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Beneficial Effects of a Novel Thyromimetic on Lipoprotein Metabolism

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SUMMARY

Although L-triiodothyronine (L-T_3) lowers cholesterol, this hormone is not used to treat hypercholesterolemia because of its cardiotoxic effects. Thyromimetics, such as the novel compound CGS 23425, that mimic the beneficial but lack the detrimental effects of T_3, may be useful in the treatment of hypercholesterolemia. To show that CGS 23425 has no cardiotoxicity, atrial contractility and force were both measured and found to be unchanged in rats treated with up to 10 mg/kg drug. The lipid lowering actions of this drug resulted in a 44% decrease in low-density lipoprotein (LDL) cholesterol in hypercholesterolemic rats treated with 10 μg/kg of the compound. Normal rats required a higher dose of 1000 μg/kg to elicit a similar 50% reduction in LDL cholesterol. Both CGS 23425 or T_3 (10 nM) increased the specific binding of ^{125}I-labeled LDL to Hep G_2 cells and increased LDL receptor number by 44 and 49%, respectively. These data indicate that CGS 23425 enhances hepatic clearance of serum LDL cholesterol. Normal and fat-fed animals treated with the drug showed a dose-dependent increase in apolipoprotein AI, a protein that promotes the efflux of cholesterol from peripheral tissues. Transient transfection of a rat apolipoprotein AI promoter-chloramphenicol acetyltransferase construct, in human hepatoma cells, showed a dose-dependent increase in chloramphenicol acetyltransferase activity with EC_{50} values of 2×10^{-12} M and 10^{-10} M for thyroid hormone receptors β1 and α1, respectively, with maximal responses at 10^{-7} M. These data indicate that CGS 23425 is a thyromimetic that increases apolipoprotein AI expression via thyroid hormone receptor. In summary, CGS 23425 ameliorates hypercholesterolemia by increasing apolipoprotein A1 and the clearance of LDL cholesterol. Therefore, a compound like CGS 23425 may be useful for the prevention and reversal of atherosclerosis.

The administration of thyroid hormones or related analogs lowers plasma cholesterol in hypothyroid patients (1, 2). This beneficial effect of the thyroid hormones (L-T_3 and L-T_4) and thyromimetics arises from their actions on nuclear receptors for L-T_3. The liganded receptors regulate the expression of several hepatic genes involved in cholesterol metabolism. For example, L-T_3 increases the expression of LDL receptors and several lipolytic enzymes (2–6). Unfortunately, the natural thyroid hormones cannot be used therapeutically to treat hypercholesterolemia, in euthyroid individuals, because of their undesirable effects on the heart (7). However, synthetic thyromimetics designed specifically to eliminate or reduce the cardiac side effects and target one of the major sites of cholesterol metabolism, the liver, are expected to have a therapeutic role. Selective actions of thyromimetics on the liver may in theory arise from differences in (i) cytoplasmic binding, (ii) active transport at the plasma membrane, (iii) the activities of a putative stereospecific cytoplasm-to-nucleus transport system and/or (iv) differential binding to the two major isoforms of nuclear T_3 receptor in hepatocytes compared with other cell types (8–10). Hepatic selective thyromimetics are therefore designed to exploit one or more of these parameters.

In this report, we describe a novel thyromimetic that lowers plasma cholesterol by 60% at doses that have no effect on the heart. The reduction in plasma cholesterol is mediated by an increase in hepatic LDL receptor activity. In addition, this thyromimetic increases plasma apoAI concentrations, the major apoprotein constituent of HDL particles that have antiatherogenic properties. We anticipate that a thyromimetic such as CGS 23425 will eventually be useful in the medical treatment of hypercholesterolemia.

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Materials and Methods

Animals. Male Sprague-Dawley rats, weighing between 150 and 200 g, were maintained on a normal rat chow diet (chow-fed) or a

ABBREVIATIONS: T_3, triiodothyronine; T_4, thyroxine; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CAT, chloramphenicol acetyltransferase; apoAI, apolipoprotein AI; TR, thyroid hormone receptor; TRIAC, triiodothyroacetic acid.

chow diet supplemented with 1.5% cholesterol and 0.5% cholic acid (fat-fed) for 14 days before experiments. Groups of six chow- or fat-fed rats were treated orally by gavage with a solution of CGS 23425 or vehicle (water) orally in the doses indicated for 7 consecutive days. After the last dose, the animals were fasted for 18 hr before being killed, and blood was collected for studies outlined below.

