

# Reduction in Glucagon Receptor Expression by an Antisense Oligonucleotide Ameliorates Diabetic Syndrome in *db/db* Mice

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**Excess glucagon levels contribute to the hyperglycemia associated with type 2 diabetes. Reducing glucagon receptor expression may thus ameliorate the consequences of hyperglucagonemia and improve blood glucose control in diabetic patients. This study describes the antidiabetic effects of a specific glucagon receptor antisense oligonucleotide (GR-ASO) in *db/db* mice. The ability of GR-ASOs to inhibit glucagon receptor mRNA expression was demonstrated in primary mouse hepatocytes by quantitative real-time RT-PCR. Intraperitoneal administration of GR-ASO at a dosage of 25 mg/kg twice a week in *db/db* mice for 3 weeks resulted in 1) decreased glucagon receptor mRNA expression in liver; 2) decreased glucagon-stimulated cAMP production in hepatocytes isolated from GR-ASO-treated *db/db* mice; 3) significantly reduced blood levels of glucose, triglyceride, and free fatty acids; 4) improved glucose tolerance; and 5) a diminished hyperglycemic response to glucagon challenge. Neither lean nor *db/db* mice treated with GR-ASO exhibited hypoglycemia. Suppression of GR expression was also associated with increased (~10-fold) levels of plasma glucagon. No changes were observed in pancreatic islet cytoarchitecture, islet size, or  $\alpha$ -cell number. However,  $\alpha$ -cell glucagon levels were increased significantly. Our studies support the concept that antagonism of glucagon receptors could be an effective approach for controlling blood glucose in diabetes. *Diabetes* 53:410–417, 2004**

**I**ncreased hepatic glucose production contributes significantly to hyperglycemia in type 2 diabetic patients (1). Glucagon, a peptide hormone released by the  $\alpha$ -cell of pancreatic islets, plays a key role in regulating hepatic glucose production and has a profound hyperglycemic effect (2). After binding to the glucagon

receptor, glucagon activates adenylyl cyclase in the hepatocyte plasma membrane and triggers glycogenolysis via a cAMP-related signaling pathway. In addition, glucagon activates multiple enzymes required for gluconeogenesis, especially the enzyme system for converting pyruvate to phosphoenolpyruvate, the rate-limiting step in gluconeogenesis (3,4). It has been proposed that hyperglucagonemia is a causal factor in the pathogenesis of diabetes (5) based on the following observations: 1) diabetic hyperglycemia, from animal to human studies, is consistently accompanied by relative or absolute hyperglucagonemia (6); 2) infusion of somatostatin inhibits endogenous glucagon release, which in turn reduces blood glucose levels in dogs with diabetes induced by alloxan or diazoxide (7); and 3) chronic glucagon infusion leads to hepatic insulin resistance in humans (8).

Results from recent studies characterizing glucagon receptor knockout mice or using glucagon receptor antagonists have further suggested that interfering with glucagon's binding to its receptor could be a potentially effective approach for improving glycemic control in diabetes (9). In glucagon receptor knockout mice (10–12), blood glucose levels were significantly reduced under both fasted and fed conditions compared with levels in wild-type littermates. In addition, these knockout mice showed a marked improvement in glucose tolerance. A number of glucagon receptor antagonists have been developed recently. Some of these potent antagonists have been shown to effectively lower fasting blood glucose in mice (13). In humans, a glucagon receptor antagonist, Bay 27-9955, significantly inhibits hepatic glucose production and blocks the hyperglycemic effects caused by glucagon infusion (14). Thus, the preponderance of evidence in lean animals and normal subjects suggests that the glucagon receptor is a potential target for type 2 diabetes. This hypothesis needs to be further evaluated under diabetic conditions, both in animal and human models.

Using antisense oligonucleotides (ASOs) to reduce target gene expression is a novel approach for treating various diseases, including metabolic disorders (15,16). Studies have shown that systemic administration of ASOs to animals results in significant ASO accumulation in the liver. The glucagon receptor is expressed predominantly in the liver and, therefore, is a suitable target for applying ASO technology. In the present study, we used a specific glucagon receptor ASO (GR-ASO) to treat diabetic *db/db* mice and to assess the impact of reduced glucagon recep-

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ASO, antisense oligonucleotide; EC<sub>50</sub>, half-maximal effective concentration; FFA, free fatty acid; GLP-1, glucagon-like peptide 1; GR-ASO, glucagon receptor ASO; NIH, National Institutes of Health; RIA, radioimmunoassay.

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tor expression on the diabetic syndrome. GR-ASO treatment decreased glucagon receptor mRNA expression in liver by 83%, reduced glucagon-stimulated hepatocyte cAMP formation, significantly ameliorated the diabetic syndrome, markedly improved the glucose handling during an oral glucose tolerance test, and lowered hyperglycemic response to a glucagon challenge.

## RESEARCH DESIGN AND METHODS

### Antisense oligonucleotide design and evaluation

**Oligonucleotides.** A series of ASOs was designed to target the mouse glucagon receptor sequence (NM 008101). The oligonucleotides were evaluated for their ability to suppress glucagon receptor mRNA expression in mouse primary hepatocytes by quantitative real-time PCR. All oligonucleotides were synthesized as uniform phosphorothioate chimeric oligonucleotides, with 2'-O-methoxyethyl groups on bases 1–5 and 16–20. The oligonucleotides were synthesized using an Applied Biosystems 380B automated DNA synthesizer (PerkinElmer-Applied Biosystems) and purified as previously described (17). The active ASO and a chemistry control oligonucleotide used in these studies were designated as follows: ASO: ISIS 148359, 5'-AGCAGGCTTAGGTTGTGGTG-3', target site beginning at position 227; control oligonucleotide: ISIS 129694, 5'-GTACAGTTATGCGCGGTAGA-3'.

**Cell culture.** Primary mouse (Balb/c) hepatocytes were isolated and cultured as previously described (18). Briefly, primary hepatocytes were cultured overnight in Williams E medium supplemented with 10 mmol/l HEPES, 2 mmol/l L-glutamine, and 1× antibiotic/antimycotic (Gibco, Rockville, MD) before oligonucleotide transfection. Cells were washed with PBS, then treated for 4 h in serum-free OptiMEM with antisense or control oligonucleotides, in the presence of 2.5 μl lipofectin (Invitrogen, Carlsbad, CA) · 100 nmol/l oligo<sup>-1</sup> · ml OptiMEM<sup>-1</sup> or an equivalent amount of lipofectin alone. After oligonucleotide treatment, culture medium was replaced with complete Williams E medium and incubated for an additional 12–18 h. Total RNA was isolated using an RNeasy Mini preparation kit (Qiagen, Valencia, CA) following the manufacturer's instructions.

