weight gain, glucose, and insulin levels were assigned to either the treated or control groups. The mice were then gavaged daily with Genz-123346 or water for 10 weeks. No significant difference in food consumption was observed between the drug- versus placebo-treated animals (data not shown). In some studies, the mice that received drug exhibited decreased weight gain after the initiation of treatment, but in general, this decrease was minimal (Fig. 6*A*). Mice treated with Genz-123346 exhibited slightly lower nonfasting glucose levels after 10 weeks of treatment, although the decrease was not statistically significant (Fig. 6*B*). However, A1C levels in the drug-treated animals were significantly ($P < 0.005$) lower and equivalent to that of the lean animals (Fig. 6*C*). In addition, the treated animals exhibited a markedly improved response to glucose challenge (Fig. 6*D*). In this model, we observed increased glucose tolerance within ~4 weeks of treatment (not shown), but the longer treatment regimen was required to observe a significant reduction in A1C levels. Treatment of lean mice with Genz-123346 had no effect on glucose levels, A1C, or glucose tolerance (data not shown). Finally, nonfasting insulin levels remained elevated over the course of the study in the control animals but declined in the animals treated with Genz-123346 (Fig. 6*E*). Because β-cell function is not appreciably altered in the diet-induced obese mice (unlike in the Zucker diabetic fatty rats), the

