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Thyroid Hormone Receptor β (TRβ) and Liver X Receptor (LXR) Regulate Carbohydrate-response Element-binding Protein (ChREBP) Expression in a Tissue-selective Manner*[S]

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Thyroid hormone (TR) and liver X (LXR) receptors are transcription factors involved in lipogenesis. Both receptors recognize the same consensus DNA-response element *in vitro*. It was previously shown that their signaling pathways interact in the control of cholesterol elimination in the liver. In the present study, carbohydrate-response element-binding protein (ChREBP), a major transcription factor controlling the activation of glucose-induced lipogenesis in liver, is characterized as a direct target of thyroid hormones (TH) in liver and white adipose tissue (WAT), the two main lipogenic tissues in mice. Using genetic and molecular approaches, ChREBP is shown to be specifically regulated by TRβ but not by TRα *in vivo*, even in WAT where both TR isoforms are expressed. However, this isotype specificity is not found *in vitro*. This TRβ specific regulation correlates with the loss of TH-induced lipogenesis in TRβ$^{-/-}$ mice. Fasting/refeeding experiments show that TRβ is not required for the activation of ChREBP expression particularly marked in WAT following refeeding. However, TH can stimulate ChREBP expression in WAT even under fasting conditions, suggesting completely independent pathways. Because ChREBP has been described as an LXR target, the interaction of LXR and TRβ in ChREBP regulation was assayed both *in vitro* and *in vivo*. Each receptor recognizes a different response element on the ChREBP promoter, located only 8 bp apart. There is a cross-talk between LXR and TRβ signaling on the ChREBP promoter in liver but not in WAT where LXR does not regulate ChREBP expression. The molecular basis for this cross-talk has been determined in *in vitro* systems.

De novo lipogenesis allows the synthesis of new molecules of fatty acids from acetyl CoA. High glucose and insulin concentrations induce this process, converting the excess energy into triglycerides, a more relevant molecule for storage purposes. In rodents, both liver and WAT[3] are efficient sites for lipogenesis. The synergic actions of insulin and glucose on the expression of lipogenic genes are mediated by key transcription factors. Insulin acts mainly through SREBP (sterol regulatory element-binding protein)-1c (1), whereas carbohydrate-response element-binding protein (ChREBP) is the master factor for glucose-induced lipogenesis (2). ChREBP physiological function has mainly been studied in the liver. ChREBP$^{-/-}$ mice display a diminution in both basal and glucose-induced liver fatty acid synthesis due to the decreased expression of ChREBP glycolytic and lipogenic targets (3). Most interestingly, the ChREBP$^{-/-}$ mutation protects *Ob/Ob* mice from obesity and reduces their plasma glucose level (4), suggesting that inhibition of ChREBP might be of pharmacological interest to treat the metabolic syndrome. ChREBP is expressed in many other tissues including WAT, where its possible lipogenic role is presently unclear.

ChREBP activity is mainly regulated by post-translational modifications that control its relocation to the nucleus and its DNA binding activity (5). When active, ChREBP turns on the expression of genes harboring a ChoRE (carbohydrate-response element) in their promoters. All the genes encoding the enzymes involved in lipogenesis (FAS, ACC, *SCD1*, L-PK, *G6PD*, ME, and *Spot14*) are direct ChREBP targets. During fasting, ChREBP is inactivated and located in the cytoplasm. In contrast ChREBP mRNA level varies in a narrow range. In liver, its level doubles when animals are switched from a fasted to a fed state (6). A similar up-regulation of its expression can be observed in mouse and human hepatocytes exposed to a high glucose concentration (7). In 3T3-L1 cells, insulin, glucose, and fatty acids regulate ChREBP expression (8). In contrast to liver, ChREBP mRNA is very efficiently induced (10-fold) following refeeding in WAT (6, 8). The physiological consequence of this regulation in WAT remains unknown.

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1–3.

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[3] The abbreviations used are: WAT, white adipose tissue; LXR, liver X receptor; RXR, retinoid X receptor; LXRE, LXR-response element; ChREBP, carbohydrate-response element-binding protein; SREBP, sterol regulatory element-binding protein; TH, thyroid hormones; TR, thyroid hormone receptor; TRE, TH-response elements; FAS, fatty acid synthase; PTU, propylthiouracil; qRT-PCR, quantitative RT-PCR; m, mouse; h, human.

Thyroid hormones (TH) up-regulate lipogenesis in liver, but their roles in WAT are controversial (9–11). Their actions are mediated by the TRα and TRβ nuclear receptors, which act as transcription factors by binding to specific TH-response elements (TRE) as homodimers or heterodimers with the nuclear receptor RXR (12). Several genes involved in lipogenesis such as FAS, ACC, *Spot14*, or ME are positively regulated by TH in liver (13, 14). TRE have been identified in some but not all of their promoters. The expression patterns of TRα and TRβ are only partially overlapping (15). In liver, TRβ represents 80% of the TH-bound TR (16), whereas in WAT, both receptors are highly expressed. The phenotyping of different TR KO mice sheds light on the role of each isotype in mediating TH signal (12, 17). Importantly in the organs where they are co-expressed, their function is not necessarily redundant. Recently, two genes were described to be specifically regulated by either TRα1 or TRβ (18) in the outer hair of the developing cochlea, suggesting that each receptor might regulate its own set of targets in response to TH. The lipogenic effect of TH has been attributed to TRβ because in the liver, TH regulation of FAS, ACC, Spot14, and ME is lost in TRβ$^{-/-}$ mice (13). However, because TRα is weakly expressed in this tissue, liver might not be the most appropriate tissue to assay isotype specificity. The LXR nuclear receptors could be involved in the lipogenic action of TR. Different levels of potential cross-talk between LXRs and TRβ have indeed been described (19). For instance, LXRα expression has been previously described to be regulated by TH in mouse liver (20). At a functional level, LXRs and TRβ regulate a common set of events especially in the liver, where both receptors stimulate lipogenesis and cholesterol disposal. From a molecular point of view, these receptors can bind to identical (DR4) elements *in vitro*, although only one of these elements (in the *cyp7a1* gene promoter) has been characterized as a common LXR- and TR-response element (21). Interestingly, LXRs were recently shown to directly control ChREBP expression by binding to a DR4 element in its promoter (22). Another DR4 element located in the near vicinity was shown to mediate the positive effect of TH on ChREBP expression in the mouse liver (23).

