

Thyroid Hormone Regulation and Cholesterol Metabolism Are Connected through Sterol Regulatory Element-binding Protein-2 (SREBP-2)*

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High affinity uptake of serum-derived low density lipoprotein (LDL) cholesterol is accomplished through the LDL receptor in the liver. In mammals, thyroid hormone depletion leads to decreased LDL receptor expression and elevated serum cholesterol. The clinical association in humans has been known since the 1920s; however, a molecular explanation has been lacking. LDL receptor levels are subject to negative feedback regulation by cellular cholesterol through sterol regulatory element-binding protein-2 (SREBP-2). Here we demonstrate that the SREBP-2 gene is regulated by thyroid hormone and that increased SREBP-2 nuclear protein levels in hypothyroid animals results in thyroid hormone-independent activation of LDL receptor gene expression and reversal of the associated hypercholesterolemia. This occurs without effects on other thyroid hormone-regulated genes. Thus, we propose that the decreased LDL receptor and increased serum cholesterol associated with hypothyroidism are secondary to the thyroid hormone effects on SREBP-2. These results suggest that hypercholesterolemia associated with hypothyroidism can be reversed by agents that directly increase SREBP-2. Additionally, these results indicate that mutations or drugs that lower nuclear SREBP-2 would cause hypercholesterolemia.

The inverse correlation between serum levels of cholesterol and thyroid hormone (TH)¹ has been known from clinical observations that date back over 70 years (1). More recent studies indicate that low LDL-cholesterol uptake in hypothyroid patients is stimulated by thyroid hormone treatment (2), and studies in experimental animals have established that the levels of LDL receptor mRNA and protein in the liver are directly associated with serum thyroid hormone levels (3, 4). Although there is one report of activation of the LDL receptor promoter by the addition of thyroid hormone receptor (TR) and 3,3',5-triiodo-L-thyronine (T3) to transfected HepG2 cells, there was no putative TR site in the promoter region under study, and direct TR binding was not

evaluated (5). Even though these studies cannot rule out the existence of a functional binding site for TR, they do not address the possibility of a TR site at a remote location in the LDL receptor locus; they do suggest that the regulation by TR may be at least partly indirect.

In contrast to the lack of defined TR sites in the LDL receptor promoter, there are well defined functional sites through which cholesterol regulates gene expression (6). The key site binds the sterol regulatory element-binding proteins (SREBPs) (7). There are three major SREBPs that are encoded by two genes (8). SREBP-1a and -1c are from overlapping mRNAs encoded by one gene and the single SREBP-2 is encoded by a separate gene. All three are synthesized and first inserted into membranes of the endoplasmic reticulum and nuclear envelope where they cannot directly influence gene expression (9). When the sterol level of a cell falls, a multistep maturation process is initiated by a sterol-dependent alteration in membrane trafficking that culminates with the proteolytic release of SREBPs from the membrane (10). The soluble SREBPs enter the nucleus and activate overlapping sets of genes that are primarily involved in lipid metabolism (8). SREBP-2 preferentially activates the LDL receptor and other genes directly involved in cholesterol homeostasis and SREBP-1 products primarily activate genes of fatty acid metabolism (8).

The biological effects of thyroid hormone are through hormone-dependent changes in gene expression (11). TR complexes with retinoid X receptor (RXR), and the heterodimer binds to specific DNA sites in target genes (12). The liganded receptor then helps to initiate a multistep process that ultimately results in gene activation. The current studies indicate that SREBP-2 is directly regulated by TH, and we propose that SREBP-2 is an important link between TH and cholesterol metabolism.

Our studies demonstrate that SREBP-2 mRNA and nuclear protein levels are both down-regulated when mice are deprived of thyroid hormone. LDL receptor mRNA is also repressed under these conditions consistent with previous studies (3). We further show that an independent feeding regimen that increases nuclear SREBP-2 independently of TH results in both a restimulation of LDL receptor mRNA expression and a significant decline in serum cholesterol. We also demonstrate that the SREBP-2 promoter is activated directly by TR in a ligand-dependent manner and that there is at least one TR-binding site in the 5'-flanking DNA. These studies identify SREBP-2 as a direct thyroid hormone target and indicate that increased nuclear SREBP-2 can reverse the hypercholesterolemic effects of hypothyroidism independently of TH itself. Furthermore, our results indicate that genetic modifications or pharmacological treatments that lower SREBP-2 levels would result in hypercholesterolemia.

