Hepatic Glucocorticoid Receptor Antagonism Is Sufficient to Reduce Elevated Hepatic Glucose Output and Improve Glucose Control in Animal Models of Type 2 Diabetes


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ABSTRACT

Glucocorticoids amplify endogenous glucose production in type 2 diabetes by increasing hepatic glucose output. Systemic glucocorticoid blockade lowers glucose levels in type 2 diabetes, but with several adverse consequences. It has been proposed, but never demonstrated, that a liver-selective glucocorticoid receptor antagonist (LSGRA) would be sufficient to reduce hepatic glucose output (HGO) and restore glucose control to type 2 diabetic patients with minimal systemic side effects. A-348441 \[(3\beta,5\beta,7\alpha,12\alpha)-7,12\text{-dihydroxy-3-\{2-\[(4-[\{11\beta,17\beta\}-17\text{-hydroxy-3-oxo-17-prop-1-ynylestra-4,9-dien-11-yl\} phenyl\]methyl\}amino\}ethoxy\}cholan-24-oic acid\] represents the first LSGRA with significant antidiabetic activity. A-348441 antagonizes glucocorticoid-up-regulated hepatic genes, normalizes postprandial glucose in diabetic mice, and demonstrates synergistic effects on blood glucose in these animals when coadministered with an insulin sensitizer. In insulin-resistant Zucker fa/fa rats and fasted conscious normal dogs, A-348441 reduces HGO with no acute effect on peripheral glucose uptake. A-348441 has no effect on the hypothalamic pituitary adrenal axis or on other measured glucocorticoid-induced extrahepatic responses. Overall, A-348441 demonstrates that an LSGRA is sufficient to reduce elevated HGO and normalize blood glucose and may provide a new therapeutic approach for the treatment of type 2 diabetes.

Glucocorticoids are steroidal hormones that play an essential role in whole body glucose homeostasis, as well as in adaptive responses to physiological, metabolic, and immune-related stress (Baxter and Rousseau, 1979). It has been known since the 1920s that extracts of the adrenal glands contain a substance (now identified as cortisol) that modu-

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ABBREVIATIONS: PEPCK, phosphoenolpyruvate carboxykinase; GR, glucocorticoid receptor; GIR, glucose infusion rate; RU-486, RU-38486 (mifepristone); HbA\text{c1c}, glycosylated hemoglobin; HPA, hypothalamic pituitary adrenal; LSGRA, liver-selective glucocorticoid receptor antagonist; A-348441, \[(3\beta,5\beta,7\alpha,12\alpha)-7,12\text{-dihydroxy-3-\{2-\[(4-[\{11\beta,17\beta\}-17\text{-hydroxy-3-oxo-17-prop-1-ynylestra-4,9-dien-11-yl\} phenyl\]methyl\}amino\}ethoxy\}cholan-24-oic acid\]; HGO, hepatic glucose output; HPMC, hydroxypropylmethylcellulose; TAT, tyrosine aminotransferase; RT-PCR, reverse transcription-polymerase chain reaction; RPC, rat prednisolone challenge; ACTH, adrenocorticotropic hormone; DHT, delayed-type hypersensitivity; DNFB, 2,4-dinitrofluorobenzene; q.d., once daily.
developing insulin resistance and type 2 diabetes is substantially elevated (Niemann et al., 1985; Chu et al., 2001). The opposite situation also holds true for patients with adrenal atrophy or those with Addison’s disease, where lower circulating glucocorticoid levels or activity translate into improved insulin sensitivity and glucose tolerance (Armstrong and Bell, 1996).

The primary metabolic target organ of glucocorticoid action is the liver, where glucocorticoids activate the transcription of key gluconeogenic enzymes, including phosphoenolpyruvyl carboxykinase (PEPCK) and glucose-6-phosphatase (Barthel and Schmoll, 2003). Transgenic mice that overexpress PEPCK display a diabetic phenotype characterized by increased basal hepatic glucose output and decreased hepatic insulin sensitivity (Friedman et al., 1997). Conversely, liver-specific GR knockout mice are prone to hypoglycemia and are resistant to streptozotocin-induced type 1 diabetes due to impaired induction of gluconeogenesis (Opherk et al., 2004). Transgenic animals with a defect in the dimerization domain of the glucocorticoid receptor (GR<sup>dim/dim</sup>) also demonstrate impaired transactivation of gluconeogenic genes in the liver (Reichardt et al., 1998). Pharmacological evidence using the prototype systemic GR antagonist RU-38486 (mifepristone, RU-486) further validates the role of glucocorticoids in glucose regulation. Treatment of obese, diabetic ob/ob mice for 21 days with RU-486 normalizes postprandial glucose values and reduces hyperglycemia (Gettys et al., 1997).

Human data also suggest a regulatory role for GR in the pathogenesis of type 2 diabetes. Genetic diseases in humans with loss of function mutations in key gluconeogenic enzymes lead to significant reductions in hepatic glucose output and episodes of hypoglycemia (Rallison et al., 1979; van den Bergh, 1996). Humans with increased GR expression in skeletal muscle show insulin resistance (Reynolds et al., 2002), and skeletal muscle myoblasts from male individuals show a correlation between GR expression and insulin resistance (Whorwood et al., 2002). In normal human subjects, a single dose of RU-486 reduces hepatic glucose output (Garrel et al., 1995), and in severe cases of Cushing’s syndrome, long-term treatment with RU-486 improves glucose metabolism as demonstrated by reductions in glycosylated hemoglobin (HbA<sub>1c</sub>) from 11.5 to 6.9% (Chu et al., 2001).

