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Effects of L-Triiodothyronine and the Thyromimetic L-94901 on Serum Lipoprotein Levels and Hepatic Low-Density Lipoprotein Receptor, 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase, and Apo A-I Gene Expression

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ABSTRACT. The mechanisms by which thyroid hormone (triiodothyronine (T_3)) and a thyromimetic, 2-amino-3-(3,5-dibromo-4-[4-hydroxy-3-(6-oxo-1,6-dihydro-pyridazin-3-ylmethyl)-phenoxyl]-phenyl)-propionic acid (L-94901), lower plasma low density lipoprotein (LDL) cholesterol and raise plasma high density lipoprotein (HDL) cholesterol levels was investigated in thyroidectomized and sham-operated rats. Thyroidectomy resulted in a 77% increase in plasma LDL cholesterol, a 60% decrease in plasma triglycerides, and a modest reduction in HDL cholesterol. Daily oral dosing with T_3 (10–170 nmol/kg) or L94901 (100–1000 nmol/kg) for 7 days decreased plasma LDL cholesterol in thyroidectomized rats by 60–80%, respectively. This reduction in LDL cholesterol was accompanied by a dose-dependent increase in HDL cholesterol levels of up to 60%. Thus, the ratio of LDL to HDL was decreased from 1.01 to 0.12 after treatment with L-94901 and to 0.25 after dosing with T_3. In sham-operated animals, T_3 and L-94901 lowered LDL cholesterol by 61 and 46%, respectively, and increased HDL cholesterol by 25 and 53%, respectively. Immunoblotting analysis of liver membranes prepared from thyroidectomized or sham-operated rats demonstrated that LDL receptor protein levels were increased by up to eight-fold. Northern blotting analysis revealed similar large increases in hepatic LDL receptor mRNA levels that accounted for the increases in LDL receptor protein levels. Hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase mRNA, protein, and activity were increased 2- to 3-fold. The T_3- and L-94901-mediated increases in serum HDL levels were associated with 2- to 3-fold increases in apo A-I mRNA levels. In contrast with most other hypocholesterolemic agents, T_3 and L-94901 significantly increase HDL cholesterol levels in addition to decreasing LDL cholesterol levels due to induction of hepatic apo A-I and LDL receptor gene expression. BIOCHEM PHARMACOL **56**;1:121–129, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. thyromimetic; LDL; HDL; LDL receptor; HMG-CoA reductase; Apo A-I

It is well known that hypothyroidism is accompanied by high levels of circulating LDL§ cholesterol and an increased risk of atherosclerosis, whereas in hyperthyroidism, LDL cholesterol levels are decreased [1, 2]. It is also well established that administration of thyroid hormone effectively lowers plasma cholesterol levels in experimental animals [3–7] and in humans [2, 8].

The hypocholesterolemic effects of thyroid hormone are believed to be a consequence of specific enzyme and LDL receptor induction in the liver [2, 9–11], the key organ for cholesterol homeostasis [12, 13]. Indeed, recent studies have demonstrated that increased expression of the hepatic LDL receptor may underlie the hypocholesterolemic effect of the hormone [14–16]. For example, in hypophysectomized rats, administration of low doses of triiodothyronine caused a rapid increase in hepatic LDL receptor mRNA and immunoreactive protein levels that occurred within 1 hr after hormone administration [14]. Further studies showed that the thyroid hormone acts at the level of transcription to increase hepatic LDL receptor expression [15]. The effects of thyroid hormone on LDL receptor expression are direct rather than secondary to nonhepatic actions of the hormone, since increases in LDL receptor expression can be

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§ *Abbreviations:* FPLC, fast protein liquid chromatography; HDL, high-density lipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMGR, HMG-CoA reductase; LDL, low-density lipoprotein; LDLR, LDL, receptor; T_3, triiodothyronine; T_4, tetraiodothyronine; TG, triglyceride; and VLDL, very low-density lipoprotein.

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duplicated in cultured hepatic cells following treatment with thyroid hormone [16]. Although the naturally occurring thyroid hormones, T_3 and T_4, are potent hypocholesterolemic agents [2], they cannot be used therapeutically in patients with normal thyroid function because of their potential to induce cardiac side-effects [17], especially in subjects whose cardiac function may already be compromised [17].

A number of metabolites of T_3 and T_4 [1], as well as a variety of synthetic analogs [1, 4–6, 18] have been studied in an attempt to disassociate the hypocholesterolemic actions of thyroid hormone from its cardiac side-effects. One such thyromimetic, 2-amino-3-(3,5-dibromo-4-[4-hydroxy-3-(6-oxo-1,6-dihydro-pyridazin-3-ylmethyl)-phenoxyl]-phenyl)-propionic acid (L-94901), was reported to effectively lower serum cholesterol levels in experimental animals at doses that do not exhibit the cardiotoxic side-effects of thyroid hormone [1, 4]. Indeed, following single dose administration to hypothyroid rats, L-94901 was approximately 50% as effective as T_3 in binding to the hepatic thyroid hormone receptor but only 1.3% as effective as T_3 in associating with the cardiac receptor [4]. Following chronic administration to hypothyroid rats, the potency of L-94901 approached that of T_3, such that after 7 days of administration, the thyromimetic action of L-94901 in the liver (measured by induction of mitochondrial α-glycerol phosphate dehydrogenase [18]) was equal to that of T_3 on a molar basis [4]. The action of L-94901 on the heart remained low [4]. Thus, the possibility exists for utilizing agents that mimic thyroid hormone action in the liver to lower plasma cholesterol levels in humans.

As a result of its thyromimetic action in the liver, L-94901, administered orally for 7 days, markedly and dose-dependently reduced plasma cholesterol levels in hypothyroid rats made hypercholesterolemic by feeding a diet containing 1.5% cholesterol and 0.5% cholic acid [4]. This reduction in plasma cholesterol was due primarily to a decrease in non-HDL (VLDL + LDL) cholesterol with a slight concomitant increase in HDL cholesterol [4]. Equimolar doses of T_3 also produced reductions in plasma cholesterol that were of a magnitude equal to those of L-94901 [4, 5]. In addition, although less sensitive to the actions of either T_3 or L-94901, euthyroid rats made hypercholesterolemic by feeding diets containing cholesterol and cholic acid also showed similar hypocholesterolemic responses to T_3 and L-94901 [4]. The similarity between the potency of L-94901 as a thyromimetic and as a hypocholesterolemic agent [4] also indicates that the thyromimetic actions of L-94901 are at least in part responsible for its ability to reduce plasma cholesterol levels in experimental animals.