In this study, we show that TH directly activate ChREBP not only in liver (23) but also, to a higher extent, in WAT. *In vivo*, this effect is TRβ-, but not TRα-, dependent, although both TR isoforms are strongly expressed in WAT, whereas *in vitro*, both isoforms can drive the expression of a reporter gene downstream of the ChREBP promoter and bind to the same response element. Despite its capacity to up-regulate ChREBP expression, TRβ is not required for ChREBP induction in response to the fasting/refeeding protocol. TRβ acts independently of LXR. Finally, although ligands for these receptors could co-regulate the ChREBP promoter in liver, different approaches point out to a mutually exclusive binding of LXR and TR to this promoter.

EXPERIMENTAL PROCEDURES

Plasmids—The expression plasmids were all pSG5-based vectors (mouse TRα, rat TRβ1, mouse RXRα, and mouse LXRα). The different promoters were cloned in the pGL3 basic vector and/or PGL4.70(hRluc) (Promega, Charbonnières, France). The 3 kbp upstream of the mouse ChREBP transcription start site were amplified by PCR using the primers ChREBPprom, cloned in pGL3basic/PGL4.70(pChREBP). The mutants (pM1, pM2, and pM1M2) were obtained using site-directed PCR mutagenesis (with M1 and M2 primer pairs). All plasmids were sequenced (Cogenics Genome Express, Meylan, France).

Chemicals—Tri-iodothyronine (T3) and thyroxine (T4) were from Sigma-Aldrich (l'Isle D'Abeau, France), and the synthetic LXR ligand T0901317 (T09) was from Cayman Chemical (Montigny le Bretonneux, France).

Animals and Preparation of Tissue Samples—Knock-out mice were in a C57black6:129sv mixed background. TRβ$^{-/-}$, TRα$^{0/0}$ (17, 24, 25), LXR KO (26), and controls were fed *ad libitum* A04 diet (SAFE, Augy, France), and housed under recommended conditions. 3–5-month-old male mice were used unless indicated otherwise. TH deficiency in adult animals was induced as described with a PTU-containing diet (Harlan Teklad TD95125, Madison, WI) and followed or not by TH (mix of T4 and T3) injection (13). T09 was given by oral gavage once a day for 3 days (10 mg/kg of T09 in 100 μl of methyl cellulose 1%). *Pax8*$^{-/-}$ mice, which are genetically hypothyroid, were previously described to die before weaning (27); however, some spontaneously survived. These rare survivors were used for experiments. For the fasting/refeeding protocol, mice fed a regular chow diet were fasted for 24 h and either refed a 70% high carbohydrate diet (Harlan Teklad TD98090) or kept on fasting for an additional 16 h. Tissues were dissected immediately after cervical dislocation and flash-frozen in liquid nitrogen. For WAT *ex vivo* culture, peritesticular fat pads were dissected and cultured non-dilacerated in 10% charcoal-stripped fetal bovine serum (FBS), 5 ng/ml insulin complemented DMEM (Invitrogen, Cergy-Pontoise, France) for 24 h before the addition of ligands. All animal experiments were performed under animal care procedures and conducted in accordance with the guidelines set by the European Community Council Directives (86/609/EEC).

RNA Extraction and Expression Analyses by Relative Quantitative RT-PCR (qRT-PCR)—RNAs were extracted using TRIzol (Invitrogen). Total RNA was converted to cDNA using the SuperScript II retrotranscription kit (Invitrogen). qRT-PCR analyses were performed using the Quantitect SYBR Green PCR kit (Qiagen, Courtaboeuf, France) on a Stratagene machine MX3000 pro (Stratagene, La Jolla, CA). Duplicates were run for each sample. The results were analyzed according to the ΔΔCT method (28). *36B4* was always used as the reference gene, and the control group was either the non-treated cells or the WT non-injected animals unless otherwise indicated.

Cell Culture and Transient Transfection Assays—HeLa (ATCC-CCL2) and 3T3-L1 (ATCC-CL-173) cells were maintained in DMEM supplemented with 10% FBS (Invitrogen). For 3T3-L1, cells were induced to differentiate using insulin-dexamethasone-Rosiglitazone mix. To observe a better response to T3, cells were switched to DMEM medium supplemented with 10% charcoal-stripped FBS before the experiments. T3 was used at 10^{-8} M, and T09 was used at 10^{-5} M. Cells were harvested 24 h (ChIP or WAT explants) or 36 h (transient transfection assay) after ligand exposure. For transient transfections,

HeLa cells were seeded in 24-well plates and transfected with ExGen (Euromedex, Souffelweyersheim, France) following the manufacturer's recommendations and 0.5 μg of final DNA. pSG5 was added as a carrier when needed. Transfection efficiency was normalized using β-Gal activity brought by co-transfection of CMV β-Gal vector. For each experiment, triplicates of each conditions were done, and each experiment was repeated at least three times, giving similar results. Only one experiment is shown; each point represents the average for the triplicate, and the *error bar* represents their S.D.