Although these data provide compelling evidence for a role of glucocorticoids in the pathogenesis of type 2 diabetes, there have been no reported studies showing that the selective antagonism of hepatic glucocorticoid receptors is sufficient to reduce elevated hepatic glucose output or that reduced hepatic glucocorticoid activity would improve overall glucose control in models of type 2 diabetes. Systemic GR antagonism (as accomplished by RU-486) is sufficient to improve glucose metabolism, but this approach can result in symptomatic adrenal insufficiency (nausea, vomiting, and exhaustion), as well as increased levels of circulating cortisol due to counter-regulatory activation of the hypothalamic-pituitary-adrenal (HPA) axis (Lamberts et al., 1991). As a long-term treatment for type 2 diabetes, systemic GR antagonism is not a feasible therapeutic approach. On the other hand, an LSGRA would be expected to have therapeutic value. To explore and validate the hypothesis that an LSGRA would improve glycemic control, we have developed a novel, liver-targeted GR antagonist (A-348441) that combines the “address” of an enterohepatically constrained bile acid with the “message” of a potent GR antagonist (von Geldern et al., 2004). In theory, this pharmacological approach should antagonize GR in the liver, reduce HGO, improve hyperglycemia, and eliminate the secondary side effects associated with systemic GR antagonism. This report details the liver-selective pharmacology of the bile acid conjugate A-348441 and provides the first direct evidence that antagonism of hepatic GR is both possible and effective in reducing the elevated HGO that contributes to the pathogenesis of type 2 diabetes.

Materials and Methods

Experimental Compounds

All in vitro assays used dimethyl sulfoxide stock solutions of test compounds. Rosiglitazone was synthesized at Abbott Laboratories (Abbott Park, IL); RU-486 was purchased from Austin Chemical (Buffalo Grove, IL); metformin, dexamethasone, prednisolone, and 2,4-dinitrofluorobenzene were purchased from Sigma-Aldrich (St. Louis, MO); A-348441 was synthesized at Abbott Laboratories. All in vivo studies in which the aforementioned compounds were dosed used 0.2% hydroxypropylmethylcellulose (HPMC) as the oral vehicle unless otherwise noted below.

GR Binding Assay and Hepatocyte TAT Assays

GR binding assay and hepatocyte tyrosine aminotransferase (TAT) assays have been described previously (von Geldern et al., 2004).

RNA Extraction and Real-Time Quantitative RT-PCR Analysis

Tissues were snap-frozen in liquid nitrogen and homogenized in TRIzol (Invitrogen, Carlsbad, CA). Total RNA was DNase-treated to eliminate genomic DNA contamination, and 100 ng of total RNA was used as template. One-step quantitative RT-PCR kit (Platinum; Invitrogen) was used for real-time quantitative RT-PCR in Prism 7700 to determine the specific gene expression. Oligonucleotide sequences are as follows: rat TAT, forward 5’-GCATTCTCCGGAATTCA-3’; reverse 5’-GACAAGTCTGCTCCGAAT-3’; rat glucose 6-phosphatase, forward 5’-CTCACAGCATGACCACACA-3’; reverse 5’-GGCTCACGAGCTCAAGAGA-3’; rat GLUT2, forward 5’-AGTCCCTCATCCCTTGTCCG-3’; reverse 5’-AGTGGTCTGTCTAGCCTTG-3’; rat glutamine synthetase, forward 5’-AGGGTCACCCACCACATTTT-3’; reverse 5’-CCTCACATGGACCTGACACAT-3’. The RT-PCR conditions are as follows: 50°C for 30 min, 95°C for 5 min, 95°C for 15 s, and 60°C for 1 min, for 40 cycles. Gene expression levels were normalized for RNA loading using 28S as an internal control and are presented as relative fold change to control vehicle group.

Glutamine Synthetase Assay

Glutamine synthetase activity was determined in rat skeletal muscle L6 cells obtained from American Type Culture Collection (Manassas, VA) (CRL-1458) or from rat or mouse skeletal muscle tissue homogenates. Cells were pretreated with RU-486 or A-348441 for 30 min followed by incubating overnight with 100 nM prednisolone. Glutamine synthetase assay was performed using the method of Santoro et al. (2001). Wells that contained substrate without compound or prednisolone were used as the background,
whereas the wells that contained substrate and prednisolone without any compound were considered as maximal signal. Percentage of inhibition of each compound was calculated relative to the maximal signal, and IC\textsubscript{50} curves were generated.

**3T3-L1 Preadipocyte Differentiation Assay**

3T3-L1 preadipocytes were cultured and differentiated according to the protocol described by Camp et al. (2001). During differentiation, cells were treated with various concentrations of RU-486 and A-348441. At the end of the study, cells were fixed in 10% formalin and stained with Oil Red O. The plate was read on SpectraMAX (Molecular Devices, Sunnyvale, CA) at 520 nm, and percentage of inhibition of prednisolone differentiation was calculated.