To better understand the biochemical mechanisms responsible for the hypocholesterolemic actions of L-94901 and to compare the effects of L-94901 with those of T_3, we investigated the actions of L-94901 and T_3 on lipoprotein cholesterol concentrations and expression of key proteins involved in cholesterol homeostasis (e.g. LDL receptor, apo A-I, and HMG-CoA reductase) in euthyroid rats and rats made hypothyroid by surgical thyroidectomy. The results demonstrate that L-94901, like T_3, lowers serum LDL cholesterol and raises HDL cholesterol levels through a mechanism involving increased expression of the hepatic LDL receptor and apo A-I genes.

MATERIALS AND METHODS

Experimental Animals and Design

Surgically thyroparathyroidectomized and sham-operated control male Sprague–Dawley rats weighing between 200 and 250 g, from Charles Rivers, were housed in a reversed-cycle light-controlled room (light from 3:00 p.m. to 3:00 a.m.). These animals carry about 30% of their cholesterol in the form of LDL and 65% in HDL (see Table 1). Upon thyroidectomy, the portion carried in LDL increases to 50%. In many respects, these changes closely parallel the responses to thyroid dysfunction and thyroid hormone therapy in humans [8]. Considerable baseline data exist concerning the responses of these animals to T_3 and thyromimetics [4, 7, 10, 11, 14–16]. Thus, these animals are suitable experimental models.

The rats were given Purina rodent laboratory chow and 1% calcium gluconate as their drinking water for 2 weeks prior to use. Thyroidectomized animals were assigned to groups of three. They were administered, by oral gavage at 7:00 a.m., either vehicle (0.01 M of NaOH in PBS), vehicle containing T_3 (Sigma) at doses of 11, 55, or 167 nmol/kg/day, or vehicle containing L-94901 at doses of 110, 330, or 990 nmol/kg/day for 7 days. Sham-operated animals were treated similarly with either vehicle, T_3 at a dose of 167 nmol/kg/day, or L-94901 at a dose of 990 nmol/kg/day. The effectiveness of these treatments was assessed by measuring the induction of mitochondrial α-glycerol phosphate dehydrogenase mRNA, a sensitive marker of thyroid hormone function in liver [4, 18]. Northern blotting analysis revealed that both L-94901 and T_3 caused more than 10-fold increases (data not shown). Thus, under the conditions employed, L-94901 exhibited thyromimetic actions in liver.

Animals were weighed on days 1, 3, 5, and 7 to evaluate the changes in body mass following treatment and for determining liver-to-body weight and heart-to-body weight ratios. On the last day of the study, 2 hr after the last treatments (9:00 a.m.; mid-dark period of the lighting cycle), animals were anesthetized with pentobarbital, and blood samples were obtained from the abdominal aorta for determining plasma cholesterol, TG, T_4, LDL, HDL, and VLDL cholesterol levels. Livers were then removed, weighed, rinsed in 4° saline, and were apportioned for isolation of microsomes and poly A^+ RNA.

HMG-CoA Reductase Activity

Microsomal HMGR activity was determined by measuring the conversion of [^{14}C]HMG-CoA (DuPont/NEN) to [^{14}C]mevalonic acid in the presence of NADPH as previously described [13]. Microsomal protein, 50 μg, was

incubated for 30 min at 37° in a final volume of 75 μL of TEDK buffer (Tris, pH 7.5, 1 mM of EDTA, 5 mM of dithiothreitol, and 70 mM of KCl) containing 3.4 mM $NADP^+$, 30 mM of glucose-6-phosphate, 0.2 U glucose-6-phosphate dehydrogenase, 66.7 μM of [^{14}C]HMG-CoA (sp. act. 10 cpm/pmol), 15,000–20,000 cpm [^{3}H]mevalonate (0.6 to 1.2 Ci/mmol) (DuPont/NEN) as an internal standard, and 68 mM EDTA to prevent conversion of mevalonate to phosphomevalonate during incubation. The reactions were terminated and the mevalonate was converted to mevalonolactone by addition of 10 μL of 6 M of HCl and a further 30-min incubation. The labeled mevalonolactone was separated from unreacted substrate by silica gel TLC. Following development in toluene:acetone (1:1), the region of the chromatogram corresponding to R_f 0.4 to 1.0 was removed, immersed in 5.0 mL of Aquasol II liquid scintillation fluid, and counted, using a dual-channel ^{3}H/^{14}C program. HMGR activity is expressed as picomoles of mevalonate formed per minute of incubation at 37° per milligram of microsomal protein.

Plasma Lipid and Lipoprotein Analyses

Blood samples were collected in lithium heparin-containing Vacutainer tubes and were centrifuged at 800 *g* for 30 min at 4°. Resultant plasma samples were removed and analyzed for cholesterol and TG content using a Cholesterol/HP reagent kit (Boehringer Mannheim) and a Triglyceride G reagent kit (Wako). The lipoproteins in 50-μL plasma samples were separated into LDL, HDL, and VLDL fractions by FPLC using a 10 × 300 mm Pharmacia Superose-6 column. A mobile phase of 154 mM of NaCl, 1 mM of EDTA, 0.02% sodium azide (pH = 8.1) at a flow rate of 0.3 mL/min and a post-column reactor employing 2×-concentrated cholesterol reagent from the Cholesterol/HP reagent kit were used for separation and quantitation. Cholesterol concentrations in LDL, HDL, and VLDL were determined based on integration of peak areas and comparison with known standards.

Plasma T_4 Analyses

Plasma T_4 levels were determined using Abbott's fluorescent polarization immunoassay kit for quantitative determination of thyroxine. Thyroxine levels in plasma were 3.81 ± 1.17 μg/dL in sham-operated control rats; 1.29 ± 0.77 μg/dL in vehicle-treated thyroidectomized rats; 0.81 ± 0.88 μg/dL in L-94901-treated rats (300 μg/kg/day); and 0.035 ± 0.049 μg/dL in T_3-treated rats (150 μg/kg/day).

Immunoblotting Analysis

Liver microsomal membranes free of lysosomes were prepared by differential centrifugation [19]. These isolated membranes were subjected to SDS–PAGE on 7.5% slab gels. Molecular weight markers of 213, 119, 83, and 47 kDa were also applied. The separated proteins were electrophoretically transferred to PVDF Plus membranes. The membranes were blocked with 5% Carnation nonfat dry milk and incubated at room temperature for 1 hr with both a 1:1500 dilution of LDL receptor antiserum and a 1:500 dilution of HMGR antiserum. The ECL Western blotting kit (Amersham) was used for detection of immunoreactive protein that was quantitated by scanning with a laser densitometer.

cDNA Probes

Plasmid DNA containing rat mGPDH cDNA (L. P. Kozak, Jackson Laboratories) was digested with *Pst*I, and a 1300-bp fragment was isolated following agarose gel electrophoresis. This fragment was radiolabeled with [α-^{32}P]dCTP (DuPont/NEN) by random priming (Amersham Megaprime kit), and 2×10^6 cpm/mL was used for hybridization at 43° for 16 hr. The ribophorin cDNA probe was a PCR-amplified fragment provided by W. C. Soeller and M. D. Carty, Pfizer Central Research. LDL receptor, HMGR, and apo A-I cDNA probes were prepared as previously described [15, 20, 21].