**Tissue RNA isolation.** After subjects were killed, their liver, skeletal muscle, pancreas, and white and brown fat were isolated, snap-frozen in liquid nitrogen, and stored at -80°C. Total RNA was prepared from tissues as previously described (19). Briefly, total RNA was isolated by homogenization of the tissue in guanidinium isothiocyanate then by centrifugation over a cesium chloride gradient. The RNA pellet was resuspended in RNase-free water and further purified using an RNeasy Mini preparation kit (Qiagen, Valencia, CA) following the manufacturer's instructions.

**RNA expression analysis.** Target mRNA from cell culture experiments or tissue was analyzed by quantitative real-time RT-PCR, as described elsewhere (20). Briefly, ~200 ng of total RNA were analyzed in a final volume of 50 μl containing 200 nmol/l glucagon receptor-specific PCR primers, 0.2 mmol/l each dNTP, 75 nmol/l fluorescently labeled oligonucleotide probe, 1× RT-PCR buffer, 5 mmol/l MgCl<sub>2</sub>, 2 units of platinum *Taq*DNA polymerase (Invitrogen), and 8 units of ribonuclease inhibitor. Reverse transcription was performed for 30 min at 48°C followed by PCR: 40 thermal cycles of 30s at 94°C and 1 min at 60°C using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Target mRNA was normalized to total RNA determined by ribogreen fluorescence from the same RNA samples.

**In vivo animal study design.** The female *db/db* mice (C57BL/KsJ-*Lep<sup>db</sup>*; Jackson Laboratories, Bar Harbor, ME) and their lean littermates (C57BL/KsJ-*Lep<sup>+/+</sup>*) used were ages 6–7 weeks. The *db/db* mice were evenly divided into two groups based on blood glucose and body weight. The treatment group received 25 mg/kg GR-ASO in saline dosed twice per week intraperitoneally for 3 weeks. The mice in the control groups were dosed with a control ASO (which has no effect on glucagon receptor mRNA tested *in vitro*) at the same dosage and time as the GR-ASO-treated mice. After 3 weeks of treatment, a portion of mice from the control ASO- or GR-ASO-treated group were killed to collect blood samples for biochemical analysis. Various tissues were taken from these mice and stored at -80°C for determining glucagon receptor mRNA levels, liver glycogen, and triglyceride contents or for pancreatic morphology analysis. The rest of the mice from both groups were subjected to an oral glucose tolerance test, glucagon challenge, insulin tolerance test, or studies of glucagon effect in hepatocyte. As a control, the lean littermates of these *db/db* mice were treated in a manner similar to the *db/db* mice.

**Oral glucose tolerance test.** In the morning after an overnight fast, the mice received an oral glucose challenge (2 g/kg body wt, via gavage). Tail blood samples (10 μl) were collected at 0, 30, 60, and 120 min after glucose administration for measurement of blood glucose and plasma insulin.

**Glucagon challenge test.** Glucagon (300 μg/kg) was administered intraperi-

toneally to mice from both control- and GR-ASO-treated groups in the fed condition. Tail blood samples (3 μl) were collected at 0, 15, 30, 60, and 90 min after glucagon injection for measurement of blood glucose levels.

**Insulin tolerance test.** Mice received an intraperitoneal insulin injection under fed conditions (3 units/kg body wt). Tail blood samples (3 μl) were collected at 0, 15, 30, 60, and 90 min for measurement of blood glucose.

### Biochemical analyses

**Blood chemistry.** Serum levels of glucose and triglycerides and plasma levels of aspartate aminotransferase, alkaline phosphatases, and alanine aminotransferase under fed conditions were measured using a COBAS Mira Plus blood chemistry analyzer (Roche Diagnostic Systems, Indianapolis, IN). A mouse insulin enzyme-linked immunosorbent assay kit (ALPCO, Windham, NH) was used to measure insulin concentrations in blood. Plasma glucagon levels were measured using a radioimmunoassay (RIA) kit purchased from Linco (St. Charles, MO). Serum free fatty acids (FFAs) was measured using a nonesterified fatty acid C kit (Wako Chemicals, Neuss, Germany). During the oral glucose tolerance, insulin tolerance, and glucagon challenge tests, blood glucose levels were measured using a glucometer (One Touch Ultra; Lifescan, Milpitas, CA).

**Liver membrane glucagon receptor binding.** Livers obtained from *db/db* mice after 3 weeks of treatment with control or GR-ASO were frozen and used to prepare crude liver membrane by homogenization and then differential centrifugation. Final membrane suspension was in a buffer of 10 mmol/l Tris (pH 7.5). Glucagon binding was determined in a 96-well plate by combining membrane (50–150 μg) and <sup>125</sup>I-labeled glucagon (2–4 fmol) in an incubation buffer of 20 mmol/l Tris (pH 7.5); 1 mmol/l dithiothreitol; 0.1% BSA; 5 μg/ml each aprotinin, leupeptin, and pepstatin A; 50 μg/ml bacitracin; and 1 mmol/l Pefabloc (Roche Diagnostics). In nonspecific binding controls, 0.25 μmol/l unlabeled glucagon was added. The buffer combination was incubated for 90 min at room temperature then filtered. Filters were analyzed on a Packard TopCount microplate scintillation counter.

**Hepatocyte isolation.** Hepatocyte suspensions were prepared from livers of *db/db* mice after 3 weeks of treatment with either control or GR-ASO. Livers were perfused *in situ* via the portal vein with first 50 ml of liver perfusion medium (Gibco, Carlsbad, CA) then 50 ml of liver digestion medium (Gibco). Hepatocytes were purified by centrifugation of the suspension with Percoll separation medium (Sigma, St. Louis, MO). After purification, hepatocytes were re-suspended in Williams E medium with fetal bovine serum and cells were placed in collagen-coated plates.

**Hepatocyte cAMP formation assay.** To assess the effect of glucagon, forskolin, or isoproterenol on cAMP production, hepatocytes were incubated in Dulbecco's modified Eagle's medium/F12 medium containing 1 mmol/l IBMX. Glucagon, forskolin, or isoproterenol in different dosage ranges was then added to the culture medium. To stop the assay, 0.5 N HCl was added 5 min later; the cAMP content in the medium was determined using the RIA method.