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¹ The abbreviations used are: TH, thyroid hormone; LDL, low density lipoprotein; SREBP, sterol regulatory element-binding protein; mSREBP, mouse SREBP; TR, thyroid hormone receptor; T3, 3,3',5-triiodo-L-thyronine; RXR, retinoid X receptor; LXR, liver X receptor; PTU, propylthiouracil; HMG, 3-hydroxy-3-methylglutaryl; PGC, peroxisome proliferator-activated receptor- γ -coactivator; FAS, fatty acid synthase; CMX, cytomegalovirus X.

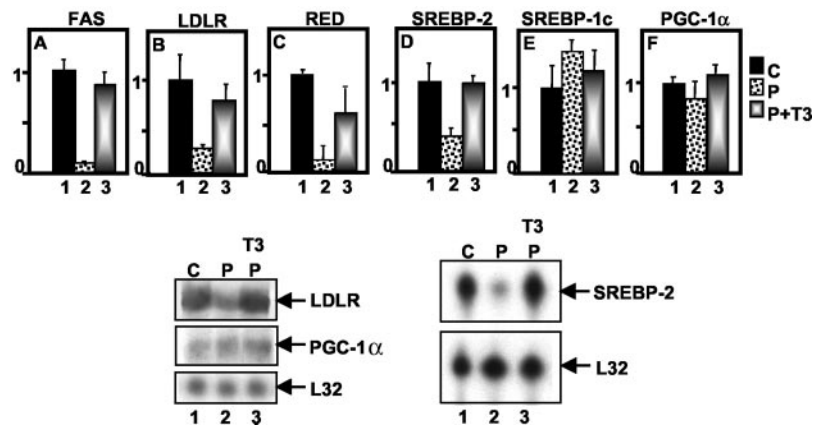


FIG. 1. Thyroid hormone regulation of SREBP-2. Total RNA was isolated from livers of mice that were fed a normal chow diet (C), an iodine-deficient diet supplemented with PTU (P), or an iodine-deficient diet supplemented with PTU and injected with T3 (P+T3) in lanes 1–3 as indicated in the figure and detailed under “Materials and Methods.” Northern analysis was performed to measure mRNA levels for FAS (A), LDL receptor (LDLR) (B), HMG CoA reductase (RED) (C), and PPAR- γ coactivator 1 α (PGC-1 α) (F). The mRNA levels were quantified using Quantity One software from Bio-Rad using densitometric scans from autoradiograms, and the intensities were normalized for L32 mRNA levels. mRNA levels for SREBP-1c (E) and SREBP-2 (D) were measured by an RNase protection assay (32). Results are expressed as -fold change relative to those of the control chow-fed animals, and the mean values obtained from individual measurements from six separate animals in each group are shown with error bars. Representative RNA blots and RNase protection experiments are shown at the bottom. Pooled RNA samples from animals in each treatment group were analyzed by either blot hybridization for LDL receptor, L32, and PGC-1 α (bottom left) or RNase protection analysis for SREBP-2 and L32 (bottom right).