Northern Blotting Analysis

Approximately 0.5 g of liver tissue was removed, placed in 10 mL of Trizol reagent (Life Technologies), and homogenized with a Polytron homogenizer, and total RNA was isolated according to the manufacturer's instructions. The quantity of RNA was determined by measuring the absorbance at 260 nm, whereas purity was assessed by the $A_{260/280}$ ratio. Poly A^+ mRNA was isolated using Clontech mRNA Separator™ Oligo(dT)-Cellulose columns and resuspended in diethyl pyrocarbonate (DEPC)-treated H_2O. Ten micrograms poly A^+ mRNA/well was separated by electrophoresis on a 1% agarose/3% formaldehyde gel in BE (20 mM of borate, 0.2 mM of EDTA) at 200 V for 2 hr. RNA was transferred to Hybond-N membranes (Amersham) by capillary action with 10× SSC (1× SSC = 0.15 M of sodium chloride + 0.015 M of sodium citrate). RNA was UV cross-linked to the membrane using a Stratalinker 1800. The blot was prehybridized in 10 mL of prehybridization solution (50% deionized formamide, 250 mM of $NaHPO_4$, pH 7.2, 250 mM of NaCl, 1 mM of EDTA, 100 μg/mL of salmon sperm DNA, 7% SDS) at 43° for 2 hr in a hybridization oven with constant rotation. Blots were washed three times at 53° with 2× SSC, 0.1% SDS; twice with 25 mM of $NaHPO_4$, 1 mM of EDTA, 0.1% SDS; twice with 25 mM of $NaHPO_4$, 1 mM of EDTA, 1% SDS; and exposed to a Fuji Phosphorimaging plate or to Kodak X-ray film [22] for 16–24 hr.

RESULTS

Alterations in Plasma Lipid and Lipoprotein Concentrations in Response to Thyroidectomy

Hypothyroidism induced by surgical thyroidectomy of rats caused marked alterations in circulating lipid and lipopro-

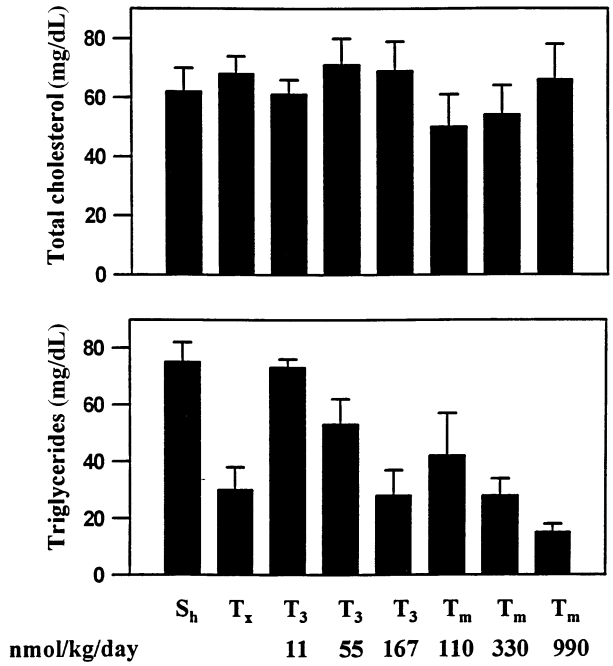

FIG. 1. Effects of T_3 and L-94901 on plasma cholesterol and TG levels in thyroidectomized rats. Values are means ± SD (N = 3). Shown are total cholesterol levels (top panel) and TG levels (bottom panel) in plasma of sham-operated control rats (S_h) and thyroidectomized rats treated for 7 days with either vehicle (T_x), T_3 (T_3), or L-94901 (T_m) at the indicated doses.

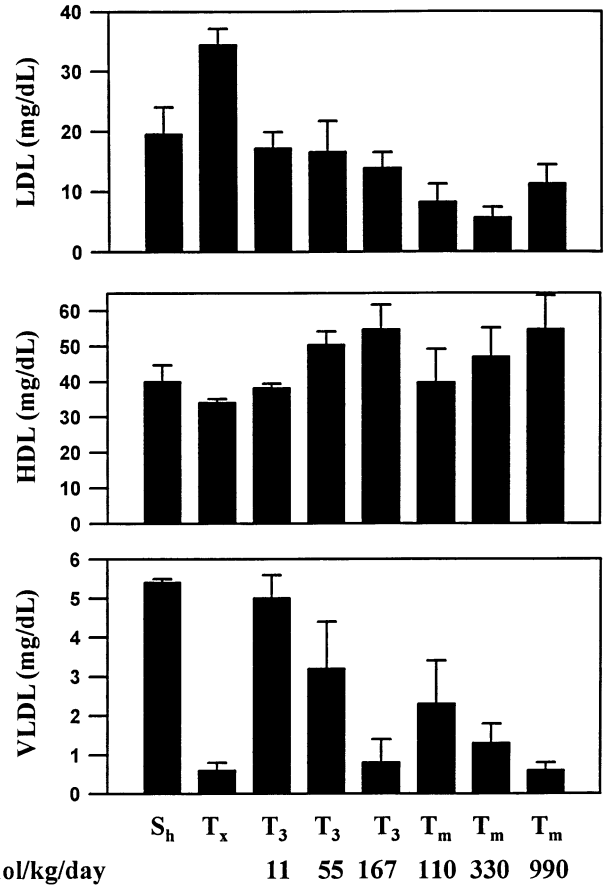

FIG. 2. Effects of T_3 and L-94901 on plasma LDL, HDL, and VLDL cholesterol levels in thyroidectomized rats. Values are means ± SD (N = 3). Shown are the LDL cholesterol concentration (top panel), HDL cholesterol concentration (center panel), and VLDL cholesterol concentration (bottom panel) in the plasma of sham-operated control rats (S_h) and thyroidectomized rats treated for 7 days with either vehicle (T_x), T_3 (T_3), or L-94901 (T_m) at the indicated doses.

tein concentrations relative to sham-operated control animals. This is similar to that previously noted in animals rendered hypothyroid either surgically or by treatment with propylthiouracil [16]. As shown in Fig. 1, total plasma cholesterol concentrations were essentially unaffected by thyroidectomy. However, plasma TG concentrations were reduced by 60%. Fractionation of serum lipoproteins into VLDL, LDL, and HDL subfractions by FPLC revealed that hypothyroidism following thyroidectomy resulted in a marked (77%) increase in LDL cholesterol levels (Fig. 2, top), with a concomitant 15% reduction in HDL cholesterol (Fig. 2, center) and 89% reduction in VLDL cholesterol (Fig. 2, bottom). Thus, the ratio of LDL cholesterol to HDL cholesterol increased following thyroidectomy from 0.49 in sham-operated control rats to 1.01 in thyroidectomized rats.

