

**HUMAN SREBP1c EXPRESSION IN LIVER IS DIRECTLY REGULATED BY
PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA (PPAR α).**

Ana Fernández-Alvarez¹, María Soledad Alvarez¹, Raúl Gonzalez^{2,3}, Carme Cucarella¹, Jordi Muntané^{2,3} and Marta Casado^{1, 3}

¹Instituto de Biomedicina de Valencia (IBV-CSIC), Jaime Roig 11 46010 Valencia, Spain; ²Liver Research Unit Reina Sofía University Hospital, Córdoba, Spain; ³Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd)

Running head: Human *SREBP1c* promoter regulation

Address correspondence to: Marta Casado, Instituto de Biomedicina de Valencia (IBV-CSIC), Jaime Roig 11, 46010 Valencia, Spain. Tel: +34 96 339 37 78; Fax: +34 96 369 08 00; Email: mcasado@ibv.csic.es

Sterol regulatory element binding proteins (SREBPs) regulate the expression of a number of enzymes which catalyze the synthesis of fatty acids, cholesterol, triglycerides, and phospholipids. SREBP1c is the most relevant isoform in the adult liver and its expression is controlled by the nutritional state. Transcriptional regulation studies into the *SREBP1c* gene, performed in the last few years, have improved our knowledge of the variability of signals that converge on its promoter region. Insulin, cholesterol derivatives, T3 and other endogenous molecules have been demonstrated to regulate the *SREBP1c* expression, particularly in rodents. The present study aimed to perform a detailed analysis of the human *SREBP1c* gene promoter structure in liver cells by focusing on responses to diverse metabolic signals. Serial deletion and mutation assays reveal that both SREBP (SRE) and LXR (LXRE) response elements are involved in SREBP1c transcription regulation mediated by insulin and cholesterol derivatives. We discovered that peroxisome proliferation-activated receptor alpha (PPAR α) agonists enhance the activity of the *SREBP1c* promoter, a DR1 element, at -453 in the human promoter, involved in this activation. Moreover, PPAR α agonists act in cooperation with LXR or insulin to induce lipogenesis. Collectively, our results identify PPAR α as a novel regulatory factor in SREBP1c regulation which plays a relevant role in the interplay between lipids and insulin metabolic regulation.

The prevalence of overweight and obesity is increasing worldwide at an alarming rate. An excess amount of body fat not only leads to reduced quality of life and immense healthcare-associated costs, but also increases risk of death. Indeed, obesity has been related to a number of cardiovascular and metabolic disorders such as hypertension, type 2 diabetes, hyperinsulinemia, dyslipidemia and atherosclerosis by defining features of the metabolic syndrome. Beyond obesity and a number of independent factors, the other etiological factor of metabolic syndrome is insulin resistance, commonly considered to be of greater priority in pathogenesis (1,2).

The discovery of Sterol regulatory element binding proteins (SREBP) was critical for our understanding of hepatic cholesterol homeostasis. SREBP1c, one of three SREBPs members of the basic helix-loop-helix (bHLH) family of transcription factors, is essential for the genomic actions of insulin on both carbohydrate and lipid metabolism (3), and plays a central role in the molecular biochemistry of metabolic syndrome. The SREBP1c expression is controlled by nutritional status. Fasting lowers SREBP1c mRNA and protein levels, whereas they are strongly induced in a fed state, followed by a compatible pattern of nutritional changes in lipogenic genes (4). Accordingly, changes in this transcription factor activity may be the key to linking insulin resistance with other obesity-associated metabolic disorders.

Liver X receptors (LXR) belong to the nuclear hormone receptor superfamily. The LXR subfamily consists of two members; LXR α and LXR β ; which are activated by oxysterols. The LXR α expression pattern is mainly restricted to the liver, adipocytes, the small intestine and macrophages, whereas LXR β is expressed ubiquitously (5). LXRs directly bind the cis elements in the *Srebp1c* promoter as heterodimers with RXR, leading to

transcriptional activation. Lipogenesis regulation by LXR is mediated through this effect on the SREBP1c expression (6).

Along with LXR, other members of this superfamily of nuclear hormone receptors, peroxisome proliferator-activated receptors (PPARs) play a major role in lipid metabolism. The PPARs family is represented by three members: PPAR α , the predominant form in the liver, PPAR δ , and PPAR γ . Different PPARs can be considered key messengers responsible for the translation of nutritional, pharmacological and metabolic stimuli into changes in the expression of those genes specifically involved in lipid metabolism (7). Like LXRs, activated PPARs also heterodimerize with RXR and alter the transcription of target genes. These heterodimers bind to specific peroxisome proliferator response elements (PPRE) consisting of a direct repeat of a hexameric DNA core recognition motif spaced by one nucleotide (8). The overexpression of PPAR α in HEK293 cells has been shown to inhibit mouse *Srebp1c* promoter activity through competition with LXR/RXR heterodimerization (9). Thus, hepatic lipid homeostasis is a result of a complex crosstalk between a number of transcription factors, including LXR, PPARs and SREBPs.

In order to understand the molecular mechanism behind the nutritional regulation of the SREBP1c expression, the *Srebp1c* gene rodent promoter and, to a much lesser extent, the human regulatory region, has been previously characterized (10,11). In the proximal region of the mice *Srebp1c* promoter, SP1, NFY, USF, SREBP and LXR-binding sites have been identified (6,12). An SRE element together with two LXREs motifs have proved indispensable for the insulin response (13). The sequence of the rat *Srebp1c* proximal promoter is 97% identical to its murine counterpart (14). Experiments carried out in Marshall B. Elam's laboratory have revealed that at least four unique transcription factor-binding elements recognized by LXR α , SREBP1, NFY and SP1 constitute the insulin-response unit of the rat *Srebp1c* promoter (14,15).

Sequence alignments show that the human SREBP1c promoter presents only 42.0% similarity to the mouse promoter, suggesting that promoters might be regulated by different pathways and mechanisms. In the present study, we extensively characterized the human proximal SREBP1c promoter by identifying the nutritional regulation mechanism in liver cells.

Moreover, we identified a PPRE element in the proximal human sequence. *In vitro* and *in vivo* studies show the direct interaction of the PPAR receptor with the human SREBP1c promoter, and propose a novel aspect of the network of transcription factors regulating human fatty acid metabolism.

EXPERIMENTAL PROCEDURES

Plasmids. A DNA fragment containing 1801 bp corresponding to the 5' upstream region of the human SREBP1c gene was amplified by PCR and cloned into the pCR2.1-TOPO vector (Invitrogen) to construct pPro1c-TOPO. A 1564 bp fragment was obtained by NcoI digestion and subcloned in the NcoI site of pGL3-basic luciferase vector (Promega) to construct the -1564/+1-luc vector. The -520/+1-luc vector was prepared by PCR from the -1564/+1-luc vector using the forward primer 5'-GGAGGGTACCAAGGCTCGCTCAGGGTGCC AGC-3' and the reverse primer GLprimer2 (Promega) to be then inserted into the KpnI/NcoI site of the pGL3-basic vector. -310/+1-luc was prepared by XhoI digestion and re-ligation from 1564/+1-luc. Mutagenesis was performed by means of the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA. USA) using pPro1c-TOPO as a template. All the constructions were confirmed by nucleotide sequencing. The expression vectors pCMX-PPAR α , pCMX-LXR α , pCMX-LXR β and pCMX-RXR α were obtained from Dr. Antonio Castrillo (Facultad de Medicina, Universidad de Las Palmas, Las Palmas de Gran Canaria, Spain).

Cell culture and luciferase assay. Rat and mouse hepatocytes in primary culture were prepared from adult rats or mice by collagenase perfusion (16) and seeded in Williams' E medium, supplemented with glutamine, 100 nM insulin, 1 μ M dexametason, 5% FBS and antibiotics. Transfection assays were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA. USA) in 6-well culture dishes with 3.8 μ g of SREBP1c reporter plasmids and 200 ng of Renilla luciferase vector (pRL-TK) 6 h after seeding. At 16 h post-transfection, hepatocytes were left for 24 h in Basal Induction Medium (Williams' E, supplemented with glutamine, 0.75% BSA, 100 nM dexametason and 20 mM glucose). When indicated, cells were treated with: a) 100 nM insulin, b) 10 μ M or 10 nM

TO901317 c) 30 μ M WY14643, and d) 1 μ M GW7647. In some experiments, cells were co-transfected with 200 ng of the indicated expression vector, while the pCMX empty vector was used to normalize the amount of DNA. Transactivation activities were measured at 24 h post-transfection in a Wallac 1420 VICTOR luminometer according to the technical manual for the Dual-Luciferase Reporter Assay System (Promega).

Human hepatocytes were prepared from the liver biopsies obtained from patients submitted to a surgical resection of a liver tumor after obtaining patients' written consent. Hepatocytes isolation was based on the two-step collagenase procedure (17). Cell viability was consistently >85%, as determined by trypan blue exclusion. Hepatocytes (8×10^6 cells; 150,000 cells/cm²) were seeded at confluence on type I collagen-coated dishes (Iwaki, Gyouda, Japan), and maintained in a DMEM-Ham-F12:William's E (1:1) medium supplemented with 26 mM NaHCO₃, 15 mM HEPES, 0.29 g/L glutamine, 50 mg/L vitamin C, 0.04 mg/L dexametasone, 2 mg/L insulin, 200 μ g/L glucagon, 50 mg/L transferrin and 4 ng/L ethanolamine containing 5% fetal calf serum for 12 h. Afterward, the medium was removed and replaced with a fresh culture medium supplemented, when indicated, with: a) 100 nM insulin, b) 10 μ M or 10 nM TO901317, c) 50 μ M WY14643, and d) 1 μ M GW7647.

