Thyroid hormone receptor beta (TRbeta) and liver X receptor (LXR) regulate carbohydrate response element binding protein (ChREBP) expression in a tissue selective manner.

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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.

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Running Title: TRβ and LXR regulate ChREBP in a tissue selective manner

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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 signalling pathways interact in the control of cholesterol elimination in the liver. In the present study 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. Since 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 eight base pairs apart. There is a crosstalk between LXR and TRβ signalling on the ChREBP promoter in liver but not in WAT where LXR does not regulate ChREBP expression. The molecular basis for this crosstalk has been determined in *in vitro* systems.

De novo lipogenesis allows the synthesis of new molecules of fatty acids (FA) from acetyl CoA. High glucose and insulin concentrations induce this process converting the excess energy into triglycerides (TG), a more relevant molecule for storage purposes. In rodents both liver and WAT 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) while ChREBP (Carbohydrate Response Element Binding Protein) 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 FA 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, 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 3T3L1 cells insulin, glucose, and FA 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.

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 pattern 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 TRKO mice shed light on the role of each isotype in mediating TH signal (17, 12). 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 $TR\beta^{-/-}$ mice (13). However since 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 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 LXRs 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 paper 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, mouse LXRα). The different promoters were cloned in the pGL3 basic vector and or PGL4.70[hRluc] (Promega, Charbonnières, France). The 3kbp 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), Thyroxine (T4) were from Sigma-Aldrich (l'Isle D'Abeau,

France), the synthetic LXR ligand T0901317(T09) from Cayman Chemical (Montigny le Bretonneux, France).

Animals and preparation of tissue samples: knock-out mice were in a C57black6:129sv mixed background. $TR\beta^{-/-}$, $TR\alpha^{0/0}$ (17, 24, 25), LXRKO (26) and controls were previously described, fed *ad libitum* A04 diet (SAFE) and housed under recommended conditions. 3 to 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 three days (10mg/kg T09 in 100µl of methyl cellulose 1%).

$Pax8^{-/-}$ mice, which are genetically hypothyroid, were described to die before weaning (27), however some spontaneously survive. These rare survivors were used for experiments.

For the fasting/refeeding protocol, mice fed a regular chow diet were fasted for 24h and either refed a 70% high carbohydrate diet (Harlan Teklad TD98090) or kept on fasting for an additional 16h.

Tissues were dissected immediately after cervical dislocation, and flash frozen in liquid nitrogen. For WAT *ex vivo* culture, peri-testicular fat pads were dissected and cultured non dilacerated in 10% Charcoal stripped fetal bovine serum (FBS), 5ng/ml insulin complemented DMEM (Invitrogen, Cergy-Pontoise, France) for 24h before 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 (QRTPCR): RNAs were extracted using Trizol (Invitrogen). Total RNA was converted to cDNA using SuperScript II retrotranscription kit (Invitrogen). QRTPCR were performed using the Quantitect Syber 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 is 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 IBMX (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 at 10^{-5}M. Cells were harvested 24h (ChIP or WAT explants) or 36h (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 0.5µg final DNA. pSG5 was added as a carrier when needed. Transfection efficiency was normalized using β-Gal activity brought by cotransfection of CMV β-Gal vector. For each experiment triplicate of each conditions were done, and each experiment have been repeated at least three times giving similar results. Only one experiment is shown and each point represents the average for the triplicate, the error bar is their standard deviation (SD).

Chromatin Immuno-precipitation 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, the anti RNA-polII (CTD4H8) from Upstate. Cells were cross-linked with 1% formaldehyde before lysis (in 1% SDS, 10mM EDTA, 50mM Tris-HCL pH 8.1) and sonication (200-700 bp DNA fragments). Lysates were diluted and precleared with herring sperm DNA (2µg/ml), BSA (2µg/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 200mM NaCl. Samples were purified (Quiagen) and analyzed by Q PCR using the primer pairs NS1, NS2 and S1.

