Regulation of hepatic triglyceride lipase by thyroid hormone in HepG2 cells

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Abstract Hypothyroidism has been reported to be associated with reduced hepatic triglyceride lipase (HTGL) activity. In order to understand the molecular mechanism by which thyroid hormone regulates HTGL activity, effects of triiodothyronine (T3) on HTGL activity, mRNA level, transcription run-on activity, and protein synthetic rate were studied in HepG2 cells. HepG2 cells treated with 1 nM T3 showed an increase in HTGL activity that was first detected at 24 h; HTGL activity continued to increase at 36 h and stayed at the elevated level at 48 and 60 h. At maximal stimulation (48 h), T3-treated cells had the following HTGL activities: 155% in spontaneously released (SR) and 224% in heparin-releasable (HR) HTGL activities (mean levels compared to control). Stimulation of HTGL activity by T3 was dose-dependent and saturable. There was, however, no change in HTGL mRNA level throughout the course of T3 treatment. The effects of T3 were reduced when transcription was blocked by actinomycin D (mean level compared to actinomycin D treatment in the absence of T3: 109% in SR and 127% in HR activities) or translation was blocked by cycloheximide (127% in SR and 122% in HR activities), but HTGL activities were still significantly higher than control. Nuclear run-on assays indicate that T3 did not change the rate of transcription of the HTGL gene. We further determined the rate of HTGL synthesis by measuring the amount of [^{35}S]methionine incorporated into newly synthesized HTGL immunoprecipitated by a monospecific anti-human HTGL antibody. We found that the T3-stimulated increase in HTGL activity was not accompanied by any change in the rate of HTGL biosynthesis. Our experimental data indicate that the T3 stimulation of HTGL activity in HepG2 cells is mediated at posttranscriptional and posttranslational levels. The partial but significant inhibition of the T3 stimulation of HTGL activity by actinomycin D and cycloheximide suggests that the effects of T3 may be mediated by other cellular processes that are more directly regulated by the hormone. This study represents the initial report on the mechanism of HTGL activation by physiological concentrations of thyroid hormone.—**Kihara, S., J. Wölle, C. Ehnholm, L. Chan, and K. Oka.** Regulation of hepatic triglyceride lipase by thyroid hormone in HepG2 cells. *J. Lipid Res.* 1993. **34:** 961–970.

Supplementary key words hepatic triglyceride lipase • triiodothyronine • hepatoma cell line HepG2

Hepatic triglyceride lipase (HTGL) plays an important role in lipoprotein metabolism. It catalyzes the hydrolysis of intermediate density lipoprotein (IDL) triglyceride to produce low density lipoprotein (LDL) (1–3), and converts high density lipoprotein $(HDL)_2$ to HDL_3 by its triacylglycerol- and phospholipid-hydrolyzing activities (4). The modification of HDL by HTGL facilitates the transfer of cholesterol from HDL to liver as a final step of reverse cholesterol transport. Therefore, reduction in HTGL activity may produce a lipoprotein profile that favors atherogenesis.

Triiodothyronine (T3) level and HTGL activity in patients with progressive coronary atherosclerosis have been reported to be significantly lower than those in patients with no progression in their coronary atherosclerosis (5). Furthermore, Johansson et al. (6) have recently reported that among young male patients with myocardial infarction, those with a normal lipoprotein pattern or hypercholesterolemia have low HTGL activities.

It is well known that hypothyroidism is an important cause of hypercholesterolemia (7–11) and reduced HTGL activity (12–17). However, the molecular mechanism by which thyroid hormone regulates HTGL activity is still unclear.

In this communication, we have examined the regulation of HTGL in HepG2 cells by T3. We selected this human cell line for study because it has been shown to have classic nuclear T3 receptors (18) and to produce authentic HTGL (19). We found that physiological concentrations of T3 stimulate HTGL activity in these cells. Further analysis indicates that the T3 stimulation of HTGL in HepG2 cells is mediated at posttranscriptional and posttranslational levels.

Abbreviations: HTGL, hepatic triglyceride lipase; T3, triiodothyronine; SR, spontaneously released; HR, heparin-releasable; CA, cell-associated; SF, serum-free control; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

MATERIALS AND METHODS

Reagents

Cell culture grade triiodothyronine (T3) was purchased from Sigma and reconstituted in 10 mM NaOH "carrier." Triolein, gum arabic, leupeptin, pepstatin A, aprotinin, Triton X-100, phenylmethylsulfonyl fluoride (PMSF), sodium heparin, and nucleotide triphosphates were purchased from Sigma. Dulbecco's modified Eagle's medium (DMEM) deficient in L-methionine, Opti-MEM, fetal bovine serum (FBS), rabbit serum, and Immunoprecipitin (formalin-fixed Staph A cells) were purchased from GIBCO. RNase-free DNase I was purchased from Pharmacia LKB Biotechnology, Inc. [^{35}S]methionine (Trans [^{35}S]-labelTH, 1048 Ci/mmol) was purchased from ICN. Tri[9,10(n)-^{3}H]oleoylglycerol, [α-^{32}P]dCTP, [α-^{32}P]CTP, and [α-^{32}P]UTP were obtained from Amersham Co. Human HTGL cDNA and human apoA-I cDNA were as described (20). Human β-actin cDNA was amplified by the following primers: 5'-GACCTGACTGACTACCTCAT GAA-3'; 5'-CTTCATGATGGAGTTGAAGGTAG-3' and subcloned into pBluescript KS.

Cell culture

HepG2 cells were given in monolayer at 37°C in an atmosphere containing 5% CO_2 and were maintained in MEM supplemented with 100 U/ml penicillin, 10 μg/ml streptomycin sulfate, 2 mM glutamine, and 10% (v/v) FBS. For all experiments, cells were seeded at a density of 2 × 10^4 cells/cm^2 (not to become confluent at the end of experiment) in serum containing medium for 24 h and then shifted to serum-free Opti-MEM for 24 h before they were given treatments. Culture medium was replaced every 12–24 h.

