Reduction of Hepatic and Adipose Tissue Glucocorticoid Receptor Expression With Antisense Oligonucleotides Improves Hyperglycemia and Hyperlipidemia in Diabetic Rodents Without Causing Systemic Glucocorticoid Antagonism

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Glucocorticoids (GCs) increase hepatic gluconeogenesis and play an important role in the regulation of hepatic glucose output. Whereas systemic GC inhibition can alleviate hyperglycemia in rodents and humans, it results in adrenal insufficiency and stimulation of the hypothalamic-pituitary-adrenal axis. In the present study, we used optimized antisense oligonucleotides (ASOs) to cause selective reduction of the glucocorticoid receptor (GCCR) in liver and white adipose tissue (WAT) and evaluated the resultant changes in glucose and lipid metabolism in several rodent models of diabetes. Treatment of *ob/ob* mice with GCCR ASOs for 4 weeks resulted in ~75 and ~40% reduction in GCCR mRNA expression in liver and WAT, respectively. This was accompanied by ~65% decrease in fed and ~30% decrease in fasted glucose levels, a 60% decrease in plasma insulin concentration, and ~20 and 35% decrease in plasma resistin and tumor necrosis factor-α levels, respectively. Furthermore, GCCR ASO reduced hepatic glucose production and inhibited hepatic gluconeogenesis in liver slices from basal and dexamethasone-treated animals. In *db/db* mice, a similar reduction in GCCR expression caused ~40% decrease in fed and fasted glucose levels and ~50% reduction in plasma triglycerides. In ZDF and high-fat diet–fed streptozotocin-treated (HFD-STZ) rats, GCCR ASO treatment caused ~60% reduction in GCCR expression in the liver and WAT, which was accompanied by a 40–70% decrease in fasted glucose levels and a robust reduction in plasma triglyceride, cholesterol, and free fatty acids. No change in circulating corticosterone levels was seen in any model after GCCR ASO treatment. To further demonstrate that GCCR ASO does not cause systemic GC antagonism, normal Sprague-Dawley rats were challenged with dexamethasone after treating with GCCR ASO. Dexamethasone increased the expression of GC-responsive genes such as PEPCK in the liver and decreased circulating lymphocytes. GCCR ASO treatment completely inhibited the increase in dexamethasone-induced PEPCK expression in the liver without causing any change in the dexamethasone-induced lymphopenia. These studies demonstrate that tissue-selective GCCR antagonism with ASOs may be a viable therapeutic strategy for the treatment of the metabolic syndrome. *Diabetes* 54:1846–1853, 2005

Diabetes is a chronic metabolic disorder and a growing medical problem of which the etiology and pathogenesis is not fully understood (1). In type 2 diabetes, insulin resistance leads to an inability of insulin to control the activity of gluconeogenic enzymes and results in significant elevation of hepatic glucose production, thus causing hyperglycemia (2). This is accompanied by abnormalities in lipid and protein metabolism, and together these metabolic perturbations can lead to serious complications including nephropathy, retinopathy, neuropathy, and coronary artery disease (3–5).

Glucocorticoids (GCs) are hormones that are synthesized in the adrenal cortex under the control of the hypothalamic-pituitary-adrenal (HPA) axis. GCs are represented mainly by cortisol and corticosterone in humans and rodents, respectively. Excessive GC action is known to cause a spectrum of clinical features such as obesity, insulin resistance, and glucose intolerance as exemplified in Cushing's Syndrome (6). GCs promote breakdown of protein and fat from storage, which causes an increased supply of free fatty acids and branched amino acids to the liver (7). In addition, GCs increase cellular concentrations of enzymes and substrates for gluconeogenesis that ultimately result in increased hepatic glucose production. In the pancreas, GCs adversely affect the β-cells and atten-

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Received for publication 23 July 2004 and accepted in revised form 10 March 2005.

ASO, antisense oligonucleotide; GC, glucocorticoid; GCCR, glucocorticoid receptor; HFD-STZ, high-fat diet–fed streptozotocin-treated; HMG, 3-hydroxy-3-methylglutaryl; HPA, hypothalamic-pituitary-adrenal; POMC, proopiomelanocortin; STZ, streptozotocin; TAT, tyrosine aminotransferase; TNF, tumor necrosis factor.

uate insulin release (8), and in peripheral tissues, GCs impair glucose uptake by skeletal muscle and adipose tissue (9,10). GCs exert their effects by binding to an intracellular glucocorticoid receptor (GCCR). Upon activation, the GCCR translocates into the nucleus and binds to glucocorticoid response elements, thus resulting in the transcriptional activation of GC-regulated enzymes such as tyrosine aminotransferase (TAT) (11) and PEPCK, the rate-limiting enzyme in the gluconeogenic pathway (12, 13). Because of its role in the transcriptional activation of gluconeogenic enzymes, pharmacological interventions to attenuate GCCR action and to consequently modulate hepatic glucose production have attracted intense interest. Both steroidal and nonsteroidal GCCR antagonists were reported earlier to show efficacy in rodent models of diabetes (14–16). For example, mifepristone (RU-486) reduced hyperglycemia by decreasing glucocorticoid-regulated transcription of gluconeogenic enzymes in *db*/*db* mice (17). Despite this positive effect, mifepristone also caused unfavorable extra-hepatic effects, including activation of the HPA axis.

Here, we report that specific reduction of GCCR mRNA expression in liver and white adipose tissue (WAT) with optimized 2′-*O*-methoxyethyl antisense oligonucleotides (ASOs) leads to significant attenuation of hyperglycemia and hyperlipidemia in rodent models of type 2 diabetes. Furthermore, GCCR ASO reduced hepatic glucose production and inhibited hepatic gluconeogenesis. Expression of GCCR and proopiomelanocortin (POMC) in the pituitary gland was unaffected by GCCR ASO treatment, and no change in circulating corticosterone levels was observed. In addition, we also showed that GCCR ASO treatment did not cause systemic GC antagonism, as reflected by a lack of effect on systemic dexamethasone-induced lymphopenia.