EMSA: mTRα1, mTRß, mLXRα and mRXRα were *in vitro* translated (TNT kit Promega). The different single-strand oligonucleotides (F) were $[\gamma\text{-}^{32}P]$ATP labelled with T4 polynucleotide kinase (Fermentas, Burlington, Ontario) before annealing with their unlabelled antisens (R).

Probes were purified and counted. 20000 cpm were used for each binding reaction. Unlabelled specific and nonspecific competitor probes were included at the indicated molar excess.

Hepatic lipogenesis: Mice were given an IP injection of deuterated water (10ml/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 TG as previously described (29). These enrichments were then used to calculate the contribution, expressed as per cent, of hepatic lipogenesis to the plasma TG pool (30).

All the primer sequences are listed in SI1.

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 SEM. Statistical relevance was determined using the one variable Anova method.

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/MMI treatment (23). Here the regulation of ChREBP was studied in the pax8 (deprived of thyroid) mutant mice and Sv129 mice treated with PTU (Figure 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) were 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 $TR\beta^{-/-}$ but not $TR\alpha^{0/0}$ mice indicating that TRβ was required at least in the two metabolic tissues studied (Figure 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 $TR\beta^{-/-}$ PTU-treated male mice. Whereas TH efficiently increased lipogenesis in WT (Figure 1-C) the response was blunted in $TR\beta^{-/-}$ mice. WAT lipogenesis was not measured due to technical limitations.

TH/TRβ and nutritional status: two independent ways to regulate ChREBP expression.

To determine the involvement of TH signalling 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 $TR\beta^{-/-}$ mice (Figure 2-A). In agreement with published data, ChREBP RNA was found only up-regulated two 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 $TR\beta^{-/-}$ mice indicating that TRβ is not required for this physiological process. We next determined whether 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 (Figure 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 TRβ/TH 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, were able to activate the 3.2 kbp ChREBP proximal promoter (figure 3-A), in the presence of TH (Figure 3-B). LXRα, 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, LXRE1 being involved for LXR response (22), LXRE2 necessary for TH response (23). These binding specificities were confirmed here by the EMSA data (figure 3-C). All three receptors bound to a 44bp probe encompassing the two LXREs. However LXR binding was competed only by a LXRE1WT but not mutated probe whereas TRβ1 or TRα binding were only competed by a LXRE2 WT but not mutated probe. The dependence on

these sites for transcriptional responsiveness to either TR or LXR was less obvious in transfection assay (Figure 3-B). The double M1M2 mutant still showed responsiveness to both compounds. This apparent discrepancy to 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 *et al* actually also observed a residual induction of similar pM1 and pM1M2 constructs by the LXR agonist T09 (22). This suggests either that beside LXRE1, some other region(s) of the promoter could mediate the response to LXR or that like 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 upstream or downstream promoter regions. TR binding was independent of T3 in agreement with the accepted model for TR action. In contrast RNA polII was enriched at the transcriptional start site only in the presence of T3 (Figure 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.

Crosstalk between TRβ and LXR signalling 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 (Figure 4 A and D). Furthermore TH was capable of activating the expression of ChREBP as well as other lipogenic genes in liver of PTU treated LXRKO mice (Figure 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 signalling pathways. In liver but not in WAT TH induction of ChREBP expression was significantly higher in LXRKO mice than in WT (4.5 fold versus 2.9 fold, respectively, Figure 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 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 cotransfection of both decreased the response to each ligand, LXR dependent activity being more affected than TR (Figure 4B 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. (Figure 4B right panel). Finally direct evidences for a mutually exclusive binding were obtained by EMSA experiments. As previously shown in figure 3 both receptors bind as RXR heterodimers to a 44-mer probe containing the two WT LXREs. These two complexes migrated at different sizes indicated on the figure. The LXR/RXR complex bound to the WT probe was gradually displaced by 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 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 signalling pathways mice or WAT explants were treated with different combinations of LXR and TR ligands (Figure 4D).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. Cotreatment with both ligands did not yield any additional effect as compared to 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 have 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 to 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 demonstrating 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 since it was abrogated in TRβ$^{-/-}$. Notably this regulation by TRβ correlates with its ability to up-regulate ChREBP expression 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 to 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$^{0/0}$ 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 studying 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 to WT (SI 3). ChREBP expression is repressed when WT but not TRβ$^{-/-}$ 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 TRβ$^{-/-}$ since similar ChREBP expression levels were found in WT under chow and TRβ$^{-/-}$ 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 *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*. previously characterized