HTGL assay

HTGL activity was quantitated by measuring the lipolytic activity toward a substrate of tri[9,10(n)-^{3}H]oleoylglycerol emulsified with gum arabic (3). The incubation medium contained triolein substrate (5 mCi/mmol triolein, 3% gum arabic), 0.1 M Tris-HCl, pH 8.8, 5% fatty acid-free bovine serum albumin (BSA), and 0.75 M NaCl in a final volume of 0.4 ml. Sample was added to start the reaction. After incubation for 60 min at 37°C, the reaction was stopped by adding 3 ml of methanol–chloroform–heptane 14:12:10 containing 0.2 mM oleic acid as a carrier. The tubes were vortexed, 0.5 ml of 0.5 M NaOH was added to each tube, and the tubes were vortexed again for 1 min and centrifuged at 1500 *g* for 15 min. Released [^{3}H]oleic acid in the upper phase was counted by scintillation spectrometry and the activity of HTGL was calculated.

In most experiments, 10 ml of culture medium was removed and adjusted to final concentrations with 10 mM potassium phosphate, pH 6.8, 10% glycerol, 90 kalikrein units/ml aprotinin, and 0.6 ml packed volume of heparin-Sepharose, and the sample was incubated for 3 h at 4°C with gentle mixing as previously described (21, 22). The heparin-Sepharose was pelleted and washed with 10 ml of phosphate-buffered saline (PBS), and bound HTGL was eluted with 2 ml of 10 mM Tris-HCl, pH 6.8, 1 M NaCl, 10% glycerol, and 1 mM EDTA. The cells were gently washed, 3 ml of the same medium containing 100 U/ml heparin was added to each dish, and the cells were incubated at 37°C for 30 min. Heparin-releasable aliquots were removed, and the cells were washed with PBS at 4°C, scraped into 1 ml of 50 mM NH_3/NH_4Cl, pH 8.1, containing 10 U heparin, and sonicated for 15 sec at 4°C. One hundred μl of the cell homogenate was taken and lysed with 0.9 ml of 0.2 M NaOH for protein assay. The remainder was centrifuged for 30 min at 12,000 *g*, 4°C, and the supernatant was used as an enzyme source.

Synthesis and isolation of cRNA probe

Anti-sense mRNA (cRNA) probe was synthesized on an isolated linear EcoRV fragment from the pIBI31 HL plasmid, containing 530-1231 internal HTGL cDNA inset and the T7 RNA polymerase binding site. The labeled transcript was synthesized according to the recommendations of the manufacturer (Promega) with the following modifications. The synthesis was carried out in 20 μl containing 0.5 μg of DNA template and a final molar ratio of [α-^{32}P]CTP (800 Ci/mmol) to cold CTP of 1:2. DNA template was removed by RNase-free DNase I digestion at 37°C for 30 min. The cRNA probe was then precipitated in 0.5 volumes of 7.5 M ammonium acetate and 2 volumes of ethanol at −20°C for 1 h. To remove unincorporated nucleotides, this ethanol precipitation step was repeated 3 times. The HTGL [^{32}P] cRNA probe was resuspended in 0.1% SDS (sp act 1.8–2.2 × 10^8 cpm/μg) and used for hybridization on the same day.

Quantitation of mRNA

Total cellular RNA was prepared by the guanidinium thiocyanate–phenol–chloroform extraction method (23). For Northern blot analysis, total RNA was electrophoresed on 1% agarose, 6.3% formaldehyde gels and transferred to nylon membrane (Hybond N, Amersham). For slot blot analyses, 0.1–10 μg of HepG2 total RNA was denatured and applied directly to membranes. Membranes were UV-cross-linked, prehybridized, hybridized with HTGL [^{32}P]cRNA, or human β-actin or apolipoprotein A-I cDNA probe labeled by random priming. Blots were washed 2 × 20 min in 1 × SSC, 0.1% SDS and 3 × 20 min in 0.1 × SSC, 0.1% SDS at 65°C. Hybridized membranes were exposed for 18 h at −80°C to Kodak XAR-5 film between two intensifying screens. Hybridization signals were quantitated by scanning the films with a densitometer and comparing slopes (obtained by linear regression analysis) of signal areas versus total RNA loaded.

Nuclear run-on transcriptional assay

Nuclei for transcription run-on assays were prepared by a modification of the technique described previously (24). HepG2 cells were suspended in buffer A (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 3 mM $MgCl_2$) and 0.5% Nonidet P-40 (v/v) and homogenized in a Dounce homogenizer. Nuclei were pelleted at 500 *g* and washed once in buffer A. The nuclear pellet was resuspended in 40% glycerol, 50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, and 0.1 mM EDTA and stored in liquid nitrogen. Nuclei isolated as described were reproducibly intact and free of cellular debris as assessed by phase contrast microscopy.

Nuclear transcription activity was determined by measurement of [α-^{32}P]UTP incorporation in RNA transcripts elongated in vitro as described by McKnight and Palmiter (25). Nuclear transcription assay was carried out in 200 μl transcription buffer composed of 35% glycerol, 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 80 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 5 mM ATP, GTP, CTP, 20 U of RNasin, and 200 μCi of [α-^{32}P]UTP at 30°C for 45 min. Nuclei were then digested with 10 μl RNase-free DNase I (7.5 U/μl) and 10 μl $CaCl_2$ (20 mM) at 37°C for 10 min. Samples were further treated with 2 μl proteinase K (10 mg/ml), 25 μl 10 × SET (5% sodium dodecyl sulfate [SDS], 50 mM EDTA, 100 mM Tris-HCl, pH 7.4) and 5 μl yeast tRNA (10 mg/ml) at 37°C for 30 min. Nuclear RNA was then extracted by a modification of the acid guanidinium–phenol method (23) followed by two ethanol precipitations.