Preparation of hepatic plasma membranes and nuclei. Livers from euthyroid male Sprague-Dawley rats were dissected free of adhering membranes and large blood vessels. Nuclei from homogenized-filtered livers were isolated by differential centrifugation (11) and the supernatant-containing plasma membranes collected. The nuclei-containing pellet was resuspended in buffer A [20 mM Tris·HCl, 0.25 M sucrose, 1 mM $MgCl_2O{\cdot}6H_2O$, 2 mM EDTA, 0.1 mM NaCl, 5% glycerol, pH 7.2] and recentrifuged. The nuclei were dissolved in 180 μl of buffer A (12) and stored at −40°.

Competitive binding assay of [^{125}I]T_3 to hepatic nuclei and plasma membranes. Nuclear binding was performed according to Stephan *et al.* (13). Briefly, 300 μg of protein were incubated with 0.3 nM [^{125}I]T_3 (1080 mCi/mg; DuPont-New England Nuclear, Boston, MA) for 50 min at 22° in a final volume of 1 ml of buffer A. Parallel incubations contained increasing concentrations of L-T_3 (Sigma, St. Louis, MO), CGS 23425 (Novartis, Summit, NJ), CGS 26214 (Novartis), D-T_4 (Sigma), or SKF-94901 (SmithKline French). Nonspecific binding was determined in the presence of 3 μM unlabeled L-T_3.

Plasma membrane binding was determined using Pliam and Goldfine's (14) method. Briefly, 90 mg of membrane protein were incubated with 0.2 nM [^{125}I]T_3 for 30 min at room temperature. Parallel incubations contained increasing concentrations of L-T_3 or the indicated thyromimetics. Nonspecific binding was determined in the presence of 6 μM unlabeled L-T_3. In both studies, bound and free radioactivities were separated by centrifugation, and radioactivity in the pellet was measured by γ-counting. The concentration of the test compounds corresponding to IC_{50} of specific binding of [^{125}I]T_3 was determined from the reciprocal plots of specific binding versus concentration of test compounds.

Serum lipoprotein determinations. Chow- or fat-fed euthyroid male Sprague-Dawley rats were treated with increasing concentrations of CGS 23425 for 7 days. Animals were fasted for 18 hr after the last dosing, and blood was collected by cardiac puncture under CO_2 anesthesia into EDTA (5%). Plasma was prepared by centrifugation, and samples were analyzed enzymatically for total, HDL cholesterol, and LDL cholesterol on a Bio-Mek automated workstation (Beckman Instruments, Palo Alto, CA) using Sigma diagnostic reagent kits. Plasma apoAI concentrations were measured using Western blot analysis and electrochemiluminescence detection as described previously (15).

Effect of CGS 23425 on the heart. Animals on a normal diet were treated with up to 40 mg/kg CGS 23425 for 7 days. The animals were weighed, and the hearts were dissected free from euthanized animals. The weight of the hearts were rapidly measured and placed in oxygenated Kreb's buffer at 28°. The right atria were isolated by dissection, hung with minimum applied tension, and allowed to equilibrate for 30 min. The spontaneous atrial rate was then determined. The left atria were isolated and used to measure maximum stimulated contractile atrial force, by creation of tension-force curves. Briefly, atria were attached to platinum electrodes in Kreb's buffer, and a resting tension of 2 g was applied. Cardiac muscle was then stimulated at 10 beats/min with a 2.5-msec duration and then allowed to equilibrate for 30 min (initial force). Tension-force curves using isometric contractile forces of 10–60 beats/min were constructed, and the maximum contractile force was measured (16).

Determination of rat apo AI promoter activity. Human fetal hepatoma cells (HuH-7 cells) were maintained in RPMI-ISE medium as previously described (17). Cells were transfected via the calcium phosphate co-precipitation method, with 2.5 μg of pAI.474.CAT (17) together with 5 μg of either pECE-hTRα or pECE-hTRβ and 2.5 μg of the bacterial plasmid pRSV-β-galactosidase, to monitor DNA uptake (17). After 24 hr of incubation, the medium was exchanged for one containing different concentrations of CGS 23425 or L-T_3. After a further 24 hr of incubation, transfected cells were harvested, and cellular protein was assayed for β-galactosidase and CAT activity as described elsewhere (17). EC_{50} values were determined directly from the graphs.