**Hepatocyte gluconeogenesis.** Hepatocytes were plated onto 24-well plates. After they were incubated overnight in Williams medium, the medium was changed by three washes of minimum essential medium/Earle's salt medium supplemented with 2 mm glutamine, 20 mmol/l HEPES, and 2 mmol/l lactic acid. These cells were then placed into 1 ml of the same medium containing 0.2 μCi of [<sup>14</sup>C]lactic acid. Next, either saline or glucagon (30 nmol/l) was added to the medium in a 10-μl volume. After being incubated for 2 h at 37°C, 0.8 ml of the medium was placed onto 2 ml of an AG1X8 formate resin. Two washes of 1 ml of water were done, and the eluates were counted in a scintillation counter.

**Hepatic glycogen and triglyceride measurement.** Hepatic glycogen content was measured as glucosyl units in micromoles per gram of wet liver. Briefly, glycogen was extracted with 30% KOH solution in boiling water bath, precipitated with ethanol, and hydrolyzed into glucose by amyloglucosidase. Subsequently, the glucose concentration was determined using a glucose kit (Trinder; Sigma). Hepatic triglyceride content was measured using a COBAS Mira Plus blood chemistry analyzer after triglycerides were extracted in liver homogenate (0.05 g/ml saline) with an equal volume of 1% sodium deoxycholate solution for ~5 h.

**Morphological analysis.** The pancreas was immersion-fixed at 4°C in 4.0% freshly prepared paraformaldehyde in 0.1 mol/l PBS (pH 7.4) with light agitation. The tissue was washed several times over 4–6 h in 0.1 mol/l PBS, routinely embedded in paraffin, and sectioned at 5 μm. Hydrated sections were stained with anti-insulin (Linco), antiglucagon (Linco), and antisomatostatin (Cortex Biochem, San Leandro, CA) followed by appropriate "ML" grade secondary antibodies labeled with either CY2, CY3, or CY5 (Jackson Immunoresearch, West Grove, PA). Sections were imaged under identical settings at the same session with a Bio-Rad MRC 1024ES confocal microscope (University of Vermont Microscope Imaging Center) and assembled using Adobe Photoshop. For α-cell quantitation, no less than five representative islets from each animal (*n* = 8 per group) were digitally imaged, the islet area

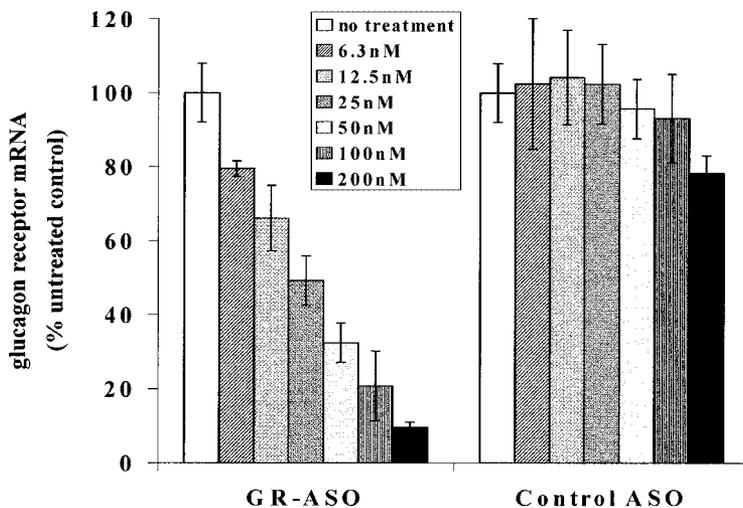


FIG. 1. Reduction of glucagon receptor mRNA expression by GR-ASO in mouse primary hepatocytes. Mouse primary hepatocytes were treated with GR-ASO or control ASO at the indicated concentrations for 4 h using lipofectin in serum-free conditions. After oligonucleotide treatment, cells were cultured in complete growth medium for an additional 12–18 h. Total RNA was isolated from triplicate samples and analyzed by real-time RT-PCR. Glucagon receptor mRNA expression was normalized to total RNA, determined by ribogreen fluorescence, in the same samples. Glucagon receptor mRNA levels are expressed as the percent of glucagon receptor mRNA levels in control animals.

was determined using National Institutes of Health (NIH) Image software, and the  $\alpha$ -cells were counted. Values were expressed as the number of  $\alpha$ -cells per micrometer squared of islet tissue. For the semiquantitative evaluation of relative glucagon levels per  $\alpha$ -cell, grey scale confocal images of the relative  $\alpha$ -cell glucagon immunofluorescence signal were analyzed in NIH Image by measuring the average pixel intensity of the perinuclear cytoplasm in a  $7.0\text{-}\mu\text{m}^2$  area. Islet core background fluorescence values were subtracted from each field. Thus, the average corrected  $\alpha$ -cell fluorescence values for each group were calculated and compared.

**Statistical analysis.** Statistical analysis was performed using the Prism program (Graphpad, Monrovia, CA) and with a one-way ANOVA with Dunnett's multiple comparison test, as well as Student's *t* test.

## RESULTS

**Evaluation of GR-ASO in vitro.** ASOs designed to be complementary to mouse glucagon receptor mRNA sequences were evaluated for their ability to suppress endogenous glucagon receptor expression in primary mouse hepatocytes in vitro, as previously described (15,21). Potent ASOs were further tested in dosage-response studies for their ability to inhibit target mRNA expression in primary mouse hepatocytes. The most potent oligonucleotide in mouse hepatocytes significantly ( $P < 0.05$ ) reduced glucagon receptor mRNA levels in a concentration-dependent manner (Fig. 1). A control oligonucleotide composed of the same chemistry and oligonucleotide length had no significant effect on glucagon receptor mRNA expression at all concentrations tested except the highest concentration, where a slight decrease in GR mRNA levels was observed. (Fig. 1).

