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Using *in vivo* electroporation to identify hepatic LDL receptor promoter elements and transcription factors mediating activation of transcription by T_3

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ABSTRACT

The technique of *in vivo* electroporation was adapted to investigate the promoter elements and transcription factors mediating the rapid induction of hepatic LDL receptor expression in response to thyroid hormone. Direct comparisons between wild type and mutant promoter constructs were made within the same animal. It was demonstrated that both TREs at bp −612 and −156 were required for the L-triiodothyronine (T_3) response. ChIP analysis showed that binding of TRβ1 to the −612 and −156 TREs was markedly stimulated by T_3 *in vivo*. Introduction of siRNAs against TRβ1/RXRα with LDL receptor promoter-luciferase construct by *in vivo* electroporation demonstrated that these transcription factors play the major physiological role in the activation of hepatic LDL receptor transcription. The findings agree with those made by transfecting H4IIE cells *in vitro* thus validating this technique for *in vivo* studies of mechanisms of transcriptional regulation. The findings reported herein also indicated, for the first time, that PPARα and USF-2 were required for maximum transcriptional activation of the LDL receptor in response to T_3 treatment.

1. Introduction

1.1. Thyroid hormone and cholesterol

Thyroid hormone acts rapidly to stimulate transcription of the hepatic low density lipoprotein (LDL) receptor several fold (Ness and Lopez, 1995). This action is critical to the serum cholesterol-lowering effect of this hormone (Ness, 1991). In fact, hypothyroidism has been associated with elevated levels of LDL cholesterol (Illingworth et al., 1981), and treatment with thyroid hormone effectively lowers LDL cholesterol (Aviram et al., 1982). This has led to the development of thyromimetics as possible anti-cholesterol drugs (Underwood et al., 1986; Leeson et al., 1989; Ness et al., 1998; Morkin et al., 2004; Valadares et al., 2009).

1.2. Use of rat hepatoma cells

In a prior investigation, rat hepatoma H4IIE cells in culture were used to identify two thyroid response elements (TREs) in the rat LDL receptor promoter (Lopez et al., 2007). The elements identified were: an upstream TRE at bp −612 and a two half-site TRE at bp −156, relative to the transcription start site (Lopez et al., 2007). Binding of thyroid hormone receptor β1 (TRβ1) to both elements was demonstrated with binding to the −612 element being much stronger than to the −156 element (Lopez et al., 2007). Mutation of the weaker −156 element did not significantly affect induction by L-triiodothyronine (T_3) (Lopez et al., 2007). However, mutation of both elements was required to completely abrogate T_3 induction of the LDL receptor promoter activity in the H4IIE cells (Lopez et al., 2007).

1.3. In vivo electroporation

The technique of efficiently transfecting rat liver cells *in vivo* using electroporation, for introduction of plasmid constructs has

Abbreviations: LDL, low density lipoprotein; TREs, thyroid response elements; T_3, L-triiodothyronine; ChIP, chromatin immunoprecipitation; TRβ1, thyroid receptor β1; siRNA, silencing RNA; RXRα, retinoic acid X receptor α; PPARα, peroxisomal proliferator receptor α; USF-2, upstream factor-2; WT, wild-type; −156Mt, −156 mutant; −612Mt, −612 mutant; DbMt, double mutant; NR, normal; Hx, hypophysectomized; fT_3, free T_3; LDLR, LDL receptor; SEM, standard error of the mean; IP, immunoprecipitation; Pol II, polymerase II; RA, 9-cis retinoic acid; SREBP, sterol response element binding protein; EMSA, electrophoretic mobility shift assays; NcoR2, nuclear receptor co-repressor 2; TRAC-1, T_3 receptor-associating cofactor 1; SMRTe, silencing mediator for retinoid and thyroid hormone receptors-extended; TRAP, thyroid hormone receptor- associated protein; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; CPT-I, carnitine palmitoyltransferase-I; ELISA, enzyme-linked immunosorbent assay; THDS, thyroid hormone depleted serum; PCR, polymerase chain reaction; ROI, region of interest.

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been developed and optimized (Heller et al., 1996; Suzuki et al., 1998). In a study using a β-galactosidase construct, it was demonstrated that 30% to 40% of liver cells expressed the β-galactosidase genetic marker (Heller et al., 1996). Thus, we employed this *in vivo* electroporation technique to study the elements and transcription factors mediating induction of hepatic LDL receptor transcription by T_3 in a live animal. Rat LDL receptor luciferase constructs, as well as siRNAs to knockdown specific transcription factors, were directly introduced into livers of live rats by *in vivo* electroporation.

2. Results

2.1. Localizing in vivo transfected promoter constructs

Using our 5 mm hexagonal array electrode, we introduced rat LDL receptor promoter-luciferase reporter gene constructs and/or siRNAs, in duplicate, into each of three liver lobes in the same animal. This allows for direct comparisons. The area transfected is limited to that inside the hexagonal array. After 24 hours, the transfected areas were removed using a 5 mm cork borer. The precise location of the transfected regions is defined by six light dots due to electrode scaring on the liver surface. Luciferase activity is restricted to the 5 mm circle as demonstrated in Fig. 1.