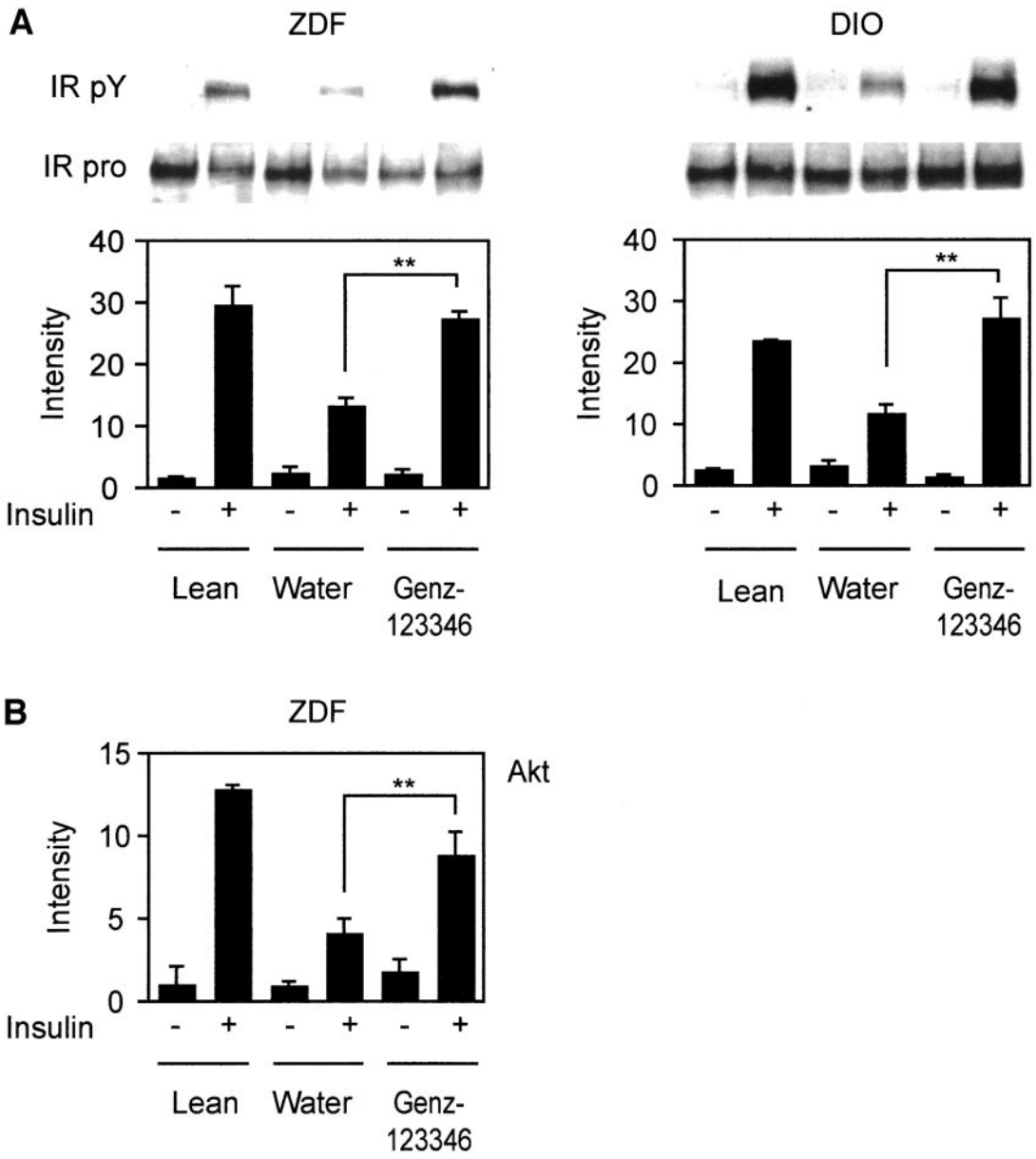

FIG. 7. ***A*****: Increased insulin-stimulated activation of the insulin receptor in Zucker diabetic fatty rats and diet-induced obese mice after treatment with Genz-123346. Rats were gavaged daily with Genz-123346 (75 mg/kg) for 6 weeks, and mice were gavaged daily with Genz-123346 (125 mg/kg) for 10 weeks. Insulin or saline was injected into the hepatic portal vein just before harvesting the muscle (see RESEARCH DESIGN AND METHODS). The β-subunit of the insulin receptor was then analyzed for the level of tyrosine phosphorylation (IR pY) or protein levels (IR pro). Representative signals from each group and the relative intensities of the phosphotyrosine bands quantitated from the Western blots are shown (arbitrary units; two to three animals per group).** ***B*****: Effect of drug treatment on Akt phosphorylation in the muscle of Zucker diabetic fatty rats. Insulin was injected as in** ***A*** **just before harvesting the tissues, and the level of Akt Thr308 phosphorylation was quantitated from the Western blots (arbitrary units; two to three animals per group.** $^{**}P < 0.005$ **(Genz-123346 Zucker diabetic fatty/diet-induced obese vs. Water Zucker diabetic fatty/diet-induced obese groups).**

lower insulin levels suggest an increase in insulin sensitivity as a result of drug treatment.

Effect of Genz-123346 on insulin signaling in muscle. To investigate the mechanism by which Genz-123346 improved glucose control and increased insulin sensitivity, we examined the phosphorylation of the insulin receptor in response to acute insulin stimulation in the treated Zucker diabetic fatty rats and diet-induced obese mice. The level of tyrosine phosphorylation of the insulin receptor from the skeletal muscle of the drug-treated Zucker diabetic fatty rats was increased significantly compared with that of the placebo-treated rats, to levels comparable with that of lean animals (Fig. 7*A*). A similar increase in insulin-stimulated muscle insulin receptor tyrosine phosphorylation was observed in the drug-treated diet-induced obese mice (Fig. 6*A*). We also examined the phosphorylation of Akt, a downstream protein activated by insulin receptor signaling. In the muscles of Zucker diabetic fatty rats, the level of Akt Thr308 phosphorylation was greatly reduced in the placebo-treated group compared with the lean animals (Fig. 7*B*). Drug treatment increased the level of Akt phosphorylation after insulin stimulation.

DISCUSSION

In summary, the GL1 synthase inhibitor Genz-123346 improved glucose tolerance and increased insulin sensitivity

in two animal models of type 2 diabetes. In Zucker diabetic fatty rats, treatment with Genz-123346 significantly lowered blood glucose levels and consequently partially prevented the normal deterioration of pancreatic β-cell function over time and preserved the ability of the islets to secrete insulin. In the high-fat–fed diet-induced obese mice, Genz-123346 essentially normalized A1C levels to that of the lean animals. The improvement in A1C levels was quite persistent, extending >4 months in the diet-induced obese mice (data not shown). Taken together, these results highlight the important role of glycosphingolipids in insulin signaling and glucose control.

