Thyroid hormone receptor-β agonists prevent hepatic steatosis in fat-fed rats but impair insulin sensitivity via discrete pathways

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Liver-specific thyroid hormone receptor-β (TRβ)-specific agonists are potent lipid-lowering drugs that also hold promise for treating nonalcoholic fatty liver disease and hepatic insulin resistance. We hypothesized that by amelioration of fatty liver, TRβ agonists (GC-1 and KB-2115) would reduce hepatic triglyceride and lipoprotein (EGP) production and diminish hepatic insulin sensitivity. GC-1 and KB-2115 treatment decreased hepatic triglyceride content by 75% and 55%, respectively. Insulin-stimulated peripheral glucose disposal was diminished because of decreased insulin-stimulated glycerol uptake and decreased hepatic insulin sensitivity. Surprisingly, GC-1 and KB-2115 increased skeletal muscle insulin signaling. In contrast, KB-2115 treatment did not induce changes in peripheral glucose uptake or lipoprotein production. Thus, although both GC-1 and KB-2115 potentiate hepatic steatosis in fat-fed rats, they each worsen insulin action via specific and discrete mechanisms. The development of future TRβ agonists must consider the potential adverse effects on insulin sensitivity.

ever, generalized thyroid hormone excess has a number of untoward effects, including tachyarrhythmias, pulmonary hypertension, osteoporosis, agitation, and even psychosis.

Thyroid hormones act primarily via three nuclear receptors, TRα1, TRβ1, and TRβ2; most tissues express both TRα and TRβ, although one isoform often predominates (1, 10, 26, 31, 55). For example, TRα1 expression predominates in the cardiac and skeletal muscle, osteoclast, and many regions of the brain. In comparison, TRβ1 predominates in liver, and TRβ2 plays a critical role in the regulation of the hypothalamic-pituitary-thyroid axis (8, 10, 19, 27). Activating TRβ in the liver could be therapeutic for metabolic diseases by lowering plasma and intrahepatic lipid content. However, natural thyroid hormones have nearly equivalent affinities for TRβ and TRα (Kd(TRα)/Kd(TRβ) = 0.7 [11]). Thus, any efforts to reap the benefits from activating TRβ with thyroid hormones will be limited by the toxicities associated with TRα activation. The tissue-specific expression of TR isoforms does provide the opportunity for selective thyroid hormone action in specific tissues. Specifically, liver-selective TRβ-specific agonists can potentially uncouple the beneficial effect of thyroid hormone on hepatic and plasma lipids without the deleterious effects on heart and bone.

Several TRβ agonists have demonstrated efficacy in decreasing plasma and hepatic lipids in both preclinical and clinical trials (2, 3, 24, 33, 51). These compounds weakly bind to and activate TRα, thus enhancing the therapeutic dose range. They can be administered at high enough doses to enhance TRβ activation without the untoward effects from TRα activation that would be seen if thyroid hormone was given at similar doses. There are multiple mechanisms that may account for the reduction in hepatic lipids, including induction of hepatic carnitine palmitoyltransferase (CPT)-1α leading to an increase in hepatic lipid oxidation (28). We hypothesized that by amelioration of fatty liver, TRβ agonists would also improve lipid-induced hepatic insulin resistance. We studied two thyroid hormone analogs: GC-1 (sobetirome), a first-generation TRβ agonist with fivefold selectivity for TRβ over TRα (51, 53), and KB-2115 (eprotirome), a more β-selective TRβ agonist that is reported to be more hepatic specific than GC-1 (3, 24).

The burgeoning epidemics of obesity and diabetes necessitate novel therapies. Thyroid hormone agonism remains a tantalizing modality (1, 54). It has the potential to mediate favorable effects on lipid metabolism and energy expenditure, decreasing plasma low-density lipoprotein (LDL) and increasing energy expenditure through futile substrate cycling (34, 35, 46) and increasing thermogenesis (21). However, generalized thyroid hormone excess has a number of untoward effects, including tachyarrhythmias, pulmonary hypertension, osteoporosis, agitation, and even psychosis.

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Materials and Methods

Animals. Male Sprague-Dawley rats (275–300 g) were received from Harlan Laboratories and acclimated for at least 3 days. Rats were housed on a 12:12-h light-dark cycle and received food and water ad libitum. Rats underwent the placement of jugular venous (for blood sampling) and carotid artery (for infusion) catheters 3–5 days before the initiation of TRβ agonist treatment. Rats were placed on a high-fat diet (Dyets 112245: 26% carbohydrate, 59% fat, 15% protein calories; Dyets, Bethlehem, PA) during the treatment period. TRβ agonists or vehicle (5% DMSO in phosphate-buffered saline) were injected intraperitoneally at a dose of 0.164 mg·kg⁻¹·day⁻¹ GC-1 or 0.1 mg·kg⁻¹·day⁻¹ KB-2115 for 10 days. Body weight was monitored three to four times weekly. Experiments were performed 16–24 h after the final injection of TRβ agonist. All procedures were approved by the Institutional Animal Care and Use Committee of the Yale University School of Medicine and the Veterans’ Affairs Connecticut Healthcare System.

