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Antisense oligonucleotides targeted against glucocorticoid receptor reduce hepatic glucose production and ameliorate hyperglycemia in diabetic mice

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Abstract

Specific blockade of glucocorticoid receptor (GCCR) action in the liver without affecting the hypothalamus-pituitary-adrenal axis could be a novel pharmaceutical approach to treat type 2 diabetes. In the present study, we applied an antisense oligonucleotide (ASO) against GCCR (ASO-GCCR) to reduce the expression of liver GCCR and examined its impact on the diabetic syndrome in *ob*/*ob* and *db*/*db* mice. A 3-week treatment regimen of ASO-GCCR (25 mg/kg IP, twice per week) markedly reduced liver GCCR messenger RNA and protein expression with no alteration of GCCR messenger RNA expression in the hypothalamus, pituitary, or adrenal gland. The ASO-GCCR treatment lowered blood glucose levels by 45% and 23% in *ob*/*ob* and *db*/*db* mice, respectively, compared with those observed in the control group. The ASO-GCCR–treated mice also showed significant enhancement of insulin-mediated inhibition of hepatic glucose production during a euglycemic-hyperinsulinemic clamp as well as marked reduction of phosphoenolpyruvate carboxykinase and glucose 6-phosphatase activity compared with control mice. The ASO-GCCR treatment did not change peripheral insulin sensitivity during the clamp. The *ob*/*ob* mice treated with ASO-GCCR had no significant difference in the plasma corticosterone and corticotropin levels compared with control mice. Lean mice receiving a similar treatment regimen of ASO-GCCR exhibited no change in blood glucose levels, oral glucose tolerance tests, or insulin tolerance tests. Our results demonstrate that selective inhibition of GCCR expression in the liver by the ASO-GCCR treatment reduced hepatic glucose production and improved blood glucose control under diabetic conditions.
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1. Introduction

Glucocorticoids play many roles in lipid and carbohydrate metabolic pathways that often lead to counteractive effects on insulin action. In the liver, glucocorticoids increase the expression of gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase. In muscle and adipose tissue, glucocorticoids induce protein catabolism and lipolysis, which in turn release amino acids from muscle and free fatty acid from fat tissue, both provide gluconeogenic substrates for hepatocytes. Glucocorticoids also promote visceral obesity, which is a key factor contributing to metabolic syndrome. It is well known that one third of patients with Cushing's syndrome suffer from glucose intolerance and overt diabetes. On the other hand, reduced glucocorticoid levels in circulation improved insulin sensitivity and blood glucose control, which has been demonstrated in adrenalectomized *ob*/*ob* mice [1,2].

Glucocorticoid effects are mediated by the glucocorticoid receptor (GCCR). The GCCR is a member of the steroid family of nuclear transcription factors. Of the 2 isoforms of the GCCR generated by alternative splicing, the GCCRα isoform modulates the expression of glucocorticoid-responsive genes, whereas the GCCRβ does not bind glucocorticoids and is transcriptionally inactive [3]. The GCCR is a potential drug target for pharmaceutical treatment of type 2 diabetes. RU-486, an antagonist of the GCCR, reduced blood glucose and insulin levels in diabetic mice [4]. In clinical studies, RU-486 normalized fasting blood glucose levels and improved performance in glucose tolerance tests [5]; however, RU-486 is also a potent progesterone antagonist and, in addition, it activates the hypothalamus-pituitary-adrenal (HPA) axis and causes increased plasma cortisol and corticotropin levels [6].

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Several nonsteroidal GCCR-specific antagonists have been reported [7]. These antagonists lowered blood glucose levels in either *db*/*db* or *ob*/*ob* mice with less effect on systemic corticosterone than RU-486 [7]. Therefore, discovery of liver-selective GCCR antagonists is a challenge for drug development focused on targeting the GCCR for indication of type 2 diabetes. A recent study demonstrated that using bile acid to link a GCCR antagonist significantly enhanced the liver-selective distribution of a GCCR antagonist and minimized its effect on HPA axis activation [8].

Antisense technology is a novel approach for reducing target gene expression both in vivo and in vitro. Previous in vivo studies have shown that systemic administration of antisense oligonucleotides (ASOs) to animals results in significant ASO accumulation in the liver and suppression of target gene expression [9-11]. In the present study, we used *ob*/*ob* and *db*/*db* mice to characterize the effects of ASO-mediated inhibition of GCCR expression in the liver and its impact on diabetic syndrome control.

2. Research design and methods

2.1. Antisense oligonucleotide design and evaluation

Rapid throughput screens were performed in vitro to identify mouse GCCR-selective ASO inhibitors. Briefly, 80 ASOs were designed to the mouse GCCR messenger RNA (mRNA) sequence. These ASOs were synthesized as 20-base phosphorothioate chimeric oligonucleotides, where bases 1-5 and 16-20 were modified with 2′-*O*-(2-methoxy)-ethyl (2′-MOE). This chimeric design has been shown to provide increased nuclease resistance and mRNA affinity while maintaining the robust RNase H terminating mechanism used by these types of ASOs [12].