Chromatin Immunoprecipitation Assays—The anti-TRα antibody was raised against a C-terminal peptide and affinity-purified with the same peptide; the anti-TRβ (TR-J52) and control IgG (normal mouse IgG) antibodies were purchased from Santa Cruz Biotechnology, and the anti-RNA polymerase II (CTD4H8) from Upstate Biotech Millipore. Cells were cross-linked with 1% formaldehyde before lysis (in 1% SDS, 10 mM EDTA, 50 mM Tris-HCL, pH 8.1) and sonication (200–700 bp DNA fragments). Lysates were diluted and precleared with herring sperm DNA (2 μg/ml), BSA (2 μg/ml), mouse IgG, and protein G-Sepharose (GE Healthcare, Saint-Cyr au Mont d'or, France). Lysates were incubated with the cognate specific antibodies or IgG and protein G-Sepharose. Beads were washed and eluted. Cross-link was reversed by overnight incubation at 65 °C in the presence of RNase A and 200 mM NaCl. Samples were purified (Qiagen) and analyzed by quantitative PCR using the primer pairs NS1, NS2, and S1.

EMSA—mTRα1, mTRβ, mLXRα, and mRXRα were *in vitro* translated (TnT kit, Promega). The different single-strand oligonucleotides (forward) were [γ-^{32}P]ATP-labeled with T4 polynucleotide kinase (Fermentas, Burlington, Ontario) before annealing with their unlabeled antisense (reverse). Probes were purified and counted. 20,000 cpm were used for each binding reaction. Unlabeled specific and nonspecific competitor probes were included at the indicated molar excess.

Hepatic Lipogenesis—Mice were given an i.p. injection of deuterated water (10 ml/kg in 0.9% NaCl isotonic water) followed by administration of drinking water enriched with deuterated water (3% v/v) *ad libitum* for 24 h. Plasma was then collected for the measurement of deuterium enrichment in plasma water and in the palmitate of plasma triglycerides as described previously (29). These enrichments were then used to calculate the contribution, expressed as percentage, of hepatic lipogenesis to the plasma triglyceride pool (30).

Statistics—For mice experiments, the data presented represent the average values for the different animals (4 or 5) from the same genotype given the same treatment. The *error bars* represent S.E. Statistical relevance was determined using the one-variable analysis of variance method.

All the primer sequences are listed in supplemental Table 1.

RESULTS

ChREBP Expression Is Regulated by TH in the Different Lipogenic Tissues in a TRβ-dependent Manner—ChREBP expression was recently shown to be regulated by TH in the liver of C57/BL6 mice treated with PTU/Methimazole (23). Here the regulation of ChREBP was studied in the *pax8* (deprived of thyroid) mutant mice and Sv129 mice treated with PTU (Fig. 1*A*). In both models, TH injection induced ChREBP mRNA level in WAT and to a lesser extent in liver. Consistently, the expression of FAS (a target of both TRs and ChREBP) and L-PK (a ChREBP-only target gene) was also enhanced by TH, suggesting that ChREBP activity (and not only expression) is also up-modulated by TH. The TH-induced regulation of ChREBP was lost in TRβ$^{-/-}$ but not TRα$^{0/0}$ mice, indicating that TRβ was required at least in the two metabolic tissues studied (Fig. 1*B*) despite the strong expression of TRα in WAT. The critical role of TRβ for TH-induced hepatic lipogenesis was demonstrated *in vivo* using wild-type (WT) and TRβ$^{-/-}$ PTU-treated male mice. Although TH efficiently increased lipogenesis in WT (Fig. 1*C*), the response was blunted in TRβ$^{-/-}$ mice. WAT lipogenesis was not measured due to technical limitations.

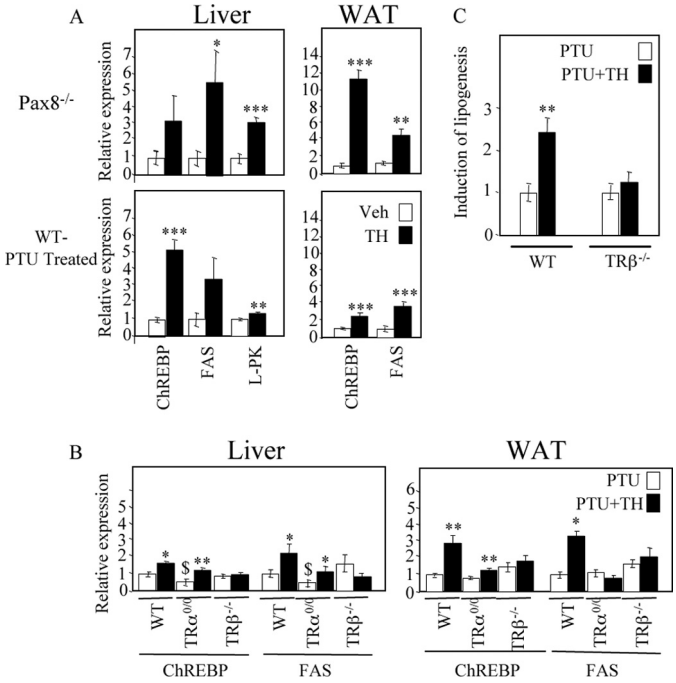

FIGURE 1. **ChREBP expression and lipogenesis are regulated by thyroid hormones in a TRβ-dependent manner.** 3-month-old males either genetically rendered (*pax8*$^{-/-}$) or chemically rendered (WT, TRβ$^{-/-}$, TRα$^{0/0}$-PTU treated) hypothyroid were injected either by PBS (*white bars*) or by TH (*black bars*). In *A* and *B*, $n = 4$ for higher panel, $n = 5$ for lower panel (*A*) and ($n = 5$) (*B*), mRNA encoding lipogenic enzymes were quantified by qRT-PCR. *Veh*, vehicle. In *C*, liver lipogenesis was measured as described under "Experimental Procedures" ($n = 5$). Results are shown as induction as compared with the PTU-treated animals of a given genotype. *Error bars* represent S.E. *Asterisks* and *dollar signs* indicate respectively statistical significance as compared with the PTU treatment of the same genotype and to the equivalent treatment in the WT group ($ or *, $p \leq 0.05$, $$ or **, $p \leq 0.005$, $$$ or ***, $p \leq 0.0005$).