**Antisense-mediated inhibition of glucagon receptor expression in vivo.** The effect of GR-ASO-mediated suppression of glucagon receptor in the *db/db* mouse was evaluated. Glucagon receptor mRNA levels were quantitated in liver, skeletal muscle, and white and brown fat of *db/db* mice treated with either control ASO or GR-ASO at a dosage of 25 mg/kg twice a week for 3 weeks by intraperitoneal injection (see RESEARCH DESIGN AND METHODS). Treatment of *db/db* mice with GR-ASO resulted in a significant (83%) decrease in glucagon receptor mRNA in liver compared with control-treated animals (Fig. 2). A substantial amount of variation in tissue glucagon receptor mRNA levels was observed in some groups. No significant reduction in glucagon receptor mRNA levels was observed in white or brown fat tissue. However, in other studies involving longer-term treatment ( $\geq 4$  weeks) with GR-ASO

in *db/db* mice, a substantial reduction ( $>80\%$ ) in glucagon receptor mRNA levels was observed in white fat tissue (B.P.M., S.B., unpublished observations).

**Effect of GR-ASO treatment on glucagon receptor binding, cAMP formation, and gluconeogenesis in isolated hepatocytes.** The glucagon receptor binding to liver membrane was determined using  $^{125}\text{I}$ -glucagon. In control ASO-treated mice, the liver membrane showed a glucagon binding of  $1.02 \pm 0.15$  fmol/mg of protein, whereas in GR-ASO-treated mice, this glucagon binding was markedly reduced to  $0.08 \pm 0.03$  fmol/mg protein ( $n = 3$  for each group;  $P < 0.05$ ).

Glucagon-stimulated cAMP formation was determined using hepatocytes isolated from control or GR-ASO-treated *db/db* mice (Fig. 3). In control hepatocytes, glucagon (0.1–100 nmol/l) induced a marked increase in cAMP

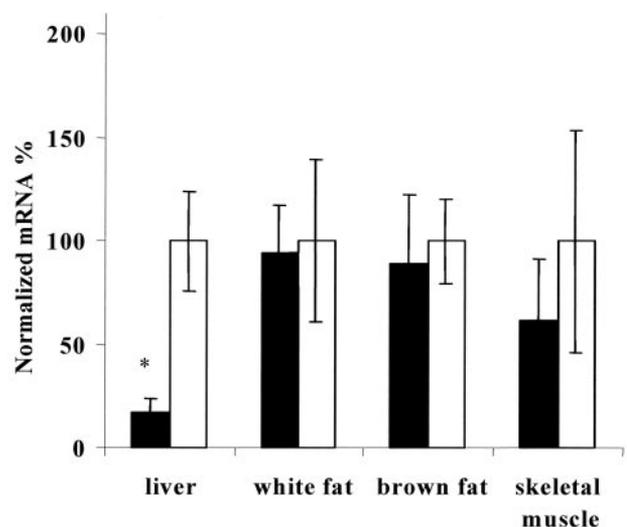
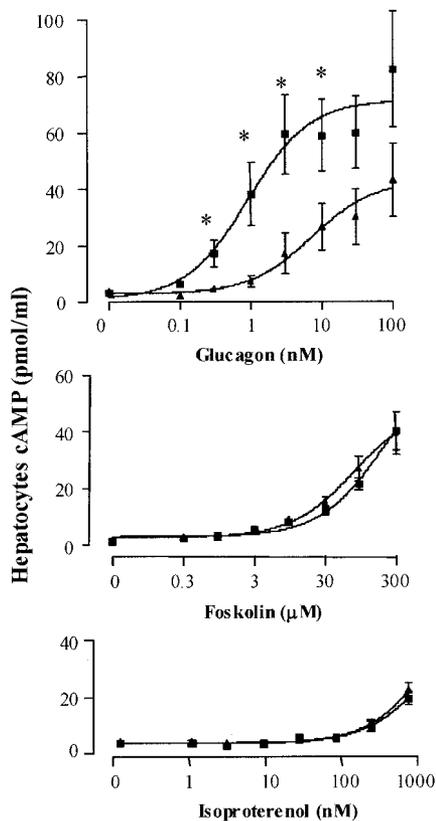


FIG. 2. Glucagon receptor mRNA expression in tissues after GR-ASO treatment of *db/db* mice. The *db/db* mice were treated with 25 mg/kg GR-ASO (■) or vehicle control (□) twice per week for 3 weeks by intraperitoneal injection. After mice were killed, total RNA was isolated from tissues for each treatment group ( $n = 8$ ) and analyzed by real-time RT-PCR. Glucagon receptor mRNA expression was normalized to total RNA, determined by ribogreen fluorescence, in the same samples. Graph represents the percent of glucagon receptor mRNA levels compared with vehicle control treatment (percent control). Statistical analysis using Student's *t* test was performed comparing GR-ASO-treated group with control ASO-treated group. \* $P < 0.001$ .

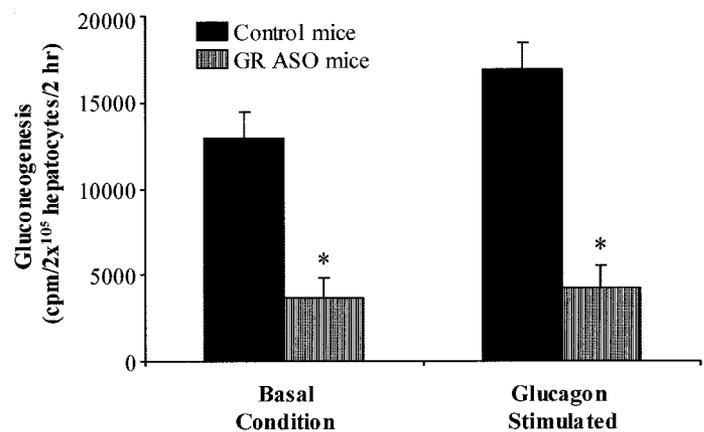


**FIG. 3.** Effect of GR-ASO on hepatocyte cAMP formation. Female *db/db* mice were treated with GR-ASO (■) or control ASO (▲) for 3 weeks. Hepatocytes were then isolated from each mouse. cAMP formation was measured as indicated in RESEARCH DESIGN AND METHODS. Data represent means  $\pm$  SE for five mice per each group. \* $P < 0.05$ , control vs. treated group at the same glucagon concentration.

formation, with a maximum value of  $96.2 \pm 20.3$  pmol/ml (half-maximal effective concentration [EC<sub>50</sub>] =  $1.39 \pm 0.53$  nmol/l). Glucagon-stimulated cAMP formation was markedly reduced in hepatocytes delivered from GR-ASO-treated mice, in which the maximum response was  $51.15 \pm 12.99$  pmol/ml, with an EC<sub>50</sub> for this effect of  $10.54 \pm 1.98$  nmol/l ( $P < 0.01$  compared with that in controls). cAMP formation in response to forskolin or isoproterenol in hepatocytes isolated from GR-ASO-treated mice was similar to that of controls, suggesting that GR-ASO treatment specifically reduced only the cAMP formation responding to glucagon stimulation.