2.1.1. Cell culture

Glucocorticoid receptor ASOs were screened in primary mouse hepatocytes for their ability to reduce GCCR mRNA expression. Primary hepatocytes were isolated as previously described and plated onto collagen-coated plates [11]. Hepatocytes were treated with ASOs and lipofectin (Invitrogen, Carlsbad, Calif) mixture for 4 hours in serum-free William's E media (Invitrogen). After 4 hours, the ASO reaction mixture was replaced with normal growth media (William's E media with 10% fetal bovine serum) and the cells were incubated under normal conditions for an additional 16 to 20 hours. The final ASO-GCCR lead was selected after detailed characterization as described above in vitro. The sequence of the mouse lead ASO-GCCR used in the current study is 5′ -GTTTGCAATGCTTTCTTCCA-3′ and hybridizes to position 410-429 of Genebank Acc# NM_008173.1.

2.1.2. Tissue RNA isolation

The animals were killed and tissue samples from the liver, adrenal gland, hypothalamus, and pituitary were immediately snap frozen in liquid nitrogen or homogenized in guanidinium isothiocyanate and stored at −80°C until processed. Total RNA was prepared from tissues as previously described [13]. Briefly, total RNA was centrifuged over a cesium chloride gradient and the RNA pellet was resuspended in RNase-free water and purified further using an RNeasy mini RNA preparation kit (Qiagen, Valencia, Calif) following the manufacturer's instructions.

2.1.3. RNA expression analysis

A detailed description of the method of analysis of total RNA has been described previously [14]. Briefly, targeted mRNA from tissue culture or animal tissues was analyzed by reverse transcriptase polymerase chain reaction (PCR) using 100 ng of total RNA in a final volume of 30 μL containing 200 nmol/L of the target of interest–specific PCR primers (described below), 0.2 mmol/L each dNTP, 75 nmol/L fluorescently labeled oligonucleotides probe, 1× RT-PCR buffer, 5 mmol/L $MgCl_2$, 2 U platinum TaqDNA (Invitrogen), and 8 U of ribonuclease inhibitor. Reverse transcription was performed for 30 minutes at 48°C followed by 40 thermal cycles of 30 seconds at 94°C and 1 minute at 60°C PCR using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, Calif). Targeted mRNA was normalized to total RNA as determined by RiboGreen fluorescence from the same RNA sample.

2.2. In vivo animal study design

2.2.1. Animals

Female *ob*/*ob* mice (C57BL/6J-*Lep*^{ob}), female *db*/*db* mice (C57BL/KsJ-*Lep*^{db}), and female lean mice (C57BL/KsJ-*Lep*$^{+/?}$) were purchased from Jackson Labs (Bar Harbor, Me). Each strain of mice (6-7 weeks old) was divided into 2 groups evenly based on averages of blood glucose and body weight. The treatment group received an intraperitoneal injection of ASO-GCCR in saline (25 mg/kg) twice per week for 3 weeks. The mice in the control groups were treated with a control ASO at the same dosage as that in ASO-GCCR–treated mice. The control ASO had the same chemistry, length, and molecular weight as the ASO-GCCR but did not reduce the expression of GCCR or any other known gene when blasted against known databases. After 3 weeks of treatment, a subgroup of mice from the control and ASO-GCCR–treated groups was killed to collect blood samples and tissues for biochemical analysis, GCCR receptor expression, and liver enzymes. The rest of the mice from both groups was subjected to oral glucose tolerance tests (OGTTs), insulin tolerance tests, dexamethasone challenge, or hyperinsulinemic-euglycemic clamp studies. To examine the effect of ASO-GCCR on HPA function, 6-week-old male *ob*/*ob* mice were randomized to 3 treatment groups based on plasma glucose levels. Animals were dosed with saline, the ASO-GCCR dissolved in saline, or a control ASO. The ASOs were given twice weekly via subcutaneous injection. Samples for hormone measurements were obtained via tail

snip after 4 weeks of treatment (around 9:00 AM) to measure plasma corticosterone and corticotropin levels.

2.2.2. Oral glucose tolerance test

Mice were fasted overnight and received an oral glucose challenge (2 g/kg body weight, oral gavage in the following morning). Tail blood samples (10 μL) were collected before and 30, 60, and 120 minutes after glucose administration for measurement of blood glucose and/or plasma insulin.

2.2.3. Insulin tolerance test

Mice received an intraperitoneal insulin injection under fed conditions (3 U/kg body weight). Tail blood samples (3 μL) were collected at 0, 15, 30, 60, 90, and 120 minutes for measurement of blood glucose.

2.2.4. Dexamethasone challenge test

Dexamethasone (100 mg/kg) was administered (intraperitoneally) to *ob*/*ob* mice treated with either control or ASO-GCCR. Twenty-four hours later, tail blood samples were collected to measure blood glucose.

