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Transcriptional regulation of human oxysterol 7α-hydroxylase by sterol response element binding protein☆

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Abstract

Oxysterol 7α-hydroxylase (CYP7B1) metabolizes oxysterols, potent regulators of lipid homeostasis. Very little is known about transcriptional regulation of human CYP7B1. The present results indicate that sterol response element binding protein (SREBP), a family of oxysterol-responsive transcription factors that stimulates cholesterol synthesis, may be an important regulator of CYP7B1. SREBP suppressed a human CYP7B1 luciferase reporter gene in several cell lines, most markedly in rat hepatoma McA-RH7777 cells. An SREBP-1-responsive region was mapped to a GC-rich sequence in the proximal CYP7B1 promoter, containing binding sites for the basal transcriptional activator Sp1. Mutagenesis of this sequence abolished SREBP-1-mediated suppression. Data indicated that SREBP does not bind this sequence but affects the gene indirectly, probably via interaction with Sp1. Our findings indicate that CYP7B1 transcription is controlled by SREBP and reveal a link between oxysterol-sensitive regulators and oxysterol metabolism. We propose that CYP7B1 is important for regulating cellular sterol content and protects against oxysterol-mediated toxicity.

Keywords: CYP7B1; SREBP; Cholesterol; Oxysterol; Sterol metabolism; Lipid homeostasis

The formation and metabolism of cholesterol are tightly regulated processes, controlled by hormones, sterol levels, and bile acid feedback inhibition [1–6]. CYP7B1 (oxysterol 7α-hydroxylase) is active in the metabolism of oxysterols, compounds that serve as important regulatory molecules as well as intermediates in cholesterol-derived biosynthetic pathways [1,5,7,8]. Oxysterols down-regulate the synthesis of cholesterol and fatty acids and increase metabolism of cholesterol to bile acids in mice [2,9]. High amounts of cellular oxysterols may result in cytotoxicity. CYP7B1 is widely expressed in many human tissues [8]. In the liver, CYP7B1 participates in the alternative (acidic) pathway of bile acid biosynthesis, catalyzing 7α-hydroxylation of 27-hydroxycholesterol [8,10,11]. This enzyme, which has attracted considerable interest in recent years, is structurally similar to cholesterol 7α-hydroxylase (CYP7A1) but has a different and broad substrate specificity [7,8,10–14]. It has been reported that 7α-hydroxylation may modulate some of the regulatory effects of oxysterols on cholesterol metabolism [15,16]. In addition to oxysterols, CYP7B1 also metabolizes certain neurosteroids and is reported to play a role in endocrine function [7,17].

Notwithstanding the important physiological role(s) of CYP7B1, very little is known about the regulation of this enzyme, particularly in humans, besides a role for the general transcriptional activator Sp1 in the basal transcription of CYP7B1 [11,18,19]. Recently, Pandak et al. [19] reported that CYP7B1 levels in primary rat hepatocytes increased when cells were incubated with cholesterol and decreased in the presence of squalestatin, a cholesterol synthesis inhibitor. This and other studies led us to hypothesize that cholesterol or oxysterols may regulate CYP7B1 expression. Our interest

☆ *Abbreviations:* CYP, cytochrome P450; CYP7B1, oxysterol 7α-hydroxylase; EMSA, electrophoretic mobility shift assay; LXR, liver X receptor; RLU, relative light units; SRE, sterol response element; SREBP, sterol response element binding protein.

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was turned towards transcriptional regulators reported to be affected by oxysterols, including liver X receptor (LXR) and sterol response element binding protein (SREBP). We could not detect any effects on human CYP7B1 transcription by LXR (M. Norlin, J.Y.L. Chiang, unpublished observation), which stimulates cholesterol elimination by induction of cholesterol 7α-hydroxylase [2,9]. However, the present study provides evidence that SREBP, a family of transcription factors that stimulate the biosynthesis of cholesterol and other lipids, may be an important transcriptional regulator of human CYP7B1.