Results and Discussion

Comparison of CGS 23425 with other thyromimetics. To compare the thyromimetic properties of various compounds, we tested their ability to compete with the binding of [^{125}I]T_3 *in vitro* to rat liver nuclei and plasma membranes (Table 1). Competition with [^{125}I]T_3 in binding to nuclei demonstrates the thyromimetic property of the compound under investigation, and competition with [^{125}I]T_3 in binding to rat liver plasma membranes indicates its stereospecificity (14). The latter feature is of interest because several cardiac effects of L-T_3 are correlated with its ability to interact directly with the plasma membrane (18). To design a thyromimetic with low toxicity we sought compounds devoid of hepatic plasma membrane binding.

The focus of our studies was a thyromimetic, CGS 23425. This compound has a chemical structure similar to a number of known and well characterized thyromimetics (Table 1). Nuclear binding studies showed that CGS 23425 bound with high affinity to hepatic nuclei (Table 1) and competed for hepatic nuclear L-T_3-binding sites about six times better than L-T_3 (Table 1). A previously described and related thyromimetic, CGS 26214 (13), competed 12 times better than L-T_3 for the binding of [^{125}I]T_3 to hepatic nuclei (Table 1). In contrast, two additional thyromimetics, D-T_4 and SKF 94901 that have hypocholesterolemic activities, were 20 and 40 times, respectively, less effective than L-T_3 in competing with the radiolabeled hormone for its nuclear binding site (Table 1).

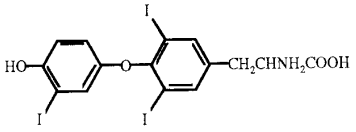
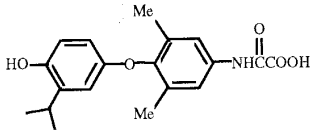
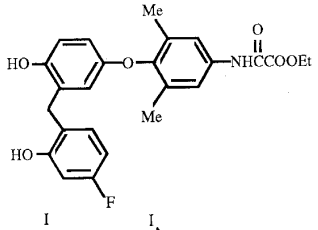
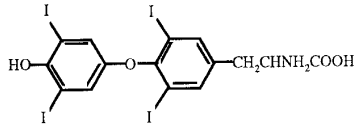
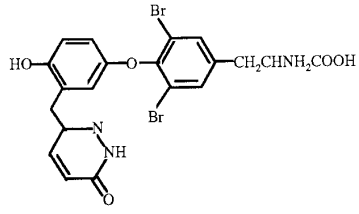
Next, we compared the relative abilities of the various compounds to compete with [^{125}I]T_3 in binding to hepatic plasma membranes. Results showed that CGS 23425 had a comparatively low affinity for the membrane compared with L-T_3; its IC_{50} was >5.0 μM, the value that is the accepted cut-off point for negligible binding (19). Similar results were obtained with CGS 26214 (Table 1). However, agents with undesirable cardiac side effects (L-T_3, D-T_4, and SKF 94901) showed considerable binding to the rat liver plasma membranes. These data suggested that CGS 23425 might act on the liver and at the same time have minimal effects on the heart.

Effect of CGS 23425 on the heart. To determine the effects of CGS 23425 on the heart, the compound was administered to normal euthyroid male rats in doses up to 40 mg/kg/day. There was no difference in the body weight and *in vivo* heart rate of control animals [(mean ± standard error) 295 ± 10 g, 404 ± 19 beats/min] compared with those treated (301 ± 14 g, 410 ± 14 beats/min) with 40 mg/kg/day. Myocardial activity, measured as heart rate of isolated atria, was reduced by 21% to 150 beats/min but only at the highest dose tested (Table 2). Additionally, there was a concomitant increase in the measured atrial force of 17%. Enhanced myocardial contractile force is accompanied by cardiac hypertrophy (20), and therefore, the observed 19% increase in total heart weight was not unexpected. At lower doses of 2.5, 10, and 20 mg/kg/day, CGS 23425 had no effect on these parameters. Together these data indicate that the compound does not appear to have cardiotoxic effects at low doses, but at the highest dose of 40 mg/kg/day, the undesirable effects on the heart begin to appear. These data are similar to those for