TH/TRβ and Nutritional Status, Two Independent Ways to Regulate ChREBP expression—To determine the involvement of TH signaling in the physiological regulation of ChREBP expression, RNA level was assessed in liver and WAT in response to a fasting/refeeding protocol in both WT and TRβ$^{-/-}$ mice (Fig. 2*A*). In agreement with published data, ChREBP RNA was found only up-regulated 2-fold in the liver (6). In contrast, a dramatic increase of its expression was observed in WAT upon refeeding. This response was also observed in TRβ$^{-/-}$ mice, indicating that TRβ is not required for this physiological process. We next determined whether

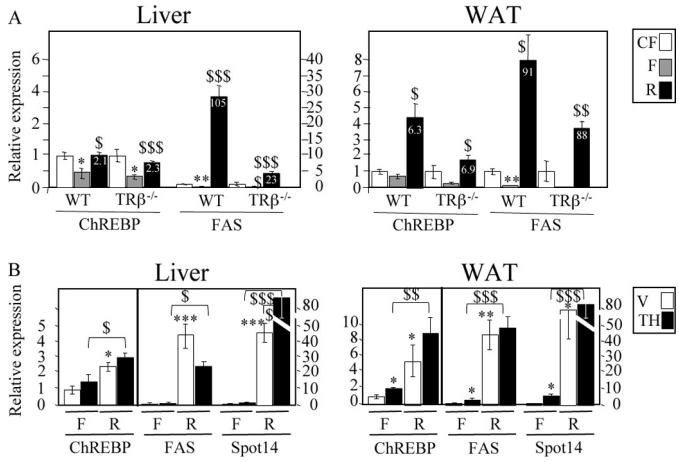

FIGURE 2. **Independent regulation of ChREBP expression by TH/TRβ and nutritional status.** WT and TRβ$^{-/-}$ 3-month-old male mice were submitted to modification of nutritional and/or TH status. In *A*, mice were either fed a regular chow diet (*CF*) or starved for 24 h and then refed (*R*) or not (*F*). ($n = 5$). In *B*, mice were starved for 24 h. One group was kept on fasting (*F*), and the other one was refed (*R*) for an additional 16 h. Half of the animals per group were injected by TH twice, once before the fast and then before the refeeding ($n = 5$). Expression of lipogenic genes was measured by qRT-PCR. *Asterisks* indicate statistical significance (*, $p \leq 0.05$, **, $p \leq 0.005$, ***, $p \leq 0.0005$) as compared with the CF group of the same genotype in *A*, to the *F/V* group in *B*). *Dollar signs* indicate statistical significance between *F* and *RF* groups in *A* and bridged groups in *B* ($, $p \leq 0.05$, $$, $p \leq 0.005$, $$$, $p \leq 0.0005$). In *A*, the value for the relative expression has been fixed to one in each genotype for the CF group. *Error bars* represent S.E.

ChREBP expression could be TH-regulated under all nutritional conditions. ChREBP, as well as FAS and Spot14 mRNAs, were induced by TH in the fasted (non-lipogenic) conditions in WAT (Fig. 2*B*). In contrast, TH failed to significantly activate these genes when mice were refed. This might be due to an already high ChREBP expression under these conditions. In the liver, the extent of ChREBP mRNA regulation is much more limited, and in contrast to WAT, the nutrition signal is dominant, blocking a potential effect of TH on the three target genes in the fasted state.

TRβ Binds to and Activates ChREBP Promoter via the Previously Described LXRE2—The results presented above identified TH/TRβ as a new way to modulate ChREBP expression *in vivo*. The mechanisms responsible for this regulation were then investigated *in vitro*. In contrast to what was observed *in vivo*, TRβ, but also TRα, when co-expressed with RXRα, was able to activate the 3.2-kbp ChREBP proximal promoter (Fig. 3*A*) in the presence of TH (Fig. 3*B*). LXRα, previously described to activate this same portion of the promoter (22), was used as a positive control. Two DR4 elements (LXRE1 and LXRE2) were described in the mouse ChREBP promoter, with LXRE1 being involved for LXR response (22) and LXRE2 being necessary for TH response (23). These binding specificities were confirmed here by the EMSA data (Fig. 3*C*). All three receptors bound to a 44-bp probe encompassing the two LXREs. However, LXR binding was competed only by an LXRE1WT but not mutated probe, whereas TRβ1 or TRα binding was only competed by an LXRE2 WT but not mutated probe. The dependence on these sites for transcriptional responsiveness to either TR or LXR was less obvious in the transfection assay (Fig. 3*B*). The double M1M2 mutant still showed responsiveness to both compounds. This apparent discrepancy with EMSA results and published data for TR (23) is likely due to the inability of the four point mutations introduced in each promoter construct to efficiently prevent TR binding. For LXR, Cha and Repa (22) actually also observed a residual induction of similar pM1 and pM1M2 constructs by the LXR agonist T09. This suggests either that besides LXRE1, some other region(s) of the promoter could mediate the response to LXR or that as for TR, the mutations introduced in LXRE1 are not disruptive enough.

ChIP experiments were performed to investigate the molecular mechanisms underlying the TR isoform specificity in the regulation of the endogenous ChREBP promoter. Differentiated 3T3L1 adipocytes in which ChREBP mRNA is also induced by TH were used. Similar to WAT, these cells express both TRα and TRβ (31). Both TRs were detected on the region containing the LXREs but not on the upstream or downstream promoter regions. TR binding was independent of T3 in agreement with the accepted model for TR action. In contrast, RNA polymerase II was enriched at the transcriptional start site only in the presence of T3 (Fig. 3*D*). Altogether, these data clearly demonstrate that both TRα and TRβ bind to the LXRE2 in the ChREBP promoter and allow its induction in the presence of T3 at least in a reporter system.