Materials and methods

Materials. Rat hepatoma cells McA-RH7777 (CRL1601), human embryonic kidney HEK 293 cells (CRL1573), and Chinese hamster ovary CHO cells (CCL61) were obtained from the American Type Culture Collection (Manassas, VA). Reagents for luciferase assay and radioactive labeling were obtained from Promega. Oligonucleotides used in electrophoretic mobility shift assay (EMSA) and mutagenesis experiments were synthesized by Invitrogen (Carlsbad, CA). Expression plasmids containing Sp1 or SREBP were kindly provided by Drs. R. Tjian (University of California, Berkeley, CA) and T. Osborne (University of California, Irvine, CA), respectively. The wild-type CYP7B1 promoter–luciferase reporter gene constructs were generated as previously described [8].

Cell culture and transient transfection assay. McA-RH7777, HEK 293 or CHO cells were cultured in 12- or 24-well tissue culture plates in DMEM F-12 medium (Life Technologies, Rockville, MD) supplemented with 10% (v/v) heat-inactivated fetal calf serum, penicillin G (100 U/ml), and streptomycin (100 μg/ml). Cells were transiently transfected with CYP7B1 promoter–luciferase reporter gene constructs, pCMV β-galactosidase plasmid (for normalizing transfection efficiency), and expression vectors containing SREBP or Sp1, using the calcium co-precipitation method [8,20]. Luciferase and β-galactosidase activities were assayed as described [8,20]. Luciferase reporter activity is expressed as relative light units (RLU) divided by β-galactosidase activity (expressed as Abs 420 nm).

Site-directed mutagenesis. Mutations were introduced into reporter constructs by PCR-based site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Complementary sets of oligonucleotides corresponding to appropriate regions of the CYP7B1 promoter were designed as PCR-primers. A reporter construct designated GCm5 and containing a mutation in GC box 2 (−12 to −7) in the proximal promoter region was based on the wild-type construct −291/+189Luc [8] and generated using a mutagenic primer (upper strand shown): $^{-22}$CGGTACGCGTGttacGAGGATAGGCG^{+4}. The mutant constructs designated GCm1 (containing a mutation in GC box 1), GCm2 (containing a mutation in GC box 3), GCm3 (containing mutations in GC boxes 1 and 3), and GCm4 (containing mutations in GC boxes 1, 2, and 3) were generated as previously described [18].

Thermal cycling was performed with the designed primers and wild-type reporter construct as template and was followed by digestion of parental template DNA with *Dpn*I. The PCRs were then transformed into supercompetent XL-1 Blue cells. All mutations were verified by sequencing.

Preparation of nuclear extracts. Nuclear extracts were prepared from McA-RH7777 cells according to methods previously described [20]. One day prior to nuclear extract preparation, the cells were transfected with expression vectors containing SREBP-1c or SREBP-2 as described in the previous section. Protein was quantified using BCA Protein Assay Reagent (Pierce) and the nuclear extracts were stored in aliquots at −70 °C.

EMSA. Custom-synthesized complementary oligomers carrying 5′ GATC overhangs were annealed and labeled by incorporating [α-^{32}P]dCTP (3000 Ci/mmol, Perkin–Elmer Life Sciences, Boston, MA) as described [20]. For binding reactions, 10 μg of nuclear extract was preincubated on ice for 15–30 min in 12 mM Hepes, pH 7.9, containing 50 mM KCl, 1 mM dithiothreitol, and 15% glycerol. Each reaction also contained poly(dI–dC), 1 μg per 2–3 μg protein, to minimize unspecific binding. Binding reactions were incubated with labeled double-stranded oligonucleotides (35,000–50,000 cpm/reaction) for 20 min at room temperature. Following incubation, the binding reactions were loaded onto a 4% non-denaturing polyacrylamide gel and DNA/protein complexes were separated by electrophoresis at 200 V in Tris–glycine buffer (25 mM Tris, 0.19 M glycine, and 1 mM EDTA) at room temperature. Gels were dried, autoradiographed, and analyzed as previously described [20].