TABLE 1

Comparison of the receptor binding properties of various thyromimetics *in vitro*

The chemical structure of L-T_3, and several thyroid hormone analogs CGS 23425, CGS 26214, D-T_4, and L-SKF94901 are shown. Hepatic nuclei and plasma membranes were incubated with ^{125}I-T_3 for either 50 min at 22°C or 30 min at room temperature, respectively, with increasing concentrations of L-T_3 or the indicated thyromimetics. Nonspecific binding was determined in the presence of 3 or 6 μM unlabeled L-T_3, respectively. Bound radioactivity was measured by γ-counting. The IC_{50} of specific binding of ^{125}I-T_3 was determined from the reciprocal plots of specific binding versus concentration of test compounds. Values for the IC_{50} represent the mean ± standard deviation of a minimum of two determinations, using 10 concentrations of competing ligand per determination.

Molecular structure and nomenclature		Nuclear IC_{50}	Membrane IC_{50}
		nM	*μM*
	L-T3	1.2 ± 0.4	0.1 ± 0.03[a]
	CGS 23425	0.2 ± 0.01	>5.0[a]
	CGS 26214	0.1 ± 0.2	>5.0[b]
	D-T4	20.5 ± 9.2	2.5 ± 0.71[a]
	SKF 94901	48.0 ± 9.0	1.0 ± 0.02[c]

[a] Present study.
[b] Yokoyama *et al.* (19).
[c] Underwood *et al.* (24).

TABLE 2

Effect of CGS 23425 on the heart

Heart weight, atrial contractility, and atrial force were used as measures of cardiac performance as described previously (23). Animals on a normal diet were treated with the indicated doses of CGS 23425 for 7 days. Heart weight was measured, and left and right atria were dissected free and used for atrial force and rate, as described in Materials & Methods. Data area means ± standard deviation for six to eight animals per treatment.

Dose	Heart weight	Atrial contractility	Atrial force
mg/kg	*% of body wt*	*beats/min*	*% of initial*
0	0.37 ± 0.01	191 ± 6	87 ± 2
2.5	0.35 ± 0.02	198 ± 10	81 ± 4
10	0.40 ± 0.01	210 ± 14	84 ± 8
40	0.44 ± 0.02[a]	150 ± 5[a]	102 ± 4[a]

[a] $p < 0.05$ by analysis of variance

CGS 26214 and SKF 94901. Neither of these thyromimetics has a cardiotoxic effect at doses up to 25 mg/kg and 1.8×10^{-5} mol/kg/dl (~100 mg/kg), respectively (21, 22). Doses of L-T_3 as low as 10 μg/kg, by comparison, showed significant cardiotoxicity in similarly treated animals (13).

Hypocholesterolemic effects of CGS 23425. Although the preceding results show that low doses of CGS 23425 had no significant effect on the heart, whether this compound lowered cholesterol levels remained unknown. To demonstrate clearly a beneficial hypocholesterolemic effect of CGS 23425, male rats fed a diet supplemented with cholic acid and cholesterol were used, because such animals show elevated levels of serum cholesterol (23). Results showed that the drug was effective at a minimum dose of 10 μg/kg/day and reduced total cholesterol levels by 60% of the untreated controls (118 ± 15 mg/dl compared with 190 ± 21 mg/dl; Fig. 1, *bottom*). Doses that exceeded 10 μg/kg/day showed no further reduction in cholesterol levels. Although male rats fed a standard diet and treated in the same manner showed a 10% decrease in serum cholesterol, they required a higher dose (1000 μg/kg/day) of CGS 23425 (Fig. 1), but this was not significantly different ($p = 0.06$) from chow-fed rats. This lack of effect of thyroid hormones and thyromimetics on serum cholesterol in rats fed a standard diet has been thoroughly documented (24).