2.2. In vivo evaluation of the −612 and −156 TREs

The *in vivo* contributions of the TREs located at bp −612 and −156, relative to the transcription start site, to T_3 stimulation of LDL receptor transcription was evaluated by introducing receptor promoter constructs into rat livers by *in vivo* electroporation. The rat wild-type (WT), −156 mutant (−156Mt), −612 mutant (−612Mt), and double mutant (DbMt) LDL receptor promoter constructs were introduced into separate liver lobes of normal (NR), hypophysectomized (Hx), and T_3-treated hypophysectomized (Hx + T_3) rats, for direct comparisons within the same animal. As shown in Fig. 2A, the promoter activity of the WT construct was 39% lower in the Hx than in the NR rats. Treating with a single dose of T_3 (after electroporation) 16 hours before euthanization caused a significant ($p<0.01$) induction (2.4-fold) in LDL receptor promoter activity as compared to the activity observed in the Hx animals (Fig. 2A). These changes in LDL receptor promoter activity directly correlated with changes in free T_3 (fT_3) (Fig. 2B) and hepatic LDL receptor mRNA levels (Fig. 2C). Fig. 3A illustrates the results for the mutation analysis experiment. For this specific experiment, the Hx rats were treated with two injections of T_3, 72 and 24 hours prior to electroporation, as indicated under Materials and methods. As shown, the activity of the WT construct was increased 8.42-fold in response to two doses of T_3, when compared to the LDL receptor promoter activity seen in the Hx animals (Fig. 3). Mutating the −612 TRE reduced the T_3-dependent stimulation of the LDL receptor promoter about 49% (Fig. 3). Similarly to the results obtained in H4IIE cells (Lopez et al., 2007), mutating the −156 TRE had no significant effect on the T_3 dependent activation of the LDL receptor promoter (data not shown). When both TREs were mutated, the stimulation by T_3 was reduced by 76% (Fig. 3).

2.3. In vivo analysis of transcription factor binding to the LDL receptor promoter

To investigate the effect of T_3 treatment on the binding of different transcription factors to the TREs *in vivo*, ChIP assays were performed as described under Materials and methods. As shown in Fig. 4, T_3

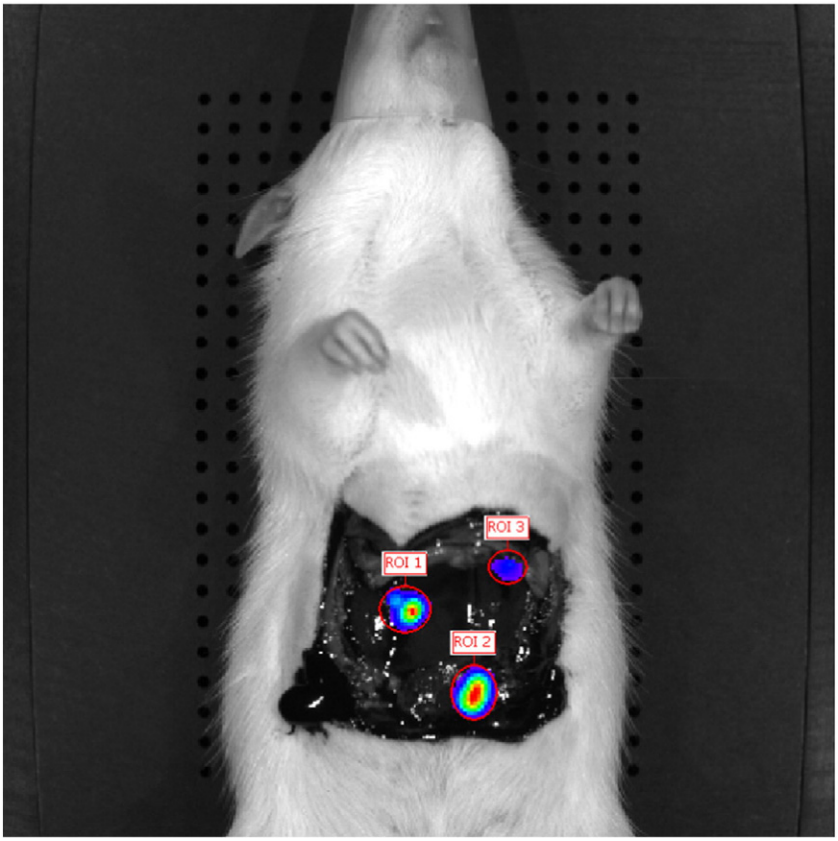

Fig. 1. *In vivo* imaging of liver sites where promoter luciferase constructs were introduced into a normal (NR) rat. Imaging was performed 24 hours after *in vivo* electroporation using a Xenogen *in vivo* Imager. Luciferase substrate was injected intraperitoneally prior to imaging. The regions of interest (ROI) are circled.