Aliquots from T3-treated and untreated nuclei were counted in a scintillation counter, and an equal number of counts from each condition was hybridized to anti-sense RNAs of human HTGL, β-actin, and apolipoprotein A-I synthesized from cDNAs cloned in pIBI31 or pBluescript KS vector using T3 or T7 RNA polymerase and immobilized on nylon membrane using a slot-blot apparatus. HTGL sense RNA was used as a negative control. Hybridization, washing, and autoradiography were carried out as described in mRNA quantitation.

Measurement of HTGL synthetic rate

HTGL synthetic rate was measured by immunoprecipitation of HepG2 cell extract using a rabbit polyclonal antibody against purified human postheparin plasma HTGL (26). The specificity of the antibody was determined by Western blot analysis, and no reactivity was detected against antithrombin III (data not presented). Immunoprecipitation of HTGL in HepG2 cells was performed by a modification of two previously published protocols (27, 28). HepG2 cells were washed with PBS at 37°C, and 3 ml of warm methionine-deficient DMEM was added to each plate. After 30 min at 37°C in a 95% air, 5% CO_2 incubation, 1 mCi of [^{35}S]methionine was added to each plate, the medium was gently swirled, and the dishes were returned to the incubator for 30 min. The medium was discarded, the plates were placed on ice and washed with PBS at 4°C, and 0.3 ml of lysis buffer containing sodium heparin at 10 U/ml was added to each dish. The cells were scraped and sonicated for 15 sec at 4°C and centrifuged for 30 min at 12,000 *g* at 4°C, and then the clear supernatant was removed and stored at −80°C.

Incorporation of [^{35}S]methionine into detergent-solubilized cellular protein was determined by precipitation of the protein in 5 μl of sample with boiling trichloroacetic acid (10% v/v) followed by quantitation of precipitated radioactivity by scintillation counting. Duplicate analyses were performed in each case. Equal amounts of trichloroacetic acid-precipitable radioactivity adjusted to 100 μl with lysis buffer were mixed with 1 ml of immunoprecipitation buffer (27) containing 1% BSA, and then 20 μl of anti-HTGL rabbit antiserum or nonimmune rabbit serum was added.

Following a 24-h incubation on a rotating rack at 4°C, 20 μl of Immunoprecipitin® was added and incubations were continued for 45 min at room temperature. The suspension was then centrifuged at 12,000 *g* for 20 sec, the supernatant was discarded, and the pellet was washed six times with immunoprecipitation buffer containing 0.1% SDS, and once with 0.0625 M Tris-HCl, pH 6.8. The pellet was dissolved in 30 μl of electrophoresis loading solution (27) and boiled for 10 min.

Samples were subjected to electrophoresis on SDS-10% polyacrylamide gel and 4% stacking gel at 25 w for 3 h. Gels were calibrated with ^{14}C-labeled protein molecular weight markers. After electrophoresis, gels were fixed for 1 h, impregnated with ENHANCE®, dried, and exposed to X-ray film (XAR-5) for 2–7 days at −80°C.

Western blot analysis

Appropriately diluted heparin-released media of HepG2 culture were reduced and fractionated by electrophoresis as described above. The gels were blotted onto nitrocellulose membranes (BA 85, Schleicher & Schuell) electrophoretically. The membranes were then exposed to an anti-HTGL antibody (26). The antibody was detected by the immunoalkaline phosphatase technique (Immun-Blot Assay Kits, Bio-Rad). Intensity of the bands was linear for HTGL released into the media from 10 μg to 100 μg cell protein. The relative densitometric values were deduced from the slopes of the curves derived from the loading of different amounts of samples on the gel.

Statistical analysis

Results are presented as mean ± SD. The difference between the two groups was examined for statistical significance by unpaired two-tailed *t* test, and significance was assigned for $P < 0.05$.

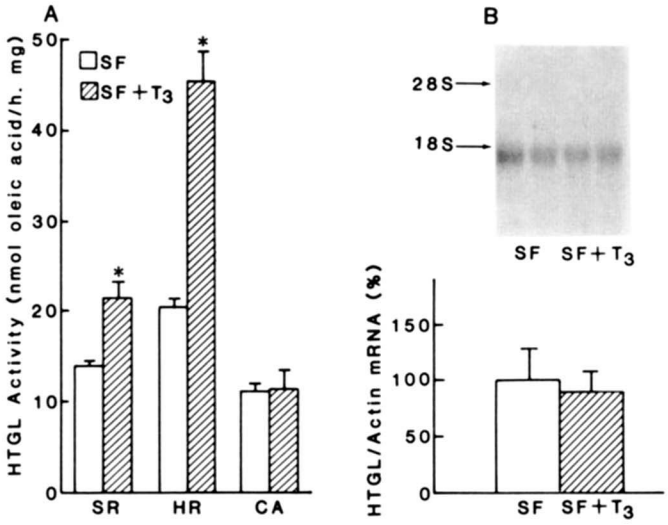

Fig. 1. Effects of T3 on HTGL activity and HTGL mRNA in HepG2 cells. HepG2 cells were plated and fed as described in Materials and Methods. Forty-eight h after addition of 1 nM T3 or carrier (six plates each), media were collected from all dishes (SR) and then the cells were washed and incubated for 30 min with heparin-containing media. Aliquots of heparin-releasable (HR) media were collected, and the cells were again washed. One-half of the plates was used to prepare extracts for assay of cell-associated (CA) HTGL activity and protein assay, and one-half was used for RNA isolation. A: Stimulation of HTGL activity by T3 in each fraction. SF: serum-free control, SF + T3; 1 nM T3 treatment. HTGL activity was expressed as nmol oleic acid/h • mg^{-1} cell protein. Bars denote SD. $^{*}P < 0.01$. B: (Top) Northern blot analysis of HTGL mRNA. Total RNA (5 μg/lane) from HepG2 cells was electrophoresed on a 1% agarose, 6.3% formaldehyde gel, transferred to a nylon membrane, and hybridized to an HTGL cRNA probe. (Bottom) Comparison of the effects of T3 on HTGL/β-actin mRNA ratio. HTGL and β-actin mRNA were determined from the slopes of linear plots of slot-blot analyses as described in Materials and Methods. Bars denote SD.