MATERIALS AND METHODS

Animal Feeding Studies—Four-week-old B6129 male mice were obtained from Taconic and maintained on a 12-h light/dark cycle (dark cycle was from 7:00 p.m. to 7:00 a.m.) with free access to food and water. The mice were allowed to adapt to the new environment at least 1 week before experiments and were randomly divided into three groups: control, TH-deprived, and TH-supplemented groups. The control group was fed *ad libitum* with normal chow diet. For the TH-deprived group, mice were fed an iodine-deficient diet supplemented with 0.15% propylthiouracil (PTU) (Harlan Teklab) for 3 weeks. On the 18th day of the iodine-deficient diet supplemented with PTU, the TH-supplemented group of mice was given 1 μ g of T3 (ICN) per gram of body weight by intraperitoneal injections daily for 4 days. For the cholestid/lovastatin study, each group of mice was fed the respective diet, and, where indicated, the diet was supplemented with cholestid (Amersham Biosciences and The Upjohn Company) (4%, w/w) and lovastatin (Purepac Pharmaceutical Co.) (0.1%, w/w) for the final 7 days. The mice were sacrificed between 8:00 a.m. and 10:00 a.m., and livers were removed and divided into pieces. One piece was used immediately to prepare nuclear protein. Others were frozen in liquid nitrogen and stored at -80°C until RNA was extracted as described below.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated from mouse livers using TRIzol (Invitrogen). Total RNA (20 μ g) were fractionated on 1% agarose-formaldehyde gels and transferred to Nytran (Schleicher and Schuell BioScience). The membranes were hybridized with ^{32}P -labeled cDNA probes (1×10^6 cpm/ml) overnight at 42°C . An 80-base *HindIII/EcoRI* fragment of rat ribosomal protein L32 cDNA (a gift from Tom Lane, University of California, Irvine) was used as a probe to normalize total amounts of RNA/lane. Every blot was rehybridized with the L32 probe for normalization. After hybridization, blots were washed and exposed to a Kodak BioMAX film followed by scanning with an HP 7400c flat bed scanner, and mRNA levels were then quantified using the Quantity one software (Bio-Rad). The following cDNA probes were used: HMG CoA reductase, a 0.6-kb *HindIII/BamHI* fragment from pBluescript mouse Red; PGC-1 α , a 1.5-kb *BglIII/NcoI* fragment from the pSV-SPORT-PGC-1 (gift of Peter Tontonoz, UCLA); FAS, a 0.2-kb *EcoRI* fragment from mouse FAS cDNA; LDL receptor, a 0.3-kb *PstI/BamHI* fragment from pBluescript mouse LDLR; 5'-deiodinase, a 0.8-kb *BamHI/EcoRI* from pCR2.1-mouse-5'DI.

RNase Protection Assay—Total RNA samples (20 μ g) were subjected to the RNase protection assay using the RPAII kit (Ambion, Inc.). ^{32}P -labeled cRNA probes were generated from pBluescript-mSREBP-1c, mSREBP-2-pGEM-T, and L32-pGEM constructs by *in vitro* transcription using the MAXIScript kit (Ambion, Inc.). The RNase protection assays were performed by adding a cRNA probe for either SREBP-2 or SREBP-1c mRNA (1×10^5 cpm) and a cRNA probe (1×10^5 cpm) for L32 mRNA. L32 cRNA probe was used to normalize total amounts of RNA/lane. After digestion with RNase A/RNase T1, protected frag-

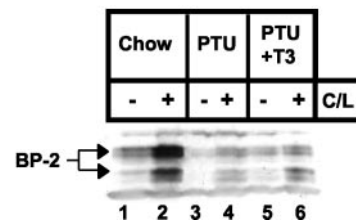


FIG. 2. Regulation of SREBP-2 protein levels by TH and cholestid plus lovastatin. Mice were fed diets identical to those described in the legend to Fig. 1, supplemented with or without cholestid/lovastatin (C/L) as indicated. Nuclear protein extracts were analyzed by 8% SDS-PAGE and Western blotting with an antibody raised against mouse SREBP-2 protein. The full-length and a major degradation product of SREBP-2 protein are marked with the arrows.

ments were resolved on 8 M urea, 5% polyacrylamide gels, dried, and processed as described above.

Protein Isolation and Blotting—Fresh slices of the same livers used above for RNA analysis were processed for nuclear proteins (13). Nuclear extracts were fractionated on 8% SDS-PAGE, transferred to nitrocellulose, and analyzed first by Ponceau S staining to confirm that equal amounts of total protein were both loaded and transferred in each lane. Then, the blot was incubated with a rabbit polyclonal antisera directed against mouse SREBP-2 (from Jay Horton, University of Texas, Southwestern) followed by a secondary antibody conjugated to horseradish peroxidase. Reactivity was then detected with the ECL kit (Pierce).

Cell Culture and Transient Transfection Assay—HepG2 cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum at 37°C and 5% CO_2 . One day before transfection, cells were seeded in 6-well plates at 350,000 cells/well. After incubation for 16 h, cells were transfected by the calcium phosphate co-precipitation method as described (14) with 2 μ g/well of the indicated SREBP-2 promoter-luciferase reporter, 2 μ g/well of the cytomegalovirus β -galactosidase plasmid as an internal control for transfection efficiency, and indicated expression vectors for CMX-hTR- β (2 μ g) or CMX-hRXR- α (0.5 μ g). Salmon sperm DNA was added to equalize total amounts of DNA. After 4–6 h, cells were treated with 10% glycerol, washed three times with phosphate-buffered saline, and refed with a serum-free minimum essential medium supplemented with 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml selenite, 5 μ l/ml defined lipid mix (Invitrogen), and 0.11% de-lipidated bovine serum albumin (Sigma) in the presence or absence of 1 μM 3,3',T3. The cells were incubated for an additional 40 h before harvest. Luciferase and β -galactosidase assays and normalization were described previously (13). Values represent the mean of duplicate \pm S.D. Each experiment was repeated at least three times.