Cross-talk between TRβ and LXR Signaling for the Regulation of ChREBP Expression—Published work described the LXRα gene as a TH target in mouse liver (20). In the present study, no significant regulation of LXRα expression by TH or T3 was detected in the different models and experiments performed (Fig. 4, *A* and *D*). Furthermore, TH was capable of activating the expression of ChREBP as well as other lipogenic genes in the liver of PTU-treated LXR KO mice (Fig. 4*A*). The induction of ChREBP expression by TH is thus LXR-independent. TRβ and LXR activate the ChREBP promoter by respectively binding to LXRE2 and LXRE1, two elements located in the close vicinity of each other. We thus assayed a potential functional interaction between the two signaling pathways. In liver but not in WAT, TH induction of ChREBP expression was significantly higher in LXR KO mice than in WT (4.5-fold *versus* 2.9-fold, respectively, Fig. 4*A*), suggesting that LXR might limit TRβ access to the promoter in WT liver. Such an increase is not observed for the regulation of other genes such as FAS, which is known to be regulated by both pathways. To document this interference for promoter binding, transfection experiments were performed in the presence of non-limiting amounts of RXR. Transfected alone, TRβ or LXR induced pChREBP activity in the presence of their cognate ligands (respectively, TH and T09). Remarkably, co-transfection of both decreased the response to each ligand, LXR-dependent activity being more affected than TR (Fig. 4*B*, *left panel*) by this inhibition. T09 and T3 displayed additive effects when both receptors were present. These observations support the fact that concomitant binding of the two receptors to a single ChREBP promoter does not occur. This mutual inhibition was also observed to a lesser extent for both TR and LXR activities when increasing amounts of the other receptor were added. (Fig. 4*B*, *right panel*). Finally direct evidences for a mutually exclusive binding were obtained by EMSA experiments. As shown previously in Fig. 3, both receptors bind as RXR heterodimers to a 44-mer probe containing the two WT LXREs. These two complexes migrated at the dif-

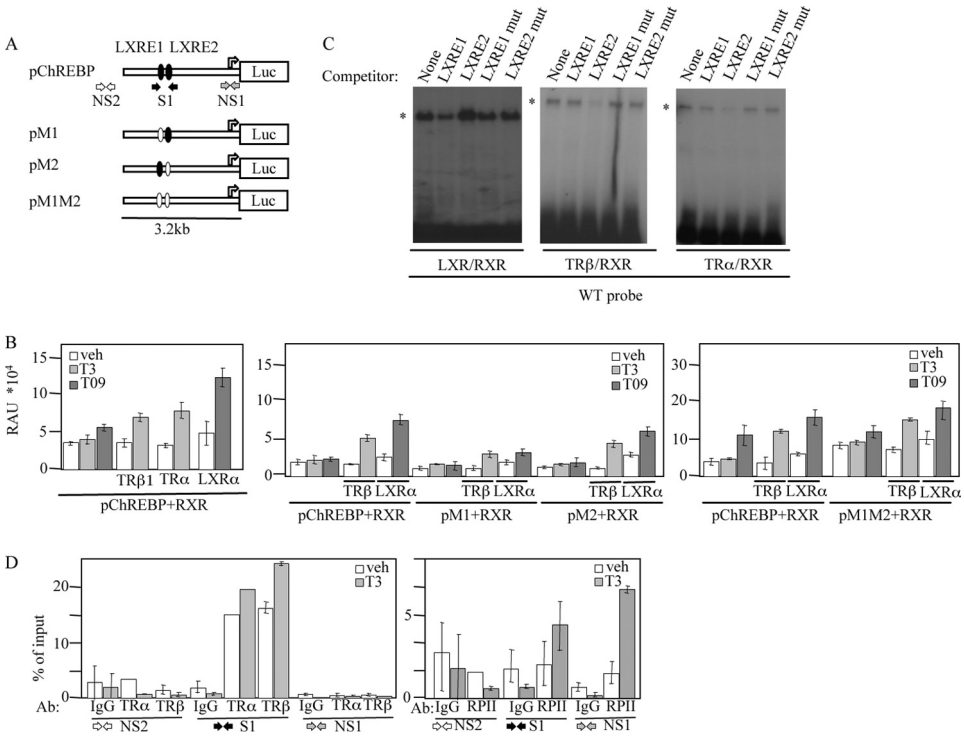

FIGURE 3. **TRα and TRβ bind to and activate ChREBP promoter via the previously described LXRE2.** *A*, scheme of the different versions of the ChREBP promoter cloned upstream of a luciferase reporter. LXRE1 and LXRE2 are pictured as *black ovals* or *white* when mutated. The *top arrow* indicates the transcription start site. The *arrow pairs* below the promoter indicate the localization of the primers used for ChIP analyzes: *white* for NS2, *black* for S1, and *gray* for NS1. The regions amplified by these three pairs are respectively the promoter portion −4100/−3900, −2558/−2384, and −203/+4. *B*, the indicated promoters were transfected with TRα, TRβ, or LXRα together with an RXRα encoding plasmid and treated with vehicle (*veh*, *white*), T3 (*light gray*), or T09 (*dark gray*). The relative luciferase activity measured is reported as arbitrary units (*RAU*). *C*, EMSA were performed using a 44-bp-long probe from the ChREBP promoter (WT probe) containing the area with the two LXREs to detect TRβ/RXRα or TRα/RXRα binding. LXRα/RXRα has been included as a control. The *asterisks* indicate the specific complexes. Competition with 100-fold excess of cold smaller fragments containing only one of the two LXREs, either WT (LXRE1 or LXRE2) or mutated (LXRE1mut or LXRE2mut), was used to assess the specificity of the binding. *mut*, mutation. *D*, ChIP experiments were performed on differentiated 3T3-L1, treated (*light gray*) or not (*white*) with T3. On the *left* are results obtained with anti-TRα (*TRα*), anti-TRβ (*TRβ*), or mouse IgG (*IgG*). On the right are results obtained with anti-RNA polymerase II (*RPII*) and mouse IgG (*IgG*). The specificity of the antibodies (*Ab*) used was verified on transfected HeLa cells (supplemental Fig. 1).The same lysates were used for all conditions, and each precipitation was done in replicates. The results shown are an average of these duplicates. Each experiment has been repeated at least twice. The primer pairs used for detection are indicated under the *arrows*. *Error bars* represent S.D.

ferent sizes indicated on the figure. The LXR/RXR complex bound to the WT probe was gradually displaced by an increasing amount of TRβ/RXR, which noticeably failed to bind the probe even at the highest amount added. We also observed that a TRβ/RXR complex was displaced by the addition of LXR/RXR. However, in both cases, the newly added complex was perfectly able to bind in a dose-dependent manner if the probe used contained a mutated version of the LXRE required for the fixation of the initially present receptor (M1 for LXR and M2 for TR). Altogether, these data strongly suggest that despite using two different LXREs, in this *in vitro* setting, concomitant binding of LXR/RXR and TR/RXR to the ChREBP promoter fragment is prevented.