RESEARCH DESIGN AND METHODS

Selection of rodent GCCR ASOs. Rapid throughput screens were performed in vitro to identify GCCR-selective ASO inhibitors for both mouse and rat. Briefly, eighty ASOs were designed to both the mouse and rat GCCR mRNA sequences. ASOs were synthesized as 20-base phosphorothioate chimeric oligonucleotides where bases 1–5 and 16–20 were modified with 2′-*O*-(2-methoxy)-ethyl residues. This chimeric design has been shown to provide increased nuclease resistance and mRNA affinity while maintaining the robust RNase H–terminating mechanism used by this class of ASOs (18).

GCCR ASOs were screened in primary mouse and rat hepatocytes for their ability to reduce GCCR mRNA expression. Primary hepatocytes were isolated as previously described and plated onto collagen-coated plates (19). Hepatocytes were treated with ASO and Lipofectin (Invitrogen, Carlsbad, CA) mixtures in serum-free William's E media (Invitrogen). After 4 h, ASO reaction mixtures were replaced with normal growth media (William's E media with 10% FBS), and the cells were incubated under normal conditions for an additional 16–20 h. GCCR mRNA was analyzed by quantitative RT-PCR after extraction of total RNA from cells using the RNeasy 96-kit (Qiagen, Valencia, CA). The method for RT-PCR analysis and primer-probe sets used is identical to that described below for in vivo sample analysis. The final GCCR ASO leads were selected for pharmacological efficacy studies in animal models.

Animal studies. Mouse experiments were conducted in 6- to 8-week-old male *ob*/*ob* (C57BL/6J-Lep^{ob}/Lep^{ob}) and *db*/*db* (C57BLKS/J-*m*-Lepr^{db}/Lepr^{db}) mice and normal lean littermates (C57BL/6J) (Jackson Laboratories, Bar Harbor, ME). Rat experiments were conducted in 7- to 8-week-old male Zucker diabetic fatty ZDF/Gmi-fa (ZDF) and male Sprague-Dawley (SD) rats (Charles River, Portage, MI). All animal experiments were performed under the institutional American Association for the Accreditation of Laboratory Animal Care guidelines. Animals were maintained on a 12-h light/dark cycle and fed ad libitum unless noted. The *ob*/*ob* and *db*/*db* mice were fed Diet 5015 and 5008 (Purina LabDiet, Richmond, IN), respectively. ZDF rats were also fed the 5008 diet, and SD rats were fed normal rodent food. Animals were acclimated for 7 days in the research facility before initiation of the experiments. The high-fat diet–fed streptozotocin (HFD-STZ) rat model was developed as described earlier with slight modifications (20). Normal 6-week-old male SD rats were fed with diet 96132 (Harlan Teklad, Madison, WI) for 2 weeks followed by a single intraperitoneal injection of 50 mg/kg streptozotocin (STZ) (Sigma, St. Louis, MO). One week after STZ injection, glucose levels were determined. All the animals were randomized to treatment groups based on plasma glucose levels. ASOs were prepared in buffered saline and sterilized by filtering through a 0.2-μm filter. Animals were dosed with saline, the lead GCCR ASO dissolved in saline, or a control ASO that had the same chemistry, length, and molecular weight as the GCCR ASO, but did not reduce the expression of GCCR or any other known gene when blasted against known databases. In *ob*/*ob* mice, efficacy of GCCR ASO was evaluated in multiple doses (6.25, 12.5, and 25 mg/kg) and the ASOs were given twice weekly via subcutaneous injection for 4 weeks. For the studies involving *db*/*db* mice, ZDF, and HFD-STZ rats, a single dose of 25 mg/kg was examined by administering twice weekly via subcutaneous injection for 4 weeks. Blood samples were obtained from mice and rats via tail snip. For fasted glucose measurements, the animals were fasted for 12 h with free access to water. All clinical parameters were measured on the Olympus AU 400^{e} Clinical Analyzer. Body composition analysis was conducted using Lunar X-ray densitometer (GE Medical Systems, Madison, WI) in *ob*/*ob* mice that were treated with 25 mg/kg GCCR ASO twice a week for 4 weeks. Liver glycogen was measured as described (21). Plasma adiponectin, resistin, tumor necrosis factor (TNF)-α, and insulin levels were measured by enzyme-linked immunosorbent assay using kits from Linco Research (St. Charles, MO) and ALPCO (Windham, NH); plasma interleukin-6 levels were measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN).

For ex vivo hepatic glucose production studies, control and GCCR ASO–treated SD rats were fasted for 24 h and administered either vehicle or dexamethasone (Bausch & Lomb, Tampa, FL) at a dose of 12.5 mg/kg. Six hours after treatment, precision-cut liver slices were prepared using a Krumdieck Tissue Slicer (Alabama Research and Development, Munford, AL) and incubated in glucose-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 0.1% BSA, 10 mmol/l lactate, 1 mmol/l sodium pyruvate, 10 mmol/l alanine, and 10 mmol/l glycerol (no-glucose-supplemented medium). After a 1-h preincubation, individual slices were transferred to separate wells of a 24-well plate containing 0.5 ml no-glucose-supplemented medium, and glucose released into the medium after 1.5 h was determined by a Hitachi 912 clinical chemistry analyzer. Liver slices were weighed, and glucose production per milligram of liver tissue was determined. For the dexamethasone challenge, normal SD rats were administered a dose of 4 mg/kg 72 h after the last ASO injection. Plasma corticosterone and ACTH levels were measured using enzyme-linked immunosorbent assay kits (ALPCO) following the manufacturer's instructions.

Tissue RNA Isolation. Forty-eight hours after the final dose, the animals were killed, and liver tissue and WAT were isolated. The tissue samples 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 (22). 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) following the manufacturer's instructions.