Electrophoretic Mobility Shift Assays. EMSAs were performed using double-stranded DNA oligonucleotides end-labeled with [γ -32P]dATP. The oligonucleotides corresponding to the DR1 binding site (5'-CCCTTCGTTAAAGGGTCAAAGCAGAGAA GTCCTGGCCC-3'), the LXRE1 binding site (5'-GGAGCTGAGGGCCAGTGACCGCCAGTAA CCCCGGCAGACGCTGG-3') and the LXRE2 binding site (5'-CGGGTTAAAGGCGGACGTCCGCTAGTAA CCCCAACCCCATTCAAGC-3') were annealed with the complementary sequence by incubation at 85°C for 10 min in 70 mM Tris-HCl pH 7.5; 13 mM MgCl₂; 1.3 mM EDTA; 1.3 mM spermidine and 6.7 mM DTT with overnight cooling. A 300 ng aliquot of the probe was labeled with 20 units of T4 polynucleotide kinase in the presence of 40 μ Ci [γ -32P]dATP at a final volume of 10 μ l for 30 min at 37°C and

purified in Sephadex G25 columns. Binding reactions were carried out for 20 min at room temperature using *in vitro* translated human PPAR α , LXR α and RXR α prepared with the TNT T7-coupled reticulocyte lysate system (Promega), 9 fmol of probe, and 2 μ g dI/dC in the binding buffer (20 mM HEPES pH 8.0, 0.1 mM EDTA, 50 mM CINA, 1 mM DTT, 5% Glycerol and 5 mM Cl₂Mg). Each protein's expression was confirmed by Western blot as previously described (18). Supershifts assays were carried out using specific α -PPAR α (sc-9000), α -LXR α/β (sc-13068) and α -RXR α (sc-553) antibodies in the reaction mix for 30 min on ice before adding the probe. DNA-protein complexes were resolved on a 6% (w/v) non denaturing polyacrylamide gels in 0.5x TBE buffer (1x TBE is 90 mM Tris, 90 mM boric acid and 1 mM EDTA). The dry gel was exposed overnight in an IP screen and analyzed in a FLA5000 (FUJIFILM).

Total RNA preparation and RT-PCR. Total RNA was isolated from hepatocytes by using Trizol reagent (Invitrogen). First-strand cDNA was synthesized from 1 μ g of total RNA using a random hexamer and expand reverse transcriptase (Roche Diagnostics, Manheim, Germany). The cDNA was used as a template for quantitative PCR using Syber Green reagent (Applied Biosystem) and specific primers for rat and mouse *Srebp1c* (forward primer 5'-CCATGGATTGCACATTGAA-3', reverse primer 5'-GCCAGGGAAAGTCACTGTCTT -3') or human *SREBP1c* (forward primer 5'-CCATGGATTGCACTTTCGAA -3', reverse primer 5'- GCCAGGGAAAGTCACTGTCTT -3'). The amount of total cDNA in the sample was analyzed in the same reaction using specific primers for mouse *Gapdh* (forward primer 5'-GTATTGGCGCCTGGTCAC-3', reverse primer 5'-AATCTCCACTTGCCACTGCA-3'), rat β -actin (forward primer 5'-TTCACCACCCAGCCATGT-3', reverse primer 5'-GTGGTACGACCAGAGGCATACA-3') or human *36B4* (forward primer 5'-AGATGCAGCAGATCCGCAT-3', reverse primer 5'-GTTCTTGCCCCATCAGCACC-3') as a control. FAS (Hs00188012_m1) and ACC (Hs00172885_m1) mRNA expression levels were quantified using the ABI 7500 fast instrument and Taqman technology (Assays-on-demand gene expression product; Applied Biosystems).

Chromatin Immunoprecipitation. ChIP assays were performed as previously described (19) using the isolated nuclei from the formaldehyde cross-linked human and rat hepatocytes. Immunoprecipitation was performed with the α -PPAR α (sc-9000), α -RXR α (sc553), α -SREBP1 (sc-8984) and α -RNA pol II (sc-899) (Santa Cruz Biotechnology, Santa Cruz, U.S.A.) antibodies. Normal rat IgG (sc-2026) was employed as a negative control for rat hepatocytes. Purified samples were analyzed by PCR. The primers used to detect target sequences were as follows: Human DR1/LXRE site (*SREBP1c* promoter) 5'-GCCAGGACTTCTCTGCTTG-3' and 5'-GGGTTGGGGTTACTAGCGGACG-3'; Human *SREBP1c* coding region (exon 9) 5'-GGCTGCTGCCCCAGT-3' and 5'-GACAAAGAGAACCAAGGAGAC-3'; Human *HMGCR* (promoter) 5'-ACGCTGATTGGGTCTATGG-3' and 5'-GTGTAAATGGCTCCGGTCAC-3'; Human α -actin (coding region) 5'-CTTCTGCCCTCCGCAGCTGA-3' and 5'-GTGAATGCCGCCGACTCCA-3'; Rat LXRE site (*Srebp1c* promoter) 5'-CTGGCGCAGTTGCGGTTAAA-3' and 5'-GCCGCGCCGCGCCCCAATAA-3'; Rat SRE site (*Srebp1c* promoter), 5'-CTGCTGATTGCCATGTGC-3' and 5'-GCTACCCCTACAGCGTCCG-3'; Rat *Srebp1* coding region (exon 4) 5'-GCCCATCCACCGACTAGCAG-3' and 5'-GGAACGGTAGCGCTCTCAA-3' and Rat β -actin (coding region), 5'-TTCACCACCCCAGCCATGT-3' and 5'-GTGGTACGACCAGAGGCATACA-3'. PCR fragments were generated with a 5-min melting step at 94°C, followed by 45 cycles of amplification (94°C for 30 s, 56°C for 30 s and 72°C for 45 s) and a terminal extension. Each ChIP assay was performed at least twice to ensure reproducibility.

Confocal Microscopy. Human hepatocytes grown in 35 mm glass bottom dishes no. 1.5 (MatTek Corp., Ashland, MA) were used for live cell imaging. Cells were treated for 24 h under basal conditions or with 1 μ M GW7647, 100 nM insulin, or a combination of both. Cells were fixed in 4% PFA pH 7 for 10 min and washed three times with PBS 1X. Nile red staining was performed using the modified protocol described previously (20). Briefly, fixed cells were incubated for 4 h with 1 μ g/ml of Nile Red solution and washed in PBS 1X overnight.

Afterward, cells were stained for 10 min with DAPI 8 μ g/ml to visualize cell nuclei. Confocal images were obtained with a Leica TCS SP2 Spectral microscope and a 63X/1.40 NA oil objective (Leica Microsystems, Wetzlar, Germany). Fluorescence intensity was measured with the ImageJ program.

Statistical analysis. Values are expressed as mean \pm SD (n ranged from 3 to 5 independent experiments). Statistical significance was estimated with the Student's two-tailed t-test for unpaired observations. A p-value of less than 0.05 was considered significant.

RESULTS

Sequence alignment of rodents and human *SREBP1c* promoters. A comparative analysis of the human, rat, and mouse genomic sequences at the 5' upstream position from the SREBP1c isoform transcriptional start site reveals a high degree of similarity, particularly in the first 300 bp (Fig. 1). However, some critical differences along the promoter region can be identified. First, the human sequence presents an expansion of 67 bp that contains at least five SP1 binding sites. Second, the E-box present in the mouse and rat promoters is absent in the human sequence. Third, the rat and mouse promoters present a unique SRE binding site, whereas an additional SRE element is found 100 bp upstream in the human promoter, as previously described (10). Two human exclusive regulatory sequences were observed in the distal region: a previously described PDX1 binding site (21) and a DR1 binding site predicted by an *in silico* analysis.

We first confirmed the identity of the basal transcription regulatory elements, which were predicted on the basis of the proximal promoter region's similarity between species. A deletion analysis of 1500 bp of the human *SREBP1c* promoter cloned in a luciferase reporter vector was performed in transiently transfected rat primary hepatocytes. A construct containing 300 bp, which includes one SRE site, the NFY site and all the SP1 sites, was able to maintain almost 60% of the activity observed with the complete 1500 bp promoter luciferase construction (Fig. 2). The -520/+1 vector, which lacks only the PDX1 binding site, showed no diminished activity. Furthermore, a construction with a 158 bp deletion without the SRE2, the NFY and all the proximal SP1 binding sites, displayed no luciferase activity. This deletion

analysis indicates that the PDX1, DR1 and LXRE sites are not relevant for the human *SREBP1c* promoter's basal activity in hepatocytes.