Alterations in Plasma Lipid and Lipoprotein Concentrations in Thyroidectomized Rats Treated with Thyroid Hormone

When thyroidectomized rats were treated with thyroid hormone (T_3) for 7 days at doses that ranged between 10 and 170 nmol/kg/day to reverse their hypothyroidism, total plasma cholesterol levels were essentially unaffected (Fig. 1). Fractionation of serum lipoproteins into LDL, HDL, and VLDL subfractions by FPLC revealed that low doses of thyroid hormone normalized levels of LDL cholesterol (Fig. 2, top). This treatment also eliminated reductions in HDL cholesterol (Fig. 2, center), VLDL cholesterol (Fig. 2, bottom) and plasma TG (Fig. 1, bottom) levels, and decreased the LDL cholesterol to HDL cholesterol ratio to 0.45.

Higher doses of T_3 (55 and 167 nmol/kg/day) further reduced LDL cholesterol levels to below the basal levels seen in sham-operated control rats, resulting in up to a 60% reduction in circulating LDL cholesterol levels relative to vehicle-treated control thyroidectomized animals (Fig. 2, top). This reduction was accompanied by a dose-dependent increase in circulating HDL cholesterol levels of up to 60% (Fig. 2, center). The ratio of LDL cholesterol to HDL decreased from 1.01 in vehicle-treated thyroidectomized rats to 0.32 and 0.25 in thyroidectomized rats treated with 55 and 167 nmol/kg/day of T_3, respectively, values below that noted in sham-operated control rats.

Plasma TG levels in thyroidectomized rats changed in a biphasic manner after T_3 administration. Low-dose T_3 (11 nmol/kg/day) normalized the hypotriglyceridemia induced by thyroidectomy (Fig. 1), whereas higher concentrations of T_3 suppressed circulating TG levels in a dose-dependent

TABLE 1. Alterations in plasma lipid and lipoprotein levels in euthyroid rats treated with T_3 or L-94901

Parameter	Vehicle (mg/dL)	T_3* (mg/dL)	T_3* % Control	L-94901† (mg/dL)	L-94901† % Control
Total cholesterol	62.0 ± 8.0	59 ± 8	95	72 ± 5	116
LDL cholesterol	19.5 ± 4.5	7.7 ± 3.4	39	10.5 ± 1.8	54
HDL cholesterol	40.0 ± 4.7	50 ± 5.6	125	61 ± 3.9	153
VLDL cholesterol	5.4 ± 0.1	2.5 ± 0.8	46	0.6 ± 0.1	11
Triglycerides	75.0 ± 7.0	47 ± 14	63	16 ± 3	21

Values are means ± SD (N = 3).
*Treated with 167 nmol/kg/day of T_3.
†Treated with 990 nmol/kg/day of L-94901.

manner by up to 64% relative to vehicle-treated thyroidectomized animals (Fig. 1). A similar pattern of VLDL cholesterol normalization by low-dose T_3 followed by subsequent dose-dependent reduction of VLDL cholesterol levels (up to 85%) was also noted following T_3 treatment (Fig. 2).

These observations indicate that low-dose T_3 returns animals to a near-euthyroid state and reverses the dyslipidemia induced by thyroidectomy, whereas higher doses of T_3 that render animals hyperthyroid have additional favorable effects on lipid and lipoprotein metabolism.

Alterations in Plasma Lipid and Lipoprotein Concentrations in Thyroidectomized Rats Treated with the Thyromimetic L-94901

When thyroidectomized rats were treated with the thyromimetic L-94901 for 7 days at doses ranging between 110 and 1000 nmol/kg/day, alterations in plasma lipid and lipoprotein levels similar to those noted following T_3 supplementation were observed. For example, all doses of L-94901 reduced LDL cholesterol in thyroidectomized rats to well below the basal levels seen in sham-operated control animals, resulting in up to an 84% reduction in circulating LDL cholesterol levels relative to vehicle-treated thyroidectomized rats (Fig. 2, top). This reduction in LDL cholesterol was similarly accompanied by an increase in HDL cholesterol of up to 60% (Fig. 2, center). The LDL to HDL ratio decreased from 1.01 in vehicle-treated thyroidectomized rats to 0.21 and 0.12 in thyroidectomized rats treated with 110 and 330 nmol/kg/day of L-94901, respectively. TG and VLDL cholesterol concentrations in thyroidectomized rats were reduced further following administration of higher doses of L-94901 such that maximal reduction in TG and VLDL cholesterol levels of 80 and 89%, respectively, were noted (Figs. 1 and 2).

These observations indicate that, like T_3, the thyrominetic L-94901 favorably modulates plasma lipid and lipoprotein concentrations such that LDL cholesterol is reduced, HDL cholesterol is increased, the ratio of LDL cholesterol to HDL cholesterol is reduced, and VLDL cholesterol and TGs are reduced.

Alterations in Plasma Lipid and Lipoprotein Concentrations in Euthyroid Rats Treated with T_3 or L-94901

The alterations in plasma lipid and lipoprotein concentrations in euthyroid rats treated with T_3 or L-94901 mirrored the effects noted in thyroidectomized rats. The absence of changes in total plasma cholesterol levels following 7 days of treatment of euthyroid rats with T_3 or L-94901 was a consequence of concomitant reductions in LDL and VLDL cholesterol and increases in HDL cholesterol (Table 1). TG levels were again reduced in a manner that paralleled reductions in VLDL cholesterol (Table 1). Ratios of LDL cholesterol to HDL cholesterol were likewise reduced from 0.48 in control animals to 0.15 following treatment with 167 nmol/kg/day of T_3 and to 0.17 after treatment with 990 nmol/kg/day of L-94901, again reflecting favorable alterations in lipoprotein composition.

These results indicate that the thyromimetic L-94901, like T_3, is capable of favorably affecting circulating lipid and lipoprotein concentrations in euthyroid as well as hypothyroid animals.