As a complementary way to analyze the interference between LXR and TR signaling pathways, mice or WAT explants were treated with different combinations of LXR and TR ligands (Fig. 4*D*). The efficiency of the different treatments was validated by measuring the expression levels of known LXR or TR targets in the two considered systems. In WAT explants, all genes behaved as expected with strong induction of ABCA1, SREBP1c, and ApoE by T09, whereas ChREBP and FAS were stimulated by T3. Surprisingly, in these same samples, LXR ligand failed to induce ChREBP expression. Co-treatment with both ligands did not yield any additional effect as compared with treatment with individual ligand for any of the target tested. This suggests that TR and LXR mainly possess a non-overlapping set of targets in WAT. In liver, treatments were also efficient, with an increase of both ChREBP and FAS by TR and LXR ligand alone. In this condition, ChREBP induction by T09 does not reach statistical significance, but lack of strong induction has already been described by others (32).

Co-treatment with T09 and TH led to a significant increase in ChREBP as well as FAS liver expression as compared with TH treatment alone. This suggests that the two signals can be additive in this organ. For SREBP1-c and ABCA1, the situation is more complex. In PTU-treated mice, no activation was detected by T09 alone, and TH repressed expression of both genes. Nonetheless, T09 strongly increased their expression in TH-treated animals. Altogether, these data demonstrate that TRβ and LXR are both active in the two lipogenic tissues, WAT and liver, although their target genes are different *in vivo* and depend on the tissue considered.

DISCUSSION

In this report, we show that in mice, ChREBP is a new direct TH target not only in liver, which is in agreement with recently published data (23), but also to a much higher extent in WAT. Careful dissection of the molecular mechanism of ChREBP regulation allowed us to demonstrate that TRβ, but not TRα, is required for this activity *in vivo* and interferes with LXR signaling.

TH Stimulate ChREBP Expression in a TRβ-dependent Manner in Liver and WAT—TH have been long known to regulate energy metabolism and lipogenesis in the liver (9–11), yet their lipogenic effect in other tissues such as WAT was still controversial. Measurement of *in vivo* hepatic lipogenesis demonstrates that TH induction of this process is TRβ-mediated because it was abrogated in TRβ$^{-/-}$. Notably, this regulation by TRβ correlates with its ability to up-regulate ChREBP expres-