**In Vivo Compound Evaluation**

**Animals.** All protocols involving rodents were approved in advance by the Abbott Institutional Animal Care and Use Committee, and all studies were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care-approved barrier facility. Unless noted, all animals were maintained on normal 12:12-h light/dark cycle and were given food and water ad libitum. Dog study protocols were approved in advance by the Vanderbilt University Association for Assessment and Accreditation of Laboratory Animal Care committee and were conducted in Association for Assessment and Accreditation of Laboratory Animal Care-approved facilities.

**Rat Prednisolone Challenge Model.** Overnight fasted 150-g male CD rats (Charles River Laboratories, Inc., Wilmington, MA) were orally dosed with HPMC vehicle, A-348441, or RU-486 60 min before an oral challenge with prednisolone at 10 mg/kg. Five hours after the prednisolone challenge, rats were euthanized with CO\textsubscript{2} and bled via cardiac puncture for evaluation of blood lymphocytes and plasma drug levels. Liver biopsy punches (7 mm) were harvested for evaluation of TAT, hepatic glycogen, and GR antagonist levels. Additional liver tissue, retroperitoneal fat, skeletal muscle, kidney, and skin from an ear biopsy were also removed for isolation and evaluation of mRNA in selected assays. Hepatic TAT activity was determined spectrophotometrically (Diamandstone, 1966) and normalized for hepatic protein content by the method of Lowry et al. (1951). Hepatic glycogen content was evaluated as described previously (Keppler and Decker, 1994). Prednisolone-induced elevations in blood glucose were evaluated in separate studies using a similar pretreatment protocol, except that rats were bled via tail snip at selected times after the prednisolone challenge, and plasma glucose was measured using a Medisense Precision G (Abbott Laboratories) glucometer. Plasma drug levels were evaluated from EDTA-treated blood samples, and liver homogenates were extracted with tert-2-ethyl methyl ether. Samples were quantitated on a PE Scieix API 2000 liquid chromatograph/tandem mass spectrometer (Concord, ON, Canada). Blood lymphocytes were measured using an Abbott Cel-Dyn 3700.

**Mouse HPA Activation Model.** Nonfasted CD-1 male rats (Charles River Laboratories, Inc.), weighing approximately 25 g, were dosed with HPMC vehicle, RU-486 (30–100 mg/kg), or A-348441 (30–300 mg/kg) at 8:00 AM when diurnal levels of corticosterone were low. Two hours later, mice were briefly anesthetized with 95% CO\textsubscript{2}, 5% O\textsubscript{2} bled by cardiac puncture, and then immediately euthanized with 100% CO\textsubscript{2}. Plasma was subsequently isolated and analyzed for corticosterone by liquid chromatograph/mass spectrometry, and for adrenocorticotropic hormone (ACTH) levels by enzyme-linked immunosorbent assay (ALPCO Diagnostics, Windham, NH).

**Rat DTH Model.** Male CD rats (Charles River Laboratories, Inc.), weighing approximately 225 g, were initially sensitized on shaved abdomens with 50 \(\mu\)l of a 2% 2,4-dinitrofluorobenzene (DNFB; Sigma, St. Louis, MO) solution in 95% acetone/5% olive oil on Day 1, and followed with a similar, secondary DNFB sensitization on Day 2. On Day 7, rats were orally dosed with vehicle or antagonist 2 h prior to a topical DNFB challenge on the ear. A submaximal dose of dexamethasone (0.03 mg/kg) was orally administered to all rats except vehicle controls, 1 h before the ear challenge. Rats were topically challenged on both sides of the right ear with 20 \(\mu\)l of a 0.4% DNFB solution in 95% acetone, 5% olive oil on each side of pinna. Rats were euthanized on the following day, after which 7.5-mm punch biopsies from treated ears were harvested and weighed as an index of edema.

**Euglycemic-Hyperinsulinemic Clamp in Zucker fa/fa Rats.** Male Zucker fa/fa rats and their lean controls were obtained from Charles River Laboratories, Inc., at 6 to 8 weeks of age and allowed to acclimate in groups of four to five per cage 1 week before initiation of the study. Seven days before experimentation, catheters were implanted in the left common carotid artery and right jugular vein and advanced to the aortic arch and superior vena cava, respectively. After surgery and before clamp procedure, rats had regained their presurgical body weights. On the day of the study, conscious overnight fasted rats underwent a euglycemic-hyperinsulinemic clamp by constant infusion of insulin at 4 mU/kg-min (regular insulin, Humulin R; Eli Lilly & Co., Indianapolis, IN, in 0.1% bovine serum albumin) and variable glucose to maintain euglycemia (50% dextrose; Abbott Laboratories). A single, oral 100-mg/kg dose of A-348441 or HPMC vehicle was given 2 h before the initiation of the clamps. Approximately 20-\(\mu\)l arterial samples were taken every 5 min during the study to permit adjustment of the GIR for maintenance of euglycemia. During the final 30 min of the 90-min clamp, arterial blood samples (300 \(\mu\)l) were taken at 60, 75, and 90 min for determination of glucose specific activity. Specific activities and GIRs were averaged since it was determined that specific activity was stable over the 30-min sampling period for each rat. To determine whole body glucose fluxes, 3-[\(\text{H}\)]glucose was used as the tracer (PerkinElmer Life and Analytical Sciences, Boston, MA). 3-[\(\text{H}\)]Glucose is a nonrecirculating radioisotope as the \(\text{H}\) atom is lost to water at the glucose isomerase step. The 3-[\(\text{H}\)]Glucose was mixed in saline and infused at 0.01 ml/min (0.06 \(\mu\)Ci/min) after a bolus of 7.2 \(\mu\)Ci. This procedure is similar to that described previously (Pospisilik et al., 2002). Total glucose production (\(R_{p}\)) and utilization (\(R_{d}\)) were determined by the equations for isotope dilution during a constant infusion of tracer, according to the method of Deboldo et al. (1963). During a euglycemic-hyperinsulinemic clamp and steady-state conditions, endogenous \(R_{p}\) equals \(R_{d}\) minus the GIR. The endogenous \(R_{p}\) is representative of hepatic glucose production with a minor contribution from the kidneys.