2.2.5. Hyperinsulinemic-euglycemic clamp

After 3 weeks of treatment, the clamps were performed in *db*/*db* mice as described previously [15] using [3-^{3}H]glucose (NEN Life Science Products, Boston, Mass) for the estimation of insulin effect on hepatic glucose production and glucose distribution rate.

2.3. Biochemical analyses

2.3.1. Blood chemistry

Plasma levels of glucose, triglyceride, cholesterol, aspartate aminotransferase (AST), alkaline phosphatase (ALP), and alanine aminotransferase (ALT) under fed conditions were measured using a COBAS Mira Plus blood chemistry analyzer (Roche Diagnostic Systems, Indianapolis, Ind). ELISA kits for insulin, corticosterone, or corticotropin assay (ALPCO, Windham, NH) were used to measure hormone concentrations in plasma. Plasma glucagon was measured using a radioimmunoassay kit from Linco (St Charles, Mo). During the OGTT, insulin tolerance test, and dexamethasone challenge test, blood glucose levels were measured using a glucometer (One Touch Ultra, Lifescan, Milpitas, Calif).

2.3.2. Hepatic glycogen and enzyme activity measurement

Hepatic glycogen content was measured as glucosyl-units in micromoles per gram of wet liver [16]. Briefly, glycogen was extracted with 30% KOH solution in a boiling water bath, precipitated with ethanol, and hydrolyzed into glucose by amyloglucosidase. Subsequently, glucose concentration was determined using a glucose kit (Trinder, Sigma, St Louis, Mo). Hepatic glucose 6-phosphatase activity was measured as previously described [17]. Briefly, phosphate converted from graded series of glucose 6-phosphate by glucose 6-phosphatase was detected calorimetrically and V_{max} and K_m were calculated. Hepatic PEPCK activity was measured using a nicotinamide adenine dinucleotide (NADH)-coupled system to quantitate the conversion of phosphoenolpyruvate into oxaloacetate and its subsequent conversion to malate [18]. Activity was expressed as milliunit per milligram protein in liver supernatant (1 mU = 1 nmol oxaloacetate produced/min). The protein content was determined using the Bradford assay kit (BioRad, Hercules, Calif) with bovine serum albumin as a standard.

2.3.3. Liver GCCR expression by Western blotting

Western blot analysis was performed using a standard procedure. The primary antibody was GR (M-20, Santa Cruz

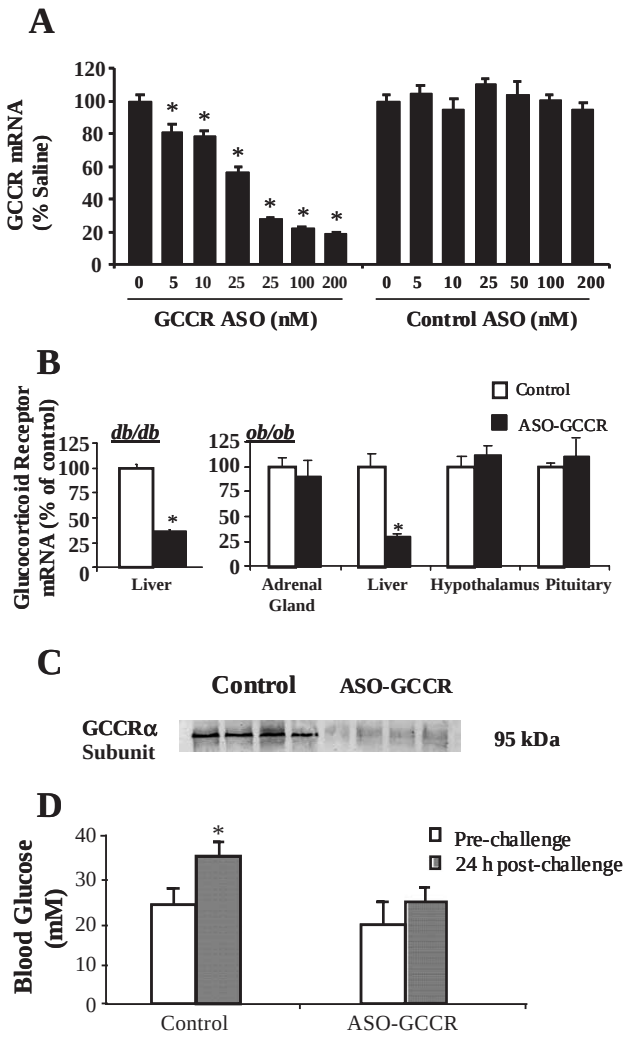

Fig. 1. The ASO-GCCR treatment reduced liver GCCR expression in mRNA and protein levels and decreased the hyperglycemic effect of the dexamethasone challenge. A, In vitro inhibition of GCCR mRNA expression in mouse hepatocytes. Data are expressed as mean ± SEM of mRNA levels relative to untreated cells (n = 3, *P < .05, ASO-GCCR treatment vs corresponding untreated group by analysis of variance). B, The GCCR mRNA expression in the liver, hypothalamus, pituitary, and adrenal gland of *db*/*db* and *ob*/*ob* mice after 3 weeks of treatment. C, Western blotting of immunoreactive GCCRα in *db*/*db* mice liver after 3 weeks of treatment. D, Blood glucose levels at 24 hours after dexamethasone challenge in *ob*/*ob* mice.

