Biochemical and Biophysical Research Communications 430 (2013) 336–339

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

CCAAT/enhancer binding protein β deletion increases mitochondrial function and protects mice from LXR-induced hepatic steatosis

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ARTICLE INFO

Article history:
Received 30 October 2012
Available online 15 November 2012

Keywords:
LXR agonist
Lipogenesis
Steatosis
Mitochondrial function
Cholesterol efflux

ABSTRACT

Drugs designed specifically to activate liver X receptors (LXRs) have beneficial effects on lowering cholesterol metabolism and inflammation but unfortunately lead to severe hepatic steatosis. The transcription factor CCAAT/enhancer binding protein beta (C/EBPβ) is an important regulator of liver gene expression but little is known about its involvement in LXR-based steatosis and cholesterol metabolism. The present study investigated the role of C/EBPβ expression in LXR agonist (T0901317)-mediated alteration of hepatic triglyceride (TG) and lipogenesis in mice. C/EBPβ deletion in mice prevented LXR agonist-mediated induction of lipogenic gene expression in liver in conjunction with significant reduction of liver TG accumulation. Surprisingly, C/EBPβ$^{-/-}$ mice showed a major increase in liver mitochondrial electron chain function compared to WT mice. Furthermore, LXR activation in C/EBPβ$^{-/-}$ mice increased the expression of liver ATP-binding cassette transporter ABCG1, a gene implicated in cholesterol efflux and reducing blood levels of total and LDL-cholesterol. Together, these findings establish a central role for C/EBPβ in the LXR-mediated steatosis and mitochondrial function, without impairing the influence of LXR activation on lowering LDL and increasing HDL-cholesterol. Inactivation of C/EBPβ might therefore be an important therapeutic strategy to prevent LXR activation-mediated adverse effects on liver TG metabolism without disrupting its beneficial effects on cholesterol metabolism.

1. Introduction

The nuclear liver X receptors (LXRs) LXRα and LXRβ are sterol sensors that bind oxysterols and act as potent transcriptional switches for the coordinated regulation of genes involved in the control of hepatic lipid and cholesterol metabolism. They control genes that regulate cholesterol transport, catabolism, and absorption [1], as well as the transcriptional control of triglyceride (TG) synthesis. This latter effect is partly mediated by sterol regulatory element-binding protein-1c (SREBP1c) activation [2,3] and the up-regulation of its downstream target genes (e.g. lipoprotein lipase (LPL) [4], fatty acid synthase (FAS) [5], and stearoyl-CoA desaturase 1 [6]). Additionally, LXR activation increases the carbohydrate response element-binding protein (ChREBP) to coordinate lipogenesis [7] and hepatic fatty acid synthesis. In rodents but not in humans, LXRs enable bile acid synthesis by activation of cholesterol 7α-hydroxylase (CYP7A1) [8]. Two nonsteroidal LXR synthetic ligands, GW3965 and T0901317, have been created and in atherosclerotic mouse models their activation resulted in decreased atherosclerosis [9,10]. Unfortunately, most LXR agonists, while lowering cholesterol, have the concomitant induction of lipogenic genes that leads to hypertriglyceridemia and liver steatosis, which have limited further clinical development.

The transcriptional control of liver gene expression also includes the CCAAT/enhancer binding protein family member C/EBPβ, a b-ZIP transcription factor essential for adipose differentiation and native immunities [11,12]. Recently, we showed that C/EBPβ deletion in mice reduces methionine–choline deficient diet-induced lipogenesis and abnormal lipid accumulation in liver [13]. We also demonstrated that C/EBPβ deletion in Lepr$^{db/db}$ mice reduced hepatic steatosis, adiposity, and diabetes [14]. However, whether C/EBPβ participates in LXR-mediated induction of lipogenesis or cholesterol metabolism remains unexplored.

In the present study we show that C/EBPβ$^{-/-}$ mice are protected from chronic LXR agonist-mediated abnormal TG accumulation in liver, along with reduced lipogenic gene expression, using T0901317, a potent and selective agonist of LXRα and LXRβ. Unexpectedly, C/EBPβ deletion increased the expression of genes and proteins involved in mitochondrial function, which may play

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http://dx.doi.org/10.1016/j.bbrc.2012.11.027

an important role in protection from steatosis. Moreover, the present studies demonstrate that C/EBPβ deletion does not interfere with the therapeutic potential of LXR activation on cholesterol metabolism, suggesting it may have potential as a therapeutic target for both cholesterol and lipid homeostasis.