**Euglycemic-Euinsulinemic Clamps in Conscious Normal Fasted Dogs.** Each experiment consisted of an equilibration period (−140 to −40 min), a basal period (−40 to 0 min), and an experimental period of 4 to 5 h. After a 60 h fast, dogs underwent a euglycemic, euinsulinemic clamp procedure. The clamp consisted of infusion of somatostatin (0.8 \(\mu\)g/kg/min) and replacement of basal glucose amounts intravenously. The insulin infusion rate was adjusted between −140 and −40 min to maintain euglycemia; average infusion rate was 243 \(\mu\)U/kg/min and was not changed after −40 min. Glucagon was infused intravenously at 0.57 ng/kg/min from −140 min to 270. After the clamp was established, blood samples were obtained (−40 and 0 min) for baseline measurements, followed by an experimental period of intraduodenal infusion of either HPMC vehicle (\(n=6\)) or A-348441 (10 or 100 mg/kg; \(n=6\)) over a 15-min period. Peripheral glucose was infused at variable rates as needed to maintain euglycemia during the experimental period (0−270 min). Total glucose appearance and disappearance rates were determined using 3-[\(\text{H}\)]Glucose, as described previously (Moore et al., 2004). ob/ob Mouse Model of Type 2 Diabetes. Male B6.V(Lepr\textsuperscript{ob/ob}) (ob/ob) mice and their lean littermates (The Jackson Laboratory, Bar Harbor, ME) were group housed and allowed free access to food (Purina 5015) and water. Mice were 6 to 7 weeks old at the start of each study. On day 0, animals were weighed and postprandial glucose levels determined (Medisense Precision X glucometer; Abbott Laboratories). Mean postprandial glucose levels did not differ significantly from group to group (\(n=10\)) at the start of the studies.
Animals were orally dosed bid with HPMC vehicle or A-348441 at 30 and 100 mg/kg, b.i.d. (60 and 100 mpk/day) at approximately 8:00 AM and 5:00 PM each day. On day 5 of the study, 2 h after the ninth dose, postprandial blood glucose was measured via tail snip, after which mice were euthanized via CO2; blood samples (EDTA) were taken by cardiac puncture and immediately placed on ice for measurement of plasma drug levels and other blood parameters. Blood samples were then spun, and plasma was removed and frozen until further analysis. The following plasma parameters were run according to instructions by the manufacturer and consisted of alanine aminotransferase/aspartate aminotransferase for hepatic enzymes (GO- and GP-transaminase kit; Sigma-Aldrich), free fatty acids (NEFA C kit; Wako Chemicals, Neuss, Germany), triglyceride and cholesterol lipid measurements (Infinity kits; Sigma-Aldrich), and immunoreactive insulin by enzyme-linked immunosorbent assay (American Laboratory Products Co., Windham, NH).

In synergy studies, mice were orally dosed q.d. in the morning (approximately 8:00 AM) with HPMC vehicle or rosiglitazone (0.03 mg/kg) for a 7-day lead in period. Starting on day 7, animals were dosed for 14 additional days with HPMC vehicle alone, A-348441 (30 mg/kg), rosiglitazone (0.03 mg/kg), or a combination of rosiglitazone (0.03 mg/kg) and A-348441 (30 mg/kg). Drug concentrations of dosing solutions were adjusted so that each group received the same dose volume (10 ml/kg). Postprandial glucose levels were determined 16 h after the last oral dose.

Data and Statistical Analysis. All in vivo data are represented as group means ± S.E.M. Data were analyzed for statistical significance by ANOVA, with Dunnett’s post hoc comparison using InStat program (GraphPad Software Inc., San Diego, CA). Comparisons were considered statistically significant at p ≤ 0.05.