Table 1
Effect of ASO-GCCR treatment on lean or diabetic mice

Mice	Body weight gain (g/3 wk)	Blood glucose (mmol/L)	Plasma triglycerides (mg/dL)	Plasma cholesterol (mmol/L)
Lean, control	2.3 ± 0.1	8.2 ± 0.2	ND	ND
Lean, ASO-GCCR	2.4 ± 0.2	7.8 ± 0.2	ND	ND
db/*db*, control	2.3 ± 0.5	34.0 ± 1.9	276 ± 15	4.0 ± 0.3
db/*db*, ASO-GCCR	4.9 ± 0.4*	26.3 ± 1.7*	166 ± 9*	3.8 ± 0.2
ob/*ob*, control	7.3 ± 0.4	31.0 ± 5.9	222 ± 35	5.7 ± 0.4
ob/*ob*, ASO-GCCR	6.4 ± 1.2	16.9 ± 2.1*	143 ± 7*	6.1 ± 0.3

Lean or diabetic mice received either ASO-GCCR or control ASO. Blood was collected via orbital bleeding at the end of the study to measure the plasma parameters (see Research Design and Methods). Mice were weighed on the first day and last day of the study to calculate the body weight gain. n = 8. ND indicates not determined.

* $P < .01$, compared with that in control mice of the same strain.

Biotechnology, Inc, Calif), a rabbit polyclonal antibody raised against the amino terminus of GR α of mouse origin. The second antibody was peroxidase-conjugated goat anti-rabbit IgG (Pierce, Rockford, Ill).

2.4. Statistical analysis

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

3. Results

3.1. Evaluation of ASO-GCCR in vitro

After extensive characterization of multiple candidate ASOs, a lead mouse ASO was selected for in vivo studies. The mouse ASO-GCCR caused a dose-dependent reduction in the expression of GCCR mRNA in primary mouse hepatocytes (Fig. 1). A control ASO composed of the same chemistry and oligonucleotide length had no effect on GCCR mRNA expression.

3.2. Antisense-mediated inhibition of GCCR expression in vivo

The effects of the ASO-GCCR treatment on GCCR mRNA levels in various tissues were examined in diabetic mice after 3 weeks of treatment (Fig. 1B). The ASO-GCCR treatment significantly decreased mRNA levels of GCCR in the liver by 71% and 64% in *ob*/*ob* and *db*/*db* mice, respectively. Glucocorticoid receptor mRNA levels in the hypothalamus, pituitary, and adrenal gland in the *ob*/*ob* mice showed no changes compared with control mice. Liver GCCR protein expression was examined using an