Plasmids—The 5'-deletion mutants of the SREBP-2 promoter were

FIG. 3. LDL receptor mRNA is activated by cholestid/lovastatin in TH-deprived mice. Total RNA (20 μ g) from aliquots of the same livers used for nuclear protein analysis in Fig. 2 were analyzed by blot hybridization for LDL receptor (A) or 5'-deiodinase (C) and by RNase-protection assay for SREBP-2 (B) as indicated. Samples were analyzed as described in the legend to Fig. 1.

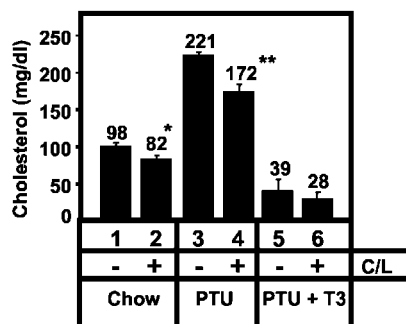
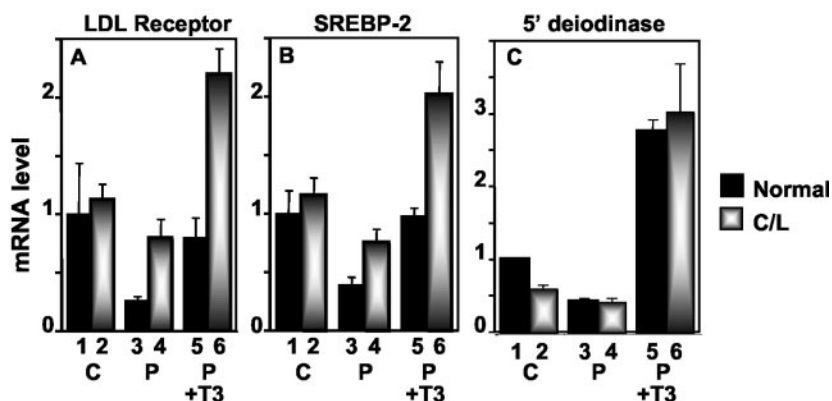


FIG. 4. Elevated serum cholesterol in TH-deprived mice is reversed by treatment with cholestid and lovastatin. Serum levels of total cholesterol for the animals used for Figs. 2 and 3 were measured. Student's *t* test of the data indicates a *p* = 0.006 for the difference between samples 1 and 2 and a *p* = 0.004 for the difference between samples 3 and 4.

constructed by PCR-based amplification and cloning upstream of the luciferase gene in pGL3-Basic. Mouse genomic DNA isolated from B6129 mouse liver was used as a template for PCR. The following forward primers were used: -1019/SREBP-2.Luc, 5'-ACGATGCCCTGATATTCTACTGTTTGTG-3'; -1509/SREBP-2.Luc, 5'-AGAAACCTGTCTCTTGCTCAGTTCT-3'; -2053/SREBP-2.Luc, 5'-CACCAGAAGAGGGCAGTACAGTCTGTTACA-3'. The reverse primer for all was 5'-GCTTGGCGCAGATGCCCTGTGAGGTCAG-3'. *SacI* and *HindIII* were added to the forward and reverse primers, respectively, for cloning purposes. The PCR fragments were cloned into *SacI/HindIII* sites of pGL3-Basic. -4316/SREBP-2.Luc was generated by cloning of a PCR fragment from -4316 to -1704 into *SacI/EcoRV* sites of -2053/SREBP-2.Luc. The primers were as follows: forward primer, 5'-ATCTGTTCCAGCATCCATATGGTAGCTT-3'; reverse primer, 5'-AGACTAACAAAGGGCTGGATATCAGAC-3'. Mouse cDNA was produced from total RNA using cDNA Cycle kit (Invitrogen) and used to generate cDNA fragments for type I 5'-deiodinase and SREBP-1c by PCR. A 5'-deiodinase PCR fragment was ligated into pCR2.1. pBluescript mouse SREBP-1c was constructed by blunt-end ligation of a PCR fragment into the *SmaI* site of pBluescript KS. The following primer pairs were used: 5' deiodinase, 5'-GCAAGGTGCTAATGACGCTGTTC-3' and 5'-AGACATCTGTACTGGTGTGGGAAAG-3'; mSREBP-1c, 5'-ATCGGCGCGGAAGCTGTCGG-3' and 5'-TTGTTGATGAGCTGGAGCATGT-3'. Mouse SREBP-2-pGEM-T was a generous gift from Jay Horton (University of Texas, Southwestern). CMX-hTR- α and CMX-hTR- β were from Dr. Bruce Blumberg, University of California, Irvine, and CMX-hRXR- α and pCMX-hLXR- α were from Dr. Peter Tontonoz (UCLA).