Considering that glucagon plays an important role in gluconeogenesis in liver, we next examined the effect of GR-ASO treatment on gluconeogenesis by measuring the conversion of lactic acid to glucose in isolated hepatocytes from ASO-treated mice. Under basal conditions, gluconeogenesis from hepatocytes isolated from mice treated with GR-ASO was reduced by  $\sim 70\%$  compared with control groups (Fig. 4). Glucagon stimulation increased gluconeogenesis by 132% in control mice, but had no effect on gluconeogenesis in hepatocytes isolated from GR-ASO-treated mice.

**Effect of GR-ASO treatment on the diabetic syndrome in *db/db* mice.** After 3 weeks of treatment with GR-ASO, *db/db* mice showed a significant decrease in fed serum glucose, FFAs, and triglyceride levels compared with *db/db* mice treated with control ASO (Table 1). Fed



**FIG. 4.** Effect of GR-ASO on hepatocyte gluconeogenesis. Female *db/db* mice were treated with GR-ASO or control ASO for 3 weeks. Hepatocytes were isolated from each mouse. Gluconeogenesis was measured as indicated in RESEARCH DESIGN AND METHODS. Data represent means  $\pm$  SE for four hepatocyte preparations per each group. \* $P < 0.05$ , control vs. treated group at the same condition.

plasma insulin levels were increased approximately two-fold relative to controls, whereas serum glucose levels in GR-ASO-treated mice were reduced by 25% relative to controls. Plasma insulin levels in overnight-fasted control ASO- and GR-ASO-treated groups were similar ( $603 \pm 36$  vs.  $515 \pm 32$  pmol/l), whereas fasting blood glucose levels in GR-ASO-treated mice remained significantly lower than that of control ASO-treated mice ( $12.5 \pm 1.4$  vs.  $17.3 \pm 1.4$  mmol/l;  $P < 0.05$ ). Plasma glucagon levels in GR-ASO-treated mice were 10-fold higher than that of control-treated mice under fed conditions and fivefold higher than control-treated mice under fasted conditions, suggesting a compensatory response to the reduced expression of glucagon receptor.

We next applied an oral glucose tolerance test to determine the effect of GR-ASO treatment on glucose excursion in *db/db* mice. As shown in Fig. 5A, *db/db* mice treated with GR-ASO showed a remarkable improvement in blood glucose control during the glucose challenge, as reflected by a significant decrease in the blood glucose area under the curve in GR-ASO-treated mice ( $3,141 \pm 176$  mmol  $\cdot$  l<sup>-1</sup>  $\cdot$  120 min<sup>-1</sup>) compared with control mice ( $3,914 \pm 123$  mmol  $\cdot$  l<sup>-1</sup>  $\cdot$  120 min<sup>-1</sup>;  $P < 0.01$ ). Plasma insulin levels during the oral glucose tolerance test showed no statistically significant difference between GR-ASO-treated and control groups at any of the time points, suggesting that improved glucose handling could be the result of a diminished influence of glucagon on hepatic glucose production. Evidence supporting this notion was provided by the glucagon challenge test. After being administered glucagon, *db/db* mice in the GR-ASO-treated mice showed a diminished hyperglycemic response compared with control mice, whose blood glucose levels significantly increased 15–60 min after glucagon injection (Fig. 5B).

Although GR mRNA levels were reduced by 83% in liver of treated *db/db* mice, these mice did not display any indications of hypoglycemia during an insulin tolerance test, in which 3 units/kg of insulin were given (Fig. 5C). We also tested the effects of GR-ASO treatment in euglycemic lean mice. Treatment with GR-ASO (25 mg/kg, i.p., twice per week for 3 weeks) reduced fed blood glucose levels in lean mice ( $7.8 \pm 0.1$  vs.  $8.5 \pm 0.2$  mmol/l in control groups;

TABLE 1  
Effect of 3-week treatment with GR-ASO on diabetic end points in *db/db* mice

	Serum glucose (mmol/l)	Serum triglyceride (mg/dl)	Serum FFA (mmol/l)	Plasma insulin (pmol/l)	Plasma glucagon (ng/l)	Body weight gain (g)
Control ASO	31.7 ± 2.1	455 ± 49	1.07 ± 0.10	2,650 ± 600	130 ± 10	7.0 ± 0.6
GR-ASO	24.1 ± 2.8*	174 ± 14†	0.69 ± 0.05†	6,167 ± 633†	1,411 ± 157†	7.0 ± 1.0

Data are means ± SE, with *n* = 12 for each group. Diabetic mice received a 3-week treatment with either control ASO or GR-ASO. Blood samples were collected at the end of study from mice in the fed state biochemical parameters were analyzed as described in RESEARCH DESIGN AND METHODS. \**P* < 0.05, †*P* < 0.01 compared with the same parameter in the control ASO-treated group.

*P* < 0.01), but had no marked changes in fasting blood glucose levels (4.7 ± 0.7 [control group] vs. 4.9 ± 0.2 [treated group] mmol/l). When the GR-ASO-treated lean mice underwent an oral glucose tolerance test, their blood glucose levels were not significantly different from those of controls (Fig. 6A). The treated lean mice also showed no significant difference in response to a glucagon challenge compared with the control mice (Fig. 6B). However, after insulin (1 unit/kg, i.p.) was administered, GR-ASO-treated mice showed a significant delay in the recovery of blood glucose levels compared with control ASO-treated lean mice (Fig. 6C). The lowest blood glucose level observed during this insulin tolerance test was ~4.44 mmol/l, and hypoglycemia was never observed in these mice during the test.