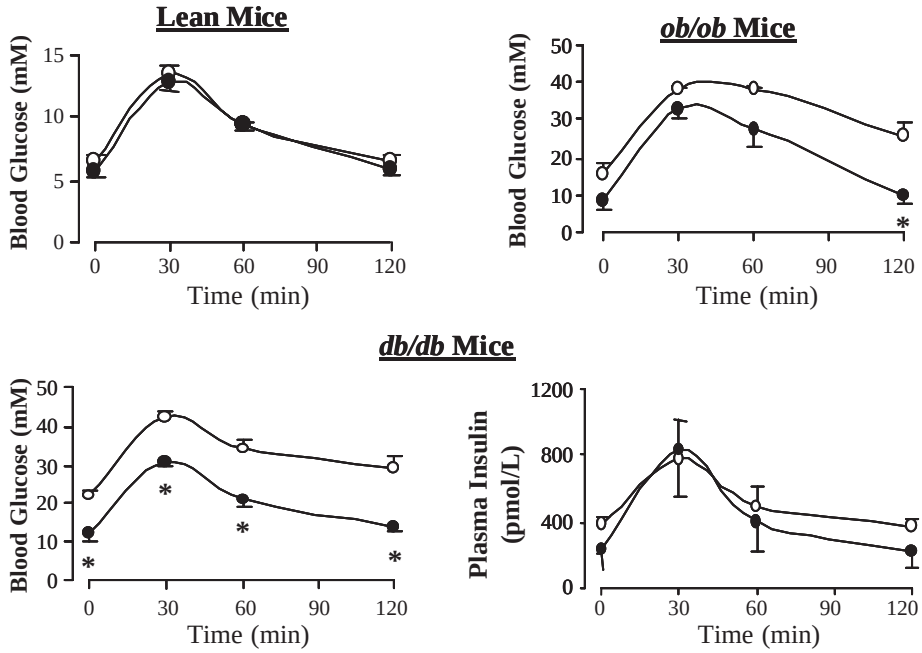

Fig. 2. The ASO-GCCR treatment improved glucose tolerance test without significant effect on insulin secretion. Mice were overnight fasted and a glucose bolus was administered via gavage (2 g/kg). Blood glucose levels were measured before and 30, 60, and 120 minutes after the glucose challenge using a glucometer. In *db*/*db* mice, blood samples of 20 μL were also collected from tail bleeding to measure plasma insulin. Open circles represent control; filled circles, ASO-GCCR treatment. n = 8 for each group of mice. Asterisk represents $P < .05$, compared with that in control mice of the same strain.

anti-GCCRα isoform antibody. Western blotting showed that in the GCCR-treated *db*/*db* mice, the GCCRα immunostaining at 95 kDa was markedly decreased compared with that in the liver of control mice (Fig. 1C), indicating a marked reduction of GCCRα protein expression. Consistent with the reduced expression of GCCR, these ASO-GCCR–treated *ob*/*ob* mice displayed a blunted blood glucose response to dexamethasone challenge (Fig. 1D).

3.3. Effects of ASO-GCCR treatment on diabetic syndrome in ob/ob and db/db mice

The levels of blood glucose, plasma triglyceride, and cholesterol in diabetic mice were measured after 3 weeks of ASO treatment. In ASO-GCCR–treated *db*/*db* mice, blood glucose levels were reduced by 23% compared with control ASO–treated mice ($P < .01$). The effects of ASO-GCCR on blood glucose were more dramatic in *ob*/*ob* mice, displaying a 45% reduction compared with that in control mice ($P < .01$; Table 1). The ASO-GCCR also decreased plasma triglyceride levels in both *db*/*db* and *ob*/*ob* mice by 35% to 40% of those observed in control mice ($P < .01$). No changes in plasma cholesterol levels occurred in either *db*/*db* or *ob*/*ob* mice after 3 weeks of ASO treatment. Although ASO-GCCR showed a potent glucose-lowering effect in diabetic mice, it had no effect on blood glucose levels in lean mice treated with the same dosage (Table 1).

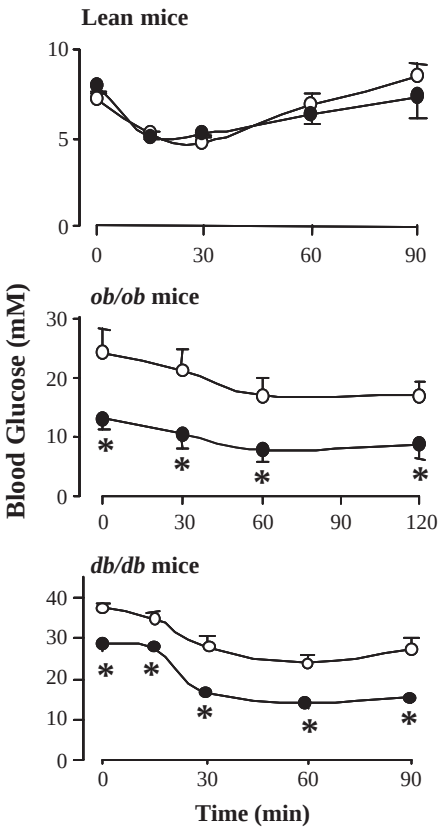

Fig. 3. Effect of ASO-GCCR treatment on insulin tolerance test. Mice received an insulin injection (3 U/kg IP). Blood glucose levels were measured at indicated time points after insulin administration. Open circles represent control; filled circles, ASO-GCCR treatment. n = 8 for each group of mice. Asterisk represents $P < .05$, compared with that in control mice of the same strain.

Table 2
Effect of ASO-GCCR on endogenous glucose production in *db*/*db* mice

	Control	ASO-GCCR
Basal condition		
Blood glucose (mmol/L)	16.9 ± 2.0	9.4 ± 2.4*
Plasma insulin (pmol/L)	1140 ± 435	1320 ± 435
Endogenous glucose production [mg/(kg body weight · min)]	85.6 ± 14.6	51.2 ± 10.3
Hyperinsulinemic condition		
Blood glucose (mmol/L)	7.5 ± 0.7	7.3 ± 1.0
Plasma insulin (pmol/L)	18300 ± 3150	16950 ± 2850
Glucose infusion rate [mg/(kg body weight · min)]	18.0 ± 6.0	50.4 ± 7.3**
Glucose disappearance rate [mg/(kg body weight · min)]	60.4 ± 3.9	57.0 ± 8.2
Endogenous glucose production [mg/(kg body weight · min)]	38.5 ± 7.2	6.5 ± 3.6**

Hyperinsulinemic-euglycemic clamps were conducted in *db*/*db* mice after the 3-week treatment of ASO-GCCR. Data represent mean ± SE (n = 5-7).
* $P < .05$, compared with that in control mice.
** $P < .01$, compared with that in control mice.