a similar situation in some cells of the inner ear where *prestin* and *KCNQ4* were specifically regulated by TRβ and TRα, respectively (18). 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 coregulators 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 signallings 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 similar response element *in vitro*, and thus might control the same target set. In this paper we showed that LXRα is not regulated by TH in any of the systems tested (Figure 4). Furthermore we demonstrated a transcriptional activation of ChREBP by TH and its persistance in LXRKO mice. Therefore the requirement of LXRs in this TH-controlled pathway can be excluded. Moreover in the liver of these LXRKO 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 base pairs, 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 response to T09 or TH are decreased in cells co-transfected with both LXR and TR as compared to each alone, supporting the idea that randomly some copy of the transfected promoters bind LXR, the others TR.

In this context the additive effect of TR and LXR ligands on ChREBP expression in liver might at first appears 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 while *in vitro* these receptors share the capacity to activate transcription through the same response elements, validation of this observation on natural promoters is far 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 (figure 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 have 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, 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 under non lipogenic condition 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 LXRKO mice (SI4). Other factor 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 since TH injection in those conditions failed to do so and TRβ signalling 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 since 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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FOOTNOTES

We would like to thank several people: D.J. Mangelsdorf (Howard Hughes Medical Institute, Dallas, TX) for providing the LXRα/β knockout mice, N. Aguilera and A. Couzon for taking care of our mice at the PBES (ENS Lyon, France), D. Jeantet for the genotyping, F. Flamant and L. Canaple for critical reading of the manuscript. This work was supported by SIGNATOR ANR-06-BLAN-0232-01, Crescendo Contract LSHM-CT-2005-018652, Cascade, Contract FOOD-CT-2004-506319, the Fondation pour la Recherche Médicale Grant INE2000-407031/1 and the Fondation BNP-Paribas.

FIGURE LEGENDS

Figure 1 *ChREBP expression and lipogenesis are regulated by thyroid hormones in a TRβ dependent manner*. 3-month old males either genetically (pax8$^{-/-}$) or chemically rendered (WT, TRβ$^{-/-}$, TRα$^{0/0}$ - PTU treated) hypothyroid were either injected by PBS (white bars) or TH (black bars). In A) (n=4 for higher panel, n=5 for lower panel) and B) (n=5) mRNA encoding lipogenic enzymes were quantified by QRTPCR. In C) liver lipogenesis was measured as described in Material and methods (n=5).

Results are shown as induction as compared to the PTU treated animals of a given genotype. Stars and dollar signs indicate respectively statistical significance as compared to 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 *** $p \leq 0.0005$).

Figure 2 *Independent regulation of ChREBP expression by TH/TRβ and nutritional status*.WT and $TR\beta^{-/-}$ 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 24h and then refed (R) or not (F). (n=5). In B) mice were starved for 24h. One group was kept on fasting (F), the other one was refed (R) for an additional 16h. Half of the animals per group were injected by TH twice, one before the fast then before the refeeding (n=5). Expression of lipogenic genes was measured by QRTPCR. Stars indicate statistical significance (* $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$) as compared to the CF group of the same genotype in A), to the F/V group in B). $ 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.