RNA expression analysis by quantitative real-time RT-PCR. A detailed description of the method of analysis of total RNA has been described previously (23). Briefly, targeted mRNA from tissue culture or animal tissues was analyzed by RT-PCR using 100 ng total RNA in a final volume of 30 μl containing 200 nmol/l of the target-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 units of platinum *Taq*DNA (Invitrogen), and 8 units of ribonuclease inhibitor. Reverse transcription was performed for 30 min at 48°C followed by 40 thermal cycles of 30 s at 94°C and 1 min at 60°C PCR using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Target mRNA was normalized to total RNA as determined by RiboGreen fluorescence from the same RNA sample.

Primer probes. The following primer probe sets were used for real-time RT-PCR analysis. Mouse GCCR: forward primer (FP) 5′-CGGGACCACCTCC CAAA-3′, reverse primer (RP) 5′-CCCCATAATGGCATACCGAA-3′, and probe primer (PP) 5′-TCTGCCTGGTGTGCTCCGATGAAG-3′. Rat GCCR: FP 5′-AA ACAATAGTTCCTGCAGCATTACC-3′, RP 5′-CATACAACACCTCGGGTTCAA TC-3′, and PP 5′-ACCCCTACCTTGGTGTCACTGCT-3′. Rat POMC: FP 5′-CG AGGCCTTTCCCCTAGAGT-3′, RP 5′-ACGTGCTCCAAGCCATCAG-3′, and PP 5′-CAAGAGGGAGCTGGAAGGCGAGC-3′. Rat TAT: FP 5′-AAGCAGAAGACG GAGGATGC-3′, RP 5′-CCGACCTGACTGAAGAGGATG-3′, and PP 5′-TCTCCC ACCCATTTCCTCGTGCTG-3′. Rat PEPCK: FP 5′-ACAGGCAAGGTCATCATG CA-3′, RP 5′-TGCCGAAGTTGTAGCCAAAGA-3′, and PP 5′-ACCCCTTCGCTA TGCGGCCC-3′. Rat 3-hydroxy-3-methylglutaryl (HMG)-CoA: FP 5′-ATCCTGG