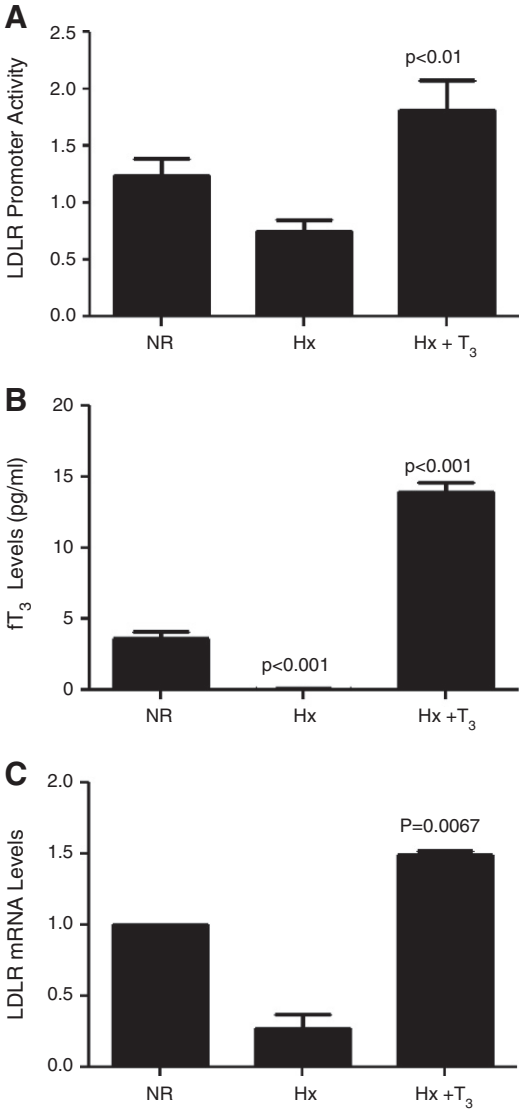

Fig. 2. Effects of T_3 on the expression of the LDL receptor gene in rat livers. **A**. *In vivo* reporter gene studies using electroporation. For this experiment, 40 μg of the WT LDL receptor (LDLR) promoter construct were injected at different sites into the livers of NR and hypophysectomized (Hx) rats. Some of the Hx rats received 1 dose of T_3 16 hours before euthanization (Hx + T_3). Preparation of liver lysates and measurement of luciferase activity were carried out as described under Materials and methods. The data are presented as mean LDLR promoter activity ± SEM for six electroporation sites for at least two animals. The *p* value was obtained by comparing the Hx and Hx + T_3 conditions. **B**. Levels of fT_3 in serum samples obtained from the same animals used in (**A**.) were determined using ELISA. The data are presented in term of pg/dl. *p* Values were obtained by comparing NR versus Hx and Hx versus Hx + T_3, respectively. **C**. Effect of T_3 on hepatic LDLR mRNA levels. Total RNA samples prepared from the same rats employed in (**A**.) were analyzed by real-time RT-PCR using LDLR specific primers. The data are represented as mean relative LDLR mRNA levels ± SEM where the value of receptor mRNA for the normal sample was set to 1.0. The *p* value was obtained by comparing the Hx and Hx + T_3 conditions.

treatment significantly enhanced the binding of TRβ1 to both TRE sites. The degree of binding enhancement was about 3.65- and 3.0-fold for the −612 and −156 motifs, respectively, similar to the binding enhancement seen with the positive control, RNA Pol II (Fig. 4). Binding of RXRα, by itself, to the −612 and −156 elements, was minimal (data not shown). However, T_3 treatment enhanced the binding of TRβ1 together with RXRα to about 2-fold for both TRE sites (Fig. 4). Interestingly, significant binding enhancements were also observed for PPARα and USF-2, two transcriptional factors also known to bind to these promoter elements (Adams et al., 2010; Boone et al., 2009; Jackson-Hayes et al., 2003).

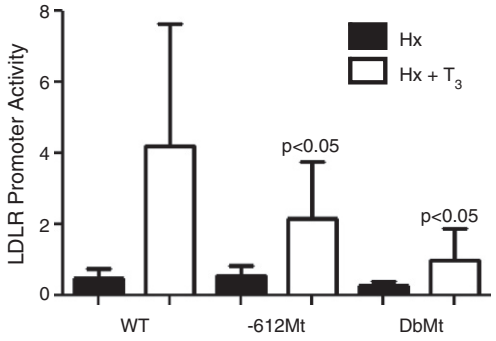

Fig. 3. *In vivo* contributions of the −612 and −156 TREs to the T_3-dependent activation of the LDLR promoter. For this experiment, 10 μg of the WT, −612Mt, and DbMt LDLR promoter constructs were electroporated into the livers of Hx and Hx + T_3 rats. Two doses of T_3 were given 72 and 24 hours before electroporation as described under Materials and methods. Lysate preparation and luciferase assays were carried out. The data are presented as mean LDLR promoter activity ± SEM for at least four electroporation sites per each treatment condition. *p* Values were obtained by comparing to the WT construct.