Insulin regulation is mediated by the SRE and LXRE sites. The insulin-mediated regulation of the SREBP1 pathway has been extensively studied in recent years. This hormone is the most important factor to specifically regulate the SREBP1c isoform, which reflects the relevance of this protein in the interplay between glucose and lipid metabolism (3). Although it is clear that insulin activates the murine *Srebp1c* promoter by acting on SRE sites, and is mediated by SREBP itself, there has been some controversy about the relevance of LXRE sites in this regulation (10). In order to clarify this issue, we analyzed the insulin-mediated regulation of the human *SREBP1c* promoter in rat primary hepatocytes. The single and double mutants for all the relevant binding sites in the promoter were generated on the -1564/+1-luc construction. Each construct's activity was studied under basal conditions or after 100 nM insulin stimulation by luciferase assays in transiently transfected rat hepatocytes. Insulin increased WT human promoter construction activation by 1.75 fold (Fig. 3A). The single and double SRE mutants in the human promoter abolished the insulin response. The same effect was seen in the single and double LXRE mutants, or in the 1c310 construction which lacks both LXREs and one SRE binding site. This result suggests that human *SREBP1c* promoter regulation by insulin was mediated by both the SRE and LXRE elements in the liver, and that the absence of any of these sites abolishes *SREBP1c* activation. Deletion of the NFY binding site also led to a loss of insulin response, but this effect was probably associated with the diminished basal activity in this mutant.

Both LXRE sites are necessary for complete promoter activation. Activation of the *Srebp1c* mouse promoter at the LXRE sites is mediated by LXR nuclear receptors, as previously shown (6). To study this regulation on the human promoter, we performed a luciferase assay in transiently transfected rat hepatocytes with various human *SREBP1c*-reporter vectors. The -1564/+1-luc construction showed a more than 4-fold activation in the presence of LXR synthetic agonist TO901371 (Fig. 3B). This activation lowered by 50% when one of the LXRE sites

was deleted, and it was completely abolished when both sites were muted. The same effect was seen in the -310/+1 construct which does not contain the LXRE binding sites. The SRE double mutant and the NFY mutant displayed a normal LXR response in spite of their diminished basal activity. This experiment demonstrates that the two LXR binding sites are functional for the human *SREBP1c* promoter stimulatory effect of TO901371, and in the same way as in the rat and mouse promoters.

*The human *SREBP1c* promoter is activated by PPAR α agonists.* The *in silico* analysis of the human *SREBP1c* promoter predicted a DR1 motif, recognized as a PPAR/RXR or a HNF4 binding site according to the Motifviz and Matinspector programs, respectively. A comparative analysis with different databases revealed that the DR1 site present in SREBP1c is very similar to that of the active PPRE in the malic enzyme promoter's regulatory region (22). Although there are several members of the PPARs family, PPAR α is the most abundant in the liver. To evaluate the relevance of PPAR α on the *SREBP1c* promoter regulation, we performed a reporter assay using rat hepatocytes transiently transfected with human *SREBP1c* luciferase constructs. The cells were treated with two PPAR α agonists: WY14643 and GW7647. Both agonists increased the *SREBP1c* promoter activity of either the -1564/+1 or the -520/+1 constructs. No activation was observed using the -310/+1-luciferase vector which lacks DR1 and both LXREs motifs (Fig. 4A).

Regulation of the SREBP1c expression by PPAR α agonists was assessed in human hepatocytes primary culture by a quantitative PCR analysis after 24 h of treatment with GW7647. The SREBP1c expression significantly increased by 2-fold in the presence of the PPAR α agonist (Fig. 4B). As expected, a similar effect was seen when cells were treated with 100 nM insulin for 24 h. Moreover, the PPAR α agonist increased the insulin effect with more than a 7-fold activation in the presence of both GW7647 and insulin.

To determine whether the activation of *SREBP1c* by PPAR α is due to its binding to the predicted DR1 site in the human promoter, we performed an EMSA assay using *in vitro* synthesized PPAR α and RXR α proteins. A PPAR/RXR heterodimer was found to bind to the DR1 probe with partial competition in the presence of the anti-PPAR α antibody and an

extinction of binding in the presence of the anti-RXR antibody. When adding synthesized RXR α protein alone, the presence of a single retarded band indicated that an RXR homodimer was also capable of binding to this sequence *in vitro* (Fig. 4C). An EMSA assay was performed using a PPRE probe as a positive control and a mutated PPRE as a negative control; the expression of each recombinant protein was confirmed by Western blot analysis (data not shown).

The gel shift data were supported with a ChIP assay using human primary hepatocytes. Monolayer cells were fixed and total chromatin was extracted 24 h after incubation with 1 μ M GW7647. The binding of PPAR α to the SREBP1c promoter was analyzed. An RNA pol II antibody was used as a control of the active gene expression, while an RXR α antibody was used as a positive control. After DNA purification, we analyzed the binding of PPAR α , RXR α and RNA pol II by PCR. Both PPAR α and RXR α were seen to bind to the SREBP1c promoter (Fig. 4D). We analyzed the binding of PPAR α and RXR α to the HMGCR promoter, which contains an active PPRE and, as expected, presented a positive binding to both proteins. No enrichment was seen when we used primers to amplify the α -actin or the SREBP1c coding region. The α -RNA polymerase antibody mainly precipitated in the coding chromatin fragments of HMGCR and SREBP1c, and was absent in α -actin, which was not expressed under the experimental conditions (Fig. 4D).

LXRE-mediated activation of the SREBP1c promoter by PPAR α agonists. Considering that the human SREBP1c expression was induced by PPAR α agonists in human hepatocytes and that the SREBP1c promoter was able to bind to PPAR/RXR heterodimers *in vitro* and *in vivo*, we analyzed the regulation of the human SREBP1c promoter in the presence of the PPAR α and RXR α overexpressions. The overexpression of RXR alone activated the promoter in rat primary hepatocytes, while the PPAR α overexpression was only able to produce a slight, but non significant, increase in SREBP1c activity. It was the co-expression of both proteins that brought about the greater activation of the human SREBP1c promoter (Fig. 5A). To evaluate whether SREBP1c activation was present only in the human promoter, we performed the same analysis using a rat Srebplc promoter luciferase reporter vector. An unexpected result was obtained given that the PPAR/RXR heterodimer

overexpression also activated the rat Srebplc promoter (Fig. 5A).

Taking into account the activation of the rat promoter by PPAR/RXR and the absence of a DR1 binding site in the sequence, we considered an LXRE binding site implication in this activation. We performed a shift assay to analyze the binding of PPAR/RXR and LXR/RXR to two separate probes that contained all the SREBP1c promoter LXRE sites (Fig. 5B), and observed an *in vitro* binding of the LXR/RXR heterodimer to both probes (lanes 4 and 9), which was partially competed by the anti-LXR antibody (lanes 5 and 10). Surprisingly, when we incubated both probes in the presence of PPAR and RXR, a retarded band appeared (lanes 1 and 6), which was totally competed with the α -RXR antibody (lanes 3 and 8). In the presence of the α -PPAR α antibody however, we observed competition for the PPAR/RXR heterodimer only with the LXRE2 probe (lane 7). This result suggests that the PPAR/RXR heterodimer is capable of binding *in vitro* to the LXRE2 site, but not to the LXRE1 site. Moreover, the PPAR α agonist significantly increased the liver SREBP1c endogenous expression in rat and mouse cultured hepatocytes treated with 1 μ M GW7647 for 24 h (Fig. 5C).

To determine the *in vivo* binding of PPAR α to the rat Srebplc promoter, we performed a ChIP assay using rat primary hepatocytes. Monolayer cells were fixed and total chromatin was extracted 24 h after incubation with 1 μ M GW7647. The binding of PPAR α and SREBP1 to the Srebplc promoter was analyzed. An RNase pol II antibody was used as a control of the active gene expression. A positive binding of PPAR α to the SREBP1c promoter's LXRE region was observed (Fig. 5D). SREBP1 binding was detected mainly in the SRE binding site together with RNA pol II, which also precipitated with the SREBP1c and β -actin-coding chromatin fragments. No PPAR α binding was observed in the absence of GW7647 (data not shown).

Cross-regulation between PPAR and LXR on the SREBP1c promoter. We have demonstrated above an interaction between PPAR α and the LXRE binding site in the SREBP1c promoter. We next studied the cross-regulation between LXR and PPAR α , which participate together in SREBP1c activation. We evaluated the effect of LXR knocking on human SREBP1c promoter activity by transfecting WT and LXRx/β double

knockout (DKO) mouse hepatocytes with the human *SREBP1c* reporter promoter construct. The -1564/+1 construct of the human *SREBP1c* promoter was activated in the presence of the LXR and PPAR α agonists, TO901317 and GW7647, respectively. The LXR-mediated response was abolished in the DKO hepatocytes and was rescued when an LXRx expression vector was added to the transfection mix, as expected. However, we observed a diminished but still significant activation of the human promoter in the DKO hepatocytes in the presence of 1 μ M GW7647, with a normal response noted in the presence of the exogenous LXRx protein (Fig. 6A).