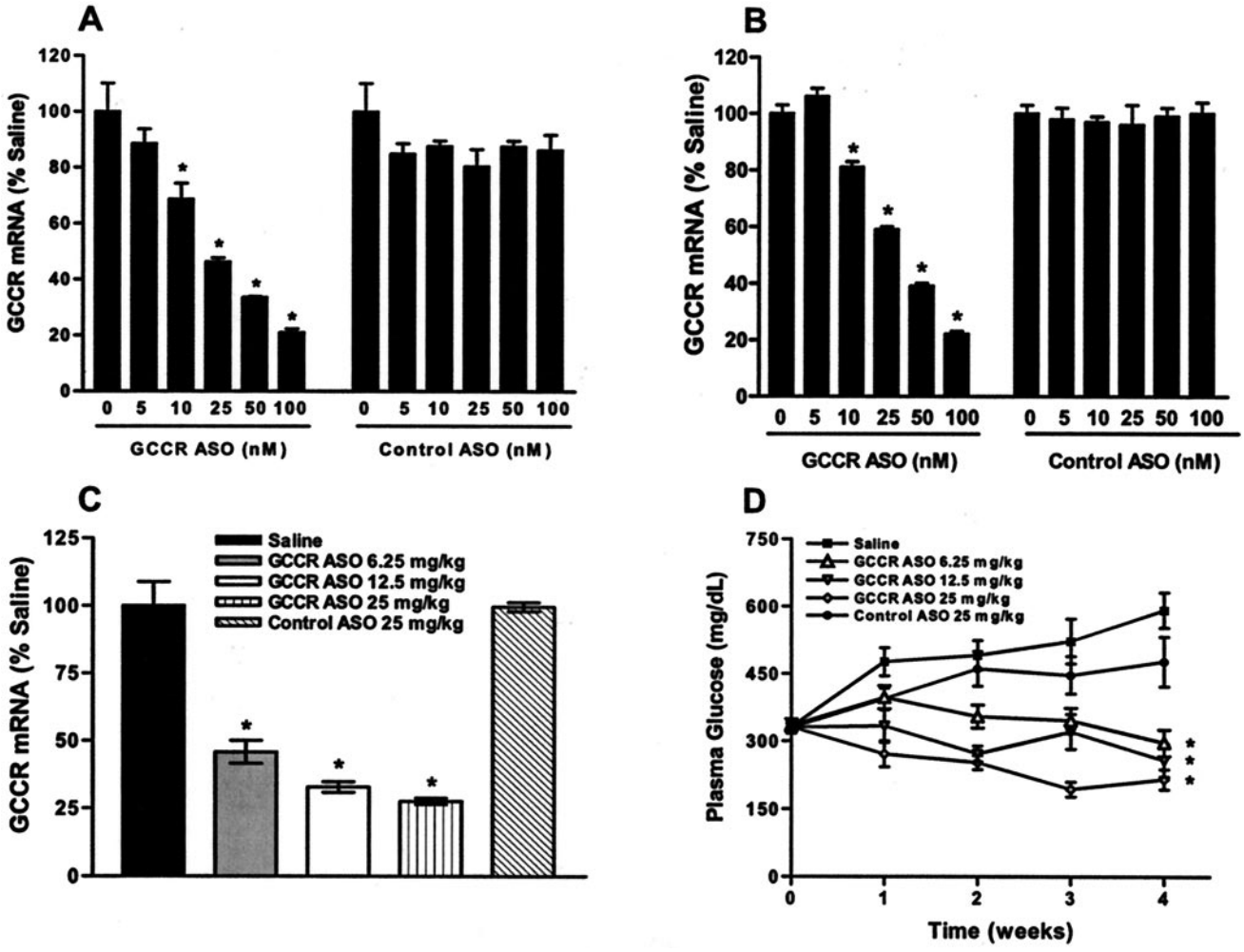

FIG. 1. In vitro reduction of GCCR mRNA expression in mouse and rat hepatocytes. Mouse (*A*) or rat (*B*) primary hepatocytes were treated with the indicated ASO concentration for 4 h as described in RESEARCH DESIGN AND METHODS. After ASO treatment, cells were cultured in growth medium for an additional 16–20 h. Total RNA was extracted and GCCR mRNA expression was assessed by RT-PCR. GCCR expression was normalized to total RNA in the same samples. Data are expressed as means ± SE of mRNA levels relative to untreated cells ($n = 3$). *$P < 0.05$, GCCR ASO treatment vs. corresponding untreated group by ANOVA. *C*: GCCR ASO specifically reduces GCCR mRNA levels in liver from *ob/ob* mice. Mice were dosed with saline, GCCR ASO 6.25 mg/kg, 12.5 mg/kg, and 25 mg/kg, or control ASO 25 mg/kg subcutaneously twice a week for 4 weeks. Total mRNA was prepared and analyzed for GCCR expression by RT-PCR ($n = 7$ per group). Data are expressed as means ± SE relative to saline-treated mice. *$P < 0.05$, GCCR ASO treatment vs. saline treatment by ANOVA. *D*: Reduction of GCCR expression lowers plasma glucose. *Ob/ob* mice were dosed as described above, and plasma was analyzed for glucose as described in RESEARCH DESIGN AND METHODS. Data are expressed as means ± SE. *$P < 0.05$, GCCR ASO treatment vs. saline treatment by ANOVA.

GCCCCACGT-3′, RP 5′-TGGTGCCAACTCCAATCACA-3′, and PP 5′-CACCCT TGACGCTCTGGTGGAATGTC-3′.

Statistical analysis. All results are expressed as means ± SE. Statistical significance was calculated with the Student's *t* test or ANOVA followed by Bonferroni or Dunnett's post hoc test as appropriate using Prism software (Graphpad, San Diego, CA). An α value of $P < 0.05$ was considered statistically significant.

RESULTS

Identification and characterization of potent mouse and rat GCCR ASOs. After extensive characterization of multiple candidate ASOs, lead ASOs for each species (mouse and rat) were selected for in vivo studies. Both mouse GCCR ASO (Fig. 1*A*) and rat GCCR ASO (Fig. 1*B*) potently reduced the expression of GCCR mRNA in primary hepatocytes. A control ASO composed of the same chemistry and oligonucleotide length had no effect on GCCR mRNA expression.

GCCR ASO treatment reduces GCCR expression in vivo. Based on the in vitro results, we investigated the effects of the lead murine GCCR ASO to suppress GCCR mRNA levels in murine models of type 2 diabetes. After 4 weeks of systemic administration of GCCR ASO to *ob*/*ob* mice, a dose-dependent reduction of hepatic GCCR mRNA expression was observed (Fig. 1*C*). Similarly, in *db*/*db* mice, GCCR ASO treatment resulted in a significant reduction of GCCR mRNA levels in the liver (saline 100 ± 5 vs. GCCR ASO 29 ± 4, $P < 0.05$). In both *ob*/*ob* and *db*/*db* mice, GCCR mRNA levels were significantly reduced by 40–50% in WAT (data not shown). The control ASO had no effect on GCCR mRNA expression in either of these tissues.

GCCR ASO treatment lowers plasma glucose levels in *ob/ob* and *db/db* mice. In addition to reducing the level of GCCR mRNA, GCCR ASO decreased plasma glucose and circulating lipid levels in diabetic models. In saline and control ASO-treated *ob*/*ob* mice, hyperglycemia continued to worsen throughout the study duration, whereas GCCR ASO–treated animals showed a significant dose-dependent

TABLE 1
Effect of GCCR ASO on adipocytokines and insulin

	Resistin (ng/ml)	TNF-α (pg/ml)	Insulin (ng/ml)	Adiponectin (μg/ml)	Interleukin-6 (pg/ml)
Treatment					
Saline	22 ± 1.4	42 ± 2.3	43 ± 5.9	8.73 ± 0.14	6.27 ± 0.74
GCCR ASO	18 ± 0.9	27 ± 0.8*	16 ± 1.4*	8.72 ± 0.35	5.37 ± 1.22

Data are means ± SE.

reduction in plasma glucose levels (Fig. 1*D*). A similar glucose-lowering effect was also observed in *db/db* mice treated with GCCR ASO compared with the saline-treated group (saline 510 ± 21 mg/dl vs. GCCR ASO 303 ± 19 mg/dl, $P < 0.05$). Because of GCCR's role in regulating gluconeogenesis, we monitored the effects of GCCR ASO on fasted glucose levels. A significant reduction in fasted glucose levels was observed in *ob/ob* mice (saline 321 ± 16.2 mg/dl vs. GCCR ASO 220 ± 8.3 mg/dl, $P < 0.05$) and *db/db* mice (saline 320 ± 26.9 mg/dl vs. GCCR ASO 204 ± 24.6 mg/dl, $P < 0.05$). In both models, control ASO did not show a significant effect on fasted glucose levels. The effects of GCCR ASO were not accompanied by changes in liver glycogen level, food intake, or body weight in these mice (data not shown).

Reduction of GCCR expression resulted in a significant lowering of plasma triglycerides in *db/db* mice as compared to saline-treated controls (saline 218.3 ± 12.3 mg/dl vs. GCCR ASO 122.5 ± 10.3 mg/dl, $P < 0.05$), but such a decrease was not observed in *ob/ob* mice (saline 121.1 ± 4.5 mg/dl vs. GCCR ASO 116.3 ± 6.2 mg/dl). No significant effects on plasma cholesterol were observed in *ob/ob* or *db/db* mice after GCCR ASO treatment (data not shown).