The ASO-GCCR effect on body weight gain was different between *db*/*db* and *ob*/*ob* mice. The ASO-GCCR significantly increased body weight gain in *db*/*db* mice but had no impact on the body weight of *ob*/*ob* mice. The body weight gain in lean mice treated with ASO-GCCR was also the same as that in control ASO–treated lean mice (Table 1).

Oral glucose tolerance tests were conducted in diabetic and lean mice after 3 weeks of treatment (Fig. 2). The ASO-GCCR–treated *ob*/*ob* and *db*/*db* mice showed a marked improvement in glucose excursion. Compared with the control mice, the blood glucose area under the curve during OGTT was decreased by 31% and 38% in ASO-GCCR–treated *ob*/*ob* and *db*/*db* mice, respectively. In lean mice, ASO-GCCR–treated mice showed a glucose response during OGTT similar to that of control ASO–treated mice, suggesting that ASO-GCCR had no effect on glucose excursion in normal mice. In addition to blood glucose, we also monitored plasma insulin levels during OGTT in *db*/*db* mice. Both ASO-GCCR–treated and control ASO–treated *db*/*db* mice showed increased insulin release after glucose stimulation, whereas no difference was observed between the control and ASO-GCCR–treated groups. This result indicated that improved glucose tolerance in diabetic mice is unlikely caused by changes in insulin secretion but by an improvement in insulin sensitivity.

To evaluate the effects of the ASO-GCCR treatment on insulin sensitivity, we performed insulin tolerance tests in diabetic or lean mice after treatment with ASO-GCCR or control ASO for 3 weeks. The ASO-GCCR treatment had no effect on insulin-mediated glucose lowering in lean mice because the blood glucose levels in both control and ASO-GCCR–treated groups showed similar changes after insulin administration (Fig. 3A). However, in both *ob*/*ob* and *db*/*db*

Table 3
Effect of ASO-GCCR on liver glycogen content and enzyme activities in *db*/*db* mice after 3-week treatment (n = 8)

Treatment	Liver glycogen (glucose, μmol/g tissue	PEPCK (mU/mg protein)	Glucose 6-phosphate	
			V_{max} [μM/(μM · mg protein)]	K_m (mmol/L)
Control	353 ± 15	4.7 ± 0.2	2.19 ± 0.11	2.5 ± 0.2
ASO-GCCR	346 ± 20	3.3 ± 0.3*	1.14 ± 0.09**	2.2 ± 0.1

Liver tissues were collected from *db*/*db* mice after the 3-week treatment and hepatic glycogen. Enzyme activities of PEPCK and glucose 6-phosphate were measured as described in Research Design and Methods.

* $P < .05$, compared with that in control mice.

** $P < .01$, compared with that in control mice.

mice, the ASO-GCCR treatment resulted in a significant decrease in blood glucose levels upon insulin challenge (Fig. 3B and C).

The hyperinsulinemic-euglycemic clamp was then conducted to determine the possible target tissue in which insulin sensitivity had been improved by the ASO-GCCR treatment. We observed a 40% reduction in the rate of endogenous glucose production under basal conditions in ASO-GCCR–treated *db*/*db* mice compared with that in control mice (Table 2). When plasma insulin levels were raised to ~17 nmol/L by insulin infusion, endogenous glucose production in both control and ASO-GCCR–treated mice was markedly inhibited by insulin, but a stronger effect in ASO-GCCR–treated mice was observed. Hyperinsulinemia also increased glucose uptake into peripheral tissues. However, control and ASO-GCCR–treated mice showed similar insulin-stimulated glucose distribution rates under clamp conditions. Thus, the increased glucose infusion rate necessary to maintain blood glucose levels in ASO-GCCR–treated mice was mostly caused by a significant reduction of endogenous glucose production rather than glucose uptake by muscle or adipose tissues (Table 2).

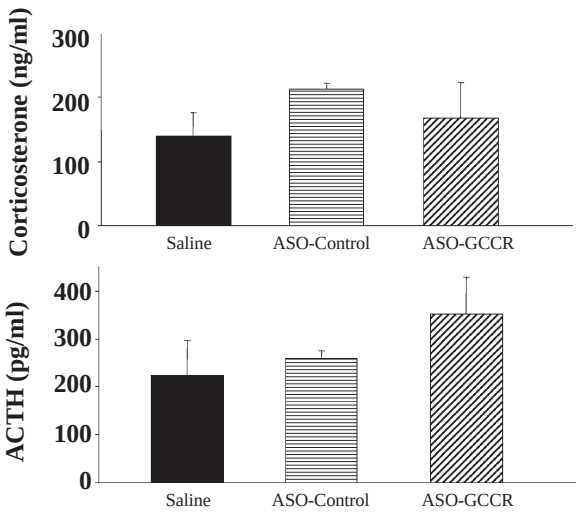

Fig. 4. Effect of ASO-GCCR treatment on corticotropin and corticosterone levels in *ob*/*ob* mice. After 4 weeks of treatment, groups of mice were killed and the plasma levels of corticosterone and corticotropin were measured using ELISA methods.