Electrophoretic Mobility Shift Assays—hTR- α , hTR- β , hLXR- α , and hRXR- α proteins were produced using the TNT-coupled transcription/translation system (Promega). The sequences of one strand of the complementary oligonucleotide probes were as follows: SREBP-2, 5'-CGGACCTGATGCCCTTTTCTGACCTTGGGCACTGGGCATATG-3'; DR-4, 5'-CTAGAGCTTCAGGTCACAGGAGGTCAGAGAGCT-3'; mutant TRE, 5'-TCCCGCTATGCAAATCTGAGCTGCTG-3'. The complementary oligonucleotides were annealed, and, where indicated, they were labeled with [γ - 32 P]ATP by T4 polynucleotide kinase. Binding reactions were carried out at 4 $^{\circ}$ C for 20 min in 20 μ l of binding buffer containing 12.5 mM Hepes (pH 7.6), 50 mM KCl, 1 mM MgCl₂, 5% glycerol, 4 μ g of poly(dI-dC), 5 μ g of nonfat dry milk, and 5% (w/v) Ficoll. T3 or cold DNA

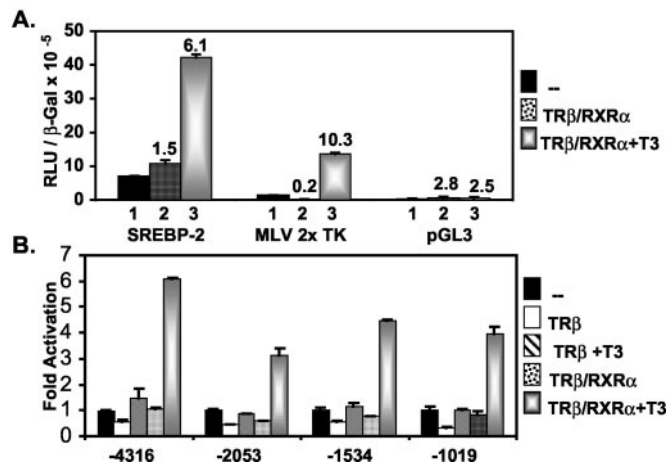


FIG. 5. SREBP-2 promoter is directly activated by TR. A, HepG2 cells were transfected with SREBP-2 promoter-luciferase fusion construct or various control plasmids. Each promoter-luciferase fusion construct was transfected alone or together with expression constructs for TR- β plus RXR- α . Cells were cultured in serum-free minimal medium, and 1 μ M T3 was included as indicated. Results are expressed as corrected luciferase light units divided by β -galactosidase activity. The fold change relative to cells transfected with each luciferase reporter alone is also indicated on each bar graph. The data represent the mean of duplicates for three individual experiments and include error bars. RLU, relative light units. B, a similar experiment was performed as in A, except different truncation derivatives of the SREBP-2 promoter were used.

competitors were added to the binding buffer at the concentrations indicated in the legend to Fig. 6. The protein-DNA complexes were analyzed on a 5% polyacrylamide gel with 0.5 \times TBE buffer (89 mM Tris borate, 2 mM EDTA). The gels were dried and analyzed by autoradiography.

Serum Cholesterol Measurements—Serum total cholesterol levels were measured using the Infinity cholesterol reagent kit (Sigma, catalog no. 401-25P) and Cholesterol standards (Sigma, catalog no. C0534).

RESULTS

To study the effects of thyroid hormone depletion in an animal model system, mice are fed an iodine-deficient diet and PTU, which inhibits the 5'-deiodinase enzyme required to convert the T4 form of TH into the more bio-active T3 isoform (15). Under these conditions, serum TH levels fall significantly, and the effect can be reversed by injecting the animals with exogenous T3 (16). Concomitant with the changes in serum T3, the level of mRNA for putative thyroid hormone target genes in the liver decline, and they return to normal when T3 is added back (17).