**Effect of GR-ASO treatment on liver glycogen and triglyceride content.** Treatment with GR-ASO for 3 weeks resulted in an increase in whole liver tissue weight that was significantly higher than in control mice (3.1 ± 0.1 vs. 2.0 ± 0.1 g; *P* < 0.01). The plasma levels of aspartate aminotransferase and alkaline phosphatase were not markedly different from those of controls, but the level of plasma alanine aminotransferase was significantly increased (Table 2). This result was likely attributable to the effects of glucagon receptor inhibition as control ASO-treated mice did not display a significant increase in liver weight. To examine the possible cause for this

increased liver weight, we measured liver glycogen and triglyceride content in both control and treated *db/db* mice. Compared with the control ASO-treated mice, the glycogen content in GR-ASO-treated mice was reduced by 17 and 44% in the randomly fed and overnight fasted states, respectively (Table 2). In contrast, hepatic triglyceride content was 80 and 93% greater in GR-ASO-treated mice than in control ASO-treated mice in the fed and overnight fasted states, respectively (Table 2).

**Effect of GR-ASO treatment on pancreatic islet morphology.** After 3 weeks of treatment with GR-ASO, plasma glucagon levels were found to be ~10-fold higher in *db/db* mice compared with mice receiving control ASO. We processed the pancreas from these mice and prepared sections for multiple-labeling immunofluorescence and morphometric analyses to determine if there were potential morphological changes after ASO treatment that may provide insight into the apparent pancreatic islet α-cell hypersecretion of glucagon. Pancreatic islets from all mice displayed a normal cytoarchitecture with central β-cells surrounded by a loose mantle of α- and δ-cells (Fig. 7). No changes in islet size between the groups were observed (data not shown). Furthermore, islets from the GR-ASO-treated mice exhibited no changes in the number of α-cells compared with islets from control mice (Fig. 8); thus, α-cell hyperplasia, as has been recently reported in glucagon receptor knockout mice (11), was not observed.

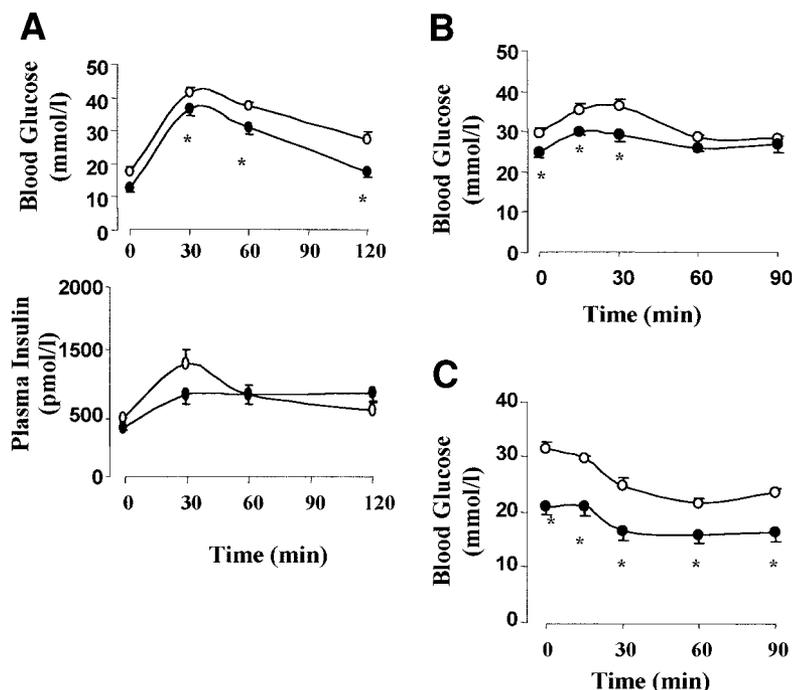


FIG. 5. Effect of GR-ASO treatment on *db/db* mice. Female *db/db* mice were treated with GR-ASO (●) or control ASO (○) for 3 weeks and then subjected to an oral glucose tolerance test (A), glucagon challenge test (B), or insulin tolerance test (C). Tail blood samples were collected at different time points to measure blood glucose and plasma insulin as described in RESEARCH DESIGN AND METHODS. Data represent means ± SE for eight mice per each group. \**P* < 0.05, control vs. treated group at the same time points.

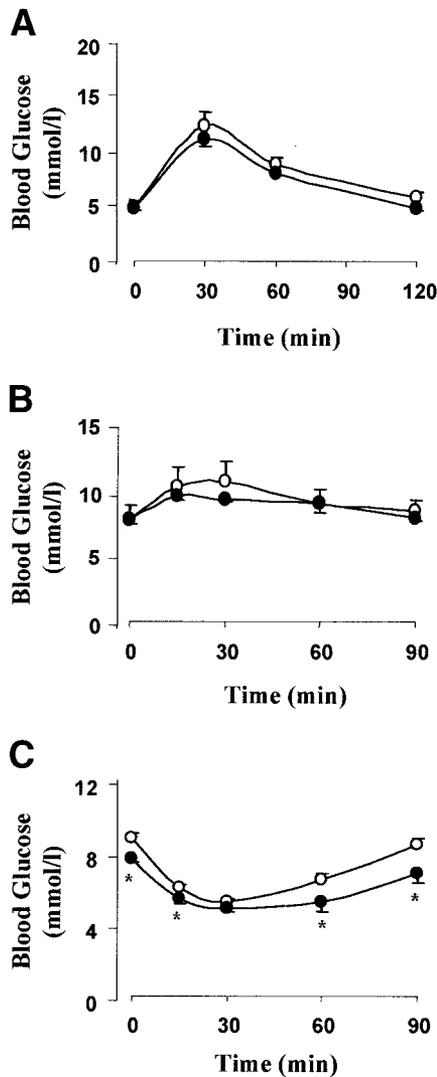


FIG. 6. Effect of GR-ASO treatment in lean mice. Female C57BL/6J mice were treated with GR-ASO (●) or control ASO (○) for 3 weeks and then subjected to an oral glucose tolerance test (A), glucagon challenge test (B), or insulin tolerance test (C). Tail blood samples were collected at different time points to measure blood glucose using a glucometer. Data represent means  $\pm$  SE for eight mice of each group. \* $P < 0.05$ , control vs. treated group at the same time points.

However, using confocal microscopy with semiquantitative image analysis, the relative  $\alpha$ -cell glucagon immunoreactivity was increased 1.8-fold in GR-ASO-treated mice compared with control ASO-treated control mice (Figs. 7 and 8). No discernible differences in the pattern or intensity of insulin or somatostatin immunostaining were ob-

served among the animal groups, although compared with normal nondiabetic mice, most  $\beta$ -cells in the *db/db* mice were relatively insulin depleted.