To further examine the mechanisms underlying the effect of ASO-GCCR on endogenous glucose production, we measured the liver glycogen content and the activities of 2 major hepatic gluconeogenesis enzymes, PEPCK and glucose 6-phosphatase (Table 3). Glycogen content in liver tissue from ASO-GCCR–treated mice showed no changes compared with that in control mice. However, the activities of both PEPCK and glucose 6-phosphatase in ASO-GCCR–treated mice were significantly reduced compared with those in control mice.

3.4. Effects of ASO-GCCR treatment on plasma levels of corticotropin, corticosterone, and glucagon

The effect of ASO-GCCR on the HPA axis was examined by a study comparing the plasma corticosterone and corticotropin levels among groups of mice treated with saline, control ASO, or ASO-GCCR for 4 weeks. Neither corticosterone nor corticotropin showed significant differences among these 3 groups (Fig. 4). We also measured the plasma glucagon level in mice treated with saline or ASO-GCCR and no differences between the groups were observed (76.6 ± 13.0 pmol/L in the saline group and 84.9 ± 8.0 pmol/L in the ASO-GCCR group).

3.5. Effects of ASO-GCCR treatment on liver weight and plasma levels of AST, ALP, and ALT

Liver weight and plasma levels of AST, ALP, and ALT in control and ASO-GCCR–treated mice were measured in this study. The ASO-GCCR treatment did not produce any significant change in liver weight or plasma enzyme levels in *ob*/*ob*, *db*/*db*, or lean mice (Table 4).

Table 4
Effect of ASO-GCCR on liver weight and plasma levels of enzymes

Mice	Liver weight (g)	AST (U/L)	ALP (U/L)	ALT (U/L)
Lean, control	1.26 ± 0.05	112 ± 13	121 ± 10	99 ± 12
Lean, ASO-GCCR	1.29 ± 0.06	156 ± 26	141 ± 10	147 ± 24
db/*db*, control	1.81 ± 0.17	96 ± 5	76 ± 5	71 ± 9
db/*db*, ASO-GCCR	2.21 ± 0.10	122 ± 18	93 ± 2	89 ± 15
ob/*ob*, control	3.29 ± 0.16	444 ± 57	197 ± 25	247 ± 30
ob/*ob*, ASO-GCCR	3.58 ± 0.14	528 ± 36	208 ± 26	270 ± 31

Liver tissues and plasma from ASO-GCCR–treated and control ASO–treated mice were collected to measure the liver weight and plasma levels of enzymes (n = 8).

4. Discussion

Our present study showed that ASO-GCCR treatment significantly reduced liver GCCR expression, decreased blood levels of glucose and triglycerides, improved glucose tolerance, and increased insulin sensitivity in *ob*/*ob* and *db*/*db* mice. The hyperinsulinemic-euglycemic clamp results further demonstrated that improved insulin sensitivity is mostly a consequence of inhibition of hepatic glucose production rather than of changes in glucose distribution to peripheral tissues.

Increased hepatic glucose production, which consists of gluconeogenesis and glycogenolysis, contributes significantly to hyperglycemia in type 2 diabetes. Liver PEPCK and glucose 6-phosphatase are 2 key enzymes that mediate gluconeogenesis. Glucocorticoids enhance the expression and activities of both enzymes, which in turn increase hepatic gluconeogenesis [19]. It is well known that glucocorticoids antagonize the effect of insulin and stimulate transcription of both PEPCK and glucose 6-phosphatase genes. The PEPCK gene promoter contains a glucocorticoid response unit that includes 2 accessory factor binding sites and 2 GCCR binding sites, all of which are required for a maximal glucocorticoid response. Orphan receptors COUP-TF and hepatocyte nuclear factor-4 were shown to serve as accessory factors required for the induction of PEPCK gene transcription by glucocorticoids [20]. The mechanisms of glucocorticoids-mediated induction of glucose 6-phosphatase gene expression are less clear. Hepatocyte nuclear factor-1 may be involved in the regulation of glucose 6-phosphatase gene transcription by cAMP/glucocorticoids and insulin [21]. Although it is clear that PEPCK functions as a key enzyme in hepatic gluconeogenesis, whether inhibition of this enzyme could reduce gluconeogenesis and decrease fasting blood glucose levels is uncertain. No hypoglycemia after a 24-hour fast has been observed in liver-specific PEPCK knockout mice [22]. Further studies have demonstrated that hepatic gluconeogenesis in these liver-specific PEPCK knockout mice was markedly impaired [23]. However, these mice are able to maintain euglycemia by increasing extrahepatic gluconeogenesis as well as by reducing whole-body glucose use [22]. In our current study, we observed a 30% decrease in PEPCK activity in ASO-GCCR–treated diabetic mice accompanied with a reduction in blood glucose levels both under fed and fasting conditions. Two possible explanations should be considered when comparing the effects of PEPCK activity on blood glucose levels in ASO-GCCR–treated mice and liver-specific PEPCK knockout mice. First, the 3-week ASO treatment might not be long enough to induce the compensation of extra-liver gluconeogenesis as observed in PEPCK knockout mice [22]. Second, in ASO-GCCR–treated mice, glucose 6-phosphatase activity was also markedly reduced. Glucose 6-phosphatase is another key enzyme in glucose homeostasis that catalyzes the terminal steps in gluconeogenesis and glycogenolysis. Inhibition of this enzyme would not only reduce gluconeogenesis but should also reduce glycogenolysis, which will also lower the rate of hepatic glucose output.