2.4. In vivo siRNA studies

To evaluate the relative involvement of endogenous TRβ1, PPARα, and USF-2 in mediating transcriptional activation of the hepatic LDL receptor gene by T_3, siRNAs to knockdown these transcriptional factors were utilized. The siRNAs were introduced by *in vivo* electroporation together with the WT LDL receptor promoter construct into euthyroid rats. The LDL receptor promoter activity obtained in the presence of the siRNAs was directly compared with the promoter activity seen in the presence of saline. As shown in Fig. 5A, the receptor promoter activity was reduced to about 25% of control in the presence of siRNAs against TRβ1. LDL receptor mRNA levels were reduced to about 35% of control by the same siRNAs (Fig. 5B). Knockdown of PPARα and USF-2 using siRNAs significantly reduced LDL receptor promoter activity to about 35% of control (Fig. 5A), while LDL receptor mRNA levels were reduced to about 50% of control (Fig. 5B).

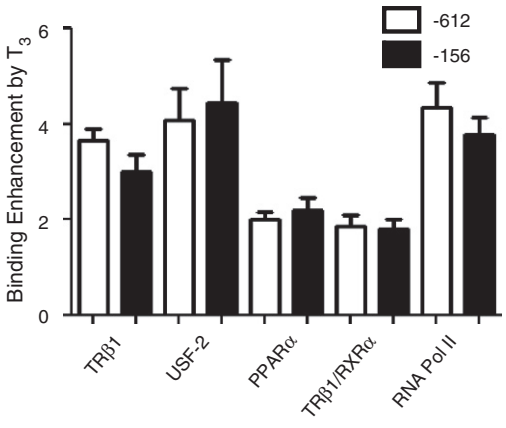

Fig. 4. *In vivo* binding of transcriptional factors to the LDL receptor promoter. ChIP assay followed by real-time PCR analysis were performed on liver samples obtained from Hx and Hx + T_3 rats. Negative (IgG) and positive (RNA Pol II) control antibodies were used. PCR data were normalized to input DNA and presented as mean binding enhancement by T_3 ± SEM, where the binding to chromatin from livers of Hx + T_3 rats was expressed relative to the binding to chromatin from livers of Hx rats. All the differences shown were found to be statistically significant ($p<0.05$), relative to the total input DNA. Samples from at least four animals were considered for each treatment condition.

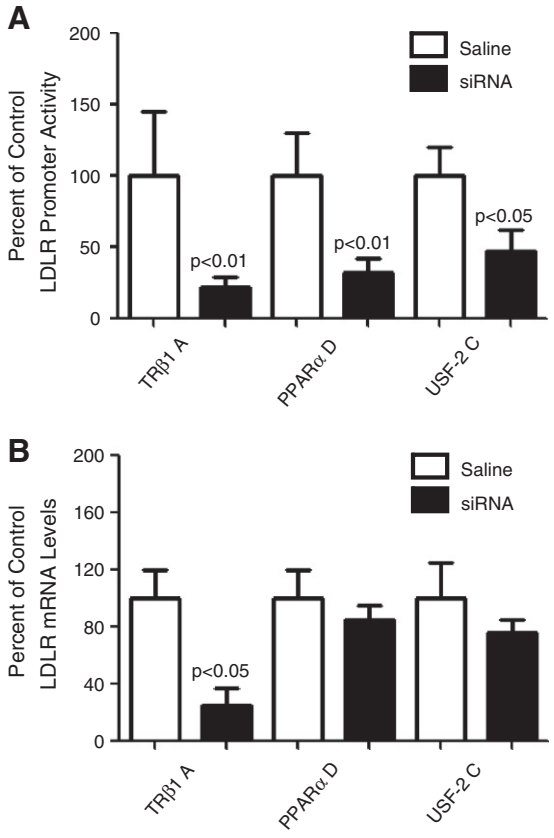

Fig. 5. Effects of *in vivo* siRNA knockdown of transcriptional factors on LDL receptor gene expression. Studies were performed in NR rats. Six sites, two in each liver lobe, were injected with the WT LDLR promoter construct and the indicated siRNAs or saline (negative control), followed by electroporation of the injection site. **A**. Liver punches were excised 24 hours later for lysate preparation followed by luciferase assays. **B**. Liver samples adjacent to the electroporation sites were used in the preparation of RNA samples that were analyzed using real-time PCR. In both panels, the data are reported as mean ± SEM for at least eight animals per treatment condition. *p* Values were obtained by comparing to the activity of the WT construct in the presence of saline.