A luciferase assay with the WT and LXRE double mutant constructions of the human *SREBP1c* promoter was performed to further analyze the interaction between PPAR α and LXR. This assay revealed that TO901317 activation was eliminated in the double LXRE mutant construction, but GW7647 significantly activated both the WT and the mutant constructions. However, the double LXRE mutant led to diminished activation in the presence of the PPAR α agonist if compared to the WT. This result corroborates the data obtained in DKO mice, demonstrating that LXR is necessary for *SREBP1c* promoter maximum activation in the presence of a PPAR α ligand (Fig. 6B). Furthermore, a synergic effect with combined LXR and PPAR α agonists was observed in the human promoter, but not in the rat promoter (Fig. 7A). This difference between species was also observed when using the PPAR α and LXR overexpressions (data not shown). To corroborate this synergic effect produced by the LXR and PPAR α agonists, we analyzed the activation of endogenous *SREBP1c* in cultured human hepatocytes treated with the respective agonists. The SREBP1c gene expression was activated 2-fold in the presence of 1 μ M GW7647. A concentration of 10 nM TO901317 activated *SREBP1c* to the same extent, and boosted GW7647 activation with a 5.5-fold induction in the presence of both agonists (Fig. 7B).

Lipogenesis activation by PPAR α agonists. To evaluate the effect of the PPAR α -mediated activation of SREBP1c on the lipogenic pathway, we analyzed the expression of fatty acid synthase (FAS) and acetyl-coA carboxylase (ACC) lipogenic genes. GW7647, insulin, and TO901317 slightly activated both FAS and ACC

in human hepatocytes; an additive effect was seen with insulin and GW7647, and also with GW7647 and TO901317 (Fig. 8A and B). This effect on GW7647-mediated insulin lipogenic potentiation was confirmed by confocal microscopy. Freshly isolated human hepatocytes were treated as indicated previously, and were subjected to Nile red and DAPI staining. Under these conditions, we observed a cytosolic lipids accumulation in the presence of 100 nM insulin with a synergic effect when GW7647 was also present (Fig. 8B). Fluorescence intensity in the insulin- and GW7647-treated hepatocytes was significantly enhanced (data not shown).

DISCUSSION

Insulin and lipids mediators are signaling molecules that regulate cellular responses in a wide range of physiological and pathological nutritional disorders. The SREBP1c transcriptional factor is a convergence point from starving to post-prandial phases. To coordinate these signals, the promoter region of SREBP1c contains multiple and complex regulatory elements modulated by cholesterol, fatty acids and carbohydrates (11). In this report, we have identified the most relevant regulatory sequences in the human *SREBP1c* promoter structure.

We have clearly demonstrated that the insulin-responsive elements in the *SREBP1c* promoter involve both LXRE and SRE binding sites, which are essential for maximum activation in hepatocytes. Different binding sites, such as LXRE, SRE, SP1 and NFY, have been shown to be involved in the regulation of the mouse and rat *Srebp1c* promoters in different cell lines during a full insulin response (15). It is important to stress that SP1 and NFY are responsible for almost 60% of basal activity; this sometimes makes it difficult to elucidate their implication in insulin activation. An SRE binding site implicates an auto-regulatory loop mediated by SREBP1c itself and by other SREBP family members. The presence of a second active SRE binding site in the human promoter suggests that this loop plays an important role in human metabolism regulation. Studies performed in skeletal muscle have demonstrated that this loop is more relevant for insulin regulation than LXR (10). However, our results prove the essential role of LXR signaling in insulin regulation in hepatocytes. Different mechanisms have been described to be implicated in the insulin mediated activation of LXR and its target genes.

In addition, insulin has been seen to activate the LXR expression in the liver (23), while others have hypothesized that insulin may stimulate the production of an oxygenated sterol ligand for this nuclear receptor (13). Furthermore, physiological glucose concentration activates LXR in the liver and induces the expression of LXR target genes, whose efficacy is similar to that of oxysterols, the well-known LXR ligands (24). LXR could, therefore, be a transcriptional switch that integrates hepatic glucose metabolism and fatty acid synthesis.

Many aspects of hepatic lipid metabolism are under the transcriptional control of PPAR α . It is well-established that an impaired PPAR α function is associated with hepatic lipid accumulation. Moreover, synthetic agonists for PPAR α have been explored for the treatment of fatty liver disease (25). The discovery of a DR1 putative binding site in the human promoter prompted us to study the human *SREBP1c* promoter's response to PPAR α ligands. We found a positive response to WY14643 and GW7647 agonists together with activation in the presence of an exogenous PPAR expression. Moreover a synergic effect was observed in the presence of insulin or LXR agonists. PPAR α seems to be an activator of the *SREBP1c* promoter *in vivo*, as predicted by chip-chip analyses (26).

Crosstalk between PPAR and LXR at the transcriptional level has been previously demonstrated. An active PPRE element present in the LXRx promoter region is able to respond to PPAR agonists (27). Given the fact that there are two LXRE active sites in the *SREBP1c* promoter, we considered the possibility of an indirect activation of this promoter mediated by LXRx activation. However, *SREBP1c* promoter activation in the presence of the PPAR α /RXR heterodimer was seen in either hepatocytes from double LXR knockout mice (DKO) or rat hepatocytes transfected with the LXRE double mutant luciferase construction. These experiments demonstrate a direct activation mediated by PPAR itself.

The effect of the PPAR α nuclear receptor on the control of *Srebp1c* mouse promoter activity has been previously studied (9). The authors showed that PPAR α inhibited the mouse *Srebp1c* promoter's LXR-mediated activation in HEK293 and HepG2 cells; moreover, they observed no activation in rat hepatocytes treated with WY14643. The authors suggested that this inhibition was mediated by

the sequestration of RXR avoiding the LXR-mediated activation. This discrepancy may relate to the use of fasted mice in Yosikawa's study. König et al have also shown that PPAR α agonists reduce triglycerides synthesis in rat hepatoma cells, which is partially caused by inhibited SREBP1 activation (28). The above experiments were performed under low glucose conditions to mimic the fasted condition. However, our experiments were performed in cells maintained at high glucose concentrations or in hepatocytes obtained from *ad libitum*-fed animals. Our results suggest that PPAR α -mediated *SREBP1c* promoter activation is dependent on the presence of the LXR nuclear receptor, and probably on nutritional status. Recently, Chakravarhy et al. have identified a physiologically relevant endogenous ligand for PPAR α in the liver that induces the expression of those genes involved in fatty acid metabolism, but only in the presence of active fatty acid synthase (29). Furthermore, the SREBP1c mRNA levels were lower in the livers of PPAR α -null mice than in the WT mice (30). Accordingly, the level of SREBP1c significantly increased in fenofibrate-treated WT mice (31), but not in PPAR α -null mice (32). Thus it is feasible to hypothesize that PPAR α gene expression regulation in general, and the SREBP1c expression in particular, may depend on an acting ligand nature.

PPAR α has been shown to govern the expression of numerous genes involved in fatty acid oxidation, ketogenesis, gluconeogenesis, cholesterol catabolism and lipoprotein metabolism (33). However, the data obtained from transgenic mice (34) and our results achieved with human hepatocytes report that PPAR α does not merely serve as a transcriptional activator of fatty catabolism, but plays a much broader role in lipid metabolism. An important question to clarify is why a transcription factor, whose primary role is to coordinate fatty acid oxidation in the starved state, is required to facilitate fatty acid synthesis during the re-feeding period. We have shown that PPAR α agonists cooperate with insulin to induce hepatic lipid synthesis. Our results also demonstrate that LXR is essential for SREBP1c regulation by insulin through its LXR elements present in the promoter. Moreover, the maximum effect of PPAR α is obtained when the LXR element remains unmodified. PPAR α may be required for the production of an endogenous

LXR ligand. In line with this, the original PPAR α dependence of insulin action in PPAR α -deficient mice was abolished under conditions in which LXR activation was not limiting (35).

Important differences between rodents and humans have been previously demonstrated in terms of gene regulation by PPAR and LXRs, especially in the field of macrophage homeostasis (36). It is important to bear in mind not only that the expression levels of PPAR and LXR can differ between humans and mice, but levels of RXR expression may vary and could influence the activity of both LXR and PPAR, and their crosstalk (7).

Ours results provide an insight into the role of the insulin-, LXR-, and PPAR-mediated

regulation of human SREBP1c and its relation with nutritional status. PPAR α and LXR act as lipid-glucose sensors and bind to ligands deriving from either intracellular metabolism or dietary lipids. Endogenous ligands for PPAR include fatty acids and eicosanoids, whereas metabolites of oxidized cholesterol activate LXRs. Moreover, it has been demonstrated there is no single endogenous ligand for PPAR α , thus PPAR could play a role as a nutritional status sensor in the cell to regulate the SREBP1c dependent pathway by controlling the fasting/fed transition.

