Selective thyroid receptor modulation by GC-1 reduces serum lipids and stimulates steps of reverse cholesterol transport in euthyroid mice

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Thyroid hormones [predominantly 3,5,3′-triiodo-l-thyronine (T3)] regulate cholesterol and lipoprotein metabolism, but cardiac effects restrict their use as hypolipidemic drugs. T3 binds to thyroid hormone receptors (TRs) α and β. TRβ is the predominant isoform in liver, whereas T3 effects on heart rate are mediated mostly by TRα. Drugs that target TRβ or exhibit tissue-selective uptake may improve plasma lipid levels while sparing the heart. Here, we asked how the TRβ- and liver uptake-selective agonist GC-1 influences cholesterol and triglyceride metabolism in euthyroid mice. GC-1 treatment reduced serum cholesterol levels by 25% and serum triglycerides by 75% in chow-fed mice and also attenuated diet-induced hypercholesterolemia. GC-1 reduced plasma high-density lipoprotein cholesterol levels; increased expression of the hepatic high-density lipoprotein receptor, SR-BI; stimulated activity of cholesterol 7α-hydroxylase; and increased fecal excretion of bile acids. Collectively, these results suggest that GC-1 stimulates important steps in reverse cholesterol transport. Use of TRβ and uptake selective agonists such as GC-1 should be further explored as a strategy to improve lipid metabolism in dyslipoproteinemia.

Strategies that decrease plasma cholesterol levels should prevent and reduce cardiovascular disease (1). Several lipid-lowering drugs are now available (2–4). Most, such as the hydroxymethyl glutaryl CoA (HMG CoA) reductase inhibitors (statins), or bile-acid-binding agents, reduce cholesterol levels within liver. This activates sterol regulatory element-binding proteins (SREBPs) (4), thereby lowering low-density lipoprotein (LDL) receptor (LDLR) expression and promoting clearance of LDL cholesterol. Nevertheless, there is a demand for novel therapies to optimize plasma lipids: treatments that promote reverse cholesterol transport (RCT), efflux of cholesterol from peripheral tissues, and excretion of cholesterol should be of particular value (2–4).

Several hormones, such as insulin, growth hormone, and thyroid hormone (TH), influence lipid metabolism in part by SREBP-independent pathways (5–7). Thus, modulation of these pathways could offer novel therapies. TH reduces circulating cholesterol (8). Plasma cholesterol levels are often elevated in hypothyroidism due to reduced LDL turnover, which may be associated with normal or increased triglycerides (9). Conversely, hyperthyroid patients have reduced plasma cholesterol, also associated with elevated or normal plasma triglycerides (10). TH action is mediated by nuclear receptors (11, 12). Separate genes encode TH receptor (TR) subtypes α and β, which differ slightly in structure and substantially in tissue distribution. TRα regulates heart rate and the speed and force of systolic contraction (13). TRβ exerts several metabolic effects (14), facilitated by high expression in liver (15, 16).

Because of strong cardiac effects of TRα activation (17), use of TH as hypolipidemic drugs carries significant risks (18). However, TH derivatives that bind TRβ selectively or exhibit liver-selective uptake could improve plasma lipids without affecting heart rate (19). Thus, the TRβ and liver uptake-selective agonist GC-1 and the TRβ-selective agonist KB141 reduce plasma lipids in animal models, without obvious adverse effects (19–21).

The present studies elucidate how GC-1 regulates cholesterol and triglycerides in normal mice. GC-1 reduced serum cholesterol and triglyceride levels, induced up-regulation of the hepatic high-density lipoprotein (HDL) receptor SR-BI, and increased bile acid synthesis and excretion. This suggests that GC-1 stimulates several steps in RCT, and that GC-1 might be a candidate for treatment and prevention of cardiovascular disease in humans.

Materials and Methods

Animals and Experimental Set-Up. Studies were approved by the institutional Animal Care and Use Committee. One hundred eighteen male C57BL/6 mice, 3 months old (M&B, Ry, Denmark), were kept in standardized conditions with free access to water and normal chow (Lactamin, Kimstad, Sweden). 3,5,3′-triiodo-l-thyronine (T3) (Sigma) or GC-1 (22) was administered i.p. in 20% DMSO or propylene glycol for 8 days, once daily at 9:00 a.m.; controls received vehicle. In one experiment, nine groups of six mice were treated with vehicle, or 5.4, 24, 48, and 97 nmol/kg per day of T3 or GC-1. The lowest dose of T3 induces euthyroidism in hypothyroid mice (23). In another experiment, one group of seven mice was given no additional supplementation; three groups of seven mice received 10% corn oil and 2% cholesterol (cholesterol diet); and three groups of seven mice received 10% corn oil, 2% cholesterol, and 0.5% cholic acid (cholic acid diet). For this experiment, mice were treated with vehicle or 97 nmol/kg per day GC-1 or T3. Food was withdrawn 5 h before kill. Blood was drawn by cardiac puncture under light isoflurane anesthesia. Animals were killed by cervical dislocation. Livers were immediately frozen in liquid nitrogen.