2. Materials and methods

2.1. Mice and diets

All mice were housed in colony cages in a pathogen-free barrier facility operating a 12-h light/12-h dark cycle. The breeding and care of these animals were in accordance with the protocols approved by the Animal Care Research Committee of the University of Colorado Denver. The generation and genotyping procedures for C/EBPβ$^{-/-}$ mice have been described previously [15]. Homozygous C/EBPβ$^{-/-}$ and WT male mice were used for LXR agonist treatment. At 12 weeks of age, mice were fed ad libitum a cereal-based powdered diet (Teklad 7001; Harlan Teklad, Indianapolis, IN) with or without 0.025% (wt/wt) LXR agonist T0901317 (Cayman Chemical, Ann Arbor, MI) for 4 days.

2.2. Blood and hepatic lipids

Mice were fasted for 4 h and blood samples were collected by retro-orbital blood sampling. Mice were then sacrificed and liver tissue was collected. Serum was separated by centrifugation and stored at −80 °C until analyzed. Serum TG, total cholesterol, HDL-cholesterol, and LDL-cholesterol levels were determined using enzymatic kits (Sigma–Aldrich, St. Louis, MO and Roche Diagnostics Corp., Indianapolis, IN) according to the manufacturer's procedures. Total liver lipids were extracted following a modified method [16] from Folch et al. [17] and then assayed for TG.

2.3. Histological analyses

Frozen liver sections were fixed in paraformaldehyde and stained with Oil Red O solution (Sigma–Aldrich). Stained liver sections were washed thoroughly with distilled water prior to microscopic observation. Images were captured using an Olympus camera mounted on an Olympus upright microscope.

2.4. Quantitative real-time PCR

Total RNA was isolated from relevant tissues using RNeasy Plus kit (Qiagen, Valencia, CA). Reverse transcription and quantitative real-time PCR were performed as previously described [18]. RNA expression data were normalized to levels of reference genes ubiquitin C and GAPDH using the comparative threshold cycle method.

2.5. Protein extraction and Western blot analysis

Livers (~200 mg) were homogenized and proteins were extracted from whole-cell lysates and mitochondrial/nuclear fractions as described previously [19]. Western blot analysis was performed as described previously [13]. Primary antibodies used in this study were complexes I, II, and NDUFS3 (Life Technologies, Grand Island, NY), and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

2.6. Statistical analysis

Statistical comparisons between groups were made using Student's *t* test. All values are reported as mean ± SEM and differences were considered to be statistically significant at *P* values ⩽0.05.

3. Results and discussion

3.1. T0901317 administration prevents liver lipid accumulation in C/EBPβ$^{-/-}$ mice

Reports have shown that LXR agonist ingestion induces severe hepatic steatosis [20,21]. To directly assess the effect of T0901317 administration on lipid metabolism in liver, we measured the levels of intracellular TGs in liver. LXR agonist administration significantly increased the liver TG levels 2.5-fold in WT mice, whereas the livers of C/EBPβ$^{-/-}$ mice supplemented with LXR agonist showed substantial reduction (around 55%) of lipid accumulation in the liver both at baseline and in response to LXR agonist treatment compared to WT mice on same dietary treatment (Fig. 1A). Consistent with lower hepatic TG content in C/EBPβ$^{-/-}$ mice, Oil Red O staining of liver sections for neutral lipids showed no hepatic steatosis in mice on control diet, whereas LXR agonist supplementation markedly increased lipid accumulation in WT mice as shown by increases in both the number and size of liver fat droplets (Fig. 1B). By contrast, livers of C/EBPβ$^{-/-}$ deficient mice were protected from LXR agonist-mediated increase in liver fat droplets (Fig. 1B). This result indicates that C/EBPβ deletion protected these mice from LXR activation-mediated abnormal accumulation of liver TG.