REFERENCES

1. Grundy, S. M., Brewer, H. B., Jr., Cleeman, J. I., Smith, S. C., Jr., and Lenfant, C. (2004) *Circulation* **109**(3), 433-438
2. Reaven, G. M. (2005) *Annu Rev Nutr* **25**, 391-406
3. Foufelle, F., and Ferre, P. (2002) *Biochem J* **366**(Pt 2), 377-391
4. Horton, J. D., Goldstein, J. L., and Brown, M. S. (2002) *J Clin Invest* **109**(9), 1125-1131
5. Janowski, B. A., Grogan, M. J., Jones, S. A., Wisely, G. B., Kliewer, S. A., Corey, E. J., and Mangelsdorf, D. J. (1999) *Proc Natl Acad Sci U S A* **96**(1), 266-271
6. Yoshikawa, T., Shimano, H., Amemiya-Kudo, M., Yahagi, N., Hasty, A. H., Matsuzaka, T., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Kimura, S., Ishibashi, S., and Yamada, N. (2001) *Mol Cell Biol* **21**(9), 2991-3000
7. Rakhsandehroo, M., Hooiveld, G., Muller, M., and Kersten, S. (2009) *PloS one* **4**(8), e6796
8. Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A., and Evans, R. M. (1992) *Nature* **358**(6389), 771-774
9. Yoshikawa, T., Ide, T., Shimano, H., Yahagi, N., Amemiya-Kudo, M., Matsuzaka, T., Yatoh, S., Kitamine, T., Okazaki, H., Tamura, Y., Sekiya, M., Takahashi, A., Hasty, A. H., Sato, R., Sone, H., Osuga, J., Ishibashi, S., and Yamada, N. (2003) *Mol Endocrinol* **17**(7), 1240-1254
10. Dif, N., Euthine, V., Gonnet, E., Laville, M., Vidal, H., and Lefai, E. (2006) *Biochem J* **400**(1), 179-188
11. Raghow, R., Yellaturu, C., Deng, X., Park, E. A., and Elam, M. B. (2008) *Trends Endocrinol Metab* **19**(2), 65-73
12. Amemiya-Kudo, M., Shimano, H., Yoshikawa, T., Yahagi, N., Hasty, A. H., Okazaki, H., Tamura, Y., Shionoiri, F., Iizuka, Y., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Sato, R., Kimura, S., Ishibashi, S., and Yamada, N. (2000) *The Journal of biological chemistry* **275**(40), 31078-31085
13. Chen, G., Liang, G., Ou, J., Goldstein, J. L., and Brown, M. S. (2004) *Proc Natl Acad Sci U S A* **101**(31), 11245-11250
14. Deng, X., Cagen, L. M., Wilcox, H. G., Park, E. A., Raghow, R., and Elam, M. B. (2002) *Biochem Biophys Res Commun* **290**(1), 256-262

15. Cagen, L. M., Deng, X., Wilcox, H. G., Park, E. A., Raghow, R., and Elam, M. B. (2005) *Biochem J* **385**(Pt 1), 207-216
16. Casado, M., Callejas, N. A., Rodrigo, J., Zhao, X., Dey, S. K., Bosca, L., and Martin-Sanz, P. (2001) *Faseb J* **15**(11), 2016-2018
17. Ferrini, J. B., Ourlin, J. C., Pichard, L., Fabre, G., and Maurel, P. (1998) *Methods Mol Biol* **107**, 341-352
18. Fernandez-Alvarez, A., Tur, G., Lopez-Rodas, G., and Casado, M. (2008) *FEBS Lett* **582**(2), 177-184
19. Fernandez-Alvarez, A., Soledad Alvarez, M., Cucarella, C., and Casado, M. (2010) *The Journal of biological chemistry* **285**(16), 11765-11774
20. McMillian, M. K., Grant, E. R., Zhong, Z., Parker, J. B., Li, L., Zivin, R. A., Burczynski, M. E., and Johnson, M. D. (2001) *In vitro & molecular toxicology* **14**(3), 177-190
21. Tarling, E., Salter, A., and Bennett, A. (2004) *Biochem Soc Trans* **32**(Pt 1), 107-109
22. Castelein, H., Gulick, T., Declercq, P. E., Mannaerts, G. P., Moore, D. D., and Baes, M. I. (1994) *The Journal of biological chemistry* **269**(43), 26754-26758
23. Tobin, K. A., Ulven, S. M., Schuster, G. U., Steineger, H. H., Andresen, S. M., Gustafsson, J. A., and Nebb, H. I. (2002) *The Journal of biological chemistry* **277**(12), 10691-10697
24. Mitro, N., Mak, P. A., Vargas, L., Godio, C., Hampton, E., Molteni, V., Kreusch, A., and Saez, E. (2007) *Nature* **445**(7124), 219-223
25. Fernandez-Miranda, C., Perez-Carreras, M., Colina, F., Lopez-Alonso, G., Vargas, C., and Solis-Herruzo, J. A. (2008) *Dig Liver Dis* **40**(3), 200-205
26. van der Meer, D. L., Degenhardt, T., Vaisanen, S., de Groot, P. J., Heinaniemi, M., de Vries, S. C., Muller, M., Carlberg, C., and Kersten, S. (2010) *Nucleic Acids Res* **38**(9), 2839-2850
27. Tobin, K. A., Steineger, H. H., Alberti, S., Spydevold, O., Auwerx, J., Gustafsson, J. A., and Nebb, H. I. (2000) *Mol Endocrinol* **14**(5), 741-752
28. Konig, B., Koch, A., Spielmann, J., Hilgenfeld, C., Hirche, F., Stangl, G. I., and Eder, K. (2009) *European journal of pharmacology* **605**(1-3), 23-30
29. Chakravarthy, M. V., Lodhi, I. J., Yin, L., Malapaka, R. R., Xu, H. E., Turk, J., and Semenkovich, C. F. (2009) *Cell* **138**(3), 476-488
30. Patel, D. D., Knight, B. L., Wiggins, D., Humphreys, S. M., and Gibbons, G. F. (2001) *Journal of lipid research* **42**(3), 328-337
31. Oosterveer, M. H., van Dijk, T. H., Tietge, U. J., Boer, T., Havinga, R., Stellaard, F., Groen, A. K., Kuipers, F., and Reijngoud, D. J. (2009) *PloS one* **4**(6), e6066
32. Le Jossic-Corcos, C., Duclos, S., Ramirez, L. C., Zaghini, I., Chevillard, G., Martin, P., Pineau, T., and Bournot, P. (2004) *Biochimica et biophysica acta* **1683**(1-3), 49-58
33. Mandard, S., Muller, M., and Kersten, S. (2004) *Cell Mol Life Sci* **61**(4), 393-416
34. Chakravarthy, M. V., Pan, Z., Zhu, Y., Tordjman, K., Schneider, J. G., Coleman, T., Turk, J., and Semenkovich, C. F. (2005) *Cell metabolism* **1**(5), 309-322
35. Hebbachi, A. M., Knight, B. L., Wiggins, D., Patel, D. D., and Gibbons, G. F. (2008) *The Journal of biological chemistry* **283**(8), 4866-4876
36. Rigamonti, E., Chinetti-Gbaguidi, G., and Staels, B. (2008) *Arterioscler Thromb Vasc Biol* **28**(6), 1050-1059

FOOTNOTES

We thank Dr. David J. Mangelsdorf (Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, USA) for his generous gift of double LXR knockout mice, Dr. Domingo Baretino for providing us with the RXR antibody, and Benito Alarcon for his technical assistance in the confocal analysis.

This work has been supported by the Spanish Ministry of Science and Innovation [SAF2006-06760; SAF2009-12602], and the Carlos III Health Institute [Red de Centros FIS-RECAVA RD06/0014/0025]. A. F-A. has been supported by the Carlos III Health Institute [Red de Centros FIS-RECAVA RD06/0014/0025]. M.S.A is supported by a fellowship from CSIC (Spanish National Research Council) (JAE-predoc).

FIGURE LEGENDS

Figure 1. Sequence analysis of the 5' flanking sequence of the human *SREBP1c* promoter. Sequence alignment of the proximal promoter region of human, rat and mouse *SREBP1c*, including the transcriptional start site (bold letters). A schematic representation of previously demonstrated binding sites in mice and rats, and the homolog sites in the human sequence are shown. Human specific predicted binding sites are also shown.

Figure 2. Identification of the minimal human *SREBP1c* promoter region. Serial of luciferase reporter constructs containing various lengths of the human *SREBP1c* promoter, from -1564 to +1, were analyzed in primary culture rat hepatocytes. The d158-luc construction has a deletion of 158 bp, which contains NFY, SRE2, and multiple SP1 sites corresponding to the region responsible for basal activity in mice and rats. Firefly and Renilla luciferase activities were measured in cell lysates. The results represent relative Firefly/Renilla luciferase activities by considering the -1564/+1 construct as 100% fold activation. Bars represent the mean ± SE of four separate experiments.

Figure 3. Nutritional regulation of the human *SREBP1c* promoter. Insulin- and LXR-mediated regulation of the human *SREBP1c* promoter were analyzed using -1564/+1-luc (1c1500), -310/+1-luc (1c310) plasmids or mutants of the different cis-elements in the-1564/+1-luc vector. The relevance of each predicted binding site for insulin and TO901317 (LXR agonist) regulations was analyzed by luciferase assays. Activities of the WT and mutated constructs transfected in rat primary hepatocytes under basal conditions (gray bars) and 100 nM insulin (black bars), (A) or under basal conditions (gray bars) and 10 μM TO901317 (black bars) (B), are shown. The results represent relative Firefly/Renilla luciferase activities (RLU) considering the wild-type construct under basal conditions as 100% fold activation. Values are the mean ± SE of four separate experiments. Differences between the control and the TO901317 treatment significantly differ for all the constructions except for dmLXRE and 1c310 (* p<0.05; ** p<0.01). A scheme of the predicted binding sites in the human promoter is shown.