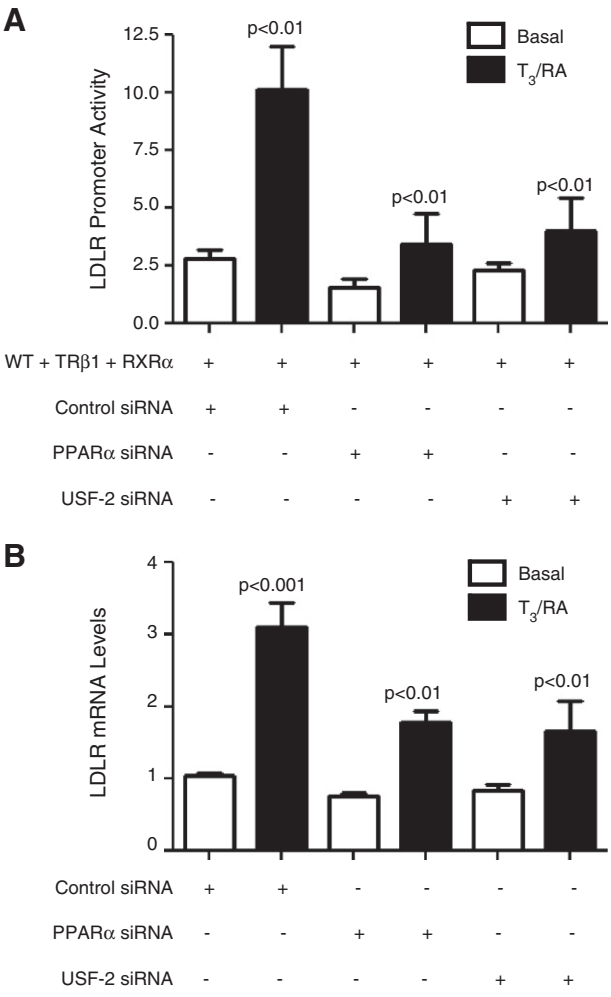

Fig. 6. *In vitro* confirmation of the role of PPARα and USF-2 on the T_3-dependent activation of the LDL receptor gene. Studies were performed in H4IIE cells grown in a thyroid hormone deficient medium. **A**. Promoter analysis experiment. Cells were co-transfected with the WT LDL receptor promoter construct, the TRβ1-pCMVS4 and RXRα-pRcRSV expression plasmids, the Renilla plasmid, and the indicated siRNAs using Fugene 6 transfection reagent. Lysing of the cells was performed 48 hours after transfections. Some cells were treated with T_3 (1 μM) and 9-cis retinoic acid (RA; 1 μM) for 16 hours prior to lysing. Luciferase assays were carried out as described in Materials and methods. **B**. Analysis of mRNA levels. Cells were transfected with control siRNAs or with siRNAs for either PPARα or USF-2 as described above. Treatment with T_3 and RA was carried out as described in A. Cells were used in the preparation of RNA 24 hours after transfection. RNA samples were analyzed using real-time PCR. In both panels, the data are presented as mean ± SEM for three samples per treatment condition. *p* Values for the samples transfected with the control siRNA and treated with T_3/RA were obtained by comparing to the control siRNA samples no treated with the hormones. All the other *p* values were obtained by comparing to the control siRNA sample treated with T_3/RA.

2.5. Confirmation of the PPARα and USF-2 siRNA studies in H4IIE cells

To confirm the involvement of PPARα and USF-2 in the T_3-dependent transcriptional activation of the hepatic LDL receptor gene, knockdown studies using siRNAs against these two transcription factors were carried out in H4IIE cells. The LDL receptor promoter activity and receptor mRNA levels obtained in the presence of the siRNAs against PPARα and USF-2 were directly compared with the results obtained in the presence of a negative control siRNA. In the presence of T_3/9-cis retinoic acid (RA), the receptor promoter activity was reduced to about 30% of control when siRNAs against PPARα and USF-2 where introduced (Fig. 6A). In agreement, LDL receptor mRNA levels were reduced to about 50% of control by the same siRNAs (Fig. 6B).

3. Discussion

3.1. Direct activation

The present study demonstrated that T_3 activates transcription of the hepatic LDL receptor *in vivo* through TREs located at −612 and −156, with the −612 element being the major element. These results are in agreement with previous *in vitro* findings made using rat liver hepatoma cells (Lopez et al., 2007). T_3 activation of LDL receptor transcription through these sites via the TRβ1, the major thyroid hormone receptor in liver (Angelin and Rudling, 2010), explains the rapid (within 1 hour) activation of receptor transcription previously reported (Ness and Lopez, 1995; Ness and Zhao, 1994). T_3 acts directly rather than via the sterol response element binding protein (SREBP) pathway to activate hepatic LDL receptor transcription (Lopez et al., 2007; Costet, 2010), since it was demonstrated that T_3 failed to restore SREBP-2 levels within the 2-hour time period when transcription of the LDL receptor is fully activated (Lopez et al., 2007).

3.2. Requirement for both TREs

Previously, binding of TRβ1 to the −612 and −156 sites of LDL receptor promoter was demonstrated *in vitro* using electrophoretic mobility shift assays (EMSA) (Lopez et al., 2007). *In vitro*, the binding of TRβ1 to the −612 site was found to be stronger than the binding to −156 (Lopez et al., 2007). Herein, ChIP assays demonstrated that

in vivo, T_3 treatment enhanced TRβ1 binding to both TRE elements to a similar extent. Interestingly, the binding of TRβ1 by itself to the LDL receptor TREs was shown to be 1.83-fold higher than the binding of TRβ1/RXRα heterodimers.