Increased LDLR and HMGR Expression in Thyroidectomized Rats Treated with T_3 or L-94901

Thyroid hormone has been shown previously to induce LDLR gene expression in hypophysectomized rats [14, 15]. It has been suggested that the T_3-induced reduction in non-HDL cholesterol that is observed in hypothyroid rats made hypercholesterolemic by diet [4] is a consequence of increased LDLR-mediated removal of circulating apoB-containing lipoproteins [2, 9–11, 16]. In view of the profound LDL cholesterol-lowering effects of T_3 and L-94901 in euthyroid and thyroidectomized rats noted above, we asked whether expression of the LDLR was induced in these animals.

When hepatic microsomal membranes from thyroidectomized rats treated with either vehicle, T_3, or L-94901 were subjected to immunoblotting analysis and probed simultaneously with antisera specific for LDLR and for HMGR, the expected bands at about 160 kDa (LDLR) and 100 kDa (HMGR) were observed. Both T_3 and L-94901 caused

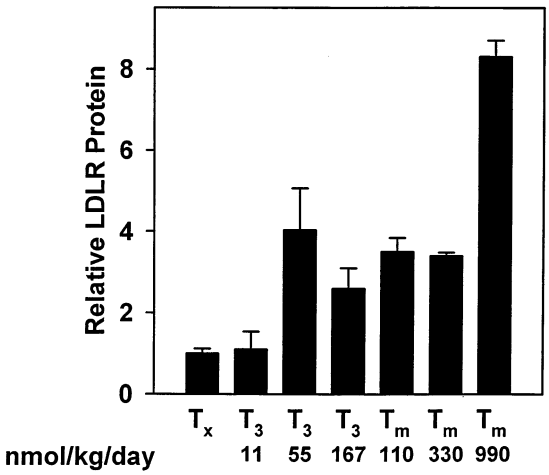

FIG. 3. Effects of T_3 and L-94901 dose on hepatic LDL receptor immunoreactive protein in thyroidectomized rats. Values are means ± SD (N = 3) relative to the vehicle-treated T_x control. The dose of T_3 or L-94901 (T_m) is given in nmol/kg/day.

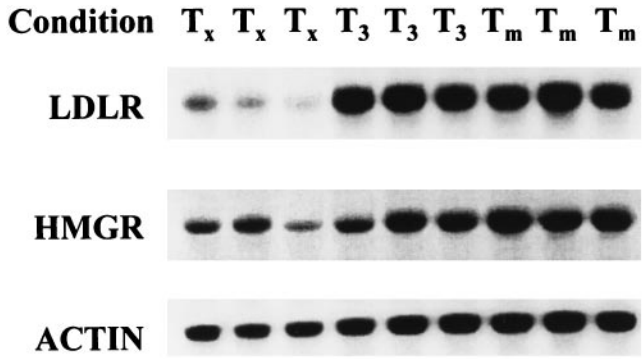

FIG. 5. Effects of T_3 and L-94901 on hepatic LDL receptor and HMG-CoA reductase mRNA levels in thyroidectomized rats. A representative Northern blot of poly A^+ RNA isolated from livers of thyroidectomized rats treated for 7 days with vehicle (T_x), 167 nmol/kg/day of T_3 (T_3), or 990 nmol/kg/day of L-94901 (T_m) is shown. The blot was sequentially probed for LDL receptor, HMG-CoA reductase, and β-actin.

striking increases in levels of hepatic LDLR immunoreactive protein (4- and 8-fold, respectively; Fig. 3), at the concentrations of T_3 and L-94901 evaluated. HMGR, which is induced by T_3 [23], was increased 2- to 3-fold. The increases in HMGR immunoreactive protein closely paralleled increases in HMGR activity induced by these agents (Fig. 4), indicating that the increases in HMGR activity were the result of increased enzyme amount rather than increased enzyme catalytic efficiency.

To determine whether the increases in hepatic LDLR protein were due to corresponding increases in mRNA levels, Northern blotting analysis was performed. Increases in hepatic LDLR protein concentration and in HMGR activity and enzyme concentration were closely paralleled by increases in LDLR and HMGR mRNA concentrations in the livers of thyroidectomized rats treated with T_3 or L-94901 (Fig. 5). The increase in LDLR mRNA induced by these agents, however, was considerably greater than the increase in HMGR mRNA levels (Fig. 5), consistent with the greater magnitude of increase in LDLR immunoreactive protein relative to HMGR immunoreactive protein and activity induced by these agents (Figs. 3 and 4).

These observations indicate that expression of the LDLR gene in chow-fed thyroidectomized rats is increased markedly by T_3 and the thyromimetic L-94901, and thus may be responsible for the reduction in circulating LDL cholesterol levels observed following treatment with these agents. Furthermore, the similarity between the magnitude of changes in LDLR mRNA levels and immunoreactive protein levels, and the magnitude of changes in HMGR mRNA, immunoreactive protein, and enzyme activity suggest that the effects of T_3 and L-94901 on the expression of these proteins is primarily, if not exclusively, exerted at the level of transcription.

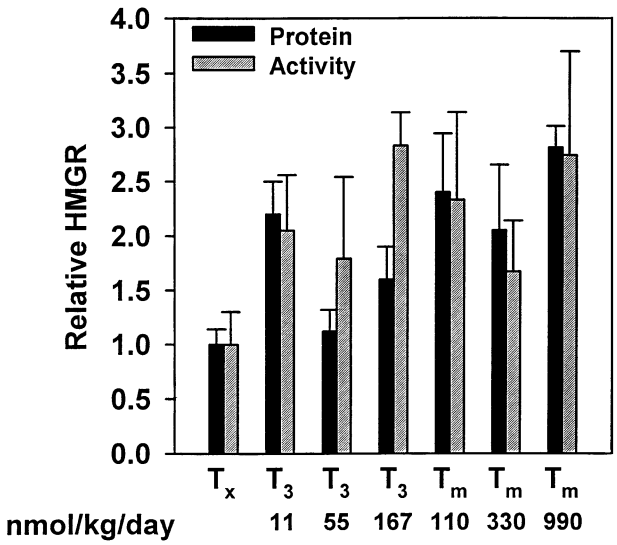

FIG. 4. Effects of T_3 and L-94901 on hepatic LDL receptor and HMG-CoA reductase immunoreactive protein and enzymatic activity in thyroidectomized rats. Values are means ± SD (N = 3) relative to the vehicle-treated thyroidectomized (T_x) control. The dose of T_3 or L-94901 (T_m) is given in nmol/kg/day.

Increased LDLR and HMGR Expression in Euthyroid Rats Treated with T_3 or L-94901

The effects of T_3 and L-94901 on the expression of the hepatic LDL receptor and HMGR were also examined in sham-operated rats. As shown in Fig. 6, treatment with T_3 and L-94901 caused marked increases in LDLR mRNA levels with only small increases in HMGR mRNA. Immunoblotting analysis revealed that LDLR protein concentrations were increased by 3.3- and 5.2-fold, respectively, in the livers of euthyroid rats treated with 167 nmol/mg/kg of T_3 or 990 nmol/kg/day of L-94901 for 7 days (Fig. 7). HMGR activity and immunoreactive protein levels were not affected significantly (Fig. 8). This is consistent with the RNA data. It also shows that LDLR is more responsive than HMGR as we have reported previously [21].