3.2. T0901317 administration reduced lipogenic gene expression in C/EBPβ$^{-/-}$ mice

In addition to increased liver TG levels, LXR agonists induce lipogenesis in liver due in part to increased SREBP1c [2,3]. To

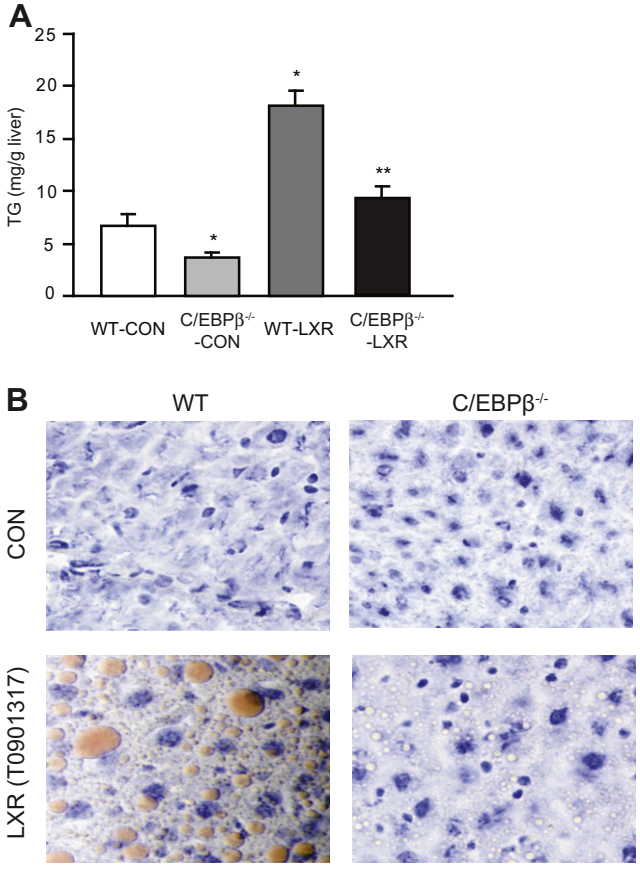

Fig. 1. (A) Liver TG. Total lipid was extracted as described in Section 2. Data are shown as mean ± SEM. *N* = 4 mice/group; $^{*}P < 0.05$ *vs.* WT-CON; $^{**}P < 0.05$ *vs.* WT-LXR (T0901317). (B) Representative photomicrographs of Oil Red O-stained liver sections are shown at 100× magnification (*N* = 4 mice/group).

investigate the effect of LXR agonist administration on lipogenic gene expression levels in liver ofC/EBPβ$^{-/-}$ mice, we measured hepatic mRNA levels in control-fed and T0901317-treated mice. Under control-fed conditions, SREBP1c was increased up to 4-fold in both WT and C/EBPβ$^{-/-}$ mice treated with LXR agonist (Fig. 2A). In contrast, expression of key lipogenic genes, FAS and SCD1, were 52–65% lower, respectively, in liver of C/EBPβ$^{-/-}$ mice, while LXR agonist administration significantly increased both genes in WT mice and in C/EBPβ$^{-/-}$ mice, but these were much higher in WT mice (Fig. 2A). Likewise, ACC1, but not ChREBP, was highly induced in WT and C/EBPβ$^{-/-}$ mice in response to LXR agonist treatment, but remained significantly lower in C/EBPβ$^{-/-}$ mice. Angptl3 is another LXR target which inhibits LPL activity, and induction of Angptl3 has been shown to contribute to the hypertriglyceridemic effect on LXR activation [22]. Angptl3 gene expression was significantly lower (41%) in C/EBPβ$^{-/-}$ mice than WT mice under basal conditions, and remained lower after LXR treatment in C/EBPβ$^{-/-}$ mice. LPL is an enzyme that hydrolyzes TGs on VLDL and thus participates in the clearance pathway of plasma VLDL TG [23]. Interestingly, LPL expression was strikingly higher in C/EBPβ$^{-/-}$ mice compared to WT mice under control-fed conditions, while in response to T0901317 treatment was 60% higher in C/EBPβ$^{-/-}$ mice than in their WT counterpart (Fig. 2A). These