Quantitative PCR. Total RNA was extracted from ~15 mg liver, ~100 mg white adipose tissue, or ~100 mg skeletal muscle using an RNaseasy mini kit (Qiagen, Valencia, CA). RNA was reverse-transcribed into cDNA with the use of the QuantiTect Reverse Transcription Kit (Qiagen). The abundance of transcripts was assessed by real-time PCR on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) with a SYBR Green detection system (Stratagene, La Jolla, CA). The expression data for each gene of interest were against β-actin as the invariant control and relative expression determined using amplification efficiencies (38). Primer sequences are shown in Table 1.

Western blotting. Tissue (~100 mg) was homogenized in 1 ml ice-cold homogenization buffer [20 mM Tris·HCl, pH 7.4, 5 mM EDTA, 0.25 mM EGTA, 10 mM Na₂HPO₄, pH 7.4, 0.1% Triton X-100, 1% protease and phosphatase inhibitor cocktails (Roche Diagnostics)] and centrifuged at 6,000 relative centrifugal force at 4°C for 15 min. The supernatant was taken, and protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA). Protein (100 μg) was loaded and resolved by SDS-PAGE using a 4–12% gradient gel and electroblotted onto a polyvinylidene difluoride membrane (DuPont, Boston, MA) using a wet-transfer cell. The membrane was then blocked for 60 min at room temperature in wash buffer containing 5% (wt/vol) nonfat dried milk and incubated overnight with primary antibody. After being washed, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad) for 60 min. Detection was performed with enhanced chemiluminescence.

Protein kinase B (Akt), phosphorylated Akt (Ser473), and phospho-PEPCK activities were measured in the cytosolic fraction by a spectrophotometric assay measuring the production of inorganic phosphate from the hydrolysis of glucose 6-phosphate or mannose 6-phosphate, as previously described (47). The latency of microsomal preparations was determined by assaying mannose 6-phosphate hydrolysis in intact vs. detergent-disrupted (0.2% sodium deoxycholate treatment for 20 min) microsomes. The glucose-6-phosphate activity of intact microsomes was corrected for the portion of activity resulting from the disrupted vesicles within untreated preparations. Reactions were carried out for 15 min at 30°C in 50 mM sodium cacodylate (pH 6.5), 2 mM EDTA, and 5 mM glucose 6-phosphate or mannose 6-phosphate, with intact microsomes at 600 μg/ml or disrupted microsomes at 60 μg/ml. Reactions were terminated by addition of phosphate detection reagent (6 parts 0.2% ammonium molybdate tetrahydrate in 1 M H₂SO₄, 2 parts 2% SDS, and 1 part 1% ascorbic acid). Samples were incubated at 45°C for 25 min to take the colorization of the phosphate detection reagent to completion, and absorbance was measured at 820 nm.

Table 1. qPCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>β-Actin</td>
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<td>Adiponectin</td>
<td>GAGCCCGAGGGGCGAGGAGCC</td>
<td>TCTCCATCAAGGCGGCCGTTA</td>
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<td>Deiodinase 1</td>
<td>TGGTTGCGAATCGAGCTGGCC</td>
<td>TACAGCCAGGAGGCTTGTCGAGT</td>
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<td>Deiodinase 2</td>
<td>AGCGACTCTTCAGAGCTGAGTA</td>
<td>GCACAGGAAATCTGCAAGAAGG</td>
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<td>G-G-Pase</td>
<td>TGGCAAGAGGAGATCTGCAAGA</td>
<td>GAGAACGCCAGGCGACAGAAATG</td>
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<tr>
<td>GLUT4</td>
<td>GCAGCGATGGATCTGAGCC</td>
<td>CGAGGAGATGGCTTAGTGGAGAT</td>
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<tr>
<td>Hairless</td>
<td>GCGCAAGGCTATCTGCTGCTGTTT</td>
<td>AACCTGTCGGCTGACACCTGGA</td>
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<tr>
<td>Malic enzyme 1</td>
<td>ATGGAAGAGGAGAATCTGCAAGA</td>
<td>GCGTGTTGCTGACATGGACAGAT</td>
</tr>
<tr>
<td>PEPCK</td>
<td>ATCGAAGCTCTCCTCTGCTTCTTCTCTCTCT</td>
<td>CGAGAAGTGGAGGTTGGTGGAGAT</td>
</tr>
<tr>
<td>PC</td>
<td>AGATAGCCTCCTCATTCCCAAGA</td>
<td>CTTCTGTCGGCTGACATGGACAGAT</td>
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<td>SERCA 1</td>
<td>GCTTCCTGAGAGGACAAATG</td>
<td>CCTGTCGATCTGAGAGGAAGATTT</td>
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<tr>
<td>Sortilin 1</td>
<td>GGGTGGGCGGAGACCGAAATG</td>
<td>CCTCCTGAGAGGAGGAAGATTT</td>
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<td>Tbp</td>
<td>GAGATCGCTGTCCTGCTCACC</td>
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<tr>
<td>Twist1</td>
<td>GGGAGGACCTGCGAGCATCCTT</td>
<td>GCTGAATGCGTCTGCTCAGGG</td>
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G-6-Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; PC, pyruvate carboxylase; Tbp, TATA box-binding protein.
Pyruvate carboxylase activity was assayed in the mitochondrial fraction after disruption by sonication. Pyruvate carboxylase activity was measured by a coupled spectrophotometric assay, coupling oxaloacetate production from pyruvate by pyruvate carboxylase to NADH oxidation by malate dehydrogenase (32). The reactions were performed at 30°C in 96-well plates with 50 mM Tris, pH 8.0, 10 mM NaHCO3, 10 mM MgCl2, 1 mM NADH, 4 mM/ml malate dehydrogenase, 10 mM ATP, 20 μM acetyl-CoA, and 25 μg/ml disrupted mitochondrial protein. Reactions were initiated with 2 mM pyruvate, and negative controls were performed without the addition of pyruvate. Absorption at 340 nm was measured every 10 s for 10 min.