In both sets of animals, the minimal effective dose of CGS 23425 was 4000 times and 40 times lower, respectively, than the cardiotoxic dose of 40 mg/kg/day (Table 2). These differ-

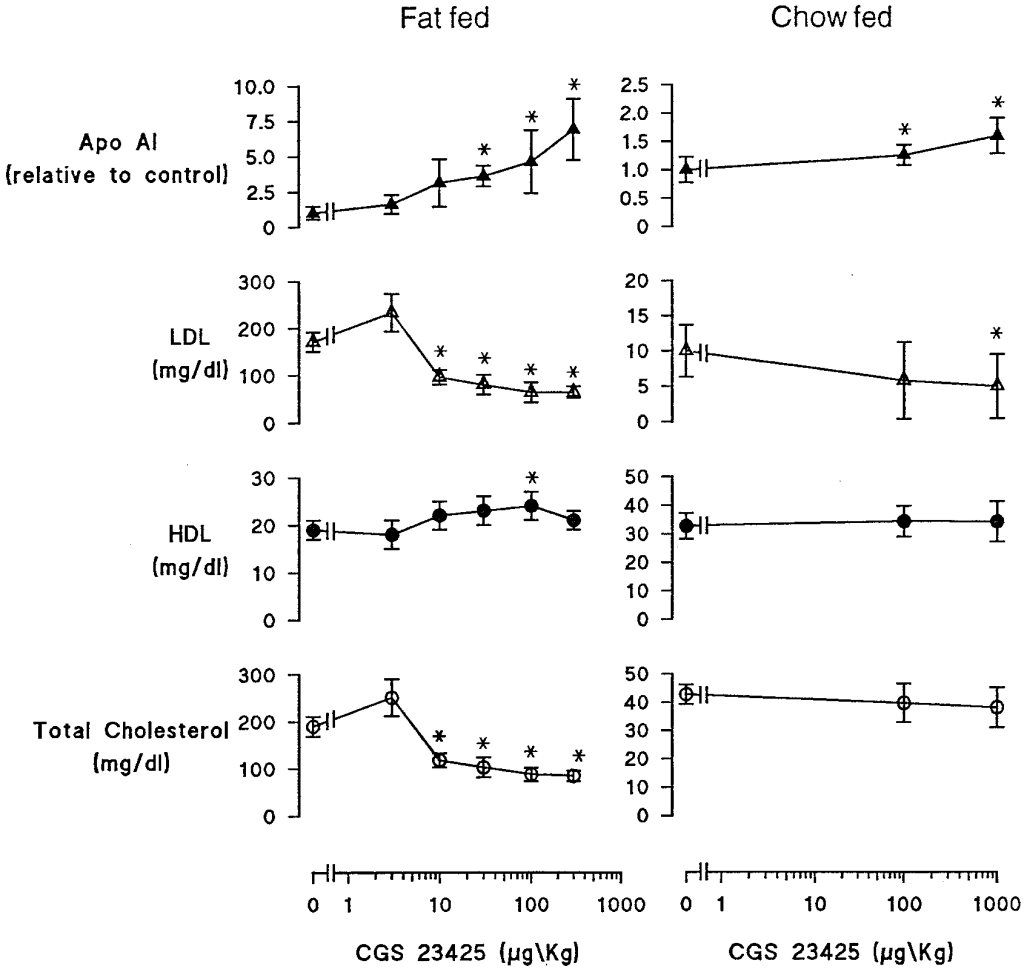


Fig. 1. Effect of CGS 234245 on serum lipoproteins. Serum apoAI, LDL cholesterol, HDL cholesterol, and total cholesterol levels in fat-fed rats (*left*) and normal fed rats (*right*) are shown. Plasma was analyzed enzymatically for total, HDL cholesterol, LDL cholesterol, and apoAI as described in Materials and Methods. Symbols represent the mean ± standard deviation for six to eight animals per datum point. *Error bars,* not shown when encompassed by the symbol (*, $p < 0.05$; Student's paired *t* test).

ences between efficacy and toxicity for CGS 23425 are slightly less than those seen for CGS 26214 (25,000 times) (21), but significantly better than SKF 94901 (33 times) (24). These observations suggest that CGS 23425 is a cardiac-sparing thyromimetic that efficiently reduces serum cholesterol in hypercholesterolemic individuals.

CGS 23425 lowers LDL cholesterol. The observed reduction in serum cholesterol may arise from changes in either the concentration of LDL or HDL. LDL particles participate in the shuttling of cholesterol from the liver to steroidogenic target tissues, whereas HDL, a key component of "reverse" cholesterol transport, does the opposite and removes cholesterol from peripheral tissues and returns it to the liver (25). Hypercholesterolemic patients would benefit from a reduction in the level of LDL with an increased abundance of HDL (26).