## DISCUSSION

Using an ASO-mediated approach to suppress target mRNA levels, we have demonstrated, for the first time, that suppression of glucagon receptor expression, primarily in liver, is sufficient to reduce blood levels of glucose, triglycerides, and fatty acids in diabetic animals. Our results support the concept that antagonism of glucagon receptors could be an effective approach for blood glucose control in diabetes.

Glucagon receptors are expressed in many tissues, with the highest levels being found in liver. Glucagon stimulates glycogenolysis via a cAMP-related signaling pathway and increases gluconeogenesis by upregulating several gluconeogenic enzymes and enhancing hepatic uptake of gluconeogenic amino acids (2–4). Under diabetic conditions, hyperglucagonemia increases hepatic glucose production and thus contributes significantly to fasting hyperglycemia. We found that chronic treatment with GR-ASO after systemic administration markedly decreased glucagon receptor expression by 83% in the liver, which, in turn, reduced the effect of glucagon receptor binding, decreased glucagon-stimulated cAMP formation, and decreased gluconeogenesis in isolated hepatocytes. It could be predicted that these changes would result in reduced glucagon-mediated hepatic glucose production, although this assumption needs to be confirmed by euglycemic-hyperinsulinemic clamp studies. Further support for this possibility was provided by the glucagon challenge test in *db/db* mice treated with GR-ASO. The GR-ASO-treated group showed a greatly attenuated hyperglycemic response to a glucagon challenge compared with the control group. This diminished effect of glucagon on hepatic glucose production also explains the amelioration of hyperglycemia in *db/db* mice treated with GR-ASO. When these mice received an oral glucose load, they showed improved glucose handling capability during the 2-h time period, even though their circulating insulin levels were comparable with those of the control group. These data are consistent with the major findings from glucagon receptor gene knockouts in lean mice reported from other laboratories (10,11), despite the fact that GR-ASO treatment only reduced the glucagon receptor mRNA expression by 83% in liver after 3 weeks of treatment.

One of the physiological functions of glucagon is to counter hypoglycemia. Under diabetic conditions, this counterhypoglycemic regulation is weakened. Therefore, reduced glucagon receptor expression could possibly in-

TABLE 2

Effect of 3-week treatment with GR-ASO on liver glycogen, triglyceride, and plasma enzymes in *db/db* mice

Treatment	Liver weight (g)	Plasma AST (mg/ml)	Plasma ALP (mg/ml)	Plasma ALT (mg/ml)	Liver glycogen ( $\mu$ mol/g wet tissue)		Liver triglyceride (mg/g wet tissue)	
					Fed	Fasted	Fed	Fasted
Control ASO	2.0 $\pm$ 0.1	117 $\pm$ 9	112 $\pm$ 7	122 $\pm$ 13	373 $\pm$ 19	222 $\pm$ 14	38.6 $\pm$ 3.5	57.9 $\pm$ 4.8
GR ASO	3.1 $\pm$ 0.1*	146 $\pm$ 11	107 $\pm$ 3	196 $\pm$ 26†	309 $\pm$ 16*	124 $\pm$ 21*	69.5 $\pm$ 6.8*	112.0 $\pm$ 6.6*

Data are means  $\pm$  SE, with  $n = 8$  for each group. Diabetic mice received a 3-week treatment with either control ASO or GR-ASO. Blood samples were collected at the end of the study. Plasma levels of aspartate aminotransferase (AST), alkaline phosphatases (ALPs), and alanine aminotransferase (ALT) were analyzed as described in RESEARCH DESIGN AND METHODS. Liver tissue samples were collected by freeze-clamp method to measure glycogen and triglyceride contents. \* $P < 0.01$ , † $P < 0.05$  compared with the same parameter in the control ASO-treated group.

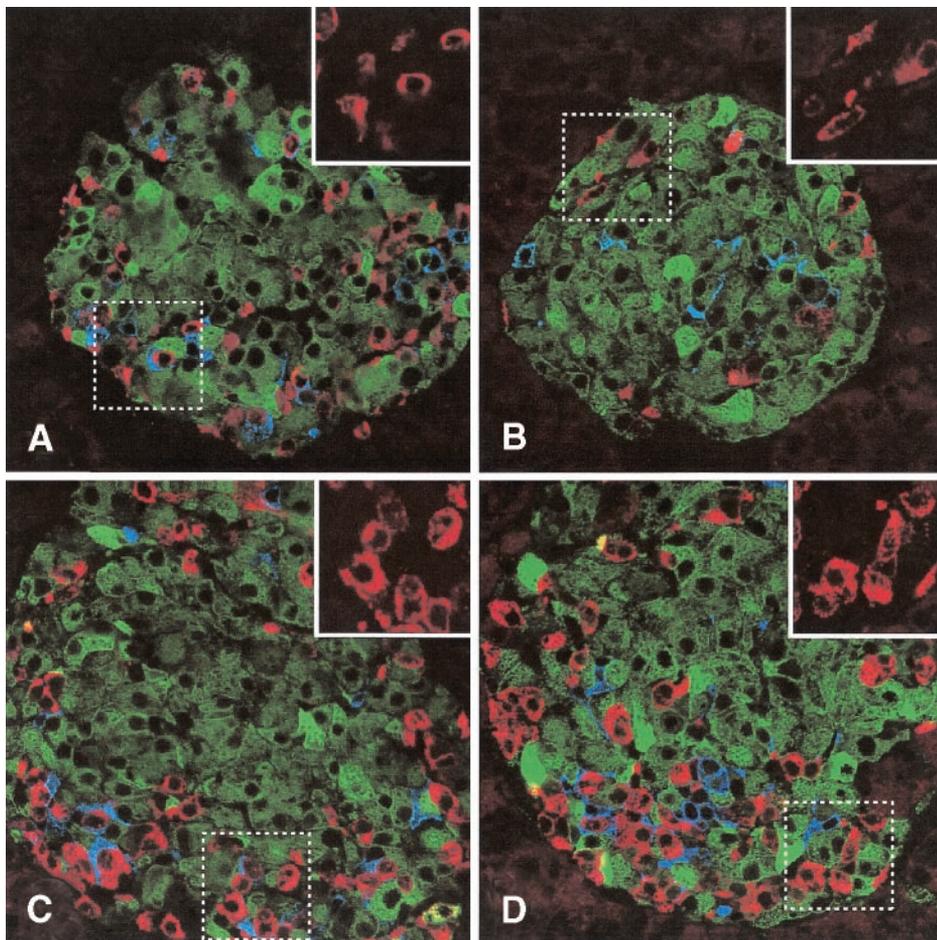


FIG. 7. Effect of GR-ASO treatment on pancreatic islet morphology. Confocal immunofluorescence showing representative islets from different *db/db* mice treated with either control oligonucleotide (A, B) or GR-ASO (C, D). Pancreas sections were stained for insulin (green), glucagon (red), and somatostatin (blue). Imaging parameters were identical for each field. Dashed area represents zoom (inset) for glucagon staining. Typical islets from mice treated with GR-ASO exhibited  $\alpha$ -cells with more intense glucagon immunofluorescent signal (red). No detectable differences in insulin or somatostatin staining were observed among the groups. Field widths are 170  $\mu$ m.