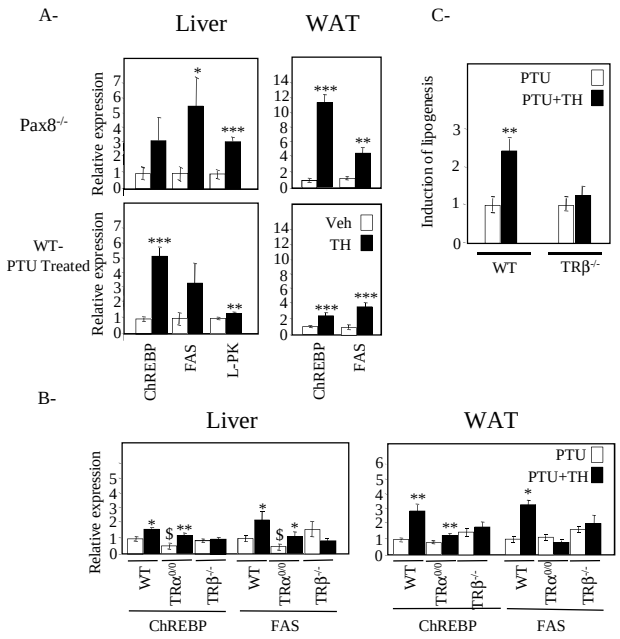
Figure 3 *TRα and TRβ bind to and activate ChREBP promoter via the previously described LXRE*2.Scheme of the different versions of the ChREBP promoter cloned upstream of a luciferase reporter. LXRE1 and LXRE2 are pictured as black ovals or white when mutated. The top arrow materializes the transcription start site. The arrows pairs below the promoter indicate the localization of the primers used for ChIP analyzes: white for NS2, black for S1, and grey for NS1. The region amplified by these three pairs are respectively the promoter portion -4100/-3900, -2558/-2384 and -203/+4. B) The indicated promoters were transfected with either TRα,TRβ or LXRα together with an RXRα encoding plasmid and treated with either vehicle (white), T3 (light grey) or T09 (dark grey). 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. 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) were used to assess the specificity of the binding. D) ChIP experiments were performed on differentiated 3T3L1, treated (black) or not (light grey) with T3. On the left are results obtained with anti-TRα (TRα), anti-TRβ (TRβ) or mouse IgG (IgG), on the right those obtained with anti-RNApolII (RPII) and mouse IgG (IgG). Specificity of the antibodies used was verified on transfected Hela cells (SI2).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 primers pairs used for detection are indicated under the arrows.

Figure 4 *Interactions between LXR and TR signalling.* A) 9-month old WT or LXRKO females were rendered hypothyroid by PTU treatment and either injected by PBS (white bars) or TH (black bars) (n=5) (left panel). Fat pads isolated from either WT or LXRDKO were kept in culture for two days in presence of the indicated ligands for the last 24h (right panel).

B) The pChREBP construct (see figure 3) was transfected together with high amount (150ng) of RXRα and the indicated combination of TRβ and LXRα (50ng each) (left panel) or an increasing amount (50, 100 and 200ng) of either TRβ1 or LXRα (right panel) depicted as the black triangle. Cells were then treated with either vehicle (white), T3 (light grey), T09 (dark grey) 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 either a WT (as described in figure 3), a M1 (WT LXRE2, mutated LXRE1) or a M2 (WT LXRE1, mutated LXRE2) probe (Indicated at the bottom of the gels). A fix amount of LXRα/RXRα (two left panels) or TRβ1/RXRα (two right panels) complex was incubated with increasing amount of the other complex respectively TRβ1/RXRα and LXRα/RXRα (x2, x4, x8 materialised by the black triangle). The two different complexes migrate at different size indicated by the arrows. D) 3-month old WT males were rendered hypothyroid by PTU treatment and either injected by 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 two days in presence of the indicated ligands for the last 24h (right panel). (n=5). For A) and D) QRTPCR analyses were performed on liver and fad pads For the four panels stars or dollars indicate statistical significance (one $p \leq 0.05$, two $p \leq 0.005$, three $p \leq 0.0005$). Stars are always for

significance between the given and control group for a genotype (PBS in A, and veh in D), dollars for the significance between the bridged groups.