Effect of GCCR ASO on body composition, plasma resistin, adiponectin, TNF-α, insulin, and interleukin-6 levels in *ob/ob* mice. Although no change in body weight was observed, densitometric analysis of body composition was performed to accurately measure changes in body fat mass. GCCR ASO significantly reduced body fat mass after a 4-week treatment period (saline 50.7 ± 0.4 vs. GCCR ASO 45.7 ± 0.5, $P < 0.05$). This reduction was also reflected as a decrease in epididymal fat pad weight (saline 5.09 ± 0.10 g vs. GCCR ASO 4.3 ± 0.12 g, $P < 0.05$). The decrease in adiposity was accompanied by a 20% decrease in plasma resistin levels, whereas circulating adiponectin and interleukin-6 levels remained unchanged. A more robust effect on lowering of TNF-α by 36% and insulin levels by 63% was observed in GCCR ASO–treated mice (Table 1).

In a separate study, lean normoglycemic mice received the GCCR ASO at a dose of 50 $mg \cdot kg^{-1} \cdot week^{-1}$ for 6 weeks. GCCR ASO caused a significant reduction in GCCR mRNA expression in the liver (saline 100 ± 5.98 vs. GCCR ASO 24.4 ± 2, $P < 0.05$) and WAT (saline 100 ± 4 vs. GCCR ASO 31 ± 6, $P < 0.05$) without causing hypoglycemia (24-h fasted levels, saline 147 ± 8 mg/dl vs. GCCR ASO 112 ± 5 mg/dl).

Effects of GCCR ASO in diabetic ZDF and HFD-STZ rats. We further investigated the effects of GCCR ASO treatment on ZDF and HFD-STZ rats. ASO treatment for 4 weeks to these animals reduced GCCR mRNA levels by

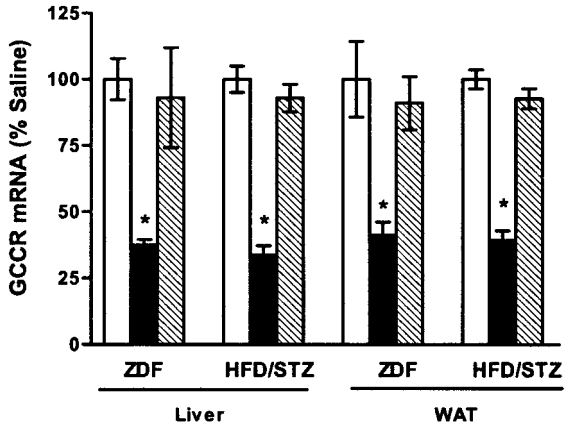

FIG. 2. GCCR ASO specifically reduced GCCR mRNA levels in liver and WAT from ZDF and HFD-STZ rats. Rats were dosed subcutaneously with saline (□), GCCR ASO 37.5 mg/kg (■), or control ASO 37.5 mg/kg (▨) twice a week for 4 weeks. Total mRNA was prepared from liver and WAT and analyzed for the expression of GCCR by RT-PCR ($n = 8$ per group for ZDF and $n = 7$ per group for HFD-STZ rats). Data are expressed as means ± SE relative to saline-treated rats. *$P < 0.05$, GCCR ASO treatment vs. saline treatment.

~60% in both liver and WAT as analyzed by RT-PCR (Fig. 2). Interestingly, no effect on fed glucose was observed in either rat model (data not shown). However, GCCR ASO treatment caused a significant lowering of fasted glucose levels in both models. In ZDF rats, the fasted glucose was reduced by ~40% (saline 255 ± 18 mg/dl vs. GCCR ASO 159 ± 21 mg/dl, $P < 0.05$). In HFD-STZ rats, the effect of GCCR ASO treatment on fasted glucose levels was more robust, reducing glucose levels by ~70% (saline 315 ± 34 mg/dl vs. GCCR ASO 99 ± 6.5 mg/dl, $P < 0.05$). Control ASO treatment had no significant effect on fasted glucose in these models. In addition, levels of plasma triglycerides and total cholesterol were significantly reduced in both models as a result of GCCR ASO treatment (Fig. 3). GCCR ASO significantly reduced plasma free fatty acids in both ZDF (saline 0.68 ± 0.01 mEQ/l vs. GCCR ASO 0.3 ± 0.02 mEQ/l, $P < 0.05$) and HFD-STZ rats (saline 0.93 ± 0.12 mEQ/l vs. GCCR ASO 0.52 ± 0.04 mEQ/l, $P < 0.05$). A

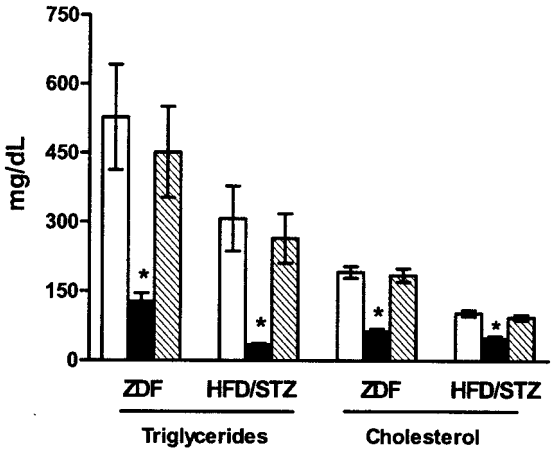

FIG. 3. Effect of GCCR ASO treatment on circulating lipids in ZDF and HFD-STZ rats. Rats were dosed with saline (□), GCCR ASO 37.5 mg/kg (■), or control ASO 37.5 mg/kg (▨) subcutaneously twice a week for 4 weeks. Blood samples were collected at the end of the study in the fed state, and lipids were analyzed as described in RESEARCH DESIGN AND METHODS. Data are expressed as means ± SE. *$P < 0.05$, GCCR ASO treatment vs. saline treatment.