HTGL activities and mRNA levels were studied in HepG2 cells treated with 1 nM T3 (**Fig. 1A**). Compared to controls, HTGL enzyme activity in T3-treated cells was 1.55 ± 0.12-fold higher in the fraction spontaneously released (SR) into the culture media, and 2.24 ± 0.16-fold higher in the heparin-releasable (HR) fraction, but it was not significantly different in the cell-associated (CA, the intracellular activity extractable from HepG2 cells after heparin treatment) fraction. Although T3 stimulated HTGL activity 2-fold compared to serum-free controls, HTGL mRNA level was unchanged by Northern blot analysis (Fig. 1B, inset). The HTGL/β-actin mRNA ratio

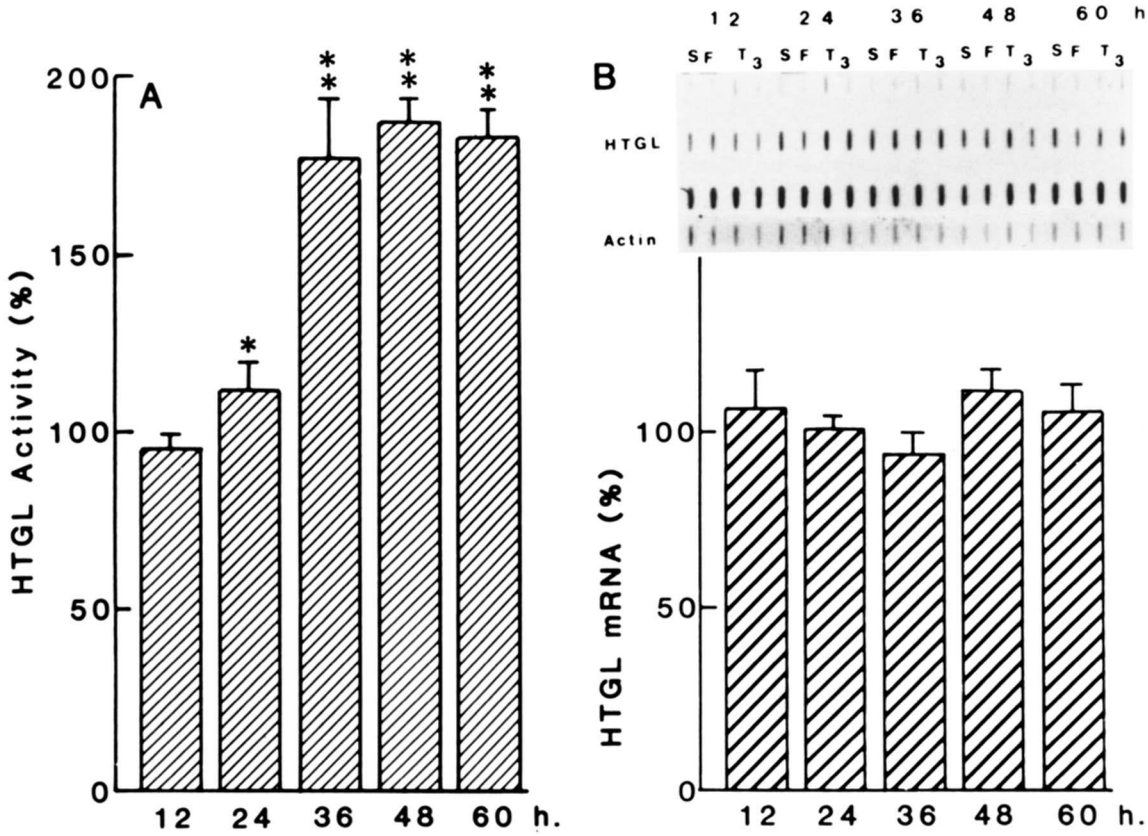

Fig. 2. Time course of the effect of T3 on HTGL activity and HTGL mRNA levels in HepG2 cells. Cells were fed as described in Materials and Methods. Plates were harvested at various times after T3 addition and assayed for spontaneously released (SR) activity (A) as well as HTGL mRNA (B). 100% of HTGL activities at 12, 24, 36, 48, and 60 h correspond to 23.9, 39.3, 46.2, 84.7, and 94.5 nmol oleic acid/h dish, respectively. Results represent data derived from three dishes. Bars denote SD. $^{*}P < 0.05$; $^{**}P < 0.01$.

as determined by quantitative slot-blot analysis from three independent experiments in T3-treated cells was 90.1 ± 17.1 (mean ± SD) % of serum-free controls.