For analysis of bile acid excretion, three groups of five chow-fed mice were treated with vehicle, 48 nmol/kg per day of GC-1, or T3 for 5 days. Feces were collected group-wise 24 h before and in the last 24 h of the experiment.

Lipid Analysis. Total cholesterol and triglycerides were determined in individual serum samples by a Monarch automated analyzer (ILS

Abbreviations: HMG CoA, hydroxymethyl glutaryl CoA; SREBP, sterol regulatory element-binding protein; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; VLDL, very low-density lipoprotein; RCT, reverse cholesterol transport; TH, thyroid hormone; TR, TH receptor; LDL, high-density lipoprotein; T3, 3,5,3′-triiodo-l-thyronine; SEC, size exclusion chromatography; C4, 7α-hydroxy-4-cholestene-3-one; CYP7A1, cholesterol 7α-hydroxylase; SHP, short heterodimerizing partner; UXR, liver X receptor.

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Laboratories Scandinavia, Sollentuna, Sweden). Serum triglyceride levels were corrected for free glycerol content. Size exclusion chromatography (SEC) of lipoproteins was performed as described in ref. 24. Hepatic lipids were extracted (25) and analyzed for cholesterol and triglycerides by using commercial kits (Roche Applied Science, Indianapolis).

Preparation of Hepatic Membranes, Immunoblotting of SR-B1, and Ligand Blot. Liver membranes were prepared as in ref. 26 and separated on 3–8% Tris-acetate gels (NuPAGE, Invitrogen). Proteins were transferred to 0.45-μm nitrocellulose filters. SR-B1 protein was detected with rabbit polyclonal antibodies (1:3,000) against mouse SR-B1 (Novus Biologicals, Littleton, CO) (27). LDL-receptor expression was determined by ligand blot in nonreduced liver membranes (28).

7α-Hydroxy-4-Cholesten-3-One (C4). C4 was analyzed in pooled serum samples by HPLC after solid-phase extraction by using 7α-hydroxy-4-cholesten-3-one as internal standard (29).

Fecal Bile Acids and Neutral Steroids. Feces were collected group-wise for 24 h. One gram of pooled dried feces was analyzed for neutral sterols (coprostanol, coprostanone, and cholesterol) and bile acids by quantitative gas-liquid chromatography (30–32).

Relative Quantitative Real-Time PCR. Total RNA was extracted by TRIZol according to instructions (Life Technologies, Gaithersburg, MD). Total RNA was treated with RNase-free DNase (Promega). First-strand cDNA synthesis was performed in triplicate for each RNA pool by using random hexamer primers and Omniscript (Qiagen, Valencia, CA). Specific primers (Tables 1 and 2, which are published as supporting information on the PNAS web site) were designed with primer express and span exon boundaries. Quantification of mRNA used Applied Biosystems PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems). Reactions were done in triplicate, with GAPDH mRNA endogenous control in all experiments.

Statistics. Data are presented as means ± SEM. One-way ANOVA, fully randomized design, was used to evaluate significant differences between groups, followed by post-hoc comparisons of group means by the HSD and LSD method (STATISTICA software, Ver. 6.0 StatSoft, Tulsa, OK). Statistical analysis was not performed for mRNA expression, because cDNA was prepared from pooled livers; SEM in these determinations shows variation in the assay.

Results

GC-1 Reduces HDL Cholesterol and Very Low-Density Lipoprotein (VLDL) Triglyceride Levels in Euthyroid Mice. We first compared effects of GC-1 and T3 administration on cholesterol and triglyceride metabolism in euthyroid chow-fed male mice. Both ligands yielded dose-dependent reductions of serum cholesterol (Fig. 1 A); the response to GC-1 was stronger than T3 at equimolar doses. Circulating cholesterol in rodents is largely contained in HDL. Chromatographic (SEC) analysis of pooled serum samples confirmed this and showed that GC-1 reduces HDL cholesterol (Fig. 1B and C). GC-1 had no effect on VLDL cholesterol, whereas T3 increased VLDL cholesterol at the high doses, 24 or 97 nmol/kg per day. As seen in hypothyroid mice (16), GC-1 but not T3 reduced serum triglyceride levels (Fig. 1D). This was due to reductions in VLDL and LDL triglycerides (Fig. 1E). Low doses of T3 increased
VLDL triglycerides, although high concentrations of T3 reversed this effect (Fig. 1F).