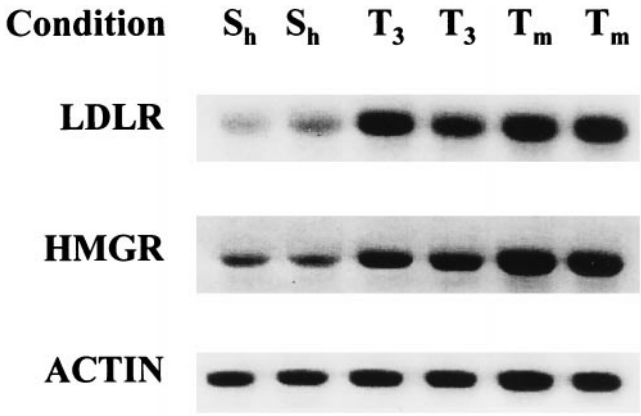

FIG. 6. Effects of T_3 and L-94901 on hepatic LDL receptor and HMG-CoA reductase mRNA in sham-operated rats. A representative Northern blot of poly A^+ RNA isolated from livers of sham-operated rats treated for 7 days with vehicle (S_h), 167 nmol/kg/day of T_3 (T_3), or 990 nmol/kg/day of L-94901 (T_m) is shown. The blot was sequentially probed for LDL receptor, HMG-CoA reductase, and β-actin.

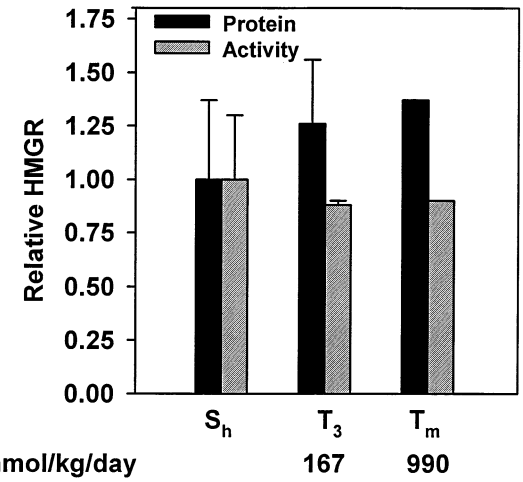

FIG. 8. Effects of T_3 and L-94901 on hepatic HMG-CoA reductase immunoreactive protein and enzyme activity in sham-operated rats. Values are means ± SD (N = 3) except for the L-94901 animal and are relative to the vehicle-treated sham-operated (S_h) control. The dose of T_3 or L-94901 (T_m) is given in nmol/kg/day.

Increased apo A-I mRNA Levels in Livers from Thyroidectomized and Sham-Operated Rats Treated with T_3 or L-94901

Apo A-I is the major apolipoprotein found in HDL. Because both T_3 and L-94901 caused increases in HDL, it was of interest to determine whether these agents might increase the expression of the hepatic apo A-I gene. When Northern blotting analysis of hepatic poly A^+ RNA isolated from thyroidectomized rats treated with either T_3 or L-94901 was performed, a 2- to 3-fold increase in the level of mature 1 kb apo A-I mRNA was observed (Fig. 9). This T_3-mediated increase in mature apo A-I mRNA levels in thyroidectomized rats agrees with previous observations made in T_3-treated hypophysectomized rats [21]. Administration of T_3 or L-94901 to sham-operated animals also increased the level of the mature form of apo A-I mRNA (Fig. 9). In this case, the increase caused by L-94901 was greater than that caused by T_3. Thus, increased expression of apo A-I may be responsible, in part, for the T_3- and L-94901-mediated increases in serum HDL levels.

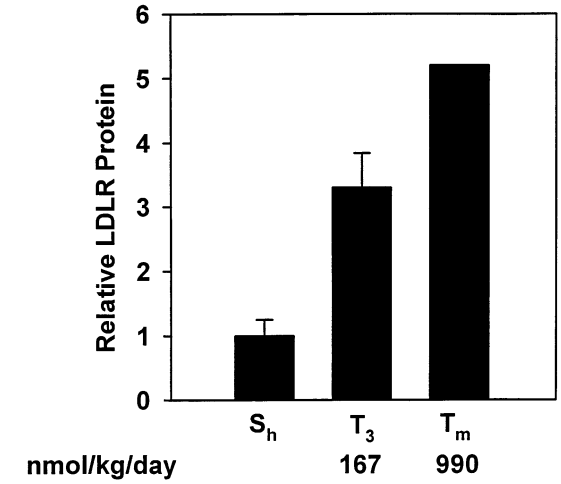

FIG. 7. Effects of T_3 and L-94901 on hepatic LDL receptor immunoreactive protein levels in sham-operated rats. Values are means ± SD, (N = 3), except for (T_m) relative to the vehicle-treated sham-operated (S_h) control. The dose of T_3 or L-94901 (T_m) is given in nmol/kg/day.

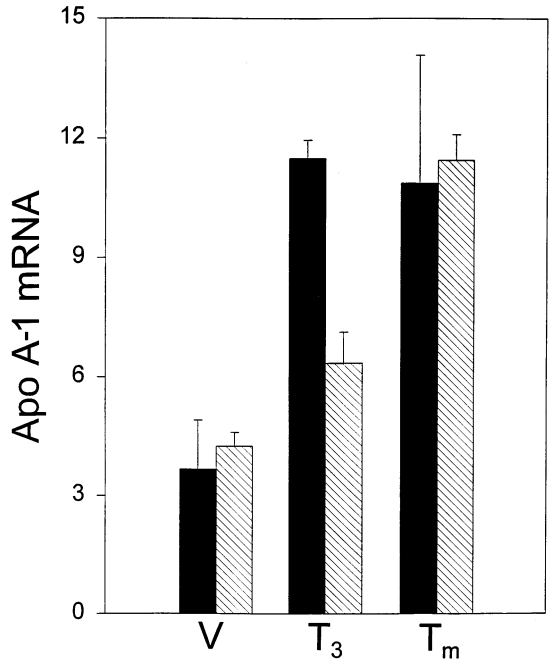

FIG. 9. Effects of T_3 and L-94901 on hepatic apo A-I mRNA levels in thyroidectomized and sham-operated rats. Values are means ± SD for 3 animals in each group. T_3 was given at a dose of 167 nmol/kg/day, while L-94901 (T_m) was given at a dose of 990 nmol/kg/day. Control animals received only vehicle (V). Apo A-I mRNA levels in arbitrary densitometric units for thyroidectomized (dark bars) and sham-operated controls (hatched bars) are presented.