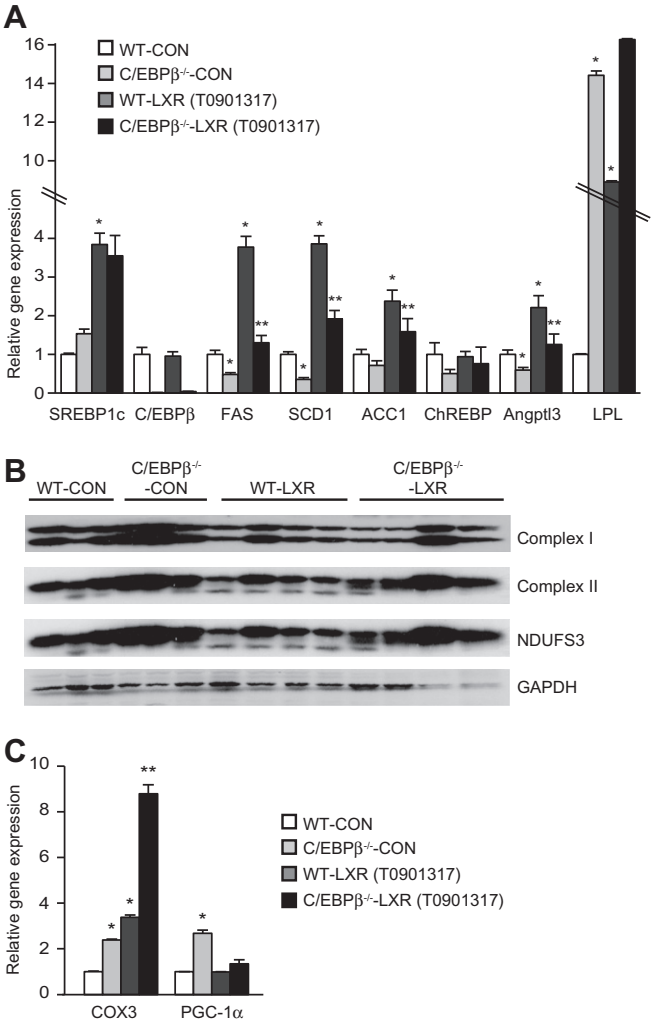

Fig. 2. (A) Expression of lipogenic genes in liver analyzed by quantitative PCR. (B) Immunoblots for mitochondrial transport chain proteins. $N = 3$ for CON groups, $N = 4$ for T0901317-treated groups. (C) Expression of mitochondrial genes analyzed by quantitative PCR. Data are shown as mean ± SEM. $N = 4$ mice/group; $^{*}P < 0.05$ *vs.* WT-CON; $^{**}P < 0.05$ *vs.* WT-LXR (T0901317).

results imply that C/EBPβ expression is required for LXR induction of target genes especially those important for lipogenesis, and is independent of SREBP1c gene expression. Together with increased LPL and reduced Angptl3, these results corroborate our data that C/EBPβ deletion prevents LXR ligand-mediated induction of liver steatosis genes, while remarkably increasing LPL gene expression.

3.3. Increased mitochondrial oxidative phosphorylation in liver of T0901317-administered C/EBPβ$^{-/-}$ mice

Given the striking increase in LPL gene expression in C/EBPβ$^{-/-}$ mice, we suspected that mitochondrial fatty acid oxidation might also help account for the reduced fatty liver in C/EBPβ$^{-/-}$ mice upon LXR activation. We measured key proteins implicated in mitochondrial function in liver. Interestingly, expression of complexes I, II, and NDUFS3 proteins were higher under basal conditions in C/EBPβ$^{-/-}$ mice, and remained higher in response to treatment with LXR agonist compared to WT mice (Fig. 2B). Consistent with the increased protein expression, gene expression of complex III (COX3) was also higher under basal condition in C/EBPβ$^{-/-}$ mice, while LXR activation increased COX3 gene expression in both WT and C/EBPβ$^{-/-}$ mice; however, COX3 remained 2.7-fold higher in the liver of LXR ligand-treated C/EBPβ$^{-/-}$ mice than LXR ligand-treated WT mice. The significant induction of COX3 gene expression and enhancement of complexes I, II, and NDUFS3 protein expression in C/EBPβ$^{-/-}$ mouse liver strongly suggests improved mitochondrial chain function in those mice and may prevent liver lipid accumulation in C/EBPβ$^{-/-}$ mice. Indeed, the present study demonstrated a significant increase in peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), a powerful transcriptional coactivator that promotes mitochondrial biogenesis and fatty acid oxidation in liver of C/EBPβ$^{-/-}$ mice under control conditions (Fig. 2C). Interestingly, a significant number of the approximately 76 known human mitochondrial genes [24] contain a potential C/EBP binding site. However, the promoter structure of most mitochondrial genes is not well studied.