Results and discussion

CYP7B1 promoter activity is suppressed by SREBP

Regulatory effects on human CYP7B1 by transcription factors in lipid homeostasis were examined using transient transfection assay. Cotransfection of a CYP7B1 promoter–luciferase reporter gene (p-2771/+189Luc) with the nuclear forms of SREBP-1a, SREBP-1c or SREBP-2, a family of transcription factors that stimulate formation of cholesterol, revealed strong suppression on CYP7B1 reporter activity. The effects of SREBP were examined in several cell lines, showing suppression in all, although we observed some differences in the magnitude of suppression and the relative effect compared between different SREBP isoforms. The most dramatic suppression was observed in McA-RH7777 (rat hepatoma) cells where all three SREBP isoforms inhibited reporter activity by about 80–95% (Fig. 1A). Strong suppression by all three SREBP isoforms (by about 70–75%) was also observed in Chinese hamster ovary (CHO) cells (Fig. 1B). In the kidney-derived HEK 293 cells, SREBP-2 inhibited promoter activity by about 40–60%, whereas SREBP-1a or -1c had no detectable effect (data not shown). The observed difference between HEK 293 cells and the other cell lines may or may not reflect tissue-specific regulatory mechanisms. Anyway, the results indicate that SREBP can affect human CYP7B1 transcription in liver and non-liver cells.

The SREBP-responsive region is located in the proximal promoter

Further studies on the effect of SREBP on the CYP7B1 promoter were carried out in McA-RH7777 cells. This cell line is reported to be particularly suitable for studying SREBP-mediated regulation due to its high SREBP-1c/1a ratio, which is similar to the physiological conditions found in the liver [21]. Although SREBP-1a

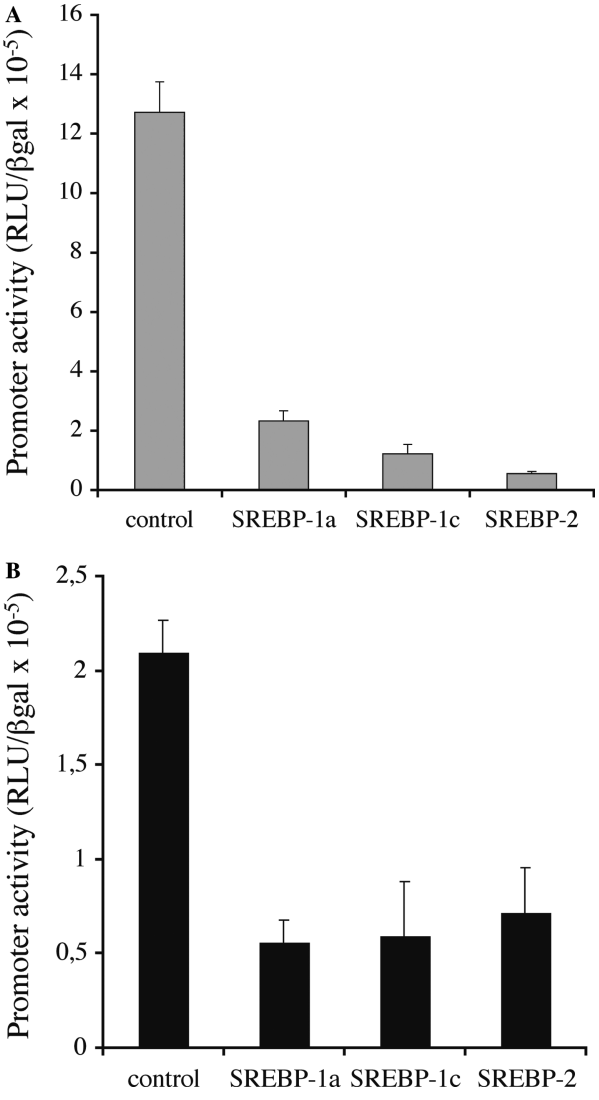

Fig. 1. Effects of cotransfection with SREBP-1a, SREBP-1c or SREBP-2 on a CYP7B1 promoter–luciferase reporter gene in (A) McA-RH7777 cells and (B) CHO cells. The CYP7B1 reporter (0.4–1 μg) was transiently transfected with expression vectors containing SREBP (0.4–1 μg). Controls were transfected with empty pcDNA3 vector instead of SREBP vector to normalize the amount of DNA transfected. The CYP7B1 reporter vector contained a fragment spanning from −2771 to +189 of the human CYP7B1 promoter sequence [8]. Error bars represent the standard deviation of the means of triplicate assays.

is the predominant form in most cell lines, SREBP-1c is the predominant form in liver and adipocytes. Effects of SREBP were studied with different deletion constructs of the CYP7B promoter–luciferase reporter gene (Fig. 2). SREBP inhibited the reporter activity of all constructs, including the one containing the shortest promoter fragment. This indicates that the sequence(s) most important for regulation by SREBP are located in the proximal promoter, in the region spanning from −86 to +176. This region contains several GC boxes, which are potential SREBP binding sites [18].