DISCUSSION

The results of this study demonstrate that both the thyroid hormone and the thyromimetic L-94901 induce the hepatic LDL receptor, leading to significant decreases in LDL cholesterol levels. In addition, they also caused substantial increases in HDL levels, likely due to induction of apo A-I. Thus, the ratio of LDL to HDL in thyroidectomized rats was reduced from 1.01 to 0.25 in response to T_3 and to 0.12 upon treatment with L-94901. Unlike the bile acid binding resins and the statins, T_3 and L-94901 significantly increased HDL levels by as much as 60%. These agents were effective in both thyroidectomized and euthyroid animals, which suggests that similar to thyroid hormone [2, 8], thyromimetics may be capable of functioning as hypolipidemic agents in hypothyroid as well as euthyroid patients fed fat and cholesterol-restricted diets. The observation that T_3 and L-94901 produce similar effects on LDL cholesterol levels in hypercholesterolemic rats [4] and normocholesterolemic animals (this study) suggests that thyromimetic therapy should be efficacious in both hyperlipidemic and normocholesterolemic subjects.

TG levels displayed a biphasic response to T_3 and L-94901. Low doses increased TG, while higher doses decreased TG levels in both thyroidectomized and sham-operated controls. The marked induction of hepatic LDLR cannot explain this response. Perhaps it results from modulation of other factors involved in controlling lipid metabolism that have been shown previously to be affected by thyroid hormone, such as lecithin cholesterol acyltransferase [2, 9], hepatic lipase [2, 10], and lysosomal acid cholesterol esterase [11]. Recent studies with the thyromimetic CGS-26214 also have demonstrated effects on postprandial lipid clearance [6], further suggesting a multifaceted modulation of TG metabolism by thyroid hormone.

The increases in hepatic LDL receptor expression promoted by T_3 and L-94901 likely occur at the level of transcription mediated by thyroid response elements in the LDLR promoter. The following observations suggest such a direct role. First, it has been demonstrated that T_3 causes rapid (within ~30 min) increases in LDLR mRNA [14] associated with apparent increases in transcription based on nuclear run-on experiments [15]. Second, a possible TRE is located just upstream from the TATA sequence in the LDL promoter [24]. Finally, a primary effect of T_3 on LDLR expression is suggested by experiments demonstrating increased LDLR activity in cultured hepatocytes [16] and Hep-G2 cells* following treatment with T_3 [16] or the thyromimetic CGS-23425.*

In contrast with the increases in LDLR gene transcription induced by T_3 and L-94901, the increases in apo A-I mRNA levels may not be due solely to increased transcription. It has been reported that within 3.5 hr after T_3 treatment, transcription of apo A-I is increased nearly two-fold [25]. However, chronic treatment with thyroid hormone decreases transcription about 50% [25]. Several studies have concluded that mRNA maturation is predominately responsible for the increase in apo A-I mRNA levels [25–28]. This is consistent with the length of time (72 hr) required to obtain maximal induction of mature apo A-I mRNA [21].

In contrast with most other hypocholesterolemic agents, the thyromimetic L-94901 caused a large induction of the hepatic LDL receptor. The various statins [lovastatin, pravastatin, fluvastatin, and rivastatin (cerivastatin)] do not increase hepatic LDL receptor immunoreactive protein levels when given to rats [29]. L-94901 also caused a modest increase in hepatic HMG-CoA reductase activity and protein (Fig. 4) and a significant increase in apo A-I gene expression (Fig. 8). Thus, thyromimetics appear to lower LDL and raise HDL levels by mechanisms that differ fundamentally from those of the statins.

Effectively lowering serum LDL cholesterol levels without inhibiting HMGR activity, as accomplished by L-94901, may be advantageous. Animals and humans that express high levels of HMGR have a greater capacity to respond to dietary cholesterol without an increase in their serum cholesterol levels [30, 31]. It has been reported that rats have 50-fold higher levels of hepatic HMGR than hamsters and are resistant to dietary cholesterol, whereas hamsters are not [31]. Also, familial hypercholesterolemic heterozygotes that express higher levels of HMGR, as reflected in plasma mevalonate levels, are more responsive to cholesterol-lowering drugs [30].

The present observations suggest that a hepatoselective thyromimetic devoid of adverse cardiovascular side-effects could be important therapeutically, not only in hypothyroid individuals but also in subjects with normal thyroid function and either normal or elevated LDL cholesterol levels with or without concomitant low HDL cholesterol or elevated TG levels. In this regard, it is of interest to note that in addition to L-94901 [1, 4], the thyromimetics CGS-26214 [6, 32] and CGS-23425* are also markedly hepatoselective and have been shown to cause reductions in LDL cholesterol and increases in HDL cholesterol in a variety of experimental animal models [6, 18, 32].

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References

1. Leeson PD, Emmett JC, Shah VP, Showell GA, Novelli R, Prain HD, Benson MG, Ellis D, Pearce NJ and Underwood AH, Selective thyromimetics. Cardiac-sparing thyroid hormone analogues containing 3′-arylmethyl substituents. *J Med Chem* **32:** 320–336, 1989.
2. Hansson P, Valdemarsson S and Nilsson-Ehle P, Experimental hyperthyroidism in man: Effects on plasma lipoproteins,

* Taylor AH, Steele RE, Stephan ZF and Wong NCW, Effect of a novel thyromimetic, CGS-23425 on lipoprotein metabolism. *10th International Congress of Endocrinology*. Book of Abstracts, Vol. I, p 665, 1996.