duce the occurrence of hypoglycemia. However, this appears to not be an issue with GR-ASO treatment based on the following observations. First, lean mice treated with GR-ASO for 3 weeks did not display evidence of hypogly-

cemia during the experimental period. Second, when we performed an insulin tolerance test to lower blood glucose, GR-ASO-treated *db/db* mice showed the same blood glucose recovery rate as that of mice treated with control ASO. Similar observations were also made in lean mice that underwent 3 weeks of GR-ASO treatment. These results suggest that additional compensatory mechanisms existed in the GR-ASO-treated mice to prevent hypoglycemia, possibly through other hormonal and neural mechanisms that regulate hepatic glucose production. The lack of hypoglycemia in glucagon receptor knockout mice also supports this conclusion (11).

In contrast to glucagon receptor knockout mice, we observed an increase in plasma insulin levels in GR-ASO-treated *db/db* mice. This effect might be the result of 1) improved blood glucose control delaying pancreatic  $\beta$ -cell deterioration in *db/db* mice, or 2) an increase in glucagon-like peptide 1 (GLP-1) levels in the pancreas as a result of increased glucagon levels observed in GR-ASO-treated mice. A small amount of proglucagon is normally processed to GLP-1 in pancreatic islet cells (11). In glucagon receptor knockout mice, pancreatic  $\alpha$ -cell hyperplasia increases the content of total GLP-1 in pancreatic extracts by >25-fold (11). Elevated islet GLP-1 levels may act in a paracrine manner to stimulate insulin release. As a consequence, blood glucose control might benefit from an increase in circulating insulin levels, which may also result in a reduction of FFA and triglyceride levels, as we observed in GR-ASO-treated *db/db* mice.

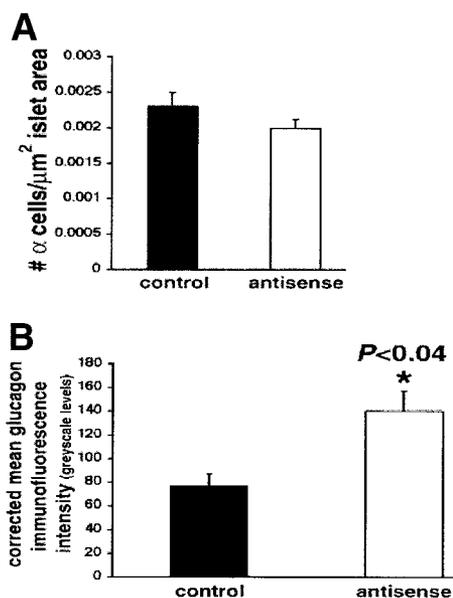


FIG. 8. Effect of GR-ASO treatment on pancreatic  $\alpha$ -cell number and relative glucagon levels. A: No significant differences in  $\alpha$ -cell numbers were found between the groups ( $n = 8$  per group). B: Relative glucagon levels of  $\alpha$ -cells as assessed by immunofluorescence intensity were significantly increased in the GR-ASO group ( $n = 3$  per group).

To explain the increase in plasma glucagon levels in GR-ASO-treated *db/db* mice, we examined pancreatic islet  $\alpha$ - and  $\beta$ -cell morphology by multiple-labeling immunofluorescence of tissue sections. GR-ASO treatment caused no gross morphological changes in islets. We did not detect increased numbers of  $\alpha$ -cells in GR-ASO-treated mice. However,  $\alpha$ -cell glucagon immunofluorescence was significantly enhanced in GR-ASO-treated mice as compared with controls. Thus, the 10-fold increase in plasma glucagon levels was likely attributable to increased glucagon secretion per  $\alpha$ -cell and not increased cell numbers. Although plasma insulin levels were increased over twofold in GR-ASO mice, no consistent changes in  $\beta$ -cell insulin staining were observed between the animal groups.

It is well known that glucagon is a potent stimulator of glycogen phosphorylase and efficiently induces glycogenolysis in the liver. However, our data showed that GR-ASO treatment in *db/db* mice did not increase glycogen content in liver, even though there was a marked reduction of glucagon-stimulated cAMP formation in isolated hepatocytes. Moreover, liver glycogen content was reduced when animals were fasted overnight. One explanation for this observation could be that glycogen synthesis is also reduced in GR-ASO-treated mice. Hepatic glycogen is synthesized by two pathways: a direct pathway via hepatic glucose uptake and an indirect pathway via gluconeogenesis. Glucagon stimulates gluconeogenesis by activating multiple enzymes that are required for this process to occur. The hepatocyte gluconeogenesis data show that blocking glucagon action in liver significantly reduces gluconeogenesis from lactic acid. This, in turn, might result in a reduced rate of glycogen synthesis, which might then buffer the consequence of reduced glycogenolysis resulting from GR-ASO treatment and prevent an increased accumulation of liver glycogen.

It is noteworthy that we observed elevated liver triglyceride levels in GR-ASO-treated mice, which is similar to the observations made in liver-specific PEPCK knockout mice (22). In these knockout mice, hepatic gluconeogenesis was severely interrupted because of the lack of PEPCK. Considering that glucagon plays an important role in regulating PEPCK expression, reduced glucagon receptor levels could reduce PEPCK activity in hepatocytes and thus somewhat mimic the consequence of PEPCK knockout mice. Another possible explanation of increased triglyceride content in liver could be related to the effect of glucagon on lipolysis in liver (9). It has been reported that a 14-day glucagon infusion decreases triglyceride content by 71% (23). However, triglyceride accumulation was not observed in glucagon receptor knockout mice (11,12). Further investigation should be pursued to fully understand these observations.

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