Because thyromimetics decrease LDL cholesterol by enhancing the clearance of these particles through increased hepatic LDL receptor number (27, 28), we asked if CGS 23425 could increase hepatic LDL receptor number and reduce serum LDL concentrations. Therefore, fractionated sera were analyzed for LDL cholesterol content using an enzymatic assay (Fig. 1, *LDL*). Results showed that LDL was significantly reduced in both fat- and chow-fed rats at the minimum effective dose. In fat-fed rats, there was a 44% reduction in LDL cholesterol at 10 µg/kg/day, and the chow-fed rats showed a 50% reduction at 1000 µg/kg/day (Fig. 1). The pattern of LDL reduction in the fat-fed rats mirrored exactly that of total cholesterol (Fig. 1, *bottom*), suggesting cholesterol reduction in these animals is primarily through receptor-mediated removal of LDL cholesterol at the liver. The lack of a hypocholesterolemic effect of low dose CGS 23425 in the chow-fed rats may arise from low levels of LDL cholesterol in the sera and/or the already high level of LDL receptor activity in these animals. Additionally, normocholesterolemic rats tend to transport cholesterol on HDL rather than on LDL particles and are less responsive to L-T_3 treatment (29), and this may account for a lack of effect of CGS 23425 in the chow-fed rats.

To test the hypothesis that CGS 23425 might increase LDL receptor number, we measured the binding of ^{125}I-LDL to HepG2 cells treated with 10 nM of the compound. Results showed a 44% increase in LDL receptor number from 81 ± 4 to 117 ± 9 ng/mg of protein (mean ± standard error; $n = 6$). This value is comparable to a 49% increase in LDL receptor number induced by 10 nM L-T_3 (81 ± 4 to 121 ± 9 ng/mg protein). These observations suggest that CGS 23425 exerts a major effect on cholesterol metabolism via its ability to enhance LDL particle removal. These data are similar to those obtained from studies with CGS 26214 (21) and SKF 94901 (30), in which serum cholesterol concentrations were reduced via increased LDL clearance.

Effect of CGS 23425 on HDL. Raising HDL in hyper- and normocholesterolemic individuals is of significant benefit in

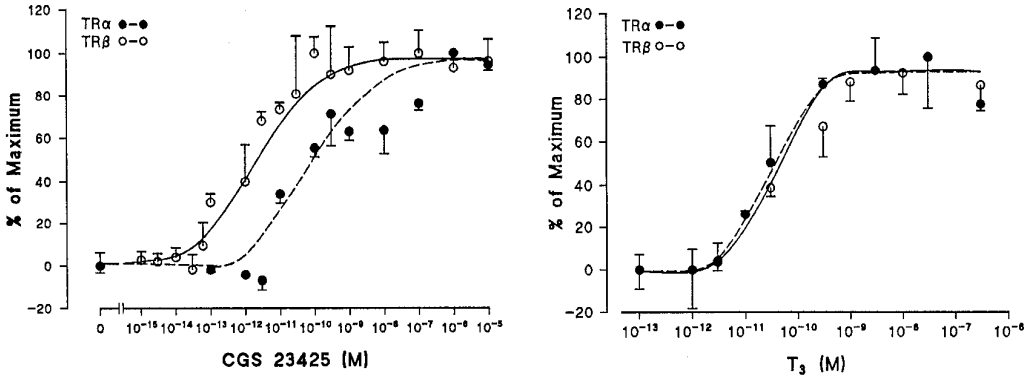


Fig. 2. Differential effects of CGS 23425 on thyroid hormone receptor isoforms. Comparison of the dissimilar effect of CGS 23425 on apoAI promoter activity in the presence of TRα and TRβ (*left*) with the similar effects of L-T_3 (*right*). Human fetal hepatoma cells (HuH-7 cells) transfected with pAI.474, CAT, and either pECE-hTRα or pECE-hTRβ were treated with the indicated concentrations of CGS 23425 or L-T_3. Cellular protein was assayed CAT activity as described in Materials and Methods. Data are mean ± standard deviation for at least four separate experiments in quadruplicate. EC_{50} values were determined directly from the graphs.

the prevention of accelerated atherosclerosis (26). Therefore, the HDL levels in the sera of these animals were measured in response to the drug. In CGS 23425-treated fat-fed rats, we observed a small but significant increase in HDL levels at 100 μg/kg/day (Fig. 1, *HDL*), whereas chow-fed rats showed no change in their HDL. These data suggest that CGS 23425 has a minimal effect on the abundance of HDL. Nevertheless, the single point showing significant elevation of HDL at 100 μg/kg/day and the known induction of apoAI protein by L-T_3 prompted us to measure serum levels of this protein. The finding of elevated levels of apoAI alone is important because of its cardioprotective effect in preventing accelerated atherosclerosis (31).