We next studied the time course of T3 treatment on HTGL activity and mRNA level in HepG2 cells (**Fig. 2A**). HepG2 cells were fed as described in Materials and Methods. At time 0, media were replaced with Opti-MEM-containing carrier or 1 nM T3. Every 12 h thereafter, plates were removed for determination of HTGL activity and mRNA. As T3 primarily affects the transcription rate of target genes and change of transcription rate of HTGL gene has been reported to be correlated with SR-HTGL activity (21, 22), SR-HTGL activity was measured at various incubation times. A small but significant increase in HTGL activity was observed at 24 h of T3 treatment. At 36 h, the HTGL activity of T3-treated cells was 1.8-fold greater than that of serum-free controls. It stayed at that level at 48 and 60 h. To determine whether the T3-stimulated increase in HTGL activity was accompanied by an increase in HTGL mRNA levels, we measured the latter by slot-blot analysis of total HepG2 RNA isolated at various times after T3 treatment. We found that there was no change in HTGL mRNA levels throughout the 60 h of T3 addition (Fig. 2B). Control hybridization using a β-actin cDNA probe showed that the total amount of RNA applied was uniform with respect to β-actin mRNA content. Therefore, the higher HTGL activity in T3-treated cells was not accompanied by any increase in steady state HTGL mRNA concentration.

The effect of varying the dose of T3 treatment on HTGL activity in HepG2 cells at 48 h is shown in **Fig. 3A.** HTGL activity in the media increased progressively with increasing concentration of T3, reaching a maximal level at 1 nM. Further increases of T3 concentration up to 100 nM did not further change HTGL activity.

HTGL activity from HepG2 cells incubated in the presence of 0.1, 1.0, and 10 nM T3 was 1.45 ± 0.04-; 1.85 ± 0.05-, and 1.89 ± 0.05-fold higher than that from serum-free control cells, respectively. Again, doses of T3 from 0.1 to 100 nM did not alter steady-state HTGL mRNA levels compared to controls (Fig. 3B).

To further define the role of gene transcription and translation in T3 stimulation of HTGL activity, SR-, HR-, and CA-HTGL activities in the presence or absence of

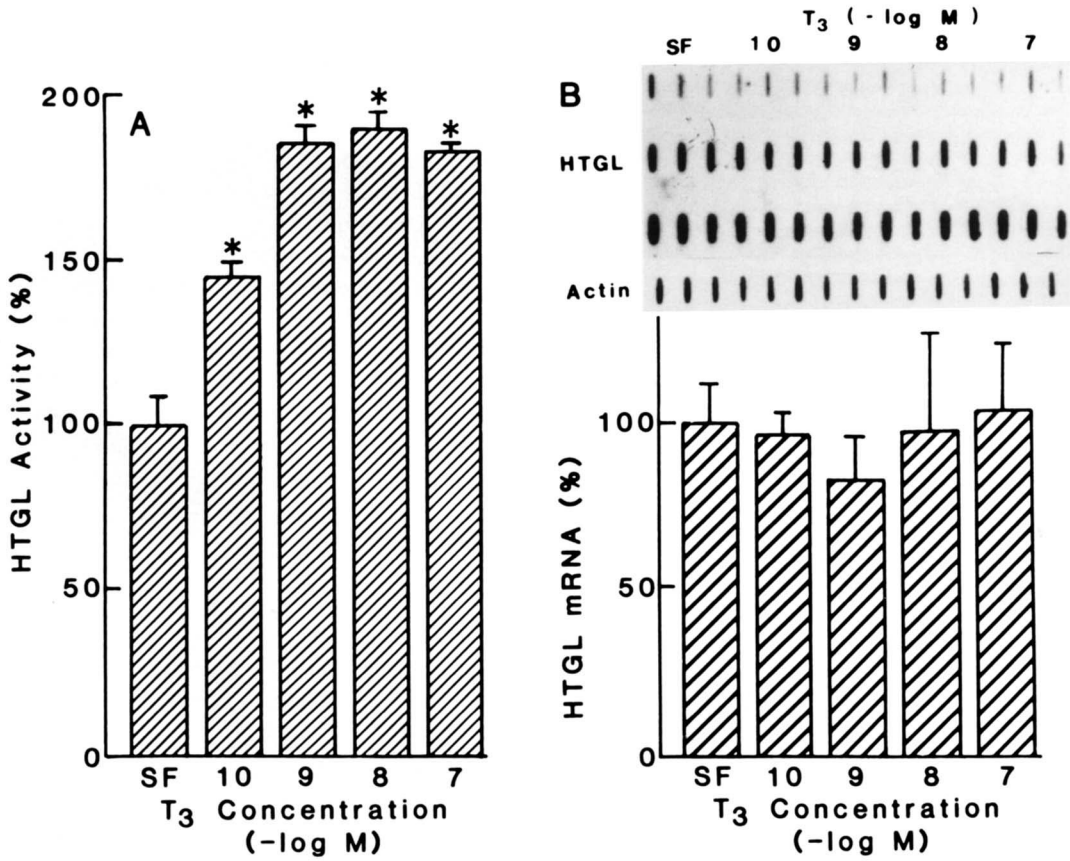

Fig. 3. Dose response of the effect of T3 on HTGL activity and mRNA levels in HepG2 cells. HepG2 cells were cultured as described in the legend of Fig. 1. HepG2 cells were treated with carrier or increasing concentrations of T3 (10^{-10}–10^{-7} M) and incubated for 48 h. Plates were then analyzed for SR-HTGL activity in the media (A), and the levels of HTGL mRNA (B) in the same cells were determined by quantitative slot-blot analysis. HTGL activity of serum-free control (SF) was 95.3 ± 8.2 nmol oleic acid/h dish. Results represent data derived from three dishes. Bars denote SD. *$P < 0.01$.