TRβ1 is a type II nuclear receptor, which in the absence of T_3, usually binds to TREs in the promoter of target genes as heterodimers with RXRα (Harvey and Williams, 2002; Yen, 2001). Under these conditions, the TRβ1/RXRα complex interacts with corepressor proteins (i.e., NcoR2, TRAC-1, or SMRTe), which assist in repressing transcription of a target gene (Harvey and Williams, 2002; Yen, 2001). When T_3 is present, the corepressor is released as a result of a conformational change in TRβ1 (Harvey and Williams, 2002; Yen, 2001). This leads to recruitment of coactivator proteins, such as TRAP (Harvey and Williams, 2002; Yen, 2001). The complex formed between the TRE motif, TRβ1/RXRα, and the coactivator, recruits RNA polymerase resulting in an increase in transcription of the target gene (Harvey and Williams, 2002; Yen, 2001). Thus, our finding that, in the case of the LDL receptor, T_3 enhances the binding of TRβ1, possibly in the form of homodimers, and to a lesser extent, of TRβ1/RXRα heterodimers, to the receptor promoter, represents a novel in vivo regulatory mechanism of transcriptional regulation by thyroid hormone. In fact, the binding of T_3 receptors in the form of monomers and/or homodimers has been reported only in *in vitro* assays (Miyamoto et al., 1993). Additional studies are required to identify corepressor and/or coactivator proteins associated with the transcriptional regulation of the LDL receptor gene in the absence and presence of T_3, respectively.

3.3. Possible role of PPARα and USF-2

In vivo and *in vitro* knockdown studies using siRNAs against PPARα and USF-2 confirmed that these two transcription factors are required for maximum T_3-dependent activation of the LDL receptor promoter. PPARα is a nuclear receptor that like the T_3 receptor, it has the ability to form heterodimers with RXRα, bind to the half-site AGGTCA, and activate transcription in response to its ligand (fatty acids) (Forman et al., 1997; Stael et al., 1998). It is possible that in the case of the LDL receptor, PPARα competes with TRβ1/RXRα for the same binding sites, and since T_3 can enhance fatty acid synthesis in the liver (Blennemann et al., 1992), the excess of fatty acids could promote PPARα binding to the LDL receptor gene. In fact, activation of PPARα has been reported to induce the expression of the hepatic LDL receptor both *in vitro* and *in vivo*. (Huang et al., 2008). Furthermore, fibrates such as fenofibrate, which are synthetic ligands for PPARα, activate LDL receptor transcription (Huang et al., 2008). It will be interesting to investigate in future studies whether PPARα/TRβ1/RXRα could form trimers on the LDL receptor TREs.

USF-2, on the other side, binds to E-box motifs (Corre and Galibert, 2005), and in agreement with this, several E-boxes have been identified around the LDL receptor TREs, especially the −156 motif (Lopez and Ness, 2006). Interestingly, USF-2 has been implicated in the T_3-dependent regulation of genes such as HMG-CoA reductase and carnitine palmitoyltransferase-I (CPT-I). In the case of the CPT-1 gene, USF-2 is able to directly interact with TR to regulate transcription. Thus, it could be possible that USF-2, PPARα, and TRβ1/RXRα are able to cooperate *in vivo* to control the transcription of the LDL receptor. There are no previous reports linking USF-2 to the transcriptional activation of the LDL receptor gene.

4. Conclusion

The findings reported herein extend the previous observations made in rat hepatoma cells to the whole animal. They indicated that both the −612 and −156 TREs are required for *in vivo* activation of hepatic LDL receptor transcription by thyroid hormone. These studies also confirmed that TRβ/RXRα are the primary transcription factors involved in this process, but showed, for the first time, that PPARα and USF-2 are required for maximum activation of the LDL receptor promoter in the presence of T_3.

5. Materials and methods

5.1. Experimental animals

NR and Hx male Sprague–Dawley rats, weighing 125 to 150 g, were purchased from Harlan (Indianapolis, IN). NR rats received Tekland rat chow and water *ad libitum*. Hx rats received Tekland Iodine Deficient chow and water *ad libitum*. Both sets of rats were housed in a reverse-cycle light-controlled room with a 12-hour light/dark period. In some experiments, Hx rats used in electroporation received a single injection of 0.1 mg/kg T_3 16 hours prior to euthanization. In other experiments, Hx rats received an injection of 1.0 mg/kg T_3 72 hours prior to electroporation and an additional injection of 0.25 mg/kg T_3 24 hours prior to electroporation. These rats were euthanized 24 hours following electroporation, at the mid-dark period (Boone et al., 2009). The animals were cared for according to the NIH guidelines set forth in the "Guide for the Care and Use of Laboratory Animals". All procedures were conducted according to protocol 3571 approved by the University of South Florida Institutional Animal Care and Use Committee.

5.2. Plasmid construction

The rat WT, −156Mt, −612Mt, and DbMt LDL promoter-luciferase reporter gene constructs were prepared as previously described (Lopez et al., 2007; Lopez and Ness, 2006). All clones were confirmed by restriction analysis followed by DNA sequencing at Genewiz, Inc. (South Plainfield, NJ).

5.3. In vivo electroporation

Ten to forty μg of the indicated LDL receptor promoter constructs were directly introduced into the livers of rats by electroporation as recently described (Lagor et al., 2007). pHRL-CMV Renilla vector (Promega, Madison, WI) was co-electroporated at a 1:2000 dilution to control for electroporation efficiency. The total volume of injected DNA was 50 μL as previously described (Boone et al., 2009). Two 5 mm electroporation sites in each of three liver lobes per rat were used. The area transfected is restricted to that inside the 5 mm circle as evidenced by imaging of live rats (Fig. 1).