Several genes involved in *de novo* lipogenesis including FAS are regulated in this manner (18). As a control, we showed that FAS displayed this pattern of regulation (Fig. 1A). Genes of cholesterol metabolism exhibit a similar pattern of expression, and in agreement with these previous observations (3) mRNA levels for HMG CoA reductase and LDL receptor follow this

classic T3 regulated pattern (Fig. 1, *B* and *C*). In contrast, expression of other genes, including those encoding SREBP-1c and the PPAR- γ coactivator (PGC-1 α) (19), is not T3-responsive (Fig. 1, *E* and *F*).

Importantly, the level of SREBP-2 mRNA followed the pattern of a typical T3-regulated gene; however, the magnitude was somewhat lower than for the other T3 target genes (Fig. 1*D*). Because SREBP-2 activates key genes of cholesterol metabolism, it is possible that the response to variations in TH is secondary to the changes in SREBP-2. This would provide a mechanism for linking cholesterol metabolism to thyroid hormone levels and would explain why genes of cholesterol metabolism follow a classic TR-regulation pattern.

If a decrease in active nuclear SREBP-2 protein was primarily responsible for the repression of LDL receptor mRNA levels in the experiments above, then an increase in nuclear SREBP-2 protein while animals were maintained in a TH-deprived state should result in reactivation of LDL receptor mRNA expression. Therefore, we repeated the experiments of Fig. 1 but we included groups of animals fed a diet containing lovastatin, an HMG CoA reductase inhibitor, and cholestid, a bile acid sequestrant. This feeding protocol is designed to simulate a low cholesterol environment in the liver, and it results in activation of genes of cholesterol metabolism (20). This regimen also increases the nuclear accumulation of SREBP-2 protein (13).

We prepared both RNA and nuclear protein from the livers of these animals. First, to evaluate whether nuclear SREBP-2 levels were altered by the PTU/low iodine diet and increased upon feeding cholestid and lovastatin, we measured the nuclear SREBP-2 protein levels by Western blotting (Fig. 2). In agreement with previous studies performed in the hamster (13), nuclear SREBP-2 levels are low when animals are fed a chow diet and they are induced by the addition of cholestid and lovastatin (Fig. 2, lanes 1 and 2). Consistent with lower levels of SREBP-2 mRNA, the PTU/low iodine diet resulted in a decrease in nuclear SREBP-2 protein (Fig. 2, compare lanes 1 and 3). Importantly, treatment of the TH-deprived animals with cholestid and lovastatin resulted in an increase in nuclear SREBP-2 protein (Fig. 2, compare lanes 3 and 4). T3 supplementation resulted in an increase above the PTU/low iodine diet (Fig. 2, lanes 5 and 6).

RNA blotting with samples from these animals demonstrated that LDL receptor mRNA was down-regulated normally by TH depletion (Fig. 3*A*, compare lanes 1 and 3). Interestingly, the level of LDL receptor mRNA was increased by the lovastatin/cholestid combination under TH-deprived conditions (Fig. 3*A*, compare lanes 3 and 4). The SREBP-2 gene is auto-regulated (21), and its expression pattern exhibited a similar regulation profile (Fig. 3*B*). For specificity, we analyzed hepatic type I 5'-deiodinase, which is a sensitive target of thyroid hormone and TR (22). Its mRNA followed a classic T3 response pattern in our experiments (Fig. 3*C*). Importantly, it was unaffected by the addition of cholestid and lovastatin (Fig. 3*C*) indicating that cholestid and lovastatin did not have a secondary affect on thyroid hormone regulation. Taken together, these data are consistent with the hypothesis that the drop in LDL receptor mRNA that occurs under TH deprivation is secondary to the decrease in SREBP-2.