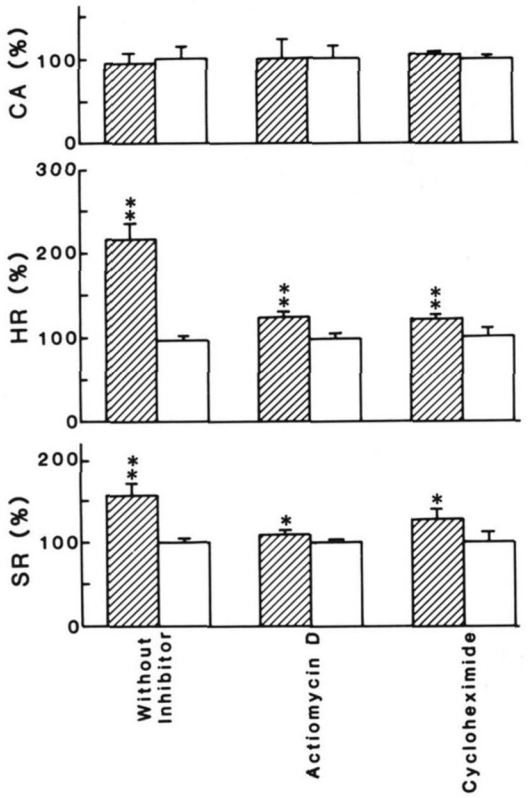

Fig. 4. Effects of actinomycin D and cycloheximide on the T3-stimulated HTGL activity. HepG2 cells were plated and fed as described in Materials and Methods. Thirty-six h after addition of 1 nM T3 or carrier (SF) with or without actinomycin D (2 μg/ml) or cycloheximide (10 μg/ml), SR, HR, and CA HTGL activities were measured. Mean HTGL activities in SF, SF plus actinomycin D, and SF plus cycloheximide in SR were 13.9, 5.9, and 4.1 nmol oleic acid/h mg; those in HR, 20.2, 12.0, and 7.2 nmol oleic acid/h mg; and those in CA, 9.2, 8.1, and 7.2 nmol oleic acid/h mg, respectively. Results represent data derived from three dishes. Bars denote SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

T3 were measured after blocking transcription with actinomycin D or protein synthesis with cycloheximide (**Fig. 4**). In the absence of inhibitors, T3 increased SR-HTGL activities to 156 ± 14%, and HR activities to 222 ± 18% compared to carrier treatment; CA activity was unaffected. The T3-stimulated increase in HTGL activity was much reduced by the addition of actinomycin D to 109 ± 3% in SR and 127 ± 5% in HR, and by addition of cycloheximide to 127 ± 10% in SR and 122 ± 3% in HR; although much smaller, these increases in HTGL activity in the presence of inhibitors are still significantly higher than controls. CA activity was unchanged in the T3-treated cells in the presence or absence of inhibitors.

To further assess HTGL regulation by T3, HTGL gene transcription rate was measured by a nuclear run-on assay (**Fig. 5A**). Nuclei were isolated from HepG2 cells incubated in the presence or absence of 1 nM T3 for 48 h. Elongation of initiated transcripts was performed in vitro using [α-^{32}P]UTP as described in Materials and Methods. Radiolabeled nuclear RNA was hybridized to HTGL sense-cRNA (as negative control), HTGL antisense cRNA, β-actin anti-sense cRNA, and apoA-I antisense cRNA immobilized on nylon membranes. No difference in either HTGL/β-actin or apoA-I/β-actin RNA ratio was observed between T3-treated samples and serum-free controls, indicating that HTGL and apoA-I transcription rates were not affected by T3. Transcription run-on assay at 24 h after T3 treatment also showed no change (data not shown). It is interesting that, although T3 did not affect the rate of apoA-I gene transcription, cellular apoA-I mRNA level was increased 2.02-fold in T3-treated cells compared to serum-free controls (Fig. 5B), suggesting that T3 stabilizes apoA-I mRNA. In contrast, T3 had no effects on HTGL transcription rate or mRNA content.

HTGL synthetic rates were measured in control and T3-treated HepG2 cells to further define the level of regulation of HTGL activity by the hormone (**Fig. 6**). In pilot experiments, the incorporation of radioactivity into total protein and HTGL protein was found to be linear up to 60 min and 30 min, respectively. Radiolabeling was performed for 30 min and the newly synthesized ^{35}S-methionine-labeled HTGL was precipitated by a

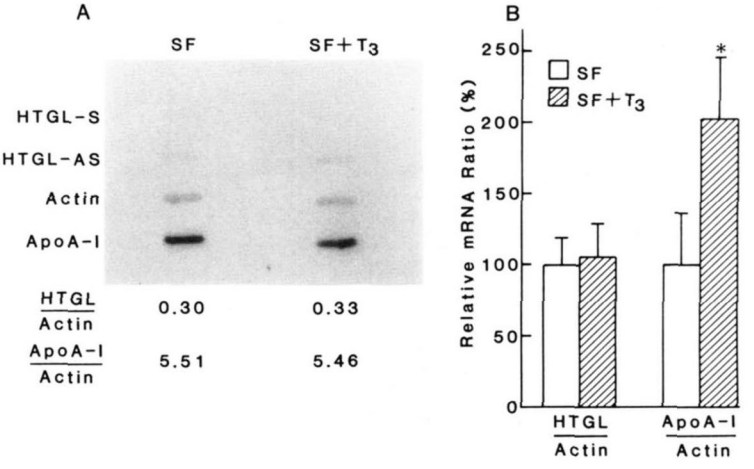

Fig. 5. Effects of T3 on (A) transcription rate and (B) mRNA levels of HTGL and apoA-I relative to β-actin in HepG2 cells. After 48 h treatment with carrier or 1 nM T3, nuclei were isolated, and transcription run-on assays were performed as described in Materials and Methods. The following RNAs were immobilized on a nylon membrane: HTGL-S, HTGL sense cRNA; HTGL-AS, HTGL antisense cRNA; actin, actin antisense cRNA; apoA-I, apoA-I antisense cRNA. SF, serum-free control. Equal counts of extracted, radiolabeled RNA from the run-on assays were hybridized, followed by washing and autoradiography. The levels of mRNA were measured as described in the legend of Fig. 1. Bars denote SD. $^{*}P < 0.05$.