5.4. Luciferase assays

The livers of electroporated animals were harvested 24 hours following electroporation. Once removed, the electroporated regions of the liver were extracted using a 5 mm cork-borer. A circle of six small dots defined the transfection sites. Approximately 100 mg of liver was placed in 2 mL of 1× Passive Lysis Buffer (Promega Corp., Madison, WI) and homogenized using a Polytron tissue disrupter. The lysate was centrifuged at 16,000 ×*g* for 5 minutes and the supernatant assayed for luciferase activity using the Dual Luciferase Assay Kit from Promega. Luciferase activity was calculated as the average ratio of firefly (reporter) to renilla luciferase (Boone et al., 2009).

5.5. Thyroid hormone assay

Blood was collected from animals at time of euthanasia and centrifuged at 16,000 ×*g* for 5 min. Supernatant was collected and used for determination of T_3 levels using the fT_3 ELISA from Calbiotech (Spring Valley, CA) (Lopez et al., 2007; Boone et al., 2009).

5.6. Real-time PCR

Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) either from liver samples distant from electroporated sites (to determine basal mRNA levels) or from adjacent sites electroporated with siRNAs (to measure the effects of those siRNAs on endogenous LDL receptor mRNA levels). The RNA samples were DNAse treated using the TURBO DNA-Free Kit (Ambion, Austin, TX) and converted to cDNA using the Reverse Transcription System (Promega) as previously described (Lopez et al., 2007). Primer sequences for the rat LDL receptor and the 18s rRNA have been previously listed (Lopez et al., 2007). The parameters for the PCR reactions were denaturation at 95 °C for 5 minutes, followed by 40 cycles of 95 °C for 15 seconds and annealing and extension at 61 °C for 1 minute. The melt curve started at 55 °C and increased by 0.5 °C each 10 seconds until it reached 95°. All samples were run in duplicate on a Bio-Rad Chromo4 DNA Engine thermal cycler using SYBR green chemistry (Lagor et al., 2007). The data were analyzed by the Comparative CT method as previously described (Lagor et al., 2007).

5.7. Chromatin immunoprecipitation (ChIP) assay

Chromatin was prepared and immunoprecipitated using the Pierce Agarose ChIP Kit (Pierce Corp, Rockford, IL). All named buffers were included in the kit. Liver samples, 100 mg each, were taken from NR, Hx, and Hx + T_3. The tissue was minced and fixed in 10 mL of 1% formaldehyde Fixation Solution for 10 minutes at room temperature. One mL of Glycine Solution was added to each tube. After rotating for 5 minutes, samples were centrifuged for 5 minutes at 720 ×*g*. The pellets were resuspended in 10 mL 1× PBS (Wash 1) and centrifuged at 720 ×*g* for 5 min. The supernatant was aspirated and Wash 1 was repeated. The cell pellets were resuspended in 1 ml ice-cold 1× PBS supplemented with 10 μL Halt Cocktail (containing protease inhibitors) and incubated on ice for 30 minutes. The cells were homogenized in a Dounce homogenizer using 10 strokes on ice to release the nuclei, transferred to a 1.5 mL tube and centrifuged at 3000 ×*g* for 5 minutes at 4 °C. The nuclei were resuspended in 1 mL of Lysis Buffer 1, vortexed for 15 seconds, and incubated on ice for 10 minutes. The tubes were centrifuged at 9000 ×*g* for 3 minutes and the supernatant was discarded. The nuclei were resuspended in 1 mL of MNase Digestion Buffer Working Solution and 2.5 μL Micrococcal Nuclease (based on optimization results), vortexed and incubated in a 37 ° C water bath for 15 minutes (mixed by inversion every 5 minutes). Ten microliters of MNase Stop Solution was added to each tube, vortexed, and incubated on ice for 5 minutes. Each tube was centrifuged for 5 minutes to recover nuclei and the supernatant was discarded. The nuclei was resuspended in 50 μL of Lysis Buffer 2 and incubated on ice for 15 minutes, vortexing for 15 seconds every 5 minutes. Each tube was centrifuged at 9000 ×*g* for 5 minutes and the supernatant (containing digested chromatin) was transferred to a new 1.5 mL tube. Chromatin size was checked by agarose electrophoresis to ensure an average size between 200 and 500 bp. Forty-five microliters of chromatin, 5 μg of antibody, and 20 μL of Agarose resin were used per each immunoprecipitation (IP) reaction. The antibodies used for the IP reactions were: USF-2 (SCBT, sc-861x), RXRα (SCBT, sc-553x), TRβ1 (SCBT, sc-33312x), and PPARα (SCBT, sc-9000x), all from Santa Cruz Biotechnology (Santa Cruz, CA). The negative (IgG) and positive (RNA Pol II) control antibodies were provided part of the Pierce Agarose ChIP Kit. The reactions containing antibodies were rotated at 4 °C for at least 15 hours. Final DNA samples were analyzed in triplicates by real-time PCR as described under real-time PCR method above with the omission of a melt curve. The primers used to the PCR reactions were: 5′-CTCTGAGTGCTATTTATGGT-3′ and 5′-CGGAGCTCCCA ACTCGTGTG-3′ for −612 and 5′-AACCTCGTCCCTAGGGCTGA-3′ and 5′-ACAGGATCACGGGTCCCACG-3′ for −156. The relative level of transcription factor binding was quantified by correcting for the amount of input DNA and negative control antibody DNA (background). Relative induction of binding was calculated as the ratio of relative binding of the factor in the Hx + T_3 chromatin preparation to the relative binding of the factor in the Hx chromatin preparation.