Tissue triglyceride isolation and measurement. Triglycerides were extracted using ~200 mg tissue. Tissues were homogenized in ice-cold 2:1 chloroform-methanol, and lipids were extracted with shaking at room temperature for 3–4 h. H2SO4 was added to ~100 mM, and samples were vortexed and then centrifuged to achieve phase separation. The organic phase was collected, dried down, and resuspended in chloroform. Triglyceride content was measured using the Genzyme Triglyceride-SL kit (Genzyme Diagnostics).

Hyperinsulinemic-euglycemic clamp studies. Clamp studies were performed after an overnight fast as previously described (42). The basal period began with a prime (2.0 mg/kg over 3 min) of 99% labeled [6,6-2H]glucose, followed by a continuous infusion at a rate of 0.2 mg·kg⁻¹·min⁻¹ for 2 h to assess basal glucose turnover. After the basal period, the hyperinsulinemic-euglycemic clamping was conducted for 120 min with a primed continuous infusion of human insulin (40 μU·kg⁻¹·min⁻¹ for 3 min/4 μU·kg⁻¹·min⁻¹) (Novo Nordisk) and a variable infusion of ~20% dextrose to maintain euglycemia (~110 mg/dl). The 20% dextrose was enriched with [6,6-2H]glucose to ~2.5% to match the enrichment in the plasma achieved after the basal period (i.e., “hot-GINF”). A 20-μCi bolus of 2-deoxy-D-[1-14C]glucose (Perkin-Elmer) was injected at 120 min in the clamp to evaluate the rate of insulin-stimulated tissue glucose uptake. At the end of the clamp, rats were anesthetized with pentobarbital sodium injection (75 mg/kg), and all tissues were taken within 3 min, frozen immediately using cooled aluminum tongs in liquid N₂, and stored at ~80°C for the subsequent analysis.

2-Deoxyglucose uptake. Tissue 2-deoxyglucose (2-DG) uptake studies were performed as previously described (17). After time ~120 min during the hyperinsulinemic-euglycemic clamp, 20 μCi of 2-deoxy-D-[1-14C]glucose were given as a single intravenous bolus. Plasma was collected at 122, 125, 130, and 140 min to determine 14C activity and plasma glucose concentration. Epididymal white adipose tissue and gastrocnemius samples were homogenized, and 2-deoxy-D-[14C]glucose 6-phosphate was separated from 2-deoxy-D-[1-14C]glucose by ion exchange chromatography. 2-DG uptake was calculated based on the intracellular 2-DG content and the plasma 2-DG area under the curve.

Biochemical analysis and calculations. Plasma glucose concentrations were measured using a Beckman Glucose Analyzer II (Beckman Coulter). Plasma insulin and glucagon concentrations were determined by radioimmunoassay using the LINCOplex Assay system (Millipore). Nonesterified fatty acids were measured using the NEFA-HR Color A and B reagent test kit (Wako Chemicals, Richmond, VA). Serum total thyroxine (T4) and triiodothyronine (T3) concentrations were measured by coated tube RIAs (Siemens Medical Solution Diagnostics, Los Angeles, CA) as previously described (14, 39). Procedures were adapted for measurements in rat serum. Thyroid-stimulating hormone (TSH) was measured using a sensitive, heterologous, disequilibrium, double-antibody precipitation RIA developed for mice (39). A series of experiments demonstrated that TRβ agonists interfere with the measurement of triiodothyronine (data not shown); thus, we were unable to assess the effect of KB-2115 and GC-1 on total T3 levels.