3.4. Reduced blood cholesterol levels and increased liver ABCG1 gene expression in T0901317-administered C/EBPβ$^{-/-}$ mice

LXR activation has been shown to lower LDL cholesterol levels in mice [25–28]. We therefore assessed the impact of LXR activation on plasma lipid levels in WT and C/EBPβ$^{-/-}$ mice. Final body weight and food intake of the mice in different groups were similar (data not shown). Total serum cholesterol levels were significantly lower in C/EBPβ$^{-/-}$ mice than WT mice (Table 1). LXR agonist T0901317 administration markedly increased total serum cholesterol levels in WT mice while LXR-mediated induction of serum cholesterol levels were lower by 40% in C/EBPβ$^{-/-}$ mice. Consistent with this observation, LDL-cholesterol levels were also significantly lower (about 29%) in C/EBPβ$^{-/-}$ mice under basal conditions and upon LXR agonist administration compared to WT mice. By contrast, LXR agonist increased serum HDL-cholesterol levels by roughly 47% in both WT and C/EBPβ$^{-/-}$ mice upon LXR activation.

To gain further insight into C/EBPβ's role in LXR-mediated regulation of liver cholesterol homeostasis, we investigated the effect of LXR activation on genes important in liver cholesterol metabolism. ABCG1, a gene important for cholesterol efflux, was 2.2-fold higher in C/EBPβ$^{-/-}$ mice compared to WT mice and remained elevated upon LXR treatment (Fig. 3). CYP7A1 expression, which is involved in the secretion of bile acids, was also enhanced to the same extent in liver of both C/EBPβ$^{-/-}$ and WT mice fed control diet plus LXR agonist. However, expression of ABCA1 was not affected in LXR-treated WT and C/EBPβ$^{-/-}$ mice.

In summary, the transcription factor C/EBPβ appears to be critical for LXR-mediated abnormal TG accumulation in liver, and

Table 1
Serum lipids in control and LXR agonist-treated mice.

	WT-CON	C/EBPβ$^{-/-}$-CON	WT-LXR (T0901317)	C/EBPβ$^{-/-}$-LXR (T0901317)
Total cholesterol (mg/dl)	93 ± 4.5	72.3 ± 5.8[a]	192 ± 2.8	145 ± 10.7[b]
LDL-cholesterol (mg/dl)	18.2 ± 7.5	5.1 ± 1.0[a]	7.3 ± 0.3	4.6 ± 0.7[b]
HDL-cholesterol (mg/dl)	47.2 ± 2.5	41.0 ± 3.3	74.2 ± 1.7	63.0 ± 6.1
TG (mg/dl)	80.5 ± 8.6	99.7 ± 19.9	66.3 ± 4.1	74.6 ± 12.4

[a] $P < 0.05$ *vs.* WT-CON; $N = 4$ mice/group.
[b] $P < 0.05$ *vs.* WT-LXR (T0901317); $N = 4$ mice/group.

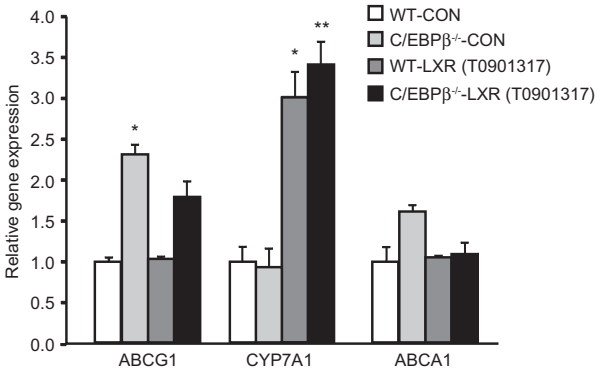

Fig. 3. Expression of genes implicated in cholesterol metabolism analyzed by quantitative PCR. Data are shown as mean ± SEM. $N = 4$ mice/group; $^{*}P < 0.05$ *vs.* WT-CON; $^{**}P < 0.05$ *vs.* WT-LXR (T0901317).

suggests that with the exception of SREBP1c and ChREBP, the target genes for LXR-induced lipogenesis depend on C/EBPβ. The reduced liver TG level was associated with a surprisingly improved mitochondrial electron transport chain function, which may have also contributed to reduced steatosis. Furthermore, the present study observed an induction of cholesterol efflux gene expression in liver and reduction of blood total and LDL-cholesterol levels in LXR-treated C/EBPβ$^{-/-}$ mice compared to LXR-treated WT mice. The results altogether suggest that inactivation of C/EBPβ might be an important therapeutic strategy to prevent the adverse effects of LXR activation on liver TG metabolism while improving the beneficial effects of LXR activation on cholesterol metabolism.

Acknowledgments

This work was supported by NIH grants R01-DK059767 to J.E.F. and P30DK048520 to the Colorado Nutrition and Obesity Research Center, Beginning grant in aid from the