TABLE 2
Plasma corticosterone (ng/ml) in diabetic rodents

	ob/ob mice	*db/db* mice	ZDF rats	HFD-STZ rats
Treatment				
Control ASO	319 ± 24	218 ± 23	30 ± 6	95 ± 23
GCCR ASO	257 ± 50	196 ± 75	35 ± 0.8	117 ± 46

Data are means ± SE.

reduction in epididymal fat pad weights by GCCR ASO was also observed in ZDF (saline 3.8 ± 0.07 g vs. GCCR ASO 2.6 ± 0.06 g, $P < 0.05$) and HFD-STZ rats (saline 2.41 ± 0.23 g vs. GCCR ASO 1.8 ± 0.63 g). The effects of GCCR ASO were not accompanied by any changes in food intake or body weight in these animals (data not shown). To understand the mechanism underlying the lipid-lowering effects of GCCR ASO, we investigated the expression of several lipogenic genes in these models. GCCR ASO treatment caused a reduction in the expression of HMG-CoA reductase, a rate-limiting enzyme in cholesterol biosynthesis (saline 100 ± 11 vs. GCCR ASO 62 ± 3.5, $P < 0.05$), thus explaining in part the effects of the GCCR ASO on cholesterol levels. The expression of several other lipogenic genes, including squalene synthase, sterol regulatory element binding protein-1c, and HMG-CoA synthase, remained unchanged (data not shown).

GCCR ASO decreased hepatic glucose production and gluconeogenesis. Ex vivo hepatic glucose production assay on liver slices from SD rats treated with GCCR ASO showed a significant reduction in basal glucose production (control ASO 0.86 ± 0.16 vs. GCCR ASO 0.35 ± 0.01 glucose [g] · h^{-1} · liver $slice^{-1}$ [mg], $P < 0.05$). To directly evaluate the effects of the ASO on GC-mediated glucose production, assays were performed in ASO-treated rats that were injected with dexamethasone 6 h before necropsy. GCCR ASO dramatically inhibited dexamethasone-induced glucose production (control ASO + dexamethasone 5.61 ± 0.68 vs. GCCR ASO + dexamethasone 0.61 ± 0.04 glucose (g) · h^{-1} · liver $slice^{-1}$ (mg), $P < 0.05$).

GCCR antagonism by ASO treatment does not stimulate the HPA axis or lead to systemic GC antagonism. Several parameters were evaluated to investigate the effect of GCCR ASO treatment on systemic glucocorticoid activity. First, plasma corticosterone was measured in all studies and was found to be unchanged in both mice and rats (Table 2). Second, the expression of GCCR and POMC mRNA in the pituitary gland from ZDF rats was measured (pituitaries were not extracted from the other models) and was shown to be unchanged with GCCR ASO treatment (Fig. 4*A* and *B*). Finally, to further address systemic effects, normal SD rats were treated with GCCR ASO twice weekly for 4 weeks and were then subjected to a dexamethasone challenge. ASO treatment significantly reduced the expression of hepatic GCCR mRNA by ~75% (Fig. 5*A*). Dexamethasone induced the transcriptional activity of TAT in liver by ~5.4-fold and in WAT by ~8.8-fold in saline-treated animals, and this induction was completely blunted in GCCR ASO–treated rats in both tissues examined (Fig. 5*B*). In addition, GCCR ASO also reduced dexamethasone-induced PEPCK expression in the liver (Fig. 5*C*). In contrast, GCCR ASO treatment had no significant effect on dexamethasone-induced lymphopenia, a marker of systemic GC effects (Fig. 5*D*). Finally, GCCR ASO did not affect basal ACTH levels (saline 9.79 ± 3.39 pg/ml vs. GCCR ASO 9.96 ± 3 pg/ml).

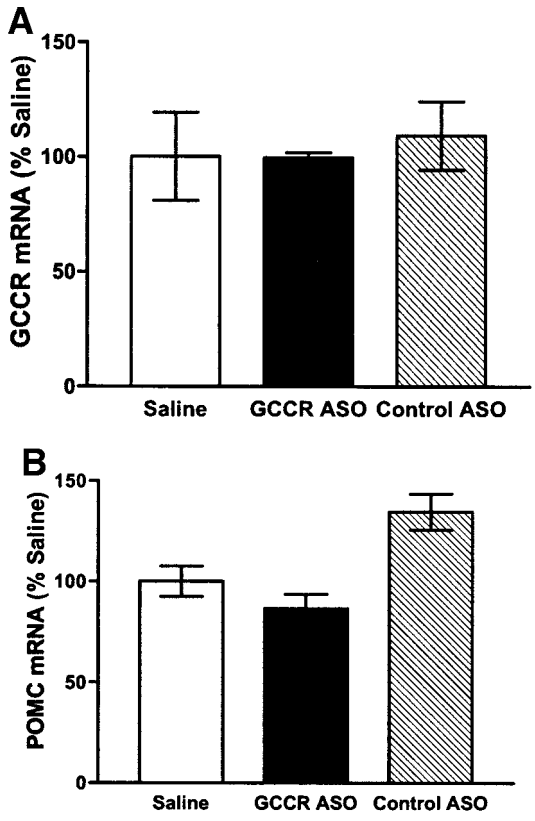

FIG. 4. GCCR and POMC expression in the pituitary gland remained unchanged after GCCR ASO treatment in ZDF rats. Rats were dosed with saline (□), GCCR ASO 37.5 mg/kg (■), or control ASO 37.5 mg/kg (▨) twice a week for 4 weeks. Total mRNA was prepared from the pituitary gland and analyzed for the expression of GCCR (*A*) and POMC (*B*) by RT-PCR (n = 4 per group). Data are expressed as means ± SE relative to saline-treated controls.