Because Genz-123346 inhibits the initial step in the synthesis of a number of glycosphingolipids, the identity of the critical glycosphingolipid(s) is unknown, although previous studies have identified several candidates capable of modifying insulin receptor activity in vitro. When different gangliosides were incubated with the partially purified insulin receptor, 2-3 sialosylparagloboside was found to be the most effective in inhibiting insulin receptor phosphorylation (23). Other investigators have shown that gangliosides GM1 and GM2 effectively inhibited insulin receptor phosphorylation in vitro (24). Tagami et al. (12) demonstrated that exogenously adding GM3 to 3T3-L1 adipocytes inhibited insulin receptor phosphorylation and impaired glucose uptake. These data suggest that, depending on the cell type, different gangliosides may be effective in interacting with and inhibiting insulin receptor activity.

Regardless of which ganglioside is most important, the mechanism by which gangliosides mediate insulin resistance may involve, based on the observed interactions with other receptor tyrosine kinases, either direct binding of gangliosides to the insulin receptor or ganglioside-mediated displacement of the insulin receptor from DRMs in the cell membrane (11). The insulin receptor has been shown to be associated with DRMs, and depletion of either cholesterol or caveolin-1, two components of DRMs, inhibits insulin signaling (25,26). Chronic exposure of 3T3-L1 adipocytes to TNF-α induces elevated expression of GM3, with a twofold increase in the levels of GM3 present in the DRMs. The insulin receptor was shown to be selectively eliminated from these GM3-enriched DRMs, and insulin signaling was inhibited (27). Additional evidence for DRMs modulating insulin receptor activity is the observation that incubating hepatocytes with an anti-GM2 antibody causes clustering of GM2 and inhibition of insulin receptor phosphorylation (26).

Given the ubiquitous expression of the insulin receptor, inhibiting glycosphingolipid synthesis is likely affecting multiple tissues involved in glucose homeostasis, including the liver, pancreas, and adipose tissue. We observed increased insulin signaling in the muscle in response to drug treatment, which is consistent with the increased insulin receptor phosphorylation and glucose uptake observed in the transgenic GM3 synthase knockout mice (13). However, the knockout mice also exhibited reduced glucose production from the liver, indicating an important role for gangliosides in this organ as well (13). Hyperinsulinemic-euglycemic clamp studies will clarify the effect of Genz-123346 on insulin-stimulated uptake of glucose into the different tissues.

Also to be determined is the relationship between the effects of elevated glycosphingolipids and the effects of ceramide, which accumulates in insulin-resistant tissues and was shown to inhibit GLUT4 translocation and block the activation of Akt and possibly IRS-1 and PI 3-kinase (28–30). Ceramide has also been implicated in the lipoapoptosis of pancreatic β-cells (31,32). Given that we observed partial protection from β-cell destruction in the drug-treated Zucker diabetic fatty rats, additional studies are needed to relate the possible effects of inhibiting glycosphingolipid synthesis on the lipoapoptotic pathway.

In conclusion, the data shown here connect what has been observed to date only in vitro (i.e., TNF-α mediated elevation of gangliosides leading to the disruption of lipid rafts and insulin resistance and the restoration of signaling by a glycosphingolipid inhibitor) to the improvement in glucose homeostasis observed in vivo in the GM3 synthase transgenic knockout mouse. In addition, the results suggest that modulating glycosphingolipid synthesis may be a novel and highly effective approach for the treatment of type 2 diabetes. The long-term consequences of chronically inhibiting ganglioside synthesis remain to be determined and may be a concern, given the important role of gangliosides in cell-to-cell interaction, cell differentiation, and cell surface signaling, especially in the brain (5). However, our results suggest that small changes in ganglioside levels, from elevated to normal, may be sufficient to restore insulin sensitivity. Thus, glycosphingolipid synthesis is a potentially important and underexploited pathway that may provide new therapeutic targets to treat diabetes and a number of other diseases.

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