5.8. siRNA knockdown study

Ten μg each of USF-2 siRNA (SABiosciences cat# SIR449799ABCD; Valencia, CA), PPARα siRNA (Dharmacon cat# LQ-080000-01-0010; Lafayette, CO), and TRβ1 siRNA (Dharmacon cat# LQ-097456-01-0010) were co-electroporated with 10 μg of WT LDL receptor promoter construct in a final volume of 50 μL using 6 sites per animal. Saline was used as the negative control for the siRNA samples. The electroporated sites were removed 24 hours later using a 5 mm cork-borer. Lysates were prepared from electroporated sites. Luciferase assays were performed as described above. Liver samples adjacent to the electroporation sites were used in the preparation of RNA samples that were analyzed using real-time PCR to measure knockdown of endogenous LDL receptor mRNA (Boone et al., 2009).

5.9. In vitro studies

For the promoter analysis experiments, cells were plated in 12-well plates at the density of 1×10^5 cells per well and incubated for 24 hours at 37 °C, 5% CO_2, in low glucose-DMEM medium supplemented with 5% (v/v) thyroid hormone depleted serum (THDS) and antibiotics. The THDS was prepared using Dowex resin as previously described (Samuels et al., 1979). Cells were co-transfected with the WT LDL receptor promoter construct and the TRβ1-pCMVS4 and RXRα-pRcRSV expression plasmids, 1 μg of each recombinant vector per well, using Fugene 6 transfection reagent, as previously described (Lopez et al., 2007; Lopez and Ness, 2006). Wells receiving siRNAs for either PPARα or USF-2 were also co-transfected with 1 μg total per well, of the indicated siRNAs (0.25 μg each of the four siRNAs provided for each transcriptional factor). Control wells were co-transfected with 1 μg per well of the universal negative control (non-targeting) siRNAs prepared by Integrated DNA Technologies (Coralville, IA). It has been shown in pilot studies that this negative control does not affect the expression of the LDL receptor gene (data not shown). Co-transfection of a Renilla plasmid (0.5 μg per well) under the control of the simian virus 40 early enhancer/promoter region was used to correct for differences in transfection efficiencies. After transfection, the cells were allowed to incubate for 48 hours at 37 °C, 5% CO_2. Sixteen hours prior to the end of the incubation period, some wells were treated with T_3 (1 μM) and 9-cis retinoic acid (RA; 1 μM). Lysate preparation was carried out as previously described [promoter and T3 paper]. Firefly and Renilla luciferase activities were measured using the Dual-Glo Luciferase Assay Kit from Promega and a SpectraMax M5 microplate reader (Molecular Devices, LLC; Sunnyvale, CA). Luciferase activity was calculated as the average ratio of firefly to renilla luciferase as described above (Boone et al., 2009).

For the mRNA analysis experiments, cells were plated in 6-well plates at the density of 8×10^5 cells per well and incubated for 24 hours at 37 °C, 5% CO_2, in low glucose-DMEM medium supplemented with 5% (v/v) THDS and antibiotics. Cells receiving siRNAs for either PPARα or USF-2 were transfected with 3 μg total per well of the indicated siRNAs (0.75 μg each of the four siRNAs provided for each transcriptional factor) using Fugene 6 transfection reagent as described above. Control wells were co-transfected with 3 μg per well of the universal negative control. After transfection, the cells were allowed to incubate for 24 hours at 37 °C, 5% CO_2. Sixteen hours prior to the end of the incubation period, some wells were treated with T_3 (1 μM) and RA (1 μM). RNA isolation and preparation

of ssDNA were completed as previously described (Lopez et al., 2007). Real-time PCR reactions were performed using 100 ng of ssDNA, the LDL receptor and 18s rRNA specific primers described above, the Applied Biosystems SYBR green PCR Master Mix, and the AB real-time PCR system. The parameters for the PCRs were: denaturation at 95 °C for 10 minutes, followed by 45 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 °C for 15 seconds, and extension at 72 °C for 30 seconds. Once again, quantitation of the results was performed using the Comparative CT method.

5.10. Statistical analysis

For the *in vivo* studies, significant differences were determined using Excel's *t*-test: Two-Sample Assuming Unequal Variances and confirmed using the nonparametric Wilcoxon matched-pairs signed rank test. For the *in vitro* studies, all the experiments were performed in triplicates. Data from the individual parameters were compared by analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparison test when applicable. A $p<0.05$ was considered significant for all tests.

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