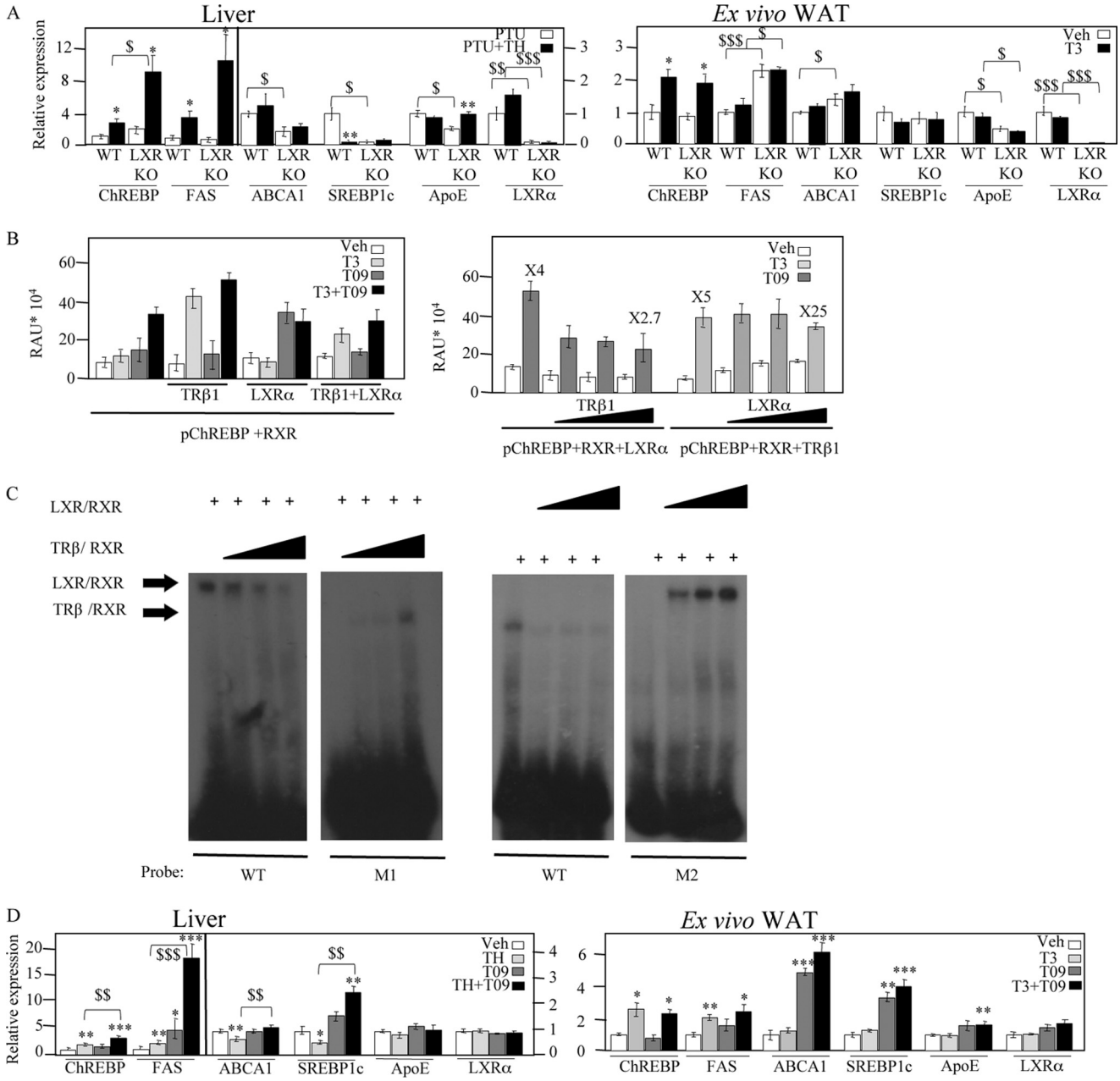

FIGURE 4. **Interactions between LXR and TR signaling.** *A*, 9-month-old WT or LXR KO females were rendered hypothyroid by PTU treatment and injected either by PBS (*white bars*) or by TH (*black bars*) ($n = 5$) (*left panel*). Fat pads isolated from either WT or LXR KO were kept in culture for 2 days in the presence of the indicated ligands for the last 24 h (*right panel*). *B*, the pChREBP construct (Fig. 3) was transfected together with high amount (150 ng) of RXRα and the indicated combination of TRβ and LXRα (50 ng each) (*left panel*) or an increasing amount (50, 100, and 200 ng) of either TRβ1 or LXRα (*right panel*) depicted as the *black triangle*. Cells were then treated with vehicle (*Veh*) (*white*), T3 (*light gray*), T09 (*dark gray*), or a combination of both (*black*). The relative luciferase activity measured is reported as arbitrary units (*RAU*). *C*, EMSA were performed to assess competition between LXR and TR for binding to the promoter using a WT (as described in the legend for Fig. 3), an M1 (WT LXRE2, mutated LXRE1), or an M2 (WT LXRE1, mutated LXRE2) probe (indicated at the bottom of the gels). A fixed amount of LXRα/RXRα (*two left panels*) or TRβ1/RXRα (*two right panels*) complex was incubated with increasing amounts of the other complex, respectively, TRβ1/RXRα and LXRα/RXRα (×2, ×4, ×8, indicated by the *black triangle*). The two different complexes migrate at different sizes indicated by the *arrows*. *D*, 3-month-old WT males were rendered hypothyroid by PTU treatment and injected by either PBS or TH. For each group, half of the animals were treated with T09 or vehicle (*left panel*). Fat pads isolated from WT mice were kept in culture for 2 days in the presence of the indicated ligands for the last 24 h (*right panel*) ($n = 5$). For *A* and *D*, qRT-PCR analyses were performed on liver and fat pads. For *panel B*, *error bars* represent S.D. *Asterisks* or *dollars* indicate statistical significance (one symbol, $p \leq 0.05$; two symbols, $p \leq 0.005$; three symbols, $p \leq 0.0005$). *Asterisks* always indicate significance between the given and control group for a genotype (PBS in *A* and vehicle in *D*), and *dollars* always indicate for the significance between the bridged groups.

sion not only in liver but also in WAT, the second most important lipogenic tissue in mice. Remarkably, regulation of ChREBP expression is particularly important in WAT as compared with liver under acute exposure to TH. In WAT, both TRβ1 and TRα1 are strongly expressed. The lack of induction by TH of the lipogenic genes in TRβ$^{-/-}$ WAT clearly demonstrates that TRβ is required for the regulation of this pathway by TH. The reduced, but significant, response to TH in TRα$^{o/o}$ WAT might suggest a possible role for TRα but is most likely due to variability in the amplitude of the response between different groups. Indeed, a similar variability (from 1.4-fold to 4-fold) has been observed within different experiments study-

ing the TH-mediated ChREBP up-regulation in WT animals. In any case, TRβ but not TRα is sufficient to drive TH-induced ChREBP expression in WAT. High basal lipogenesis was observed in the PTU-treated mutant mice as compared with WT (supplemental Fig. 2). ChREBP expression is repressed when WT but not TR$\beta^{-/-}$ mice are switched from a regular to a PTU diet, suggesting that unliganded TRβ acts as a repressor of ChREBP and might thus be considered as lipogenesis suppressor. Nonetheless, loss of ChREBP regulation is not the only explanation for higher lipogenesis in the TR$\beta^{-/-}$ because similar ChREBP expression levels were found in WT under chow and TR$\beta^{-/-}$ under different diets but were associated with very different levels of lipogenesis.

Molecular Determinants for the TRβ Specificity on TH-induced ChREBP Expression—The study of the ChREBP promoter showed that TRβ specificity of the TH response *in vivo* was not mimicked *in vitro* and identified the previously described LXRE2 as a functional TRE for both TRα and TRβ. In contrast to published data (23), mutation of the LXRE2 in the promoter did not lead to a complete loss of its TH inducibility. The particular mutations introduced in the two studies are different. Given the EMSA results, it is likely that for the present study, this mutation as well as the one introduced in the LXRE1 are disruptive enough to respectively prevent TR and LXR binding in the *in vitro* setting but not in the full promoter environment.

ChIP and EMSA experiments demonstrated that both TRα and TRβ are bound to the LXRE2, excluding a specific binding for TRβ. Using KO mice, Winter *et al.* (18) previously characterized a similar situation in some cells of the inner ear where *prestin* and *KCNQ4* were specifically regulated by TRβ and TRα, respectively. In this case, neither ChIP nor transfection experiments were performed to show a direct regulation, but both receptors bound to the two isolated TREs in EMSA. The molecular cues responsible for the TRα *versus* TRβ specificity *in vivo* remain to be determined, but altogether, the results obtained for these three genes suggest that the recruitment of co-regulators or the interaction with other transcription factors present on the promoter, necessary to efficiently stimulate transcription, might indeed be isoform-specific.