Two sequences similar to classical SREBP binding sites (SRE-like) are located in the −314 to −327 region [8]. However, deletion of these sequences (cf. constructs −343/+189Luc and −291/+189Luc in Fig. 2) did not significantly affect the SREBP-mediated effects on reporter activity.

Mutagenesis of three GC boxes in the proximal promoter results in loss of response to SREBP-1

As shown above, deletion construct analysis indicated that SREBP regulation of CYP7B1 is mediated by sequences in the proximal promoter. In a previous study from this laboratory, a cluster of three GC boxes in the proximal promoter was found to play an important role for basal transcription of CYP7B1 [18]. To examine the possible role of this region in SREBP regulation we compared the effects of SREBP on wild-type reporter and reporter constructs with mutations in these GC boxes. Mutations in one (GCm1, GCm2, and GCm5) or two (GCm3) of the GC boxes had minor effects on the SREBP-mediated response. However, mutagenesis of all three GC boxes (GCm4) resulted in substantial decrease of the response to SREBP-1a and SREBP-1c (Fig. 3). Surprisingly, this mutant retained its responsiveness to SREBP-2. This suggests that the mechanism of regulation of CYP7B1 by SREBP-1 and SREBP-2 may be different. These two SREBPs are known to have differential effects on genes in cholesterol and fatty acid synthesis. It may be concluded that the GC-rich sequences are important for regulation by SREBP-1 and that one intact GC box is sufficient to convey SREBP-mediated inhibition.

SREBP does not bind to the CYP7B1 gene

To investigate whether SREBP binds directly to the CYP7B1 gene or acts through indirect effects, we performed EMSA with five probes designed from the sequence of the proximal DNA region. These probes corresponded to promoter sequences spanning from nucleotide −30 to +180 (probe 1, −30 to +18; probe 2, +11 to +56; probe 3, +52 to +99; probe 4, +94 to +140; and probe 5, +136 to +180). A known SREBP-binding sequence from the LDL promoter [22] was used as a positive control. EMSA was carried out with radiolabeled probes and nuclear extracts prepared from McA-RH7777 cells overexpressed with either SREBP-1c or SREBP-2. EMSA with SREBP-enriched nuclear extracts showed strong binding to the LDL promoter control by both SREBP isoforms. In contrast, none of the CYP7B1 promoter sequences showed any detectable binding by SREBP-1c or SREBP-2 (data not shown). These results indicate that the suppressive effect by SREBP on CYP7B1 does not involve direct binding to the gene. Instead the observed effect may be mediated

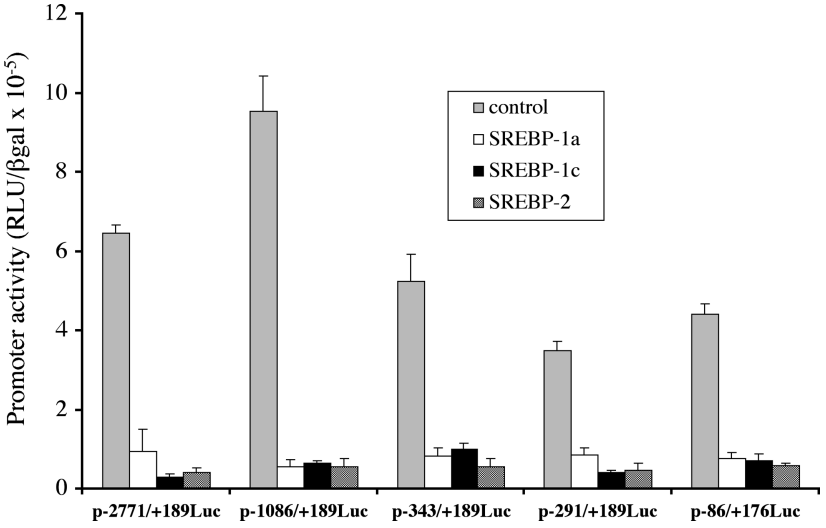

Fig. 2. Effects of SREBP on different deletion constructs of the CYP7B1 promoter–luciferase reporter gene in McA-RH7777 cells. Reporter constructs (0.8 μg) were transiently transfected with expression vectors containing SREBP (0.16 μg). Controls were transfected with empty pcDNA3 vector (0.16 μg) instead of SREBP vector to normalize the amount of DNA transfected. Error bars represent the standard deviation of the means of triplicate assays.