Statistical analysis. Statistical analysis of the data, other than thyroid function test data, was performed using Graph-Pad Prism 5.0.3. Data were compared using Student’s unpaired t-test. All data are expressed as means ± SE unless otherwise indicated. P values <0.05 were considered significant. The thyroid function tests were compared with the Mann-Whitney U-test, testing the null hypothesis that the median values of the thyroid function tests were equal. P values <0.05 were considered significant.

RESULTS

The effects of TRβ agonists on hepatic steatosis and on whole body glucose metabolism were assessed in high-fat-fed male Sprague Dawley rats treated with either vehicle or thyromimetic for 10 days. At the end of 10 days, we performed hyperinsulinemic-euglycemic clamp studies and molecular analyses.

GC-1 treatment reduces hepatic steatosis but causes hyperglycemia and insulin resistance. Ten days of GC-1 treatment did not alter body weight in high-fat-fed animals (Table 2). However, GC-1 treatment prevented the development of hepatic steatosis, as reflected by a 75% reduction in hepatic triglyceride content in GC-1-treated animals compared with vehicle-treated animals (P < 0.0001; Fig. 1A). GC-1 treatment also led to a 26% increase in fasting plasma glucose concentration (P < 0.0001; Table 2). This was associated with a threefold increase in fasting plasma insulin concentration (P = 0.0003), suggesting insulin resistance. Additionally, we observed a 19% increase in fasting plasma glucagon concentration (P = 0.04; Table 2).

Hyperinsulinemic-euglycemic clamp studies were performed to quantify changes in endogenous glucose production (EGP), insulin-stimulated glucose disposal, and hepatic insulin sensitivity. GC-1 treatment increased EGP by 56% (P = 0.01; Fig. 1D). The glucose infusion rate required to maintain euglycemia in GC-1-treated animals was decreased by 31% vs. vehicle-treated animals (P < 0.03; Fig. 1B), reflecting a decrease in insulin sensitivity. Whole body insulin-stimulated glucose disposal was similar between the two groups (Fig. 1C). Instead, GC-1 treatment resulted in hepatic insulin resistance, as reflected by impairment in insulin-mediated suppression of hepatic glucose production (Fig. 1D).

Thyroid hormone can enhance adipose tissue lipolysis, and glycerol turnover (an index of whole body lipolysis) was quantified during the basal period of the clamp. Glycerol turnover was increased by 22% in GC-1-treated rats (P = 0.0001; Table 2). However, GC-1 treatment prevented the development of hepatic steatosis, as reflected by a 75% reduction in hepatic triglyceride content in GC-1-treated animals compared with vehicle-treated animals (P < 0.0001; Fig. 1A). GC-1 treatment also led to a 26% increase in fasting plasma glucose concentration (P < 0.0001; Table 2). This was associated with a threefold increase in fasting plasma insulin concentration (P = 0.0003), suggesting insulin resistance. Additionally, we observed a 19% increase in fasting plasma glucagon concentration (P = 0.04; Table 2).

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Table 2. Fasting parameters, GC-1 vs. vehicle treatment

<table>
<thead>
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<th>Parameter</th>
<th>Vehicle</th>
<th>GC-1</th>
<th>P</th>
</tr>
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<tr>
<td>Body wt, g</td>
<td>394 ± 9</td>
<td>387 ± 11</td>
<td>0.62</td>
</tr>
<tr>
<td>Basal glucose, mg/dl</td>
<td>108 ± 2</td>
<td>135 ± 4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Basal insulin, μU/ml</td>
<td>23 ± 5</td>
<td>64 ± 7</td>
<td>0.0003</td>
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<tr>
<td>Basal glucagon, pg/ml</td>
<td>41 ± 2</td>
<td>48 ± 3</td>
<td>0.04</td>
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<tr>
<td>NEFA, mM</td>
<td>0.35 ± 0.04</td>
<td>0.47 ± 0.03</td>
<td>&lt;0.004</td>
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Values are means ± SE. NEFA, nonesterified fatty acid.
livers of GC-1-treated animals (Fig. 2, A and B). We observed no change in PEPCK mRNA expression (P = 0.21) and no change in PEPCK protein abundance. In contrast, PEPCK activity in a cytoplasmic fraction was increased 70% by GC-1 therapy (6.8 ± 0.2 mmol·mg⁻¹·min⁻¹ vs. 11.6 ± 0.9 mmol·mg⁻¹·min⁻¹, P < 0.0005). There was a trend toward decreased glucose-6-phosphatase mRNA expression (P = 0.058), protein abundance was unchanged, and catalytic subunit specific activity was unchanged (vehicle treated: 6.4 ± 1.2 pmol·µg⁻¹·min⁻¹; GC-1 treated: 6.7 ± 0.8 pmol·µg⁻¹·min⁻¹). Pyruvate carboxylase mRNA increased by 44% (P = 0.047) with GC-1 treatment, but without a significant difference in protein by Western, and difference in pyruvate carboxylase activity was not significant (vehicle treated: 6.5 ± 0.2 mmol·mg⁻¹·s⁻¹; GC-1 treated: 7.2 ± 0.2 mmol·mg⁻¹·s⁻¹, P = 0.27).