Results

In Vitro Characteristics of A-348441. The structural activity relationships surrounding the chemical synthesis and optimization of A-348441 have been described recently (von Geldern et al., 2004). A-348441 is a cholic acid conjugate of RU-486 (Fig. 1a) with high affinity for GR (Table 1; 0.27 nM for A-348441 versus 0.1 nM for RU-486). A-348441 is 50- to 5000-fold selective for GR over most steroid receptors. Like RU-486, it retains high affinity for the human progesterone receptor. In functional cellular assays using freshly isolated primary rat hepatocytes, A-348441 is a potent and efficacious antagonist of prednisolone-induced up-regulation of TAT, a classic glucocorticoid-regulated gene. In these cells, A-348441 is approximately twice as potent as RU-486, with respective IC50 values of 220 and 440 nM (Table 2). In the absence of prednisolone, A-348441 shows no agonist-like induction of hepatic TAT activity (data not shown), confirming that A-348441 is a pure GR antagonist. In cellular selectivity studies, A-348441 demonstrated selectivity in hepatocytes versus peripheral cells compared with RU-486 (Table 2). A-348441 is 12-fold less potent as an inhibitor of prednisolone-induced up-regulation of glutamine synthetase in L6 skeletal muscle myocytes compared with RU-486, and 15-fold less potent than RU-486 in a dexamethasone-stimulated model of adipocyte differentiation in 3T3-L1 cells.

In Vivo Modulation of Hepatic GR. In vivo activity of A-348441 was first characterized using a rat RPC model. In rodents as well as humans, a single dose of prednisolone will
corticoid-induced up-regulation of hepatic TAT activity by 79% at 100 mg/kg ($p < 0.01$ versus normalized vehicle prednisolone controls). Transcriptional suppression was confirmed by evaluating TAT, as well as glucose 6-phosphatase mRNA levels, in similarly treated animals (Fig. 1c), where the transcription of each gene was decreased by approximately 60% ($p < 0.01$) at the 100-mg/kg dose. A second classic hepatic response to glucocorticoids is glycogen deposition. When animals were pretreated with A-348441, there was a dose-dependent suppression in prednisolone-induced hepatic glyco-gen formation (Fig. 1d; 57% versus normalized prednisolone control; $p < 0.05$) at 100 mg/kg. These acute in vivo studies demonstrate that A-348441 suppresses glucocorticoid-induced metabolic responses involved in glucose homeostasis.

Liver Selectivity of A-348441. Consistent with the L6 myoblast and hepatocyte cell selectivity data shown in Table 2, A-348441 was inactive in vivo as an antagonist of prednisolone-induced up-regulation of glucose synthetase in skeletal muscle compared with RU-486, even at doses that effectively blocked TAT up-regulation in the liver (Fig. 2a).

### Table 2

Comparison of functional cellular activity between A-348441 and RU-486

<table>
<thead>
<tr>
<th>Cellular Assay</th>
<th>A-348441 IC$_{50}$</th>
<th>RU-486 IC$_{50}$</th>
<th>A-348441/ RU-486 Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat hepatocyte TAT</td>
<td>220 nM</td>
<td>440 nM</td>
<td>0.5</td>
</tr>
<tr>
<td>Rat skeletal muscle L6 glutamine synthase</td>
<td>250 nM</td>
<td>20 nM</td>
<td>12</td>
</tr>
<tr>
<td>Mouse 3T3-L1 preadipocyte differentiation</td>
<td>15,000 nM</td>
<td>1000 nM</td>
<td>15</td>
</tr>
</tbody>
</table>

Induce a wide range of metabolic and hormonal responses in several tissue compartments. Many of these responses occur over an 8-h interval and can be used to simultaneously evaluate hepatic and extrahepatic effects of GR antagonists. A number of hepatic genes are up-regulated by glucocorticoids, and hepatic TAT is one of the most robust responses in the liver, where gene expression and activity increased up to 10- to 15-fold over vehicle controls. This contrasted with the more modest increases in glucose 6-phosphatase or PEPCK activity in the liver, where gene expression and activity increased up to 10- to 15-fold over vehicle controls. This contrasted with the more modest increases in glucose 6-phosphatase or PEPCK mRNA expression during this time frame. As shown in Fig. 1b, when rats were predosed 2 h before the prednisolone challenge, A-348441 dose dependently antagonized the glu-
Another acute response of glucocorticoid administration that can be observed in normal fasted rats and humans (West et al., 1960) is a mild rise in blood glucose, due in part to reduced peripheral insulin sensitivity and glucose uptake in skeletal muscle (Weinstein et al., 1995; Gremlrich et al., 1997). A study conducted in normal fasted humans demonstrated a rise in blood glucose after infusion of cortisol, but it was not accompanied by an increase in hepatic glucose output (Garrel et al., 1995). This further supports the idea that this acute rise in glucocorticoid-induced blood glucose is peripheral in origin, rather than hepatic. Glucose levels in 7- to 8-week-old male CD-1 rats (Charles River Laboratories, Inc.) fasted overnight on clean bedding rose from 55 mg/dl to approximately 105 mg/dl 7 h after a single 10-μg/kg dose of prednisolone. Although 105 mg/dl does not represent frank hyperglycemia, these levels are significantly elevated versus fasted vehicle controls (p < 0.01). Predosing rats with RU-486 eliminated this glucocorticoid-induced increase in blood glucose, whereas A-348441 had no effect (Fig. 2b). This result with RU-486 is consistent with those from the study of Garrel et al. (1995) where oral administration of RU-486 before an i.v. infusion of cortisol blocked cortisol’s ability to increase blood glucose.