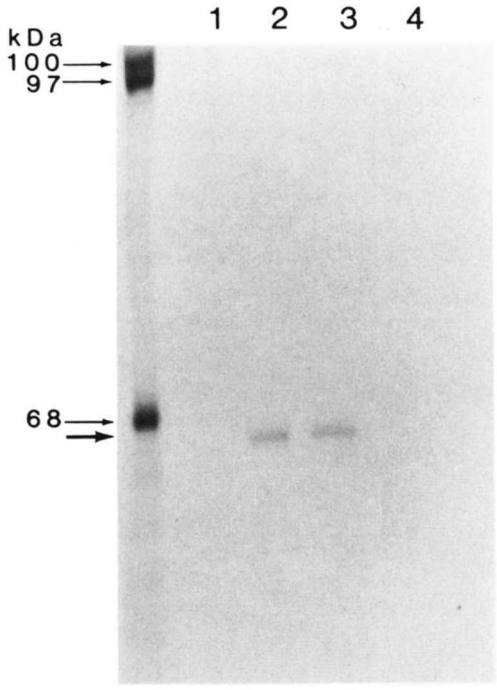

Fig. 6. SDS-polyacrylamide gel analysis of immunoprecipitated radiolabeled HTGL protein. After 48 h treatment with carrier or 1 nM T3, cells were labeled with [^{35}S]methionine for 30 min and extracts were prepared as described in Materials and Methods. Extracts were then treated with rabbit anti-human HTGL antibody (lanes 2–4) or nonimmune rabbit serum (lane 1) and immunoprecipitates were collected and analyzed by SDS-polyacrylamide gel electrophoresis. The gel was processed for fluorography as described in Materials and Methods. The arrow marks the 65-kDa position where newly synthesized HTGL protein was detected. Lane 1, T3-treated HepG2 extract (same volume as lane 3) precipitated with nonimmune rabbit serum. Lane 2, carrier-treated HepG2. Lane 3, T3-treated HepG2. Lane 4, carrier-treated HepG2 extract (same volume as lane 2) mixed with excess purified nonradioactive human HTGL before immunoprecipitation. Molecular size standards used were phosphorylase-b (100 and 97 kDa) and albumin (68 kDa).

monospecific antiserum. The immunoprecipitate was fractionated on an SDS-polyacrylamide gel and the radiolabeled enzyme was detected by fluorography. The radioactive band was identified as HTGL on the basis of the following criteria: *i)* the apparent molecular mass of 65 kD was similar to that found for HepG2 cells by other laboratories (19, 29); *ii)* the band was competed by excess purified HTGL; when labeled HepG2 extract was mixed with an excess of purified unlabeled human HTGL before addition of anti-HTGL antibody, the 65 kD band was eliminated (lane 4); and *iii)* the corresponding protein band was not observed when immunoprecipitation was performed with a nonimmune rabbit serum (lane 1).

Newly synthesized radiolabeled HTGL represents about 0.005–0.01% of the total trichloroacetic acid-precipitable ^{35}S-methionine-labeled protein in these experiments. Although SR-HTGL activity was 2-fold higher in T3-treated cells compared to serum-free control, HTGL protein synthetic rate in T3-treated cells (lane 3) was not different from control (lane 2). In three independent experiments, the HTGL synthetic rates in T3-treated cells were 92 ± 26% (mean ± SD) compared to serum-free control cells.

The effect of 1 nM T3 on HTGL immunoreactive mass in HR-medium is shown in **Fig. 7.** HR-media (from 10 to 100 μg cell protein) were loaded on gels and analyzed by Western blot. The densitometric values of HTGL bands in T3-treated cells were 174 ± 5% (mean ± SD) compared to control, indicating that the T3-stimulated increase in HTGL activity was accompanied by an increase in HTGL immunoreactive mass.

DISCUSSION

HepG2 cells secrete HTGL (19) and have nuclear thyroid hormone receptors identical to those of human fibroblast (18); it is therefore a useful model to analyze the effects of T3 on HTGL expression. In the present study, we showed that physiological concentrations of T3 increase HTGL activity 2-fold compared to serum-free control in HepG2 cells.

In dose–response experiments, maximal activation of HTGL was observed at 1 nM T3 and no further increase in activity occurred at 10 or 100 nM. It is well known that postheparin HTGL activity is markedly decreased in hypothyroidism (12–17), whereas hyperthyroidism has been reported to be associated with an increase in postheparin HTGL activity (30). The dose-dependent and saturable increase of HTGL activity by T3 suggests

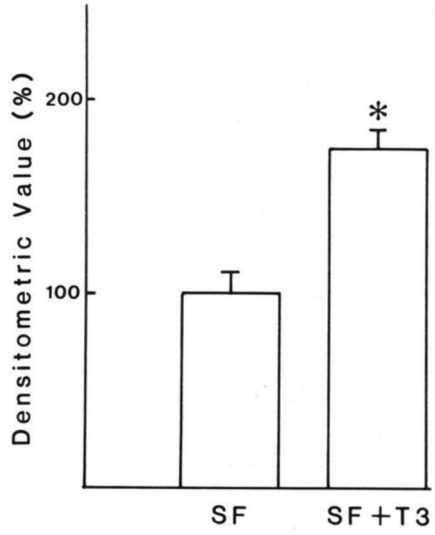

Fig. 7. Effect of T3 on HR-HTGL immunoreactive mass. HepG2 cells were cultured and HR media were collected as described in the legend of Fig. 1. The cells were used for protein assay and appropriate amounts of media were analyzed by Western blot as described in Materials and Methods. Densitometric values are arbitrary values obtained from the slopes of curves deduced from the loading of different amounts of sample on the gel. Bars denote SD. *$P < 0.01$.

a T3 hormone receptor-mediated mechanism as the physiological basis for the clinical observation.