We assessed hepatic Akt phosphorylation in basal and clamped animals to further explore the mechanism for hepatic insulin resistance following GC-1 treatment. Compared with the increase in insulin-stimulated Akt phosphorylation in vehicle-treated animals, Akt phosphorylation was completely suppressed by GC-1 treatment (Fig. 2C). Thus, although GC-1 treatment prevents the development of fatty liver in fat-fed rats, it impaired insulin-stimulated Akt activation.

**KB-2115 reduces hepatic steatosis but attenuates insulin-stimulated peripheral glucose uptake.** KB-2115 is a more TRβ-specific compound that has been shown to effectively lower plasma lipids in humans. We tested KB-2115 in high-fat-fed rats to determine if this would be a more effective therapy for nonalcoholic fatty liver disease (NAFLD) and hepatic insulin resistance. Ten days of KB-2115 (0.1 mg·kg⁻¹·day⁻¹) had no effect on body weight (Table 3). KB-2115 prevented the development of hepatic steatosis in high-fat-fed rats as effectively as GC-1 (Fig. 3A). Unlike GC-1 treatment, KB-2115 treatment did not lead to fasting hyperglycemia, although it still resulted in fasting hyperinsulinemia; insulin concentrations were almost fourfold greater with KB-2115 treatment than with vehicle treatment (Table 3). Fasting plasma glucagon concentration was unchanged.

We quantified the rate of EGP and insulin-stimulated glucose disposal with hyperinsulinemic-euglycemic clamp studies. In contrast to GC-1, KB-2115 treatment did not increase the basal rate of EGP (P = 0.24; Fig. 3D). However, KB-2115-treated high-fat-fed rats were markedly more insulin resistant than vehicle-treated high-fat-fed rats, as reflected by a 58% decrease in glucose infusion rate required to maintain euglycemia under hyperinsulinemic conditions (P = 0.0002; Fig. 3B). This difference was not attributable to a difference in hepatic insulin sensitivity, since clamped EGF was not different between KB-2115 and vehicle-treated animals (P = 0.66; Fig. 3D).

Instead, there was a 46% decrease in whole body insulin-stimulated glucose disposal compared with vehicle-treated rats (P = 0.001; Fig. 3C). This decrease in insulin-stimulated peripheral glucose disposal was accounted for by a decrease in skeletal muscle glucose uptake, as quantified by 2-DG uptake (Fig. 3, F and G). Insulin-stimulated skeletal muscle 2-DG uptake was reduced by KB-2115 treatment (68% decrease; P = 0.03), whereas epididymal white adipose tissue 2-DG uptake was unaffected. KB-2115 treatment did not cause a significant change in glycerol turnover (P = 0.39; Fig. 3E), or in plasma nonesterified fatty acid concentration (4% increase; P = 0.78; Table 3), in contrast to GC-1 treatment.
KB-2115 treatment induces small changes in gluconeogenic gene expression and hepatic insulin signaling. Hepatic gluconeogenic gene mRNA expression was evaluated in fasted rats (Fig. 4A). Glucose-6-phosphatase gene expression was increased by 50% (P < 0.027), but there were no significant changes in PEPCK and pyruvate carboxylase expression. Similar to changes seen in specific activity after GC-1 treatment, PEPCK activity was increased by 80% (P < 0.0005), glucose-6-phosphatase activity was unchanged (vehicle treated: 5.4 ± 0.4 pmol·μg⁻¹·min⁻¹; KB-2115 treated: 4.6 ± 0.5 pmol·μg⁻¹·min⁻¹), and pyruvate carboxylase activity was not significantly changed (vehicle treated: 4.2 ± 0.2 nmol·mg⁻¹·s⁻¹; KB-2115 treated: 4.5 ± 0.2 nmol·mg⁻¹·s⁻¹, P = 0.3). Hepatic insulin signaling was preserved in KB-2115-treated animals. Insulin-stimulated Akt phosphorylation was increased (P < 0.0004 vs. vehicle).
phorylation increased approximately twofold in KB-2115-treated animals compared with the greater than threefold increase in vehicle-treated animals (P < 0.25 comparing the fold increase in Akt phosphorylation; Fig. 4B).