Figure 1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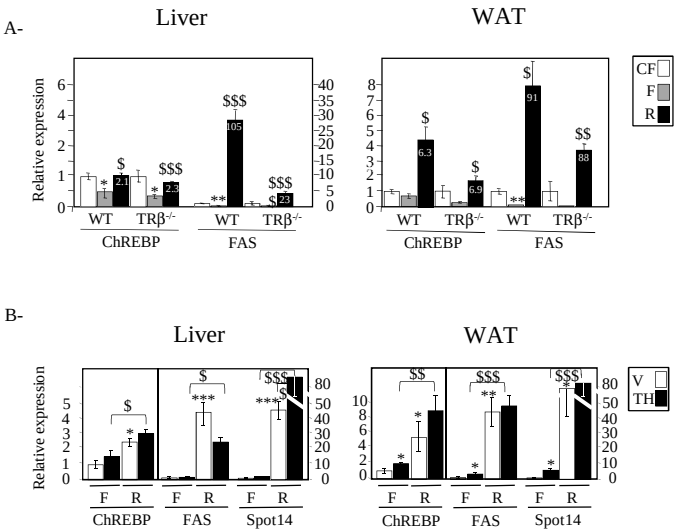
Liver
WAT
Pax8-/-
WT-
PTU Treated
Relative expression
Veh
TH
ChREBP
FAS
L-PK
C-
PTU
PTU+TH
Induction of lipogenesis
WT
TRβ-/-
B-
Liver
WAT
PTU
PTU+TH
Relative expression
WT
TRα∆/∆
TRβ-/-
ChREBP
FAS

Figure 2


A-
Liver
WAT
Relative expression
CF
F
R
WT
TRβ-/-
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Relative expression
V
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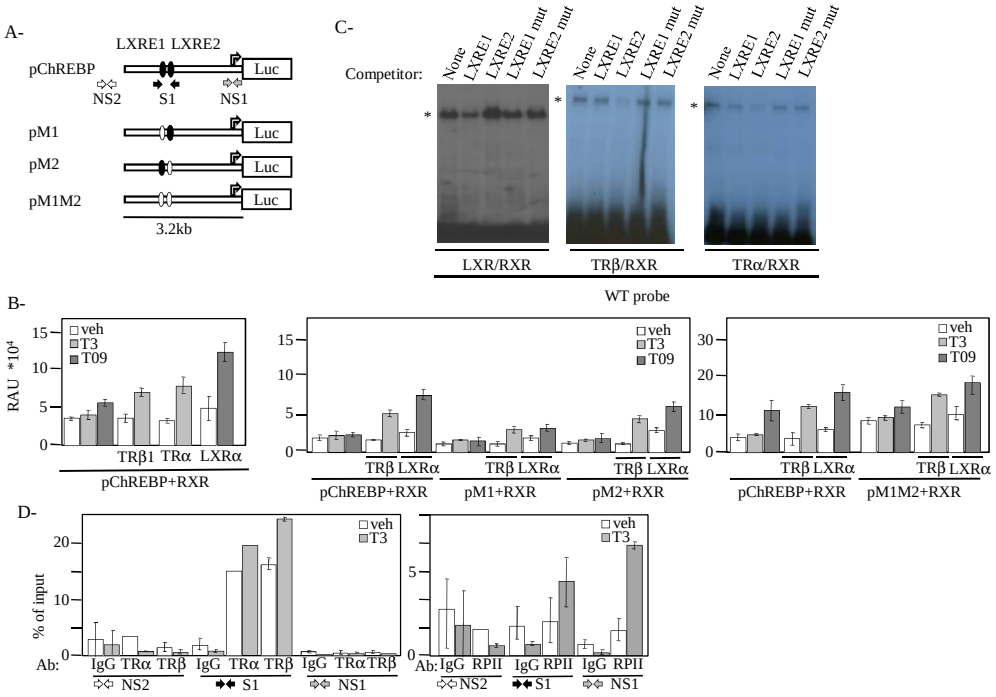
F
R
ChREBP
FAS
Spot14