Figure 4. PPAR α regulates human *SREBP1c* promoter activity. **A-** PPAR α -dependent regulation of the *SREBP1c* promoter was analyzed by luciferase assays using the -1564/+1-luc (1c1500), -520/+1-luc (1c520) and -310/+1-luc (1c310) constructs in the presence of WY14643 (WY) or GW7647 (GW) as selective PPAR α agonists. Activities of the promoter constructs transfected in rat primary hepatocytes under basal conditions (black bars), 30 μM WY (white bars) or 1μM GW (gray bars) are shown. The results represent relative Firefly/Renilla luciferase activities considering the wild-type construct under basal conditions as the reference value. Values are the mean ± SE of four separate experiments. **B-** The endogenous *SREBP1c* expression in cultured human hepatocytes was analyzed by quantitative PCR. Cells were seeded under basal conditions (control) or treated with 1 μM GW7647 (GW) and/or 100 nM insulin. Total RNA was extracted 24 h after treatment and cDNA was synthesized by reverse transcriptase. The relative amount of the *SREBP1c*/36B4 expression was determined in each condition by taking the basal condition as the reference value. The results are the mean ± SE of three separate experiments. Differences between the control and the treatments were statistically significant. **C-** Analysis of PPAR binding *in vitro* to the *SREBP1c* promoter. EMSA was performed with recombinant human proteins incubated with 32 P-oligonucleotide

encoding for the DR1 element of the human *SREBP1c* promoter. α -PPAR α or α -RXR α antibodies were used for the supershift assays. The complete EMSA gel, (left panel) shows that an equal amount of the probe was loaded in all the lanes. **D-** Analysis *in vivo* of PPAR binding to the *SREBP1c* promoter. The ChIP assay was performed in human hepatocytes treated with 1 μ M GW7647. Immunoprecipitation of samples was performed with the α -PPAR α antibody. A positive control of transcriptionally active genes was performed using the α -RNA polII antibody and a negative control with no antibody. An α -RXR antibody was used as a positive control for the PPAR α activated promoters. An analysis of the HMGCR gene was used as a positive control for PPAR α and RXR binding. The α -actin primers were used as negative control for all the antibodies. The results are representative of the PCR fragments analysed by agarose gel electrophoresis of two independent experiments.

Figure 5. PPAR α -mediated SREBP1c activation through an LXRE binding site. **A-** PPAR α -mediated regulations of the human and rat SREBP1c promoters were analyzed using rat primary hepatocytes. Cells were transiently transfected with a plasmid containing 1500 bp of a human or rat SREBP1c promoter sequence linked to the luciferase reported plasmid pGL3-basic together with the a) PPAR α , b) RXR α , and c) PPAR α and RXR α expression vectors. An empty vector was used to normalize the amounts of total transfected DNA. Firefly and Renilla luciferase activities were measured in cell lysates. The results represent relative Firefly/Renilla luciferase activities by considering the empty vector condition as 100% fold activation. Values are the mean \pm SE of four separate experiments. **B-EMSA** was performed with the recombinant human proteins incubated with 32 P-oligonucleotide encoding for LXRE1 and LXRE2 sites. α -PPAR α , α -LXR α or α -RXR α antibodies were used for the supershift assays. **C-** The endogenous SREBP1c expression in cultured rat and mouse hepatocytes was analyzed by quantitative PCR. Cells were seeded under basal conditions (control) or treated with 1 μ M GW7647 (GW). Total RNA was extracted 24 h after treatment and cDNA was synthesized by reverse transcriptase. The relative amount of the SREBP1c/GAPDH expression was determined under each condition by taking the basal condition as the reference value. The results are the mean \pm SE of three separate experiments. **D-**The ChIP assay was performed with rat primary hepatocytes. Immunoprecipitation of samples was performed with the α -PPAR α and α -SREBP1 antibodies. A positive control of the transcriptionally active genes was performed using the α -RNA polII antibody, and a negative control was normal rat IgG and no antibody. The results are representative of the PCR products analyzed by agarose electrophoresis of three independent experiments.

Figure 6. Cross-regulation between PPAR α and LXR on the SREBP1c promoter. **A-** Human *SREBP1c* promoter regulation was analyzed using the wild-type (WT) and LXR α /LXR β double-mutant mice (DKO) primary hepatocytes transfected with the -1564/+1-luc vector either with or without an LXR α expression vector. An empty vector was used to normalize the amounts of total transfected DNA. Firefly and Renilla luciferase activities were measured in cell lysates after treatment with 10 μ M TO901317 (TO; upper panel) or 1 μ M GW7647 (GW; lower panel) for 24 h. The results represent relative Firefly/Renilla luciferase activities by considering the control condition as 100% fold activation. Values are the mean \pm SE of four separate experiments. **B-Luciferase assay** was performed in the rat hepatocytes transfected with -1564/+1-luc (WT) and LXRE double mutant-luc (dmLXRE). Cells were seeded under basal conditions (control) or treated with 1 μ M GW7647 (GW) or 10 μ M TO901317 (TO). The results represent relative Firefly/Renilla luciferase activities by considering the control condition for each construction as 100%. Values are the mean \pm SE of three separate experiments.

Figure 7. Additive effect of PPAR α and LXR on SREBP1c promoter regulation. **A-**The additive PPAR α - and LXR-mediated regulations of human and rat SREBP1c promoters were analyzed using rat primary hepatocytes. Cells were transiently transfected with a plasmid containing 1500 bp of the human or rat SREBP1c promoter sequence linked to the luciferase reported plasmid pGL3-basic treated for 24 h with 1 μ M GW7647 (GW) and/or 10 nM TO901317 (TO). Firefly and Renilla luciferase activities were measured in cell lysates. The results represent relative Firefly/Renilla luciferase activities by considering the control condition as 100% fold activation. Values are the mean \pm SE of four separate experiments (* $p<0.05$). **B-** The endogenous SREBP1c

expression in the cultured human hepatocytes was analyzed by quantitative PCR. Cells were seeded under basal conditions (control) or treated with 1 μ M GW7647 (GW) and/or 10 nM TO901317 (TO). Total RNA was extracted 24 h after treatment and cDNA was synthesized by reverse transcriptase. The relative amount of the SREBP1c/36B4 expression was determined under each condition by considering the basal condition as the reference value. The results are the mean \pm SE of three separate experiments. Differences between the control and the treatments were statistically significant.

Figure 8. Lipogenic effect of PPAR α agonists in the liver. **A-** The endogenous FAS and ACC expression in human cultured hepatocytes were analyzed by quantitative PCR. Cells were seeded under basal conditions (control) or treated with 1 μ M GW7647 (GW), 10 nM TO901317 (TO), 100 nM insulin (Ins), or with a combination of GW7647 with TO901317 or insulin. Total RNA was extracted 24 h after treatment and cDNA was synthesized by reverse transcriptase. The relative amount of the SREBP1c/36b4 expression was determined under each condition by considering the control condition as the reference value. The results are the mean \pm SE of three separate experiments. ($*<0.05$). **B-**Lipids accumulation in human hepatocytes was analyzed by Nile red staining under a confocal microscope. The images are representative of at least 10 different images under each condition. All images were obtained under the same zoom, wavelength and intensity conditions.