lipoprotein lipase and hepatic lipase. *Horm Metab Res* **15:** 449–452, 1983.
3. Ness GC, Thyroid hormone: Basis for its hypocholesterolemic effect. *J Fla Med Assoc* **78:** 383–385, 1991.
4. Underwood AH, Emmett JC, Ellis D, Flynn S, Leeson B, Benson GM, Novelli R, Pearce NJ and Shaw VP, A thyromimetic that decreases plasma cholesterol levels without increasing cardiac activity. *Nature* **324:** 425–429, 1986.
5. Stephan ZF, Yurachek EC, Sharif R, Wasvary JM, Steele RE and Howes C, Reduction of cardiovascular and thyroxine-suppressing activities of L-T_3 by liver targeting with cholic acid. *Biochem Pharmacol* **43:** 1969–1974, 1992.
6. Steele RE, Wasvary JM, Dardik BN, Schwartzkopf CD, Sharif R, Leonards KS, Hu CW, Yurachek EC and Stephan ZF, CGS-26214, the thyroxine connection revisited. In: *Atherosclerosis* X (Eds. Woodford FP, Davignon J and Sniderman A), pp. 321–324. Elsevier, New York, 1995.
7. Engelken SF and Eaton RP, The effects of altered thyroid status on lipid metabolism in the genetic hyperlipemic Zucker rat. *Atherosclerosis* **38:** 177–188, 1981.
8. Aviram M, Luboshitzky R and Brook JG, Lipid and lipoprotein patterns in thyroid dysfunction and the effect of therapy. *Clin Biochem* **15:** 62–66, 1982.
9. Ridgeway ND and Dolphin PJ, Serum activity and hepatic secretion of LCAT in experimental hypothyroidism and hypercholesterolemia. *J Lipid Res* **26:** 1300–1313, 1985.
10. Hulsmann WC, Oerlemans MC and Geelhoed-Mieras MM, Effect of hypothyroidism, diabetes and polyunsaturated fatty acids on heparin-releasable rat liver lipase. *Biochem Biophys Res Commun* **79:** 784–788, 1977.
11. Severson DL and Fletcher T, Effect of thyroid hormones on acid cholesterol ester hydrolase activity in rat liver, heart and epididymal fat pads. *Biochim Biophys Acta* **675:** 256–264, 1981.
12. Bilheimer DW, Goldstein JL, Grundy SC, Starzl TE and Brown MS, Liver transplantation to provide LDL receptors and lower plasma cholesterol in a child with homozygous familial hypercholesterolemia. *N Engl J Med* **311:** 1658–1664, 1984.
13. Harwood HJ Jr, Chandler CE, Pellarin LD, Bangerter FW, Wilkins RW, Long CA, Cosgrove PG, Malinow MR, Marzetta CA, Pettini JL, Savoy YE and Mayne JT, Pharmacologic consequences of cholesterol absorption inhibition: Alteration in cholesterol metabolism and reduction in plasma cholesterol concentration induced by the synthetic saponin β-tigogenin cellobioside (CP-88818; tiqueside). *J Lipid Res* **34:** 377–395, 1993.
14. Ness GC and Zhao Z, Thyroid hormone rapidly induces hepatic LDL receptor mRNA levels in hypophysectomized rats. *Arch Biochem Biophys* **315:** 199–202, 1994.
15. Ness GC and Lopez D, Transcriptional regulation of rat hepatic LDL receptor and cholesterol 7α-hydroxylase. *Arch Biochem Biophys* **323:** 404–408, 1995.
16. Salter AM, Hayashi R, Al-Seeni M, Brown NF, Bruce J, Sorensen O, Atkinson EA, Middleton B, Bleackley RC and Brindley DN, Effects of hypothyroidism and high fat feeding on mRNA concentrations for the LDL receptor and on ACAT activity in rat liver. *Biochem J* **276:** 825–832, 1991.
17. Polikar R, Burger AG, Scherrer U and Nicod P, The thyroid and the heart. *Circulation* **87:** 1435–1441, 1993.
18. Lee Y-P and Lardy HA, Influence of thyroid hormones on L-α-glycerophosphate dehydrogenases and other dehydrogenases in various organs of the rat. *J Biol Chem* **240:** 1427–1436, 1965.
19. Ness GC, Sample CE, Smith M and Eichler DC, Characteristics of rat liver microsomal HMG-CoA reductase. *Biochem J* **233:** 167–172, 1986.
20. Chambers CM and Ness GC, Translational regulation of hepatic HMG-CoA reductase by dietary cholesterol. *Biochem Biophys Res Commun*. **232:** 278–281, 1997.
21. Ness GC, Pendleton LC, Li YC and Chiang JYL, Effect of thyroid hormone on hepatic cholesterol 7α hydroxylase, LDL receptor, HMG-CoA reductase, farnesyl pyrophosphate synthetase and apolipoprotein A-I mRNA levels in hypophysectomized rats. *Biochem Biophys Res Commun* **172:** 1150–1156, 1990.
22. Ness GC, Zhao Z and Keller RK, Effect of squalene synthase inhibition on the expression of hepatic cholesterol biosynthetic enzymes, LDL receptors and cholesterol 7α-hydroxylase. *Arch Biochem Biophys* **311:** 277–285, 1994.
23. Sample CE, Pendleton LC and Ness GC, Regulation of HMG-CoA reductase mRNA levels by triiodothyronine. *Biochemistry* **26:** 727–731, 1987.
24. Sudhof TC, Goldstein JL, Brown MS and Russell DW, The LDL receptor gene: A mosaic of exons shared with different proteins. *Science* **228:** 815–822, 1985.
25. Strobl W, Gorder NL, Lin-Lee Y-C, Gotto AM Jr and Patsch W, Role of thyroid hormones in apolipoprotein A-I gene expression in rat liver. *J Clin Invest* **85:** 659–667, 1990.
26. Strobl W, Chan L and Patsch W, Differential regulation of hepatic apolipoprotein A-I and A-II gene expression by thyroid hormone in rat liver. *Atherosclerosis* **97:** 161–170, 1992.
27. Soyal SM, Seelos C, Lin-Lee YC, Sanders S, Gotto AM Jr, Hachey DL and Patsch W, Thyroid hormone influences the maturation of apolipoprotein A-I messenger RNA in rat liver. *J Biol Chem* **270:** 3996–4004, 1995.
28. Lin-Lee Y-C, Soyal SM, Surguchov A, Sanders S, Strobl W and Patsch W, Thyroid hormone influences conditional transcript elongation of the apolipoprotein A-I gene in rat liver. *J Lipid Res* **36:** 1586–1594, 1995.
29. Ness GC, Zhao Z and Lopez D, Inhibitors of cholesterol biosynthesis increase hepatic low-density lipoprotein receptor protein degradation. *Arch Biochem Biophys* **325:** 242–248, 1996.
30. Naoumova RP, Marais AD, Mountney J, Firth JC, Rendell NB, Taylor GW and Thompson GR, Plasma mevalonic acid, an index of cholesterol synthesis *in vivo*, and responsiveness to HMG-CoA reductase inhibitors in familial hypercholesterolemia. *Atherosclerosis* **119:** 203–213, 1996.
31. Spady DK, Stange EF, Bilhartz LE and Dietschy JM, Bile acids regulate hepatic low density lipoprotein receptor activity in the hamster by altering cholesterol flux across the liver. *Proc Natl Acad Sci USA* **83:** 1916–1920, 1986.
32. Stephan ZF, Yurachek EC, Sharif R, Wasvary JM, Leonards KS, Hu CW, Hintze TH and Steele RE, Demonstration of a potent lipid-lowering activity of a thyromimetic agent devoid of cardiovascular and thermogenic effects. *Atherosclerosis* **126:** 53–63, 1996.