Figure 3


A-
LXRE1 LXRE2
pChREBP
Luc
NS2
S1
NS1
pM1
pM2
pM1M2
3.2kb
C-
Competitor:
None
LXRE1
LXRE2
LXRE1 mut
LXRE2 mut
LXR/RXR
TRβ/RXR
TRα/RXR
WT probe
B-
RAU *10⁴
veh
T3
T09
TRβ1 TRα LXRα
pChREBP+RXR
TRβ LXRα
pM1+RXR
pM2+RXR
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D-
% of input
Ab:
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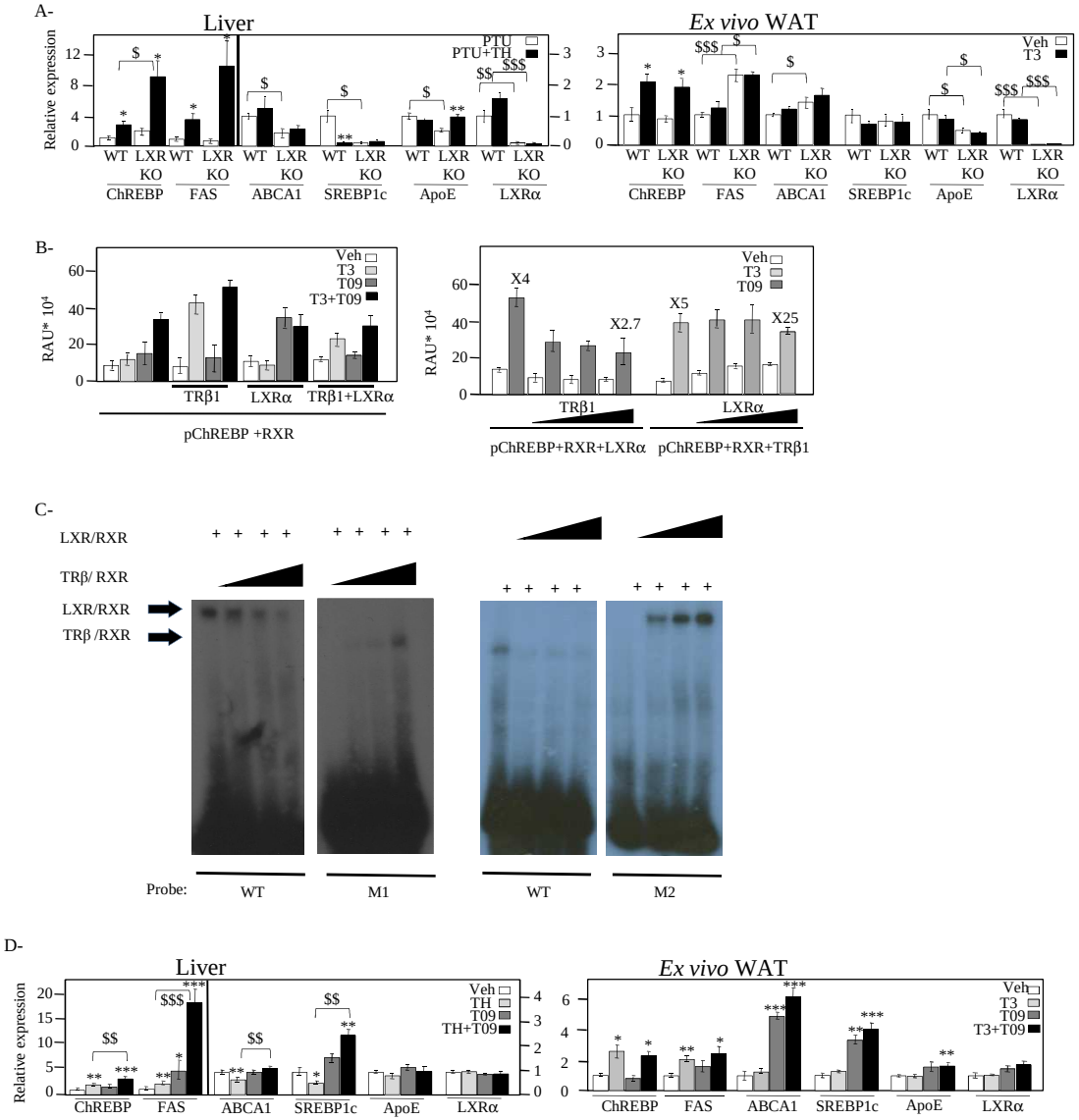
IgG RPII
NS2
S1
NS1

Figure 4



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