**GC-1 Increases Hepatic HDL Receptor Levels and Stimulates Conversion of Cholesterol to Bile Acids.** The observation that GC-1 reduced serum HDL cholesterol prompted us to assay for hepatic expression of receptors that remove cholesterol from the circulation, LDLR activity, and transcripts were not affected by GC-1 or T3 (Fig. 6 and Table 3, which are published as supporting information on the PNAS web site). However, both ligands increased levels of the HDL receptor SR-BI to +240% of control with 48 nmol/kg per day of GC-1 (Fig. 2A) and +150% with similar amounts of T3. Neither GC-1 nor T3 affected SR-BI mRNA (Table 3), suggesting this effect involves posttranscriptional mechanisms.

The large reduction of plasma HDL cholesterol induced by GC-1 and T3 was not accompanied by increased intrahepatic cholesterol (Fig. 7A, which is published as supporting information on the PNAS web site), leading us to hypothesize that increased hepatic uptake of HDL-cholesterol via SR-BI is paralleled by elimination of cholesterol as bile acids. Conversion of cholesterol to bile acids is mediated by cholesterol 7α-hydroxylase (CYP7A1), whose activity can be monitored by measuring serum levels of C4, an intermediate in bile acid synthesis (29). In agreement with earlier data showing that TRβ regulates CYP7A1 (5, 33, 34), GC-1 increased serum C4 levels, reaching +230% of control at 97 nmol/kg per day GC-1 (Fig. 2B). T3 treatment was less effective, reaching a maximal of 170% of control. These effects were paralleled by increases in hepatic CYP7A1 mRNA (Fig. 2C).

**GC-1 Regulates Expression of Genes Encoding Nuclear Transcription Factors Involved in Hepatic Lipid Metabolism.** We next examined GC-1 and T3 effects on expression of genes encoding transcription factors involved in hepatic lipid metabolism. Bile acids induce expression of short heterodimerizing partner (SHP), which inhibits CYP7A1 expression by interaction with liver receptor homologue-1 (35). GC-1 and T3 reduced SHP mRNA levels 50–60% (Fig. 2C). Thus, TRβ may stimulate bile acid synthesis by inhibiting SHP inhibition of CYP7A1 expression (36).

SREBP-2 regulates intrahepatic cholesterol balance by stimulating transcription of the gene that encodes HMG CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, and LDLR, and its closely related homolog SREBP-1c regulates genes involved in triglyceride synthesis (37). GC-1 and T3 elicited a slight up-regulation of SREBP-2 mRNA to 150% of control (Fig. 2D). Nevertheless, expression of SREBP-2 target genes, HMG CoA reductase, and LDLR (38) was unaffected (Table 3). Thus, T3 and GC-1 modulate serum lipids independently of changes in SREBP-2 mRNA. In contrast, GC-1 and T3 reduced levels of mRNA for SREBP-1c (Fig. 2D) and of at least one gene regulated by SREBP-1c, fatty acid synthetase (Table 3). Thus, GC-1 and T3 may inhibit hepatic fatty acid and triglyceride synthesis by repressing SREBP-1c gene expression.

**GC-1 Reduces HDL Cholesterol and Triglyceride Levels in Hypercholesterolemic Mice.** We next investigated effects of T3 and GC-1 in mice fed cholesterol or cholic acid that promotes hypercholesterolemia. We used 97 nmol/kg per day of GC-1 and T3, which induced the strongest effects on serum C4 and SREBP-1c mRNA in nonsupplemented animals.

The cholesterol- and cholic acid-enriched diets resulted in the expected increase in serum cholesterol levels by 50% and 250%, respectively (Fig. 3A), and GC-1 and T3 blocked these increases. The cholesterol diet increased levels of VLDL and LDL, with no effect on HDL (Fig. 3B). Here, GC-1 or T3 reduced LDL and HDL cholesterol levels, with lesser effects on VLDL levels. The cholic acid diet increased VLDL and

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**Fig. 2.** T3 and GC-1 induce hepatic expression and activity of genes involved in RCT (SR-BI and CYP7A1). (A) SR-BI protein in pooled liver membranes determined by Western blot and quantified by using IMAGE GAUGE software, Fuji Film. (B) C4 determined in pooled serum samples; results are expressed as percent change from control relative to total serum cholesterol (5.36 mg/liter), as currently recommended (62). However, GC-1 and, to a lesser extent, T3 also increased absolute C4 levels. (C and D) Effects of GC-1 and T3 on hepatic mRNAs, mean ± SEM of determinations with three separate cDNA syntheses from pooled RNA.
LDL cholesterol but reduced HDL cholesterol (Fig. 3C); GC-1 and T3 reduced cholesterol levels in all fractions. Thus, GC-1 and T3 reduce plasma HDL cholesterol levels in diet-induced hypercholesterolemia.