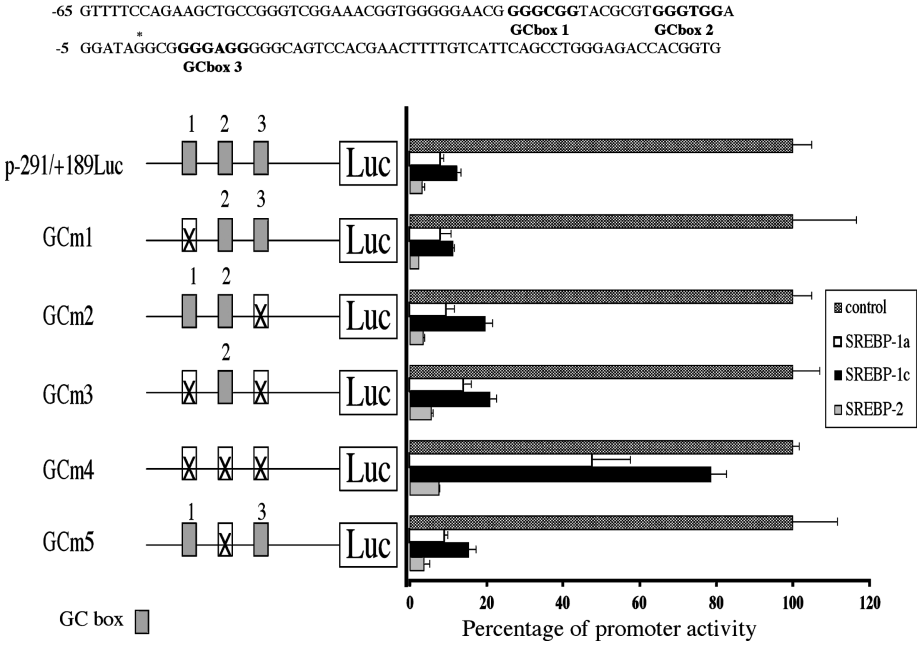

Fig. 3. Effects of SREBP on CYP7B1 reporter constructs carrying mutations in the GC boxes. The figure shows the effects of cotransfection with SREBP on the p-291/+189Luc reporter construct and its derivative mutant constructs carrying mutations in the GC boxes (−25 to +10) of the CYP7B1 promoter. The nucleotide sequence from −65 to +55 is shown. The transcription start site is indicated by *. Reporter constructs (1.3 μg) were transiently transfected with expression vectors containing SREBP (1.3 μg) in McA-RH7777 cells. Controls were transfected with empty pcDNA3 vector to normalize the amount of DNA transfected. Mutant constructs were generated by site-directed mutagenesis as described in Materials and methods. The results are shown as percentage of promoter activity. The actual promoter activity of p-291/+189Luc in the experiment shown was 5×10^6 RLU/β-gal. The basal promoter activity of GCm4, which was the lowest, was between five and ten times lower than that of p-291/+189Luc. Error bars represent the standard deviation of the means of triplicate assays.

through an indirect mechanism, most likely by interaction of SREBP with one or several other factors. This finding may be compared with a previous study on sterol 12α-hydroxylase [22] where suppression by SREBP-2 was reported to occur without direct binding to the 12α-hydroxylase promoter.

SREBP abolishes Sp1-mediated activation of the CYP7B1 promoter

The results above suggest that the suppressive effect of SREBP on CYP7B1 may be mediated by interaction with other factors. Previous data have indicated that the transcriptional activator Sp1 plays an important role for basal CYP7B1 transcription [18]. Wu and Chiang [18] identified Sp1-binding sequences in the proximal CYP7B1 promoter and found that mutagenesis of these sequences markedly reduced promoter activity. It is noteworthy that the promoter region previously shown to bind Sp1 is the same sequence as the GC-rich region shown to mediate SREBP-1 response in the current study.