It is known that glucocorticoids stimulate the expression of glycogen synthesis and increase glycogen storage in the liver. In our ASO-GCCR–treated mice, we did not observe a decrease in liver glycogen content. This result could indicate that, unlike glucagon and insulin, glucocorticoids might not be a major regulator of glycogen metabolism. However, there is a possibility that reduced activity of glucose 6-phosphatase might decrease liver glycogenolysis, thus resulting in lack of further changes in total liver glycogen content, although glycogen synthesis might be reduced in a certain degree. Further studies to measure the activity of glycogen synthesis and glycogenolysis in hepatocytes from ASO-GCCR–treated mice will clarify this issue.

Glucocorticoids promote adipogenesis and increase neuropeptide Y in the brain [24]. Blockade of glucocorticoid action by GCCR antagonism should induce changes in body weight. In our present study, we did not see changes in food intake or body weight gain in lean and *ob*/*ob* mice. This could be because (1) ASO-GCCR did not penetrate the brain and (2) there was no significant increase of plasma corticosterone level in ASO-GCCR–treated mice. However, a significant increase in body weight gain was observed in ASO-GCCR–treated *db*/*db* mice. This result might not be a direct effect of ASO-GCCR treatment but rather a consequence of improved blood glucose control in *db*/*db* mice. It is known that in *db*/*db* mice, a severe hyperglycemia reduces body weight gain. Improved blood glucose control by ASO-GCCR treatment could prevent body weight loss.

In our previous studies using ASOs against the glucagon receptor in *db*/*db* mice, we observed a significant increase in liver weight caused by triglyceride accumulation in hepatocytes, which might be a consequence of a reduction in free fatty acid use for gluconeogenesis [11]. The plasma level of ALT was also increased in mice treated with ASO against the glucagon receptor. However, in the current study, ASO-GCCR treatment did not induce significant changes in liver weight or plasma ALT levels. The difference in liver weight and plasma ALT levels between glucagon receptor ASO–treated and ASO-GCCR–treated mice might reflect differences between the metabolic pathways controlled by these 2 different hormone receptors.

The main purpose of developing a liver-selective GCCR antagonist to treat metabolic disorders is to reduce the impact of GCCR antagonism on the HPA axis. This concept has been addressed in a recent publication using a GCCR antagonist linked to bile acid, which reduces hyperglycemia in *ob*/*ob* mice with no evidence of HPA axis activation [8]. Our studies use ASO-GCCR as a novel approach to treat the diabetic syndrome without impacting the HPA axis. These results are based on the tissue selectivity of ASO-GCCR, which showed more than 70% inhibition of GCCR mRNA expression in the liver but no detectable effect on GCCR expression in the adrenal gland, pituitary, and hypothalamus.

When examining other regions of the brain, we have observed no changes in GCCR expression after ASO treatment (unpublished data).

Recently, studies highlighted the effect of cytokines on the pathogenesis of insulin resistance and type 2 diabetes [25]. Glucocorticoids are anti-inflammatory agents and, therefore, a nonselective GCCR antagonist could change plasma cytokine levels as well as inflammatory reactions. Considering that GCCR inhibition induced by ASO treatment is occurring in only a small subset of cell types/tissues, we would not expect any global change in GCCR activity. This notion is supported by our data of GCCR mRNA expression in different tissues as well as plasma corticotropin and corticosterone levels in ASO-GCCR–treated mice. However, considering that GCCR expression in white adipose tissue could be reduced by ASO treatment (unpublished data), it is possible that ASO-GCCR treatment might have some impact on cytokine production in adipose tissue. Although our results showed a significant improvement of insulin sensitivity in the liver, studies to examine the influence of ASO-GCCR on plasma cytokine levels and on insulin sensitivity in skeletal muscle and adipose tissue are required to further evaluate this therapeutic approach to type 2 diabetes.

In summary, our present study demonstrates that selective inhibition of GCCR expression in the liver by ASO-GCCR treatment could be an effective approach to reduce hepatic glucose production and improve blood glucose control under diabetic conditions.

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