**KB-2115 treatment decreases skeletal muscle GLUT4 content.** We investigated potential explanations for the decrease in insulin-stimulated skeletal muscle glucose uptake following KB-2115 treatment. First, we considered whether KB-2115 led to ectopic lipid accumulation. However, skeletal muscle triglyceride content had a tendency to be lower in the KB-2115 treatment group (Fig. 5A). Insulin signaling was evaluated by assessing insulin-stimulated Akt phosphorylation and AS160 phosphorylation (Fig. 5, B and C). Skeletal muscle insulin-stimulated Akt phosphorylation was unaffected, and there was a trend toward increased AS160 phosphorylation (P = 0.1). These data suggest that KB-2115 did not alter muscle insulin signaling. We assessed the abundance of the insulin-stimulated glucose transporter GLUT4 at the level of mRNA and protein (Fig. 5, D and E). KB-2115 treatment did not cause any difference in GLUT4 mRNA expression (P > 0.52) but resulted in an ~36% reduction in GLUT4 protein abundance (P = 0.018). The stability of GLUT4 protein is influenced by its targeting among intracellular membranes, and GLUT4 normally accumulates in a stable pool of intracellular vesicles termed “GLUT4 storage vesicles” (GSVs) (4). The TUG protein traps GSVs as an intracellular, insulin-responsive pool, and TUG depletion can accelerate degradation of GLUT4 protein (7, 57). We observed that TUG abundance was decreased by 45% in skeletal muscle of KB-2115-treated rats (Fig. 5F). This result suggested that accumulation of GLUT4 in

<table>
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<td>Basal glucagon, pg/ml</td>
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<td>NEFA, mM</td>
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Values are means ± SE.
GSVs is markedly decreased by KB-2115 treatment. In principle, a reduction in the number of GSVs could be because of impaired formation or accelerated loss of these vesicles. We therefore examined sortilin, a protein critical for GSV formation (6). Sortilin abundance was unchanged at the level of mRNA or protein (Fig. 5, $G$ and $H$), suggesting that the decrease in GSVs is not the result of reduced formation. The data support the idea that GSVs are not efficiently trapped in an insulin-responsive pool in skeletal muscle of KB-2115-treated animals and that GLUT4 degradation is consequently accelerated. We therefore examined sortilin, a protein critical for GSV formation (6). Sortilin abundance was unchanged at the level of mRNA or protein (Fig. 5, $G$ and $H$), suggesting that the decrease in GSVs is not the result of reduced formation. The data support the idea that GSVs are not efficiently trapped in an insulin-responsive pool in skeletal muscle of KB-2115-treated animals and that GLUT4 degradation is consequently accelerated.

Prominent skeletal muscle. Skeletal muscle $\text{hairless}$ expression was increased by >25-fold by GC-1 treatment ($P < 0.0001$) but was not significantly increased by KB-2115 treatment (26% increase; $P = 0.37$). Two additional thyroid hormone-regulated genes, sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) 1 and malic enzyme 1, were evaluated in KB-2115-treated skeletal muscle and found to be unaffected by treatment.

Local tissue $T_3$ levels are significantly influenced by local deiodinase 2 levels, which can further complicate the interpretation of the effects of thyroid hormone mimetics. Deiodinase 2 mRNA expression was not significantly different in either GC-1- or KB-2115-treated gastrocnemius.

Finally, to evaluate the effect of TRβ agonist treatment on the hypothalamic-pituitary-thyroid axis, thyroid function tests were measured. In contrast to the minimal effect seen when used at lower doses in humans (3, 24), 0.164 mg/kg GC-1 and 0.1 mg/kg KB-2115 in Sprague-Dawley rats suppressed endogenous hypothalamic-pituitary-thyroid axis function by both suppressing TSH and total $T_4$ (Fig. 7). GC-1 treatment led to significant differences between groups for TSH ($z = 2.76$; $UA = 0$; $P = 0.0058$) and total $T_4$ ($z = 2.76$; $UA = 0$; $P = 0.0058$). Similarly, KB-2115 treatment also led to significant differences in plasma TSH ($z = 3.73$; $UA = 3$; $P = 0.0002$) and total $T_4$ ($z = 3.92$; $UA = 0$; $P = 0.0001$).