Since Sp1 is known to interact with SREBP in the regulation of certain other genes such as the LDL receptor [23], we examined the effect of Sp1 on SREBP-mediated regulation. As would be expected, Sp1 stimulated wild-type CYP7B1 reporter activity (Fig. 4). Interestingly, cotransfection of SREBP-1c completely abolished Sp1-mediated stimulation. These results suggest that SREBP may interact with Sp1 in the regulation of CYP7B1 and that SREBP may have a dominant negative effect on CYP7B1 gene transcription. The data point towards a probable mechanism where SREBP may inhibit CYP7B1 transcription by preventing Sp1 from binding to the GC box sequence. This is consistent with the report that Sp1 has the ability to interact with several factors including SREBP [23,24].

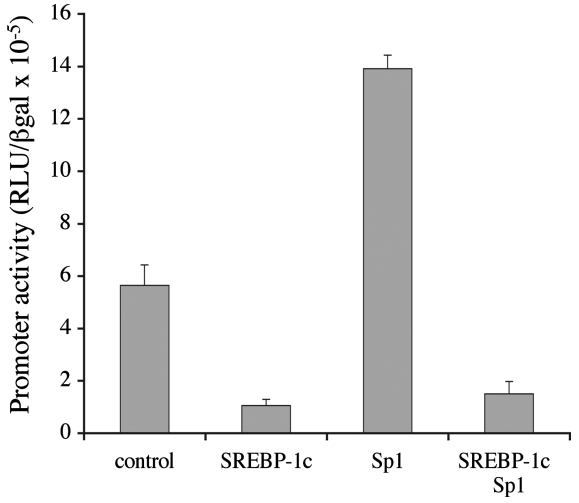

Fig. 4. Effects of cotransfection with SREBP and Sp1 on CYP7B1 reporter activity. CYP7B1/Luc reporter (0.8 μg) was transiently transfected with expression vectors containing Sp1 (0.8 μg) and/or SREBP (0.08 μg) in McA-RH7777 cells. Controls were transfected with empty pcDNA3 vector to normalize the amount of DNA transfected. The CYP7B1 reporter vector contained a fragment spanning from −2771 to +189 of the human CYP7B1 promoter sequence [8]. Error bars represent the standard deviation of the means of triplicate assays.

A proposed role for CYP7B1 in controlling the levels of cellular oxysterols

The current data showing strong SREBP-mediated suppression of the CYP7B1 promoter reveal an interesting link between oxysterol-sensitive regulatory factors and oxysterol metabolism. Although oxysterols play an important role in regulation of genes in lipid metabolism, high amounts of these reactive compounds may be toxic to the cell. For this reason, the cellular content of oxysterols may need to be tightly controlled. CYP7B1 metabolizes 25- and 27-hydroxycholesterol, two of the most abundant oxysterols in the human body. We propose that the function of CYP7B1, in liver and other tissues, might be to balance the levels of oxysterols, compounds that on the one hand are regulatory important but on the other hand potentially cytotoxic. A possible mechanism for the role of CYP7B1 in this process is outlined in Fig. 5. According to current concepts, SREBP stimulates genes in the formation of cholesterol and other lipids (Fig. 5A). Rising cellular levels of oxysterols will suppress the protease-mediated activation of SREBP [6,25] (Fig. 5B). Inactivation of SREBP by oxysterols will result in decreased expression of the genes in fatty acid and cholesterol synthesis that are normally stimulated by SREBP.