Serum apoAI protein concentrations increased in a dose-dependent manner in both experimental groups (Fig. 1, *top*). In fat-fed rats, there was a 2.4- and 5.7-fold increase in the levels of apoAI at 30 and 300 μg/kg/day, respectively. The rats on a standard chow diet also showed a significant 1.3- and 1.7-fold increase in apoAI at 100 and 1000 μg/kg/day, respectively (Fig. 1). Why CGS 23425 increases the apoAI level without marked changes in the HDL is uncertain; but because there is evidence to suggest that apoAI is a cholesterol acceptor molecule when separated from HDL, and an independent activator of lecithin:cholesterylacetyl transferase (25), then the actions of the compound on apoAI may prove to be an added benefit in the thyromimetic action of CGS 23425. Although we (17) and others (32) have previously shown L-T_3 to significantly induce apoAI expression in rats, there are no comparable human data. However, there is circumstantial evidence from genetic linkage analysis that suggests that L-T_3 is closely associated with apoAI and HDL expression in man (33). This would suggest that CGS 23425 would have a beneficial effect on human apoAI expression, HDL concentration, and HDL:LDL ratios.

Differential effects of CGS 23425 on TR isoform activity. The results of the preceding studies show that CGS 23425 increased rat apoAI abundance in a fashion similar to that of L-T_3 (17). To determine whether the increase was due to enhanced gene transcription, we measured the activity of the rat apoAI promoter in transient transfection assays using human fetal hepatoma (HuH-7) cells (17). HuH-7 cells were used for these studies because they readily respond to L-T_3 (17) and are not exposed to the hormones found in serum. CGS 23425 caused a dose-dependent increase in apoAI promoter activity with a maximal 5-fold increase (Fig. 2). However, the two major isoforms of thyroid hormone receptor, TRα1 and TRβ1, responded differently to this thyromimetic (Fig. 2). Although both reached a maximal 5-fold increase in apoAI promoter activity, the concentration required for half-maximal stimulation (EC_{50}) was lower in the presence of TRβ1. The EC_{50} for TRβ1 was $\sim 2 \times 10^{-12}$ M, and for TRα1 it was $\sim 10^{-10}$ M (Fig. 2). In comparison, L-T_3 in the same assay system produced dose-dependent curves for both isoforms of the receptor that overlapped with EC_{50} values of 6×10^{-11} M. Additionally, the maximum response to T_3 was only 3-fold. This apparent increase in apoAI promoter response is similar to that seen with TRIAC (34), where TRIAC produces increased promoter activity compared with L-T_3 and a differential effect on TRα and TRβ receptors. These changes probably reflect increased receptor occupancy compared with L-T_3. This conclusion is supported by the nuclear binding data that showed higher binding affinities for CGS 23425 to intact hepatic nuclei (Table 1). Additionally, TRIAC has also been shown to have higher affinity for TRβ receptors compared with TRα receptors (34). Another thyromimetic analog, 3,5,3′-triiodothyropropionic acid shows similar differential isoform activation effects (34), but does not produce a transcriptional response. The hepatic selectivity and lack of cardiac toxicity exhibited by CGS 23425 may be due to its differential effects on the two major isoforms of TR. This possibility is supported by the fact that the predominant TR isoform in the liver is TRβ1 and in the heart is TRα1 (35, 36).

In conclusion, the data presented here show that CGS 23425 is a thyromimetic with hypocholesterolemic activity that lacks the undesirable cardiotoxic effects of L-T_3. The mechanism of CGS 23425 action appears to involve increased LDL particle clearance by increasing the number of hepatic LDL receptors. An added beneficial effect of the compound is that it increases the levels of apoAI protein by enhancing transcription of the gene. The clear differential effects of CGS 23425 on the *trans*-activation of the apoAI promoter mediated by TRα1 and TRβ1 provides a useful tool for dissecting the actions of these receptors in response to ligand. A compound such as CGS 23425 may prove to be useful in the treatment of hypercholesterolemia.

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