DISCUSSION

GCs increase hepatic gluconeogenesis and play an important role in the regulation of hepatic glucose output. Although systemic GC inhibition can improve hyperglycemia in rodents and humans, it leads to adrenal insufficiency and stimulation of the HPA axis. The ASO chemistry used in these studies results in specific accumulation of ASO in liver and WAT without accumulation in muscle and the central nervous system (19,24–29). This unique pharmacokinetic profile of these molecules allowed us to examine the effects of tissue-specific reduction of GCCR expression on various metabolic parameters in models of diabetes. Our results demonstrate that specific reduction of the GCCR mRNA expression in liver and WAT was sufficient to cause metabolic improvements in plasma glucose and lipids without stimulating the HPA axis or resulting in systemic GC antagonism.

Pharmacological reduction of GCCR expression by ASO treatment in *ob*/*ob* and *db*/*db* mice resulted in a significant lowering of both fed and fasted glucose levels. In hyperinsulinemic *ob*/*ob* mice, a significant reduction in plasma insulin levels was observed, which is suggestive of an improvement in insulin sensitivity, since the animals were able to maintain a lower glucose concentration despite a

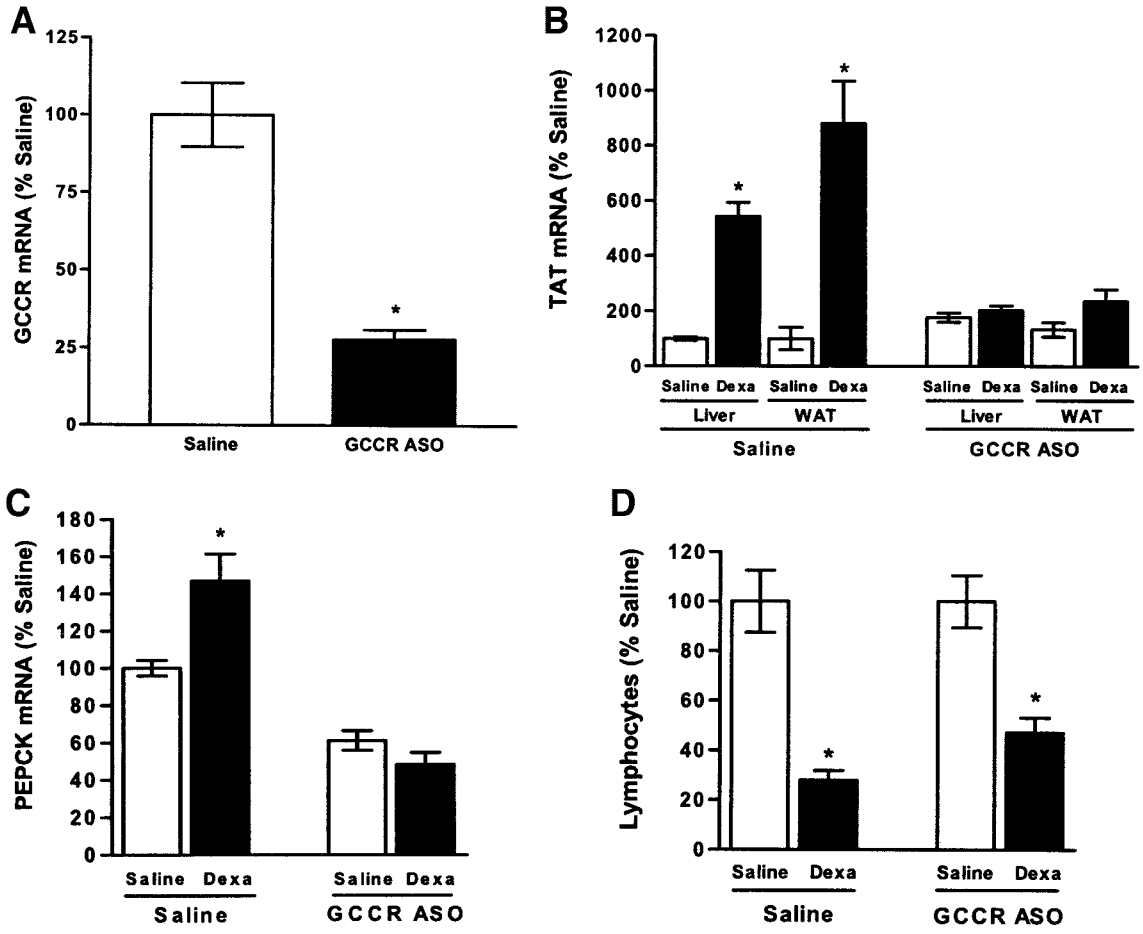

FIG. 5. GCCR ASO treatment did not result in systemic GCCR antagonism. Rats were dosed with saline (□) or GCCR ASO (■) at a dose of 50 mg/kg twice weekly for 4 weeks. A subset of animals was challenged with dexamethasone (Dexa) 72 h after the final dose. Total mRNA was prepared from liver and analyzed for the expression of the following genes by RT-PCR (n = 4 per group). *A*: GCCR expression in GCCR ASO–treated rats not challenged with dexamethasone. *P < 0.05, GCCR ASO treatment vs. saline treatment. *B*: TAT expression after dexamethasone injection in liver and WAT. *C*: PEPCK expression after dexamethasone injection in liver. *D*: Total lymphocytes measured by counting and expressed relative to the mean percentage of lymphocytes in saline-treated rats. *P < 0.05, dexamethasone treatment vs. saline treatment.

60% decrease in circulating insulin levels. In addition, TNF-α levels were decreased, which could be attributed to the reduction in fat mass. It has been known that excess GC action is involved in obesity (6), and adipose tissue is a significant source of endogenous TNF-α secretion (30). Reduction in epididymal fat weight by GCCR ASO treatment raises an interesting possibility for the treatment of weight gain, as has been suggested for adipose tissue–specific GCCR inhibition previously (31).