The increase of HTGL activity in HepG2 cells was observed 24 h after T3 addition and reached maximal level at 36 h without significant change of steady-state HTGL mRNA level relative to β-actin mRNA. In contrast, the ratio of apoA-I mRNA to β-actin was increased 2.0 ± 0.4-fold compared to serum-free control, an effect quite similar to that reported for the T3 stimulation of rat hepatic apoA-I mRNA (31–34). In the rat, the euthyroid state is associated with an approximately 2-fold increase in the level of poly(A) RNA compared to that in hypothyroidism, but no further change was observed between the euthyroid and hyperthyroid state (35, 36). The transition between the hypothyroid and euthyroid state is also accompanied by a general increase in total RNA, largely due to an increase of ribosomal RNA (35). Therefore, the diverse and highly selective effects of T3 cannot be explained by a generalized increase or decrease in mRNA population. In this study, we determined the transcription rate of the HTGL gene by nuclear run-on assays and found that T3 does not affect transcription rate of the HTGL or the apoA-I genes under the present conditions.

The regulation of malic enzyme expression by thyroid hormone has been used as a model system for thyroid hormone action (36–40). Malic enzyme mRNA rises after a lag of 2–4 h after HepG2 cells are treated with T3. It appears that the T3 regulation of malic enzyme occurs at a pretranslational level, although the possibility has not been excluded that the T3 effect is mediated by a translational product of an antecedent hepatic gene stimulated directly by the hormone. Our observations are also quite consistent with those of Peinado-Onsurbe et al. (41), who reported that neonatal rat hepatocytes incubated in the presence of T3 for 3 h did not show any change in HTGL activity. Similarly, a lag of 24 h was also seen in the stimulation of the secretion of thyroxine-binding globulin and α-1-acid glycoprotein by HepG2 cells in response to T3 treatment (42).

The T3-mediated increase in HTGL activity was statistically significant in the presence of 10 μg/ml of cycloheximide, which inhibits HepG2 protein synthesis > 95% (43). Direct measurements of HTGL transcription and translation rate indicate that the T3 regulation of HTGL enzyme activity in HepG2 cells is mediated at levels other than transcription or translation. However, the presence of actinomycin D reduced the T3 effect by 84% in SR activities and 78% in HR activities. Although the relatively long time (36 h) of exposure of the cells to the metabolic inhibitors might have affected numerous cellular processes with secondary effects on HTGL activity, these results suggest that the posttranslational effect of T3 may be mediated by an antecedent hepatic gene product directly stimulated by the hormone.

Staels et al. (33) reported that HTGL mRNA level was decreased 20% in hypothyroid rats but was unchanged in hyperthyroidism in rats. Experimental hypothyroidism in the rat decreased their food intake and body weight (44, 45). Since decrease in food intake per se has been found to be associated with low HTGL mRNA and activity, it is not clear whether the effect of hypothyroidism was mediated directly by hormone deffciency or via reduction of food intake.

Hoogerbrugge-v.d. Linden et al. (17, 46) reported that the levels of insulin-like growth factor-I (IGF-I) and HTGL were signiffcantly lower in women with primary hypothyroidism, and thyroid hormone did not restore HTGL activity in hypothyroid rats unless growth hormone was present. As thyroid hormone induces IGF-I via growth hormone, the effects of T3 may be mediated in part by IGF-I in vivo. In the present study, addition of IGF-I (50 ng/ml) or IGF-II (50 ng/ml) to the culture medium did not affect HTGL activity and mRNA level (data not shown). However, because serum-free medium contains insulin, we have not formally excluded IGF-I as a possible mediator of T3 action on HTGL in HepG2 cells.

Recently, Nozaki et al. (47) reported that T3 at the concentration of 0.2 μg/ml increased HTGL mRNA in HepG2 cells. This supraphysiological dose of T3 is about 100- to 1000-fold higher than the dose used in our study (which is within the physiological range found in vivo). Although the stimulatory effect of T3 on HTGL gene transcription in HepG2 cells can be demonstrated at these extremely high concentrations of added T3, the physiological signiffcance of this observation is unclear.

HTGL and LPL are members of the lipase gene family (48–50). LPL has been reported to be regulated by thyroid hormone at the translational level (51). Both enzymes contain at least two complex N-linked oligosaccharide chains, and glycosylation appears to be required for enzyme secretion (28, 52–55). However, unglycosylated HTGL was shown to be catalytically active in mutant HTGL molecules produced by site-directed mutagenesis (56). Thus, the loss of T3 effect in cell-associated HTGL activity is probably not a consequence of incomplete glycosylation. The T3-stimulated increase in heparin-released HTGL activity was accompanied by an increase in HTGL immunoreactive mass measured by Western blot analysis, indicating that more HTGL protein was being released in the medium. The processing of human HTGL has not been studied, but the dynamics of HTGL production is different between the human and the rat enzyme. Rat HTGL is rapidly secreted into the medium from cultured rat hepatocytes or hepatoma cells (28, 53); human HTGL, in contrast, appears to be stored in a heparin-releasable pool. We found that HTGL activity in the HR compartment was affected more than that in the SR compartment. These results suggest that T3 may stabilize HTGL protein in the heparin-releasable pool and secretion step.

In conclusion, we found that T3 stimulates HTGL activity at a posttranscriptional and posttranslational level. Our observations provide a physiological basis for the altered HDL metabolism in thyroid dysfunction.

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