The LDL receptor protein is responsible for high affinity uptake of LDL-cholesterol in the liver, and a TH-deprived state results in hypercholesterolemia because of the down-regulation in LDL receptor expression (23). To evaluate this in our studies, we measured whether the increase in LDL receptor mRNA resulted in a decrease in serum cholesterol (Fig. 4). As expected, serum cholesterol was down-regulated by cholestid and

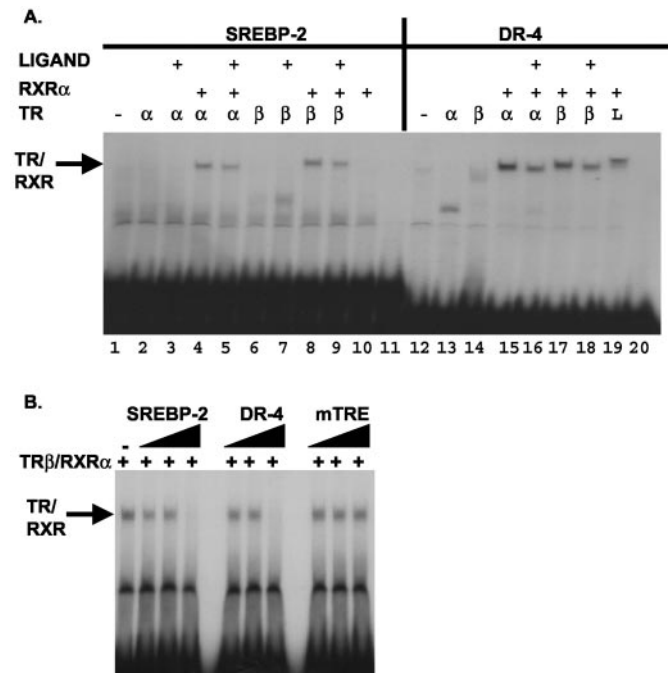


FIG. 6. TR/RXR binds directly to a site in the SREBP-2 promoter. *A*, complementary oligonucleotides encompassing the putative DR-4 site at -4260/-4221 shown in Fig. 5 were annealed and used as a substrate in electrophoretic mobility shift assays with *in vitro* translated TR- α , TR- β , RXR- α , and T3 ligand (0.1 μ M) as indicated. The probe was incubated with unprogrammed reticulocyte extract in lane 1. Free probe was loaded in lane 11. In lanes 12-20, a consensus DR-4 site was used as a probe, and LXR- α plus RXR- α was used in lane 19. The DR-4 was mixed with unprogrammed reticulocyte extract in lane 12, and free probe was loaded in lane 20. *B*, TR- β and RXR- α were translated *in vitro* and used in an electrophoretic mobility shift assay as in *A*. Unlabeled DNA competitors were added at 10-, 50-, and 100-fold molar excess as indicated. The SREBP-2 DNA used as a labeled probe was added in lanes 2-4, the consensus DR-4 site used in *A* was added in lanes 5-7, and a mutant DR-4 with four base pair mismatches relative to the consensus DR-4 (see "Materials and Methods") was used in lanes 8-10.

lovastatin in the normal chow-fed animals (Fig. 4, compare lanes 1 and 2). In the TH-deprived animals, the cholesterol level was dramatically increased as expected (Fig. 4, compare lanes 1 and 3). Importantly, inclusion of lovastatin and cholestid resulted in a significant drop in serum cholesterol levels of these otherwise TH-deprived animals. This indicates that the increase in LDL receptor mRNA resulted in functional expression of LDL receptor protein.

The studies presented so far indicate that the SREBP-2 gene might be directly activated by TH. To evaluate this, we fused 4316 bases of the 5'-flanking region of the mouse SREBP-2 gene to the luciferase reporter and used it in a HepG2 cell-based transfection assay that we established to evaluate thyroid hormone and TR regulation. When the transfected cells were cultured in a serum-free defined medium, the addition of expression vectors for TR and RXR coupled with T3 selectively induced luciferase activity ~6-fold (Fig. 5*A*). This stimulation required expression vectors and T3 hormone. A similar pattern of activation was observed for the control murine leukemia virus luciferase (MLVLuc) reporter, which has been used as a model T3-responsive promoter (24). The pGL3 vector was expressed at a very low level and showed a minimal response to TR/RXR that was not further stimulated by the addition of T3 (Fig. 5*A*).

Deletion of the 5'-flanking region of the SREBP-2 promoter from -4316 to -2053 resulted in a modest drop in T3 stimulation, and further truncation down to -1019 showed that a moderate level of stimulation by TR/RXR and T3 was retained (Fig. 5*B*). These results indicate that the SREBP-2 gene is directly

activated by TR, and it is likely that there are more than one TR-binding site spread throughout the 5'-flanking region.