To examine the mechanism(s) of action underlying the glucose-lowering effects of the GCCR ASO, we examined the direct effects of the ASO on hepatic glucose production. We demonstrated that GCCR ASO treatment directly inhibits hepatic glucose production and that inhibition of gluconeogenesis is one of the mechanisms underlying this effect and improvement in hyperglycemia. This is in agreement with previous observations that specific inactivation of the GCCR in liver by Cre/loxP method reduces the expression of gluconeogenic genes and consequently restricts gluconeogenesis and the development of hyperglycemia in an STZ-induced diabetes model (32). However, the observation that adipocytokine and insulin levels were also lowered suggests that secondary improvements in peripheral insulin sensitivity could also underlie the observed efficacy in our studies.

It is well established that mobilization of free fatty acids due to lipolysis contributes significantly to increased hepatic gluconeogenesis (7). Furthermore, TNF-α stimulates lipolysis, especially in the presence of concomitant hyperglycemia (33). Thus, a reduction in TNF-α levels could be one of the mechanisms by which GCCR ASO reduced free fatty acid levels and consequently gluconeogenesis. Glycogen levels in livers from fed and fasted animals treated with the GCCR ASO were unchanged. In contrast, we have previously demonstrated that inhibition of glycogenolysis (e.g., with ASOs against glycogen phosphorylase) causes a significant increase in hepatic glycogen levels in the same animal model (34). Thus, it appears that inhibition of glycogenolysis may not be the main cause underlying the effect of GCCR ASO in these models.

Hypoglycemia was not observed in any of these models. In lean mice, no apparent effects were observed on either fed or fasted glucose concentrations, even though the GCCR expression levels were reduced to the same extent to those observed in *ob*/*ob* or *db*/*db* mice. This suggests that the amount of reduction caused by the ASO was

sufficient to cause beneficial effects in diabetic animals, but the residual amount of target was sufficient to offset any side effects such as hypoglycemia. The lack of efficacy on fed glucose levels in ZDF and HFD-STZ rats may be related to the potency of the ASO because it resulted in only ~65% reduction of GCCR mRNA in the liver. Alternatively, glucocorticoids may not play a significant role in maintaining hyperglycemia in the fed state in these animals.

A robust effect on lowering of plasma triglycerides by GCCR reduction was evident in both rat models and *db/db* mice. Interestingly, in HFD-STZ rats, the triglyceride-lowering effect caused by GCCR reduction was sustained even in the presence of high-fat feeding during the experimental period. The cholesterol-lowering effect of GCCR ASO was evident in rats but not in the mouse models tested, which may either reflect disparate roles of glucocorticoids in the two species or inherent differences in lipid metabolism between the two species. It is known that GCs elevate and regulate HMG-CoA reductase expression levels (35,36). Thus, the reduction in cholesterol after GCCR ASO treatment is most likely mediated by a decrease in HMG-CoA reductase expression. Increasing evidence suggests that excess triglycerides (circulating or intracellular) play a causal role in obesity, insulin resistance, and type 2 diabetes (37–43). GCs stimulate secretion of triglycerides from the liver and also decrease the levels of lipoprotein lipase, thus augmenting hyperlipidemia (44–46). In addition, GCs promote differentiation of adipocytes from preadipocytes and triglyceride storage in fat cells (47). Although systemic GC excess in Cushing's syndrome is implicated in the development of visceral obesity and the metabolic syndrome, circulating GC levels appear to be normal in obese patients (48,49). It has been suggested that increased GC action within liver tissue and WAT may cause tissue-specific amplification of GC effects such as increased adipocyte differentiation, increased lipogenesis, and increased gluconeogenesis without any change in circulating GC levels (31,50,51). Our data support this notion, since local antagonism of GC in liver and WAT caused significant improvements in hyperglycemia and hyperlipidemia without any changes in circulating corticosterone levels.

A significant observation from this study is that GCCR ASO treatment had no detectable effect on the HPA axis. Regulation of the HPA axis is an important factor in the pathology of defective counterregulatory mechanisms in diabetes, since the HPA axis regulates GC secretion (52). Activation of the HPA axis induces corticotrophin-releasing hormone, which mobilizes to the pituitary gland via hypophyseal-portal circulation. It stimulates the production and processing of POMC into peptides including ACTH, which in turn act at the adrenal cortex to stimulate the secretion of corticosterone (53). It has been reported that the steroidal GCCR antagonist mifepristone (RU-38486) affects the HPA axis by increasing corticosterone levels in addition to glucose lowering in *db/db* mice (17). Conjugation of mifepristone with cholic acid demonstrated some improved hepatic selectivity and was shown to reduce glucose and lipids in animal models of diabetes without activating the HPA axis (54). In other studies, optimization of the steroidal core led to the development of RU-43044 with increased selectivity to GCCR but with limited efficacy (14). In the present study, GCCR ASO treatment did not affect the expression of either GCCR or POMC genes in the pituitary gland, nor did it change plasma corticosterone and ACTH levels. Furthermore, no change in dexamethasone-induced lymphopenia was observed, confirming the lack of an effect on the HPA axis. Recently, it was reported that specific inactivation of the GCCR in the liver by the Cre/loxP system resulted in amelioration of STZ-induced hyperglycemia and caused hypoglycemia upon prolonged fasting (32). Our results are in agreement in that we did see a reduction in fasting glucose levels; however, GCCR ASO treatment did not result in hypoglycemia during periods of fasting up to 24 h in any of the models tested in the present study. This may be because GCCR mRNA levels were only partially reduced (60–75%) in the liver by GCCR ASO treatment as opposed to a complete loss in the Cre/loxP-treated animals. In addition, inherent differences in glucose metabolism between the type 1 diabetic STZ model and the type 2 models in our study may also help explain this discrepancy.

In conclusion, our results demonstrate that tissue-specific antagonism of glucocorticoids with GCCR ASO treatment improves hyperglycemia and hyperlipidemia without causing systemic GCCR antagonism or affecting the HPA axis. These data raise the possibility that such a therapeutic strategy could be useful for the treatment of type 2 diabetes. Studies are in progress to explore additional mechanisms underlying the observed pharmacology and to further the development of GCCR ASOs for the treatment of type 2 diabetes.

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