Cholesterol and cholic acid feeding resulted in respective 6- and 12-fold increases of hepatic cholesterol (Fig. 3D). However, in line with their effects in chow-fed mice, neither TR ligand increased liver cholesterol levels and, in fact, slightly reduced liver cholesterol levels in cholic acid fed mice (Fig. 3D).

The cholesterol and cholic acid diets reduced serum triglyceride levels (Fig. 3E) and increased hepatic triglyceride levels by 5-fold (Fig. 3F). The effect on serum triglyceride levels due to cholesterol feeding was partly reversed by T3 but not GC-1, and that due to cholic acid feeding was not affected by either ligand (Fig. 3E). T3 doubled and GC-1 did not affect hepatic triglyceride levels. Thus, GC-1 does not further stimulate diet-induced triglyceride synthesis in liver.

**GC-1 Increases Hepatic HDL Receptors and Stimulates Bile Acid Synthesis in Hypercholesterolemic Mice.** Next, we examined effects of T3 and GC-1 on HDL receptor, bile acid conversion, and hepatic transcription factor expression in hypercholesterolemic mice. The cholesterol diet slightly reduced SR-BI protein levels (Fig. 4A). Here, GC-1 treatment led to a doubling of SR-BI protein levels, whereas T3 treatment led to only a minor increase. By contrast, the cholic acid diet increased SR-BI by 50%. Here, GC-1 increased SR-BI by a further 60%, whereas T3 blunted the diet-induced increase. SR-BI mRNA levels increased by 40% with both diets but, paradoxically, GC-1 and T3 reduced SR-BI mRNA compared with their respective control diets (Table 4, which is published as supporting information on the PNAS web site). Thus, GC-1 enhances SR-BI protein levels via posttranscriptional effects in both chow-fed and hypercholesterolemic mice.

Both TR ligands increased serum C4 levels, indicating that they enhance bile acid synthesis in conditions of diet-induced hypercholesterolemia. The cholesterol diet slightly increased serum C4 levels and GC-1 or T3 treatment induced a further 2-fold increase (Fig. 4B). The cholic acid diet decreased C4 by 50%. GC-1 and T3 treatments reversed this and further increased C4, leading to increases in serum C4 of 2- and 3-fold (Fig. 4B).

Changes in serum C4 did not always correlate with levels of CYP7A1 mRNA (Fig. 4C). The cholesterol diet only modestly increased serum C4 but induced CYP7A1 mRNA levels 3-fold, and GC-1 and T3 treatment partially blunted this increase. The cholic acid diet suppressed CYP7A1 mRNA below that of controls. Here, GC-1 and T3 reversed this suppression, leading to net induction of CYP7A1 mRNA.

T3 and GC-1 influenced hepatic transcription factor expression in hypercholesterolemic mice. The cholesterol diet increased SHP mRNA by 50%. GC-1 and T3 blunted this increase and reduced SHP mRNA below controls (Fig. 4C). As expected, SHP mRNA levels were greatly increased by cholic acid feeding (Fig. 4C). GC-1 and, to a larger extent, T3, reversed this induction, with SHP mRNA reduced to levels comparable to controls. Hepatic SREBP-1c mRNA increased by 150% and 200% in cholesterol- and cholic acid-fed mice (Fig. 4D), likely a result of activation of liver X receptor (LXR) α by cholesteryl (39). GC-1 and T3 treatment reversed this increase in mice on both diets (Fig. 4D). Neither ligand affected SREBP-2 mRNA (Fig. 4D).

**GC-1 Stimulates Fecal Excretion of Bile Acids.** To investigate whether TRβ stimulation increased fecal excretion of bile acids and cholesterol, animals on the standard diet were treated with GC-1 or T3 at 48 nmol/kg per day, the dose that gave the strongest induction of SR-BI (Fig. 2A). Feces were collected 24 h before and for the last 24 h of treatment. Compared with controls, animals treated with GC-1 exhibited a 45% increase and those with T3 a 30% increase in fecal bile acid excretion (Fig. 5). Neither ligand affected neutral sterol excretion (Fig 8, which is published as supporting information on the PNAS web site), suggesting that intestinal cholesterol
absorption and hepatic biliary cholesterol secretion are not affected. Thus, only the bile acid synthetic pathway was affected. Accordingly, liver CYP7A1 mRNA levels correlated with bile acid excretion (Fig. 5).