Figure 1

Figure 2

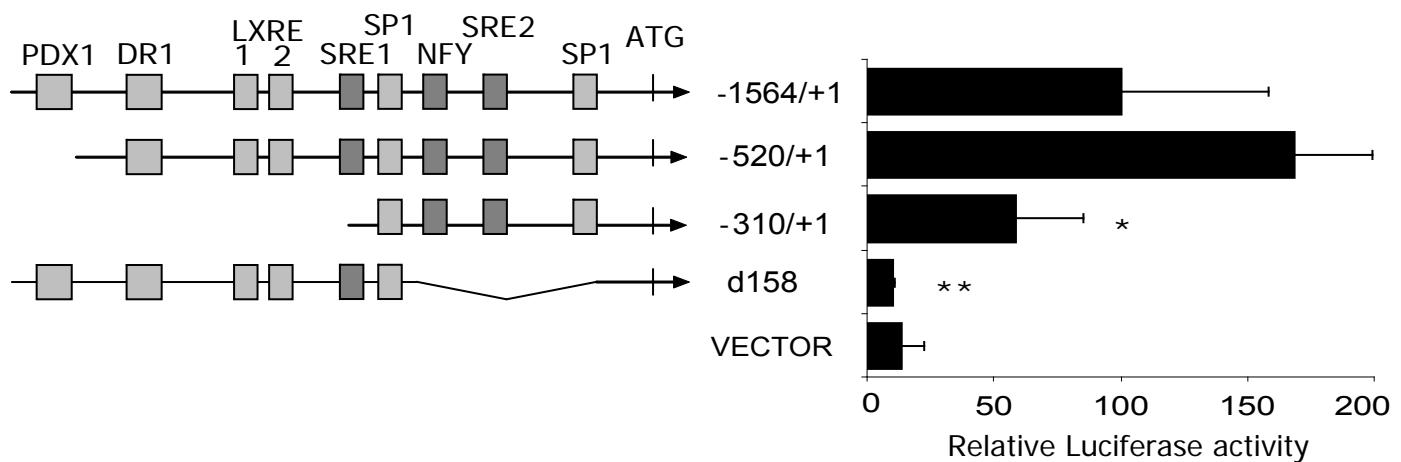
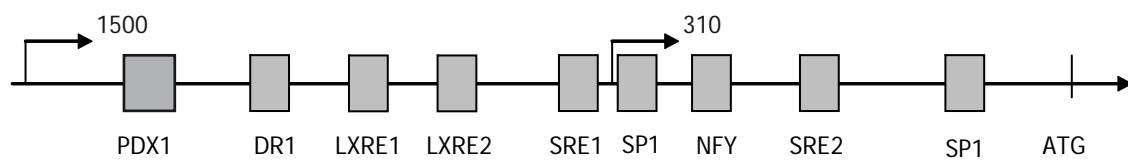
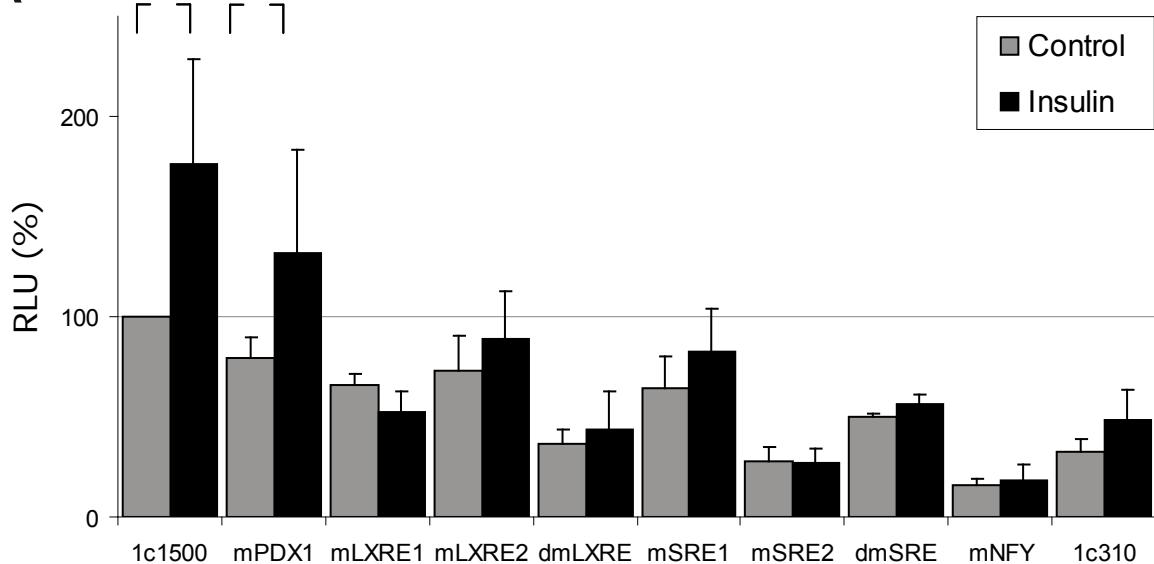


Figure 3

A



B

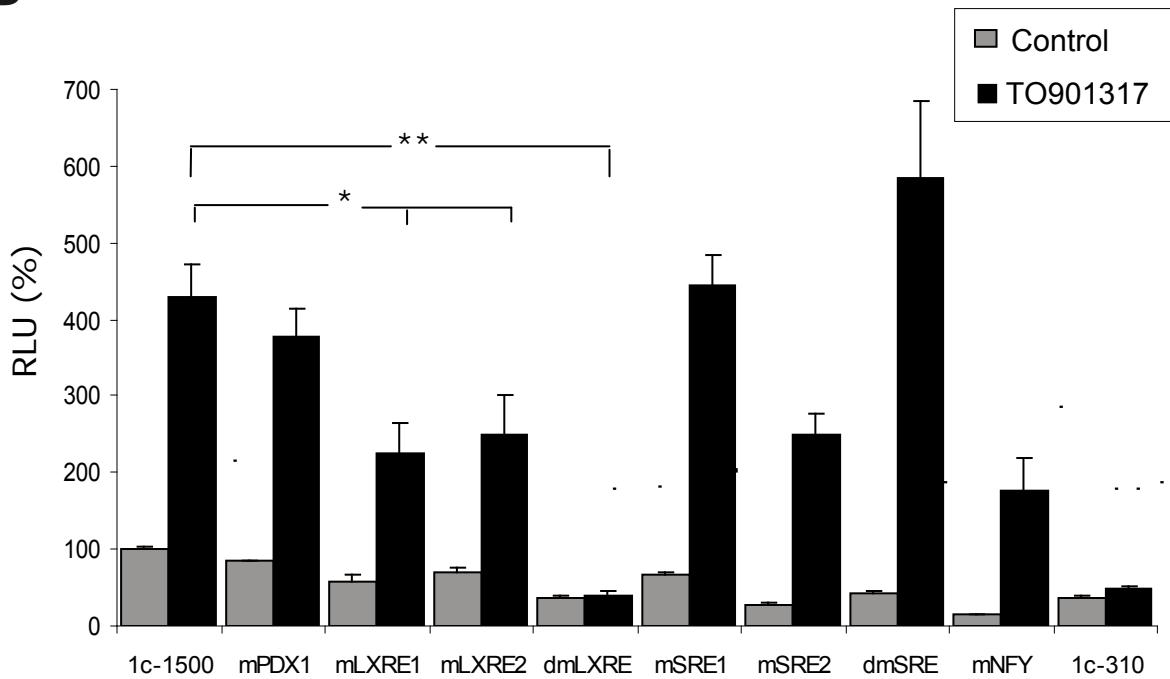
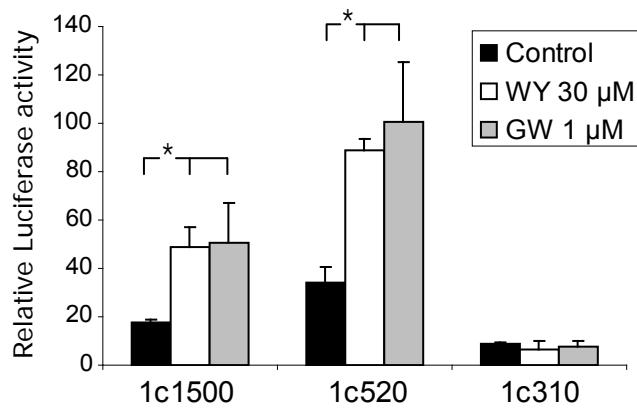
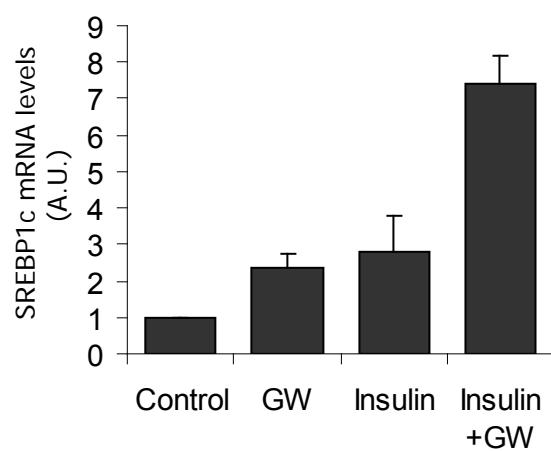