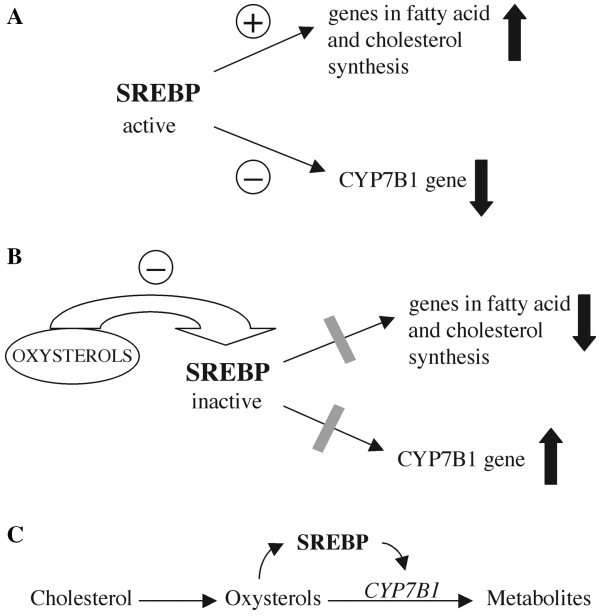

Fig. 5. Suggested mechanism for the role of CYP7B1 in balancing the levels of cellular oxysterols. (A) Low cellular oxysterol level resulting in active SREBP. (B) High cellular oxysterol level resulting in suppressed SREBP. (C) Proposed link between oxysterols, SREBP, and CYP7B1. A high cellular oxysterol level inhibits SREBP, a condition resulting in decreased expression of genes in lipid synthesis but increased expression of CYP7B1, which is needed under these conditions to metabolize the excess oxysterols. − indicates suppression and + indicates stimulation.

From the present results, it may be concluded that under conditions where SREBP is comparatively inactive, expression of CYP7B1 will increase since SREBP suppresses this gene (Fig. 5B). Consequently, as the cellular oxysterol levels rise more CYP7B1 enzyme becomes available for metabolism of these compounds. In this manner, SREBP might regulate the expression of CYP7B1 in order to balance the amount of cellular oxysterols available for regulatory purposes while maintaining them below toxic levels (Fig. 5C).

This hypothesis is in accordance with the results of previous studies on CYP7B1. (1) Pandak et al. [19] reported that CYP7B1 levels in primary rat hepatocytes increased when cells were incubated with cholesterol and decreased in the presence of squalestatin, a cholesterol synthesis inhibitor. High cellular cholesterol concentrations should lead to increased formation of cholesterol-derived oxysterols, whereas inhibition of cholesterol synthesis should lead to decreased levels of oxysterols. (2) Furthermore, a physiological role of SREBP in the regulation of CYP7B1 is supported by the findings of Repa et al. [26] on mice lacking CYP27A1, the enzyme that produces 27-hydroxycholesterol. According to this study, mice with a disrupted CYP27A1 gene have elevated levels of both SREBP-1 and -2 and significantly decreased CYP7B1 levels. (3) Stulnig et al. [27] reported that hepatic CYP7B1 expression in mice was strongly decreased (6-fold) by treatment with the synthetic LXR agonist T0901317, although the authors did not attempt to explain this finding. From our results, a possible mechanism for this effect might be surmised. We have not found any direct effects of LXR on CYP7B1 transcription, but found strong suppressive effects by the SREBPs, including SREBP-1c. Since the expression of SREBP-1c is induced by LXR [27,28], it seems very likely that activation of LXR may decrease CYP7B1 levels through stimulation of SREBP-1c transcription.

It has been reported that a human infant with a defective CYP7B1 gene suffered from severe neonatal cholestasis and died in infancy [29]. It was suggested that the condition might result from an impaired formation of bile acids, thus indicating an especially important role for CYP7B1-mediated reactions in bile acid synthesis in infancy. However, another possibility may be that the severe liver injury observed in this patient could be due to accumulation of high levels of hepatotoxic oxysterols as a consequence of the absence of CYP7B1.

CYP7B1 may be an important regulator of cholesterol homeostasis also in extrahepatic tissues. In fact, CYP7B1 mRNA expression levels are much higher in the kidney, ovary, testes, lung, pancreas, heart, and brain than in the liver [8]. Many of these tissues produce high levels of oxysterols. CYP7B1 may play a major role in metabolism of oxysterols in these tissues to render them more water soluble and less toxic for excretion in urine.

In summary, the present study provides new data on the transcriptional regulation of human CYP7B1, indicating that the transcription of CYP7B1 is controlled by SREBPs, factors involved in regulatory processes related to the maintenance of adequate cellular sterol levels. Our data strongly support an important role for CYP7B1 in controlling the levels of oxysterols in hepatic and extrahepatic cells.

Acknowledgments

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