**DISCUSSION**

Liver-specific TRβ agonists are promising therapeutic agents for treating hyperlipidemia (1, 2). While the underlying mechanisms for lowering plasma lipids are not fully established, possible mechanisms include increased LDL receptor expression (15, 29), decreased sterol response element-binding protein (SREBP)-1c expression (18, 20), increased SREBP-2 expression (45), and increased reverse cholesterol transport,
with increased cytochrome p450 7A1 activity in rodent models (15, 20). Additionally, as a result of their ability to increase hepatic lipid oxidation, hepatic and TRβ/H9252-specific thyroid hormone analogs have the potential to decrease NAFLD. Treatments targeting NAFLD may decrease the incidence of nonalcoholic steatohepatitis, which is projected to eventually overtake hepatitis C as the leading cause of liver transplantation (9). Treatment of NAFLD may also reduce the burden of diseases associated with insulin resistance, since decreased hepatic ectopic lipid accumulation should lead to decreased hepatic insulin resistance (23, 36, 37, 43). In the present set of studies, both TRβ agonists effectively prevented the development of hepatic steatosis in fat-fed rats. However, both TRβ agonists tested caused insulin resistance through discrete mechanisms.

GC-1 had notable effects on hepatic glucose metabolism. Specifically, GC-1 increased hepatic glucose output and reduced hepatic insulin sensitivity. Although thyroid hormone can induce expression of key gluconeogenic genes (16, 28), and PEPCK expression was significantly increased in a prior model of rodent thyrotoxicosis (22), changes in gluconeogenic enzymes can only partially explain the observed phenotype. There was a slight increase in the expression of pyruvate carboxylase mRNA, although without significant increases in pyruvate carboxylase protein or activity. There was no change

Fig. 5. Connecting the thyroid hormone receptor-β agonism of KB-2115 with decreased skeletal muscle glucose uptake (open bars, vehicle; filled bars, KB-2115). A: skeletal muscle tissue triglyceride content. B and C: assessment of skeletal muscle insulin signaling at the level of Akt phosphorylation (B) and AS-160 phosphorylation (C). D: skeletal muscle GLUT4 mRNA expression. E: skeletal muscle GLUT4 protein abundance measured by Western blot. F: skeletal muscle tether containing a UBX domain, for GLUT4 (TUG) protein abundance measured by Western blot (*P < 0.04 vs. vehicle). G: skeletal muscle sortilin 1 mRNA expression. H: skeletal muscle sortilin protein abundance measured by Western blot.
in glucose-6-phosphatase expression or activity. In contrast, while GC-1 did not alter cytosolic PEPCK mRNA or protein expression, there was a slight increase in enzyme activity. A similar increase was seen with KB-2115 treatment, suggesting nongenomic effects on gluconeogenesis. In addition to this increase in PEPCK activity, GC-1 also increased basal glycerol turnover. Glycerol enters gluconeogenesis at the level of dihydroxyacetone phosphate, bypassing regulatory checkpoints for

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Fig. 6. Assessment of thyroid hormone action in multiple tissues. Expression of thyroid hormone-responsive genes after GC-1 treatment (A) or KB-2115 treatment (B). Genes examined: deiodinase 1 in liver, hairless in white adipose tissue, hairless in skeletal muscle, deiodinase 2 in skeletal muscle, sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) 1 in skeletal muscle, and malic enzyme 1 (ME1) in skeletal muscle (open bars, vehicle; filled bars, thyromimetic; *\(P < 0.04\) vs. vehicle; **\(P < 0.04\) vs. vehicle; ***\(P < 0.0004\) vs. vehicle).

Fig. 7. Serum thyroid-stimulating hormone (TSH) and total thyroxine (T\(_4\)) levels after vehicle, GC-1, and KB-2115 treatment. The horizontal dashed line represents the lower limit of detection.
other three- and four-carbon precursors at the pyruvate carboxylase and PEPCk steps. Increasing glycerol flux can increase the rate of EGP (40) and may represent an additional mechanism accounting for the increase in basal EGP. In addition to the changes in basal PEPCk activity and glycerol flux, GC-1 also impaired hepatic insulin sensitivity, as reflected by the diminished suppression of EGP during the hyperinsulinemic-euglycemic clamp. This was associated with impaired insulin-stimulated phosphorylation of Akt, suggesting a defect in insulin signaling. Thus, the combined contributions of increased PEPCk activity, increased glycerol influx, and impaired insulin signaling may account for the fasting hyperglycemia and hyperinsulinemia seen in the GC-1-treated animals. In contrast, while KB-2115 also increased PEPCk activity to a similar degree, it did not alter glycerol flux or hepatic insulin sensitivity and thus did not cause fasting hyperglycemia.

KB-2115 is considered a more liver-specific agonist, and this may account for some of the differences from GC-1 (3, 24). As with GC-1, KB-2115 treatment effectively prevented the development of fatty liver in high-fat-fed rats but did so without causing fasting hyperglycemia. Still, the development of fasting hyperinsulinemia suggested that KB-2115 caused insulin resistance. Also, while KB-2115 led to a similar decrease in glucose infusion rates during a hyperinsulinemic-euglycemic clamp to those seen in the GC-1-treated rats, the underlying mechanism for insulin resistance was different. KB-2115 primarily decreased insulin-stimulated peripheral glucose disposal, which was largely attributable to decreased skeletal muscle glucose uptake.