Figure 4

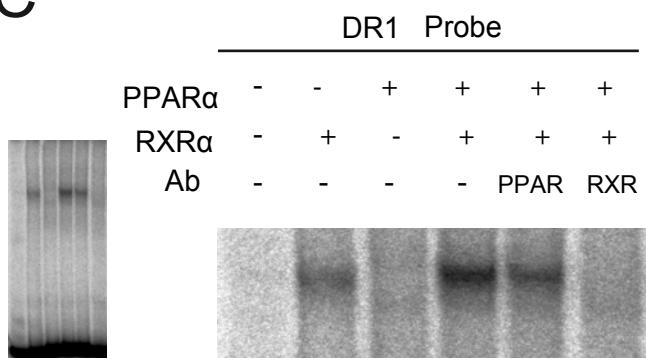
A



B



C



D

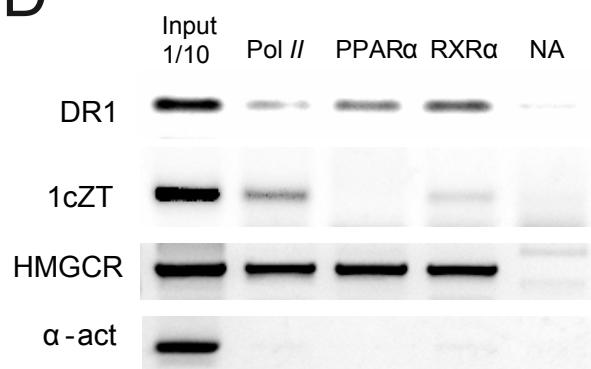
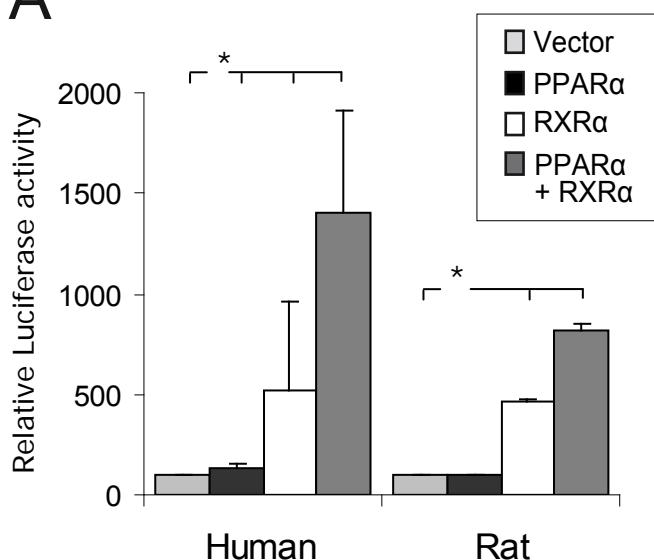
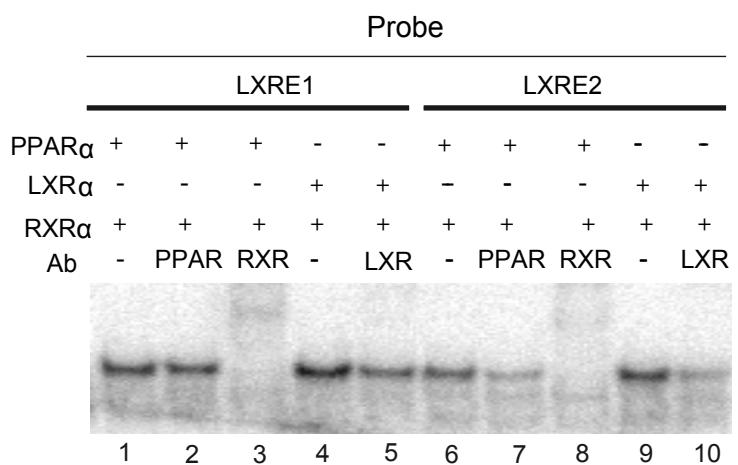


Figure 5

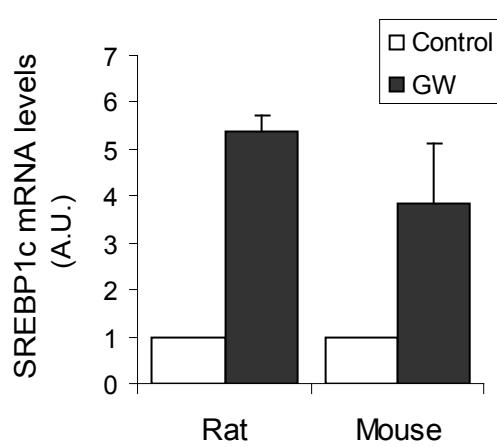
A



B



C



D

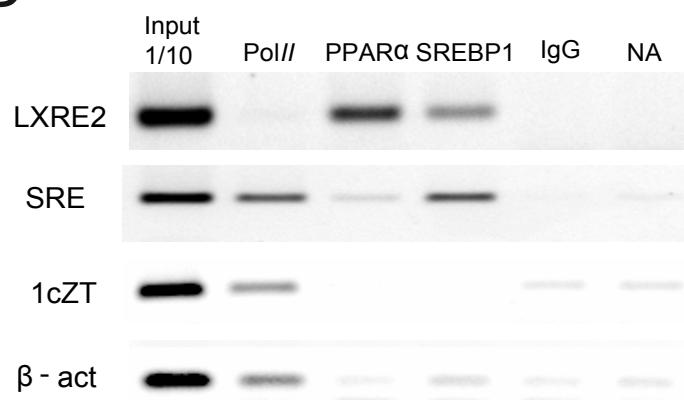


Figure 6

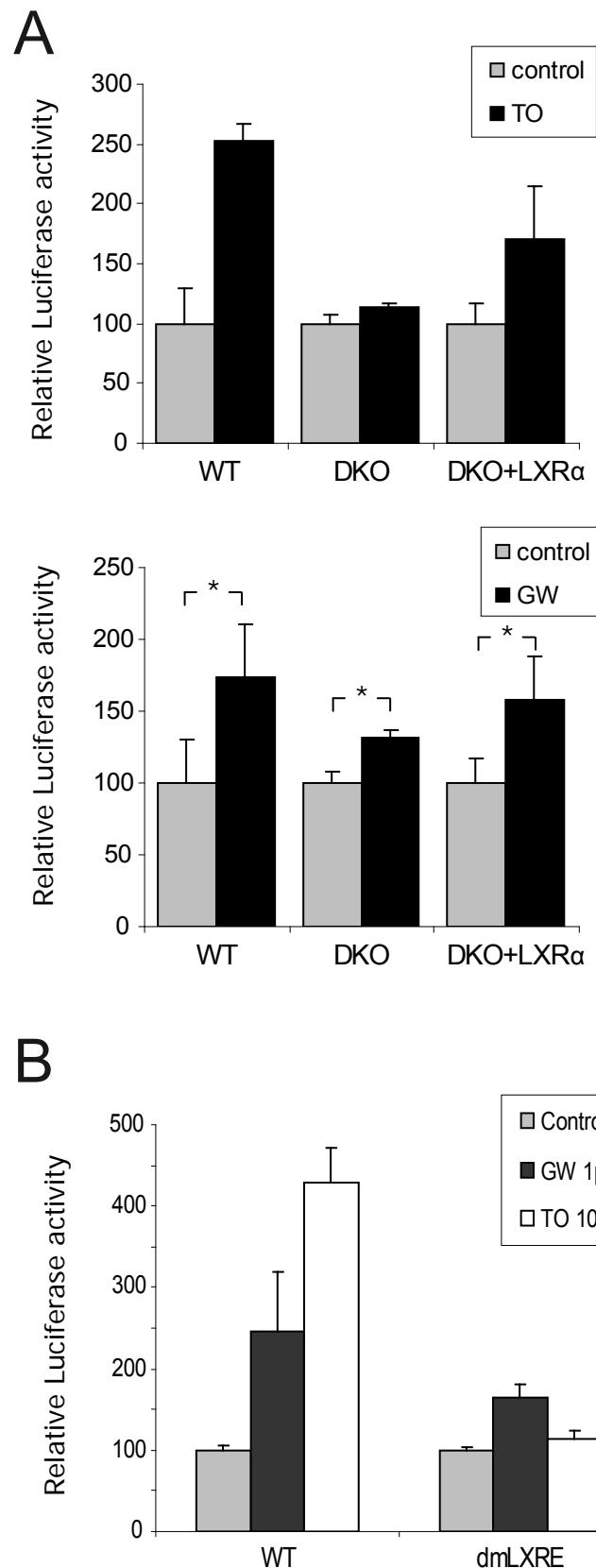


Figure 7

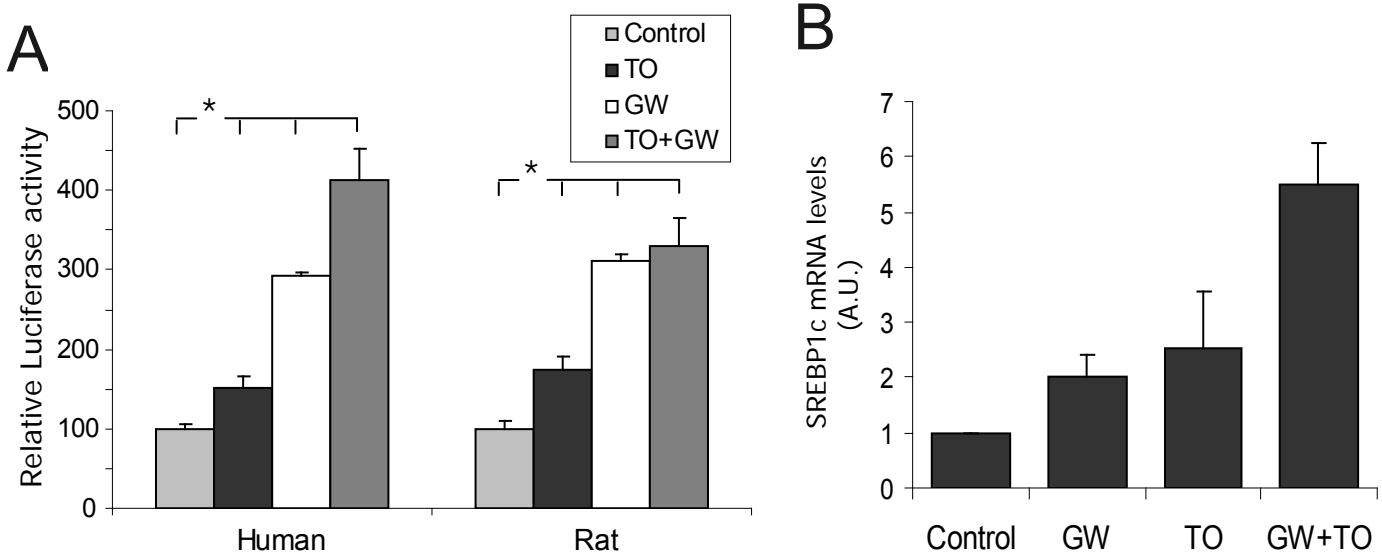


Figure 8