Interaction of TRβ and LXR Signalings in the Regulation of ChREBP Expression and Lipid Metabolism—TRβ and LXRs share a set of activities. Two hypotheses were proposed in the literature to document the mechanisms of these common functions. First, LXRα has been described as a TH target in mouse liver (20, 21). Second, these two transcription factors can recognize and bind as RXR heterodimers to a similar response element *in vitro* and thus might control the same target set. In this study, we showed that LXRα is not regulated by TH in any of the systems tested (Fig. 4). Furthermore, we demonstrated a transcriptional activation of ChREBP by TH and its persistence in LXR KO mice. Therefore, the requirement of LXRs in this TH-controlled pathway can be excluded. Moreover, in the liver of these LXR KO mice, the induction of ChREBP by TH is more important than in WT, suggesting that these receptors might limit each other's access to the promoter at least in this tissue. The proximity of the two binding sites, only separated by 8 bp, respectively, used by LXR and TR might impair the concomitant binding of the two complexes on a given copy of the promoter. Different approaches were adopted to test this hypothesis. Results from EMSA clearly show that the two complexes were not observed together on a probe. The exclusive binding of either TR or LXR was only possible when the other one was absent or not able to bind to its mutated site. In addition, responses to T09 or TH are decreased in cells co-transfected with both LXR and TR as compared with each alone, supporting the idea that randomly some copy of the transfected promoters bind LXR, whereas the others bind TR. In this context, the additive effect of TR and LXR ligands on ChREBP expression in liver might at first appear contradictory but is likely to reflect the random binding of LXR or TR in every single cell.

The situation is very different in the WAT. Strikingly and in contrast to TRβ, LXR does not regulate ChREBP in this tissue despite its strong induction of another of its targets, ABCA1. This might reflect either a lower LXR/TR expression ratio in this tissue and thus a preferential TR binding to the ChREBP promoter or a lack of LXR binding to the ChREBP promoter in WAT.

In conclusion, the possible co-regulation by TRs and LXRs has been suggested for some time, but although *in vitro* these receptors share the capacity to activate transcription through the same response elements, validation of this observation on natural promoters is unlikely to be systematic. Indeed, a number of LXR targets such as *ABCA1*, *SREBP-1c*, and ApoE, containing a well recognized DR4 element, are unresponsive to or decreased by TH, in WAT or liver, respectively (Fig. 4). In contrast, others such as the one in the *cyp7a1* promoter allow the recruitment of both (21). The ChREBP promoter is a novel situation where independent binding of TR or LXR to different DR4 elements located close to each other will prevent concomitant binding of the other. One explanation resides in what is actually called a DR4 element. The direct repetition of perfect consensus sequences separated by 4 nucleotides is very rarely found in the genome. The nature of the actual sequence might dictate the binding specificity of TRβ *versus* LXR. Finally, binding is unlikely to be sufficient, as suggested by the lack of TRα activity despite binding on ChREBP promoter in WAT and the different mode of regulation for a given receptor on a given promoter in two different tissues.

Importance of ChREBP Induction in the TH-induced Expression of Lipogenic Genes—Another question is the importance of ChREBP activation during TH-induced lipogenesis. Clearly, in both lipogenic tissues tested, ChREBP regulation is concomitant with the induction of the genes encoding the enzymes of the lipogenesis pathway. We do not have any direct evidence that ChREBP is actually required for the regulation of these genes that all contain a ChoRE. This is likely the case for genes, such as FAS or *SCD1*, in which no consensus TRE was ever found. For other genes, such as ME and *Spot14*, with characterized TRE, ChREBP and TRβ might act in an additive manner. Similar co-regulation of genes involved in the lipogenic pathways has been suggested for LXR and ChREBP.

Physiological Relevance of TH-induced Transcriptional Regulation of ChREBP—In the liver, the ChREBP protein is always highly expressed, and the regulation of ChREBP mRNA expression is thus generally not considered as a major parameter for

modulating its activity, which mainly relies on rapid post-translational modifications (5). In contrast, the ChREBP transcript level is lower in WAT, and as we show here, highly inducible by both TH and refeeding. Although lipogenesis *per se* was not measured, both stimuli induce the expression of lipogenic genes (FAS, *spot14*), suggesting that in WAT, activated ChREBP drives the same response as in liver. Under these conditions, WAT might thus contribute to the increase of whole body lipogenesis in a significant manner. It is also important to note that exogenous TH can modulate the expression of ChREBP and other lipogenic genes under non-lipogenic conditions such as fasting, supporting the hypothesis that nutritional status and TH are two independent ways to induce ChREBP levels at least in WAT.

In agreement with the absence of ChREBP up-regulation by T09 in WAT and with data published by others (6), ChREBP response to refeeding is also maintained in LXR KO mice (supplemental Fig. 3). Other factors than TRβ or LXR must then be responsible for this physiological increase of ChREBP expression.

Refeeding drives blood insulin level to rise. This hormone is thus likely to be responsible for the strong regulation of ChREBP in WAT, as shown in 3T3L1 adipocytes (8). Circulating TH levels decrease (around 50% for both T3 and T4) during short term starvation (33) and take several days to return normal. This variation of TH circulating levels is unlikely to be sufficient to amplify the stimulation of refeeding on ChREBP expression because TH injection in those conditions failed to do so and TRβ signaling was not necessary to this induction. Our hypothesis is that in other physiological situations associated with a modification of either local or circulating concentrations of TH, ChREBP would accumulate.

This work opens new perspectives because turning TRβ into a repressor in certain metabolic tissues, using a TRβ-specific ligand yet to be developed, could be one way to inhibit ChREBP expression and therefore lipogenesis induced by carbohydrate consumption. This could help improving patients with hepatic steatosis and insulin resistance (34).

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