Because there was a modest drop in T3 regulation upon deletion of the DNA from -4316 to -2053, we scanned this region for the presence of putative DR-4-like response elements, which are known to be high affinity TR sites (25). We noticed there was a recognizable match to this consensus in this sequence interval (noted in Fig. 5A), and we designed complementary oligonucleotides encompassing this putative TR site for use in a gel mobility shift assay with TR and RXR (Fig. 6). Heterodimers of each TR isoform with RXR bound efficiently to this SREBP-2 DR-4 site (Fig. 6A, lanes 1-11). Efficient binding by both TR- α and TR- β required RXR, and binding was unaffected by the presence of thyroid hormone. A similar DNA binding pattern was observed for a synthetic consensus DR-4 site (Fig. 6A, lanes 12-20). The formation of the TR/RXR-specific complex was sensitive to competition by the unlabeled SREBP-2 probe and to the consensus DR-4 site but not to DNA containing a mutated DR-4 element (Fig. 6B).

DISCUSSION

The current studies reveal a molecular mechanism for the association between hypercholesterolemia and hypothyroidism. It has been established previously that expression of the LDL receptor gene and protein was repressed when thyroid hormone levels were artificially decreased (3), but the mechanism for this drop in expression had not been defined previously. In the present report, we demonstrate that the gene encoding SREBP-2, a major transcriptional regulator of genes involved in cholesterol uptake and synthesis, is directly regulated by thyroid hormone. Both SREBP-2 mRNA (Fig. 1) and nuclear protein (Fig. 2) fall in parallel when mice are fed a diet designed to lower serum levels of TH. Nuclear SREBP-2 levels increase when the diet of chow-fed animals is supplemented with an HMG CoA reductase inhibitor and a bile acid sequestrant (13). When the TH-deprived animals in our studies were fed this same mixture, SREBP-2 nuclear protein and LDL receptor mRNA levels both increased (Figs. 2 and 3, respectively), and serum cholesterol levels decreased (Fig. 4). The activation of LDL receptor was not because of an effect of cholestid or lovastatin on TH regulation, as the mRNA level for hepatic 5'-deiodinase, which is another TR-regulated gene was not affected (Fig. 3C).

These results form the basis for a model to account for hypothyroid-linked hypercholesterolemia. The model predicts that as thyroid hormone levels fall, SREBP-2 levels decline followed closely by a drop in LDL receptor mRNA. This results in a decline in high affinity LDL cholesterol uptake in the liver resulting in hypercholesterolemia, which is fundamentally linked to thyroid hormone status. TH depletion in animals not only lowers liver LDL receptor mRNA and protein (26), but hypothyroid humans have increased serum LDL cholesterol (27) and reduced LDL uptake that can be reversed by adding T3 (2). Therefore, our model would account for the association between low serum TH levels and hypercholesterolemia in humans as well.

Because hypercholesterolemia associated with low TH levels is effectively reversed by T3 supplementation (2), the therapeutic significance of our findings is not immediately obvious. However, our data provide a mechanistic basis for the clinical association and may be helpful in designing therapeutic strategies to help treat patients with more complicated diseases such as resistance to thyroid hormone (RTH) where patients have mutations that directly inactivate the TR β gene (28).

A previous study provided evidence that the LDL receptor promoter was activated in HepG2 cells when an expression construct for TR was added along with T3 (5). However, TR binding was not directly evaluated in this earlier study and the

results observed are consistent with TR activating endogenous SREBP-2, which could then activate the LDL receptor gene. Our results indicate that TR directly activates SREBP-2, and it is well established that SREBP-2 activates the LDL receptor gene (13). Thus, our studies clearly establish an indirect mode of regulation for the LDL receptor by T3. However, further studies are required to establish whether there is also a direct effect of TR.

Our studies demonstrate that TR directly activates expression of the SREBP-2 gene, and we have identified at least one binding site for TR in the 5'-flanking sequence (Fig. 6). Based on the gradual loss in T3 responsiveness in the analysis of the promoter deletions, there are likely other functional binding sites for TR and further studies are needed to characterize these additional elements.

SREBP-2 is also autoregulated by SREBPs (21), and SREBP-2 mRNA was also up-regulated by the cholestid and lovastatin under the TH-depleted conditions shown in Fig. 3. SREBPs and TR functionally cooperate to activate both the rodent S14 and chicken acetyl-CoA carboxylase promoters (29-31). Thus, it will be interesting to determine whether SREBPs and TR functionally interact to stimulate expression of the SREBP-2 promoter as well.

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