In addition to glucocorticoid-dependent increases in TAT, plasma glucose, and hepatic glycogen, prednisolone administration results in an acute lymphopenic response indicative of its role as an anti-inflammatory agent. Within 15 min of administering prednisolone, circulating lymphocyte levels decrease, recovering to control levels approximately 12 h later. Although RU-486 dose dependently antagonized this systemic lymphopenia (64% at 100 mg/kg; p < 0.01 versus normalized prednisolone control), A-348441 had no effect on lymphocyte levels at similar doses (Fig. 2c). Comparison of plasma and liver drug levels in this RPC model 2 h after administration of 100 mg/kg A-348441 (just before prednisolone challenge) demonstrated a significantly larger concentration of drug in the liver versus plasma at both the 2-h (14 μg/g versus 0.6 μg/ml, respectively; p < 0.01) and end of study 7-h time points (66 μg/g versus 2.1 μg/ml, respectively; p < 0.01; Fig. 2d). Additional studies were performed to compare the distribution of A-348441 and RU-486 after a single 30-μg/kg oral dose with tracer amounts of radiolabeled drug in the rat over 72 h. Time-course studies demonstrated similar areas under the curve (micrograms × hour per milliliter) in plasma (RU-486, 3.7; A-348441, 7.1), brain (RU-486, 5.4; A-348441, 5.6), and kidney (RU-486, 30.2; A-348441, 48.6) but divergent areas under the curve in the liver (RU-486, 27.6; A-348441, 158.2). These data highlight that orally administered A-348441 is systematically bioavailable and is highly enriched in the liver compared with either RU-486 in the liver and A-348441 in other tissues.

One of the most sensitive hormonal responses of systemic GR antagonism is activation of the HPA axis. As glucocorticoid tone is “sensed” to be low (due to low glucocorticoid levels or the presence of a systemic GR antagonist), corticotropin releasing hormone is secreted by the hypothalamus, stimulating ACTH release by the pituitary gland, and ultimately triggering an increase in glucocorticoid secretion from the adrenal glands. When normal CD-1 mice were orally dosed with the systemic GR antagonist RU-486, ACTH levels rose significantly in a dose-dependent manner to 3 times background levels (Fig. 2e). In contrast, A-348441 had no effect on ACTH. Corticosterone responses tracked with ACTH levels; a 100-μg/kg dose of RU-486 significantly increased plasma corticosterone levels to 460 ± 18 versus 70 ng/ml ± 15 for A-348441 (p < 0.01).

A final therapeutic concern challenging the use of systemic GR antagonists is how such agents would interfere with the efficacy of steroidal anti-inflammatory therapy. We compared RU-486 with A-348441 in a glucocorticoid-sensitive model of delayed type hypersensitivity (DTH). In this rat model of T-cell mediated inflammation, RU-486 significantly reduced the oral anti-inflammatory activity of a submaximal dose of dexamethasone. In contrast, A-348441 had no effect on dexamethasone efficacy, even at a 3-fold higher dose of 300 mg/kg (Fig. 2f).

**GR-Dependent Reduction of Hepatic Glucose Output.** The studies described above demonstrate that A-348441 can antagonize glucocorticoid-induced responses in the liver in the absence of significant systemic GR antagonist activity. Our next challenge was to directly demonstrate that liver-targeted GR antagonism with A-348441 would reduce HGO, a primary component of elevated glucose levels in type 2 diabetes. We chose to evaluate A-348441 in the insulin-resistant Zucker fa/fa rat using a euglycemic hyperinsulinemic clamp to evaluate effects on HGO. In this model, insulin levels were held constant, and glucose was infused to maintain euglycemia throughout the experiment. Average glucose clamp levels in vehicle- and A-348441-treated animals were t = 60 min, 136 ± 4 mg/dl; t = 75 min, 127 ± 3 mg/dl; and t = 90 min, 129 ± 5 mg/dl; since specific activity was not changed for each animal over this 30-min period (60, 75, and 90 min), results were averaged for glucose infusion rate, production, and disposal. An acute oral dose of A-348441 significantly increased the GIR from 0.69 to 3.45 mg/kg/min, indicative of either improved global insulin sensitivity and/or reduced hepatic glucose production (Fig. 3a). The latter possibility was confirmed through the use of a tracer to track peripheral glucose uptake. A-348441 reduced endogenous glucose production (Rg; Fig. 3b) with no change in peripheral glucose uptake (Rd; Fig. 3c).

These observations were subsequently confirmed using a conscious fasted normal dog. Insulin levels for the vehicle-treated dogs during steady-state basal and experimental periods of the clamp were 4 ± 0 and 4 ± 1 μU/ml, respectively; and for the A-348441-treated group, 5 ± 1 and 5 ± 1 μU/ml each, respectively. Arterial plasma glucose levels for vehicle-treated animals during basal and experimental periods were 101 ± 2 and 102 ± 2 mg/dl, respectively; and for the A-348441-treated group, 101 ± 4 and 102 ± 3 mg/dl, respectively. Four and a half hours after a single intraduodenal dose of A-348441, GIR increased 322%, and Rg significantly decreased relative to vehicle control (Fig. 3e). Rg was unchanged over the course of the experiment (Fig. 3f), but glucose had to be infused to prevent hypoglycemia (Fig. 3d). These mechanistic studies demonstrate that acute administration of A-348441 reduces HGO across species, in normal and diseased animals, with no observable extrahepatic peripheral effects.

**Reduction of Hyperglycemia in a Mouse Model of Type 2 Diabetes.** The previous data show that A-348441 antagonizes hepatic glucocorticoid responses, including the elevated HGO characteristic of type 2 diabetes, but these studies have not addressed whether subchronic treatment...
would improve overall glucose homeostasis in an animal model of type 2 diabetes. For these studies, we chose the diabetic ob/ob mouse, which is characterized by multiple metabolic disturbances, including hyperglycemia, hyperinsulinemia, insulin resistance, dyslipidemia, and obesity. As shown in Table 3, A-348441 demonstrated a dose-dependent reduction in blood glucose over the 5-day study, normalizing blood glucose levels to those of the lean controls at the high dose. Although A-348441 had no significant effect on insulin levels during this time period, calculation of the (glucose) × (insulin) product, an index of insulin sensitivity (Levine and Haft, 1970), did significantly improve at the 100-mg/kg dose. A-348441 also significantly reduced plasma free fatty acids by 136% (p < 0.001 versus vehicle control) and plasma triglycerides by 51% (p < 0.05) compared with lean controls. No effects on body weight were observed during the study, and there were no instances of hypoglycemia or other adverse responses noted.

**Synergy of A-348441 with Rosiglitazone.** Recently, a number of combination therapies have been approved for clinical use in type 2 diabetes that take advantage of multiple drug mechanisms for improved efficacy at lower and better tolerated doses (Avandamet and Glucovance). It might be expected that combining a drug that reduced HGO along with an agent that improved peripheral insulin sensitivity would significantly improve overall glucose homeostasis beyond the effect of each drug alone. We therefore evaluated the individual and combined effects of submaximal doses of A-348441 and the thiazolidinedione peroxisome proliferator-activated receptor γ agonist rosiglitazone in the ob/ob mouse.

**Discussion**

Glycemic control is an integrated and highly regulated process, where glucose production balances glucose utilization. Glucose can be produced either through dietary ingestion of carbohydrates or released from the liver during the fasting state through glycogen breakdown or via hepatic gluconeogenesis. As glucose levels rise after a meal, insulin is released from the pancreas, leading to increased glucose disposal and decreased hepatic glucose output. In patients with

**TABLE 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body Weight</th>
<th>Glucose</th>
<th>Insulin</th>
<th>Glucose × Insulin Index</th>
<th>Free Fatty Acids</th>
<th>Plasma Triglycerides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean vehicle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ob/ob vehicle</td>
<td>24 ± 0.5***</td>
<td>165 ± 5***</td>
<td>1.1 ± 0.2***</td>
<td>171 ± 25</td>
<td>1.06 ± 0.07**</td>
<td>182 ± 12***</td>
</tr>
<tr>
<td>A-348441, 30 mg/kg</td>
<td>37 ± 0.8</td>
<td>341 ± 37</td>
<td>19.4 ± 3.0</td>
<td>7487 ± 2185</td>
<td>1.59 ± 0.14</td>
<td>355 ± 31</td>
</tr>
<tr>
<td>A-348441, 100 mg/kg</td>
<td>36 ± 0.8</td>
<td>179 ± 14***</td>
<td>21.0 ± 5.0</td>
<td>4953 ± 564</td>
<td>1.01 ± 0.06***</td>
<td>377 ± 25</td>
</tr>
</tbody>
</table>

*p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001 versus ob/ob vehicle control.
Effects of individual and combination therapy on blood glucose with rosiglitazone (Rosi, 0.03 mg/kg) and A-348441 (‘441 at 30 mg/kg) in male ob/ob mice (n = 8–10/group). *, p ≤ 0.05; **, p ≤ 0.01; and ***, p ≤ 0.001 versus vehicle ob/ob control on a given day; †, p < 0.05 versus rosiglitazone alone.

**TABLE 4**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>2.2 ± 0.5</td>
<td>2.7 ± 0.7</td>
<td>3.9 ± 0.7</td>
</tr>
<tr>
<td>ob/ob Vehicle</td>
<td>4.5 ± 0.2</td>
<td>8.0 ± 0.3</td>
<td>10.2 ± 0.8</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>4.4 ± 0.4</td>
<td>9.2 ± 0.5</td>
<td>12.4 ± 0.5*</td>
</tr>
<tr>
<td>A-348441</td>
<td>3.5 ± 0.2</td>
<td>7.6 ± 0.3</td>
<td>10.7 ± 0.4</td>
</tr>
<tr>
<td>Combination</td>
<td>N.A.</td>
<td>7.97 ± 0.4</td>
<td>10.4 ± 0.4</td>
</tr>
</tbody>
</table>

N.A., not applicable. *p ≤ 0.05 versus ob/ob vehicle control on day 21.

elevated blood glucose levels in this state. Virtually all of the increase in HGO with type 2 diabetes is due to elevated gluconeogenesis (DeFronzo, 1997). The studies presented herein demonstrate the fundamental therapeutic mechanism behind A-348441’s effects on HGO. In the fa/fa rat, a single oral dose of A-348441 significantly increased the GIR required to maintain euglycemia. This reduction in GIR resulted from a decrease in endogenous glucose production (Rd) with no change in glucose disappearance (Ra). These data suggest that an acute dose of A-344841 has no effect on peripheral insulin sensitivity. In studies where fa/fa Zucker rats were dosed for 7 days, A-348441 provides additional beneficial effects on fasting plasma glucose and peripheral glucose uptake (unpublished data), indicating that repeated exposure might have important secondary effects on insulin sensitivity.

Similar effects on endogenous glucose production in the absence of changes in Ra were also seen in the conscious fasted normal dog, supporting a hepatic site of action. Interestingly, the onset of action in the dog was almost 3 h after intraduodenal drug administration, consistent with a transcriptional mechanism of action. The full effect of A-348441 in the dog may also not have been realized since efficacy was observed only at the end of the 4- to 5-h exposure period. HGO is the sum of gluconeogenesis and glycogenolysis, and although the effects of A-348441 seem to involve both of these processes, the relative contributions of each are difficult to ascertain based on this study.

A-348441 has been shown in chronic studies to significantly reduce blood glucose, HbA1c, and lower circulating lipids in diabetic ob/ob mice (von Geldern et al., 2004). The current study evaluated subchronic effects of A-348441 on glycemic control and lipid levels in these animals to better understand the pharmacodynamics of these responses upon the initiation of therapy. The ability of A-348441 to normalize blood glucose levels and improve dyslipidemia in ob/ob mice in 5 days is striking. Glucose levels were also measured at various time points immediately after dosing, but unlike metformin (which acutely normalizes glucose levels for only 3 to 4 h after each dose), A-348441 had no acute effects (<8 h) on circulating glucose (unpublished data). The earliest reductions observed in postprandial glucose levels (16 h after last dose) were after 3 to 5 days of dosing A-348441 at 100 mg/kg, b.i.d. This time- and dose-dependent onset of action might be expected with the liver-targeted, transcriptional mechanism we have proposed for A-348441. The reduction in circulating lipids is also consistent with observations that glucocorticoids are known to increase hepatic lipid synthesis and increase circulating levels of free fatty acids and triglycerides (Diamant and Shafrir, 1975). RU-486 has similarly been shown to reduce lipids to near normal levels in patients with Cushing’s disease (Chu et al., 2001).

The novel ability of A-348441 to synergize with an antidiabetic and mechanistically distinct drug at submaximal doses demonstrates additional therapeutic utility with this approach. When low, submaximal doses of A-348441 (30 mg/kg, q.d.) and the insulin sensitizer rosiglitazone (0.03 mg/kg, q.d.) are administered in combination, blood glucose levels are significantly reduced beyond the additive effect of each agent alone. By targeting two of the major metabolic disturbances of type 2 diabetes (elevated hepatic glucose output,
and increased insulin resistance) with combination therapy, these data suggest that maximally efficacious doses of each drug are not required to achieve significant efficacy, thereby reducing the potential for adverse effects. However, even at the very low dose of 0.03 mg/kg, which lowered glucose levels by approximately 50%, rosiglitazone-induced a statistically significant increase in body weight in ob/ob mice after 21 days. When A-348441 was added to the rosiglitazone-treated animals 7 days into the study, subsequent increases in body weight gain were not observed. The reasons behind this are unclear at this time, but it is possible that A-348441 may have secondarily affected food intake, fluid balance, and adipocyte mass and/or size or increased metabolic rate in a manner that would counter rosiglitazone’s effects on body weight.

Unlike most rodent models of type 2 diabetes, humans with type 2 diabetes do not typically have elevated glucocorticoid levels despite significant increases in glucogenogenesis and HGO. It is known that circulating levels of glucocorticoids do not always determine local glucocorticoid action, since increased GR number (Reynolds et al., 2002) and tissue-specific conversion of inactive cortisone to active cortisol through 11β-hydroxysteroid dehydrogenase 1 can amplify physiologic responses (Masuzaki et al., 2001). Recent data in normal human patients have demonstrated that up to 50% of circulating cortisol is produced from nonadrenal sources such as the splanchic bed via 11β-hydroxysteroid dehydrogenase 1 (Basu et al., 2004). Relative glucocorticoid excess in the liver, as defined by either amount or activity, nevertheless remains a key feature of the elevated HGO that characterizes type 2 diabetes in humans.

A-348441 is a novel molecule that allows us to validate the concept of hepatic GR as a potential therapeutic target for type 2 diabetes. A-348441 selectively reduced glucocorticoid activity in the liver, and as a consequence, lowered hepatic glucose output, improved glucose control and reduced the dyslipidemia characteristic of diabetic animal models. Despite the well known metabolic regulatory role of glucocorticoids, the concept of antagonizing GR for type 2 diabetes has been relatively unexplored, due in part to the liabilities associated with systemic GR antagonism and also due to a void of evidence that heptatically targeted agents would be effective in reducing elevated glucose levels. Recent human data highlighting the splanchic bed as a major source of glucocorticoid production (Basu et al., 2004) raises the possibility that the glucocorticoid-rich environment of the liver may provide a constant stimulatory signal for glucose production. In type 2 diabetes, where HGO is elevated, glucocorticoids may play an even greater role in the development of type 2 diabetes than previously thought, and suggests that the development of agents such as A-348441 may provide unique therapeutic benefits as a novel treatment for type 2 diabetes.

References


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