We further explored the mechanisms that may account for the skeletal muscle insulin resistance in KB-2115-treated rats. There was no difference in skeletal muscle lipid content, and skeletal muscle triglyceride content actually tended to be lower in KB-2115-treated animals. Furthermore, insulin signaling was not perturbed, with appropriate increases in Akt and AS160 phosphorylation in response to insulin. Instead, we found a surprising decrease in GLUT4 transporter protein, which was also associated with a similar decrease in TUG abundance. These data are consistent with decreased GSV content. Thus, while skeletal muscle insulin signaling remained intact, the reduction in total GLUT4 available for insulin-stimulated translocation to the membrane reduced the maximum capacity for insulin-stimulated glucose transport into skeletal muscle, thereby accounting for the decline in peripheral glucose disposal. The decrease in GLUT4 protein is not a direct effect on GLUT4 transcription, since GLUT4 mRNA was not reduced in the KB-2115-treated animals. Rather, GSV abundance may be decreased at a posttranslational step. Abundance of sortilin, a key protein involved in GSV assembly, was unchanged. This suggests that GSV assembly was not affected by KB-2115 treatment. Instead, the data suggest that GSVs may not be appropriately sequestered in an intracellular pool, and therefore subject to increased turnover. Taken together, our data suggest that KB-2115 treatment decreases skeletal muscle GSV abundance and, as a result, decreases insulin-stimulated skeletal muscle glucose uptake in KB-2115-treated animals.

We evaluated the possibility that KB-2115 treatment may directly act in skeletal muscle. Such an effect could be via off-target binding of KB-2115 to the TRα receptor, from decreased endogenous thyroid hormone production by action of KB-2115 on the hypothalamic-pituitary-thyroid axis thereby decreasing TRH or TSH levels, or from differential interactions of this drug with coactivators and/or corepressors that interact with the thyroid hormone receptor (TR). Skeletal muscle thyroid hormone action was evaluated by assessing the expression of key downstream genes. Expression of key thyroid hormone-responsive genes, *hairless*, SERCA 1, and malic enzyme 1 (13, 49, 52), was not changed, arguing against alterations in skeletal muscle thyroid hormone action with KB-2115. Thus, the effects in skeletal muscle are likely an indirect, off-target effect of KB-2115. Interestingly, GC-1 markedly increased expression of *hairless* and induced a strong tendency toward increased expression of GLUT4, which also contains a thyroid hormone response element in its promoter (44, 50). These findings suggest that this compound acts directly in skeletal muscle, a TRα-predominant tissue. These data further demonstrate the marked differences in these two synthetic thyromimetics.

This highlights the more unexpected results of this study. Two drugs designed to be agonists of the same receptor have quite different downstream effects on physiology. The likely explanation for the difference lies in the difference in receptor specificity of the two drugs, with the action of KB-2115 more restricted to TRβ-predominant tissues than the action of GC-1. Both agents are effective in reducing hepatic steatosis, but this metabolic gain came with unique costs. GC-1, by increasing glycerol turnover (possibly through actions in adipose tissue), increasing PEPCk activity, and impairing hepatic insulin sensitivity, led to increases in basal EGP and fasting hyperglycemia. However, there was no evidence of peripheral insulin resistance, possibly because of the compensatory increase in muscle GLUT4 expression leading to muscle with normal levels of GLUT4 protein. This pattern of increased EGP and hepatic insulin resistance is similar to the pattern seen in previous reports of human and canine hyperthyroid models (12, 25, 41, 56) and mirrors the pattern seen with chloroform T4-induced thyrotoxic rats (22). In contrast, KB-2115 did not increase glycerol turnover and did not result in hepatic insulin resistance. However, via indirect mechanisms, KB-2115 led to a decrease in muscle GLUT4 abundance and decreased insulin-stimulated muscle glucose uptake.

Cardiovascular disease is still the leading cause of death globally, and new agents that can treat dyslipidemia are needed. Moreover, although NAFLD has become the most common chronic liver disease in developed countries, there are few effective therapies for NAFLD. Thus, the continued refinement and study of TRβ-specific agonists holds the promise for new and necessary therapies. The agonists studied here, GC-1 and KB-2115, have been successfully used in animal and human studies, respectively. While both potently lower hepatic lipid content and can correct dyslipidemia, we demonstrated that each compound had unique negative effects on insulin action in high-fat-fed rats, thereby questioning their therapeutic potential. These studies also suggest that attempts to selectively activate pathways in specific tissues that were intended to function as part of a coordinated whole body response may lead to surprising adverse effects, and underscores the importance of rigorous preclinical testing of novel agents, especially regarding the effects on insulin action and glucose metabolism.
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DISCLOSURES

The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

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GRANTS

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