Discussion
In the present study, we show that the selective TR modulator GC-1 induces several important steps in RCT. We find that T3 and GC-1 increase levels of hepatic SR-BI protein and, as previously reported for T3 (40, 41), CYP7A1 activity, and that this is associated with reduced circulating HDL cholesterol levels and increased fecal excretion of bile acids, with no change in fecal neutral sterol levels, a reflection of cholesterol excretion. Effects on SR-BI protein and CYP7A1 activity are observed in both normal and hypercholesterolemic mice. Thus, we propose that both ligands promote increased cholesterol influx into liver through the SR-B1 receptor, and that this cholesterol is channeled toward bile acid synthesis rather than directly to biliary cholesterol secretion.

Although GC-1 and T3 induce several key steps in RCT, the extent to which they actually promote RCT is not clear, because we did not measure cholesterol synthesis. Thus, increased release of bile acids into the intestine could be secondary to increased cholesterol synthesis. Nevertheless, although TH administration does promote cholesterol synthesis in hypothyroid animals by increasing transcription and stability of HMG CoA reductase mRNA (see ref. 42), we did not detect similar effects, likely because we used euthyroid animals (Table 3). Thus, unless T3 or GC-1 exert unexpected posttranslational effects on HMG CoA reductase, it seems unlikely that the increased excretion of bile acids is secondary to enhanced synthesis of cholesterol. Expression of the Apo A1 gene, whose product encodes the major apolipoprotein in HDL, or the ABCA1 gene, whose product secretes cholesterol onto HDL (Table 3), was also unaffected in our euthyroid mice model.

Our data are restricted to mice, so it is not clear whether similar effects occur in humans (43). However, previous studies showed that elevated TH production and administration reduce plasma HDL in humans (44–47), even though TH can induce increases in mRNA for Apo A1 in euthyroid conditions (48). Thus, it is possible that TH reduces HDL cholesterol by stimulating key steps in RCT also in humans.

TR effects upon RCT steps involve a mix of mechanisms. Increases in hepatic SR-BI protein levels result from posttranscriptional regulation; SR-BI mRNA levels were either unaffected or reduced by T3 or GC-1. This is in line with previous studies showing that such mechanisms occur (49–51). By contrast, T3 and GC-1 effects on CYP7A1 activity were generally paralleled by increases in CYP7A1 mRNA, again in line with previous studies (33, 40). The sole exception was after dietary cholesterol challenge, where T3- and GC-1-dependent increases in CYP7A1 activity were accompanied by decreases in CYP7A1 mRNA, emphasizing that multiple
mechanisms of TRα action are operative. Finally, we observe that T3 and GC-1 reduce SHP mRNA levels, raising the possibility that both ligands induce CYPTA1 expression by blocking SHP-dependent, feedback inhibition of CYPTA1 transcription.

TH lowers plasma LDL in humans and rodents (47, 52, 53), and both ligands we tested reduced cholesterol levels in all fractions in hypercholesterolemic mice. Nevertheless, although several studies show that TH induces LDLR in rodents (52, 54, 55), we did not detect changes in hepatic LDLR activity or mRNA. We speculate that our euthyroid model precludes this effect, and that other mechanisms that reduce plasma LDL may be active.

GC-1 displayed some useful properties that are distinct from T3. GC-1 elicited larger increases in SR-BI levels than T3. GC-1 also lowers serum and liver triglyceride levels via differential effects on the liver lipid metabolism and might also block the LXR agonist-induced increases in serum triglyceride levels (59, 60). However, LXR agonists elicit other actions that may be beneficial, such as inducing cholesterol removal from macrophages (61). Thus, TRβ-selective agonists might act synergistically with LXR agonists on lipid metabolism and might also block the LXR agonist-induced increases in SREBP-1c expression and triglyceride levels.

The current observation that GC-1 stimulates key steps in RCT provides a previously undescribed feature to this ligand. Coupled with previous observations of lowered plasma cholesterol, triglycerides, and lipoprotein(a) and reduced obesity, this action might amplify potential antiatherogenic effects, which are observed in the absence of deleterious effects on heart, bone, and muscle. Our data suggest that GC-1 and similar ligands deserve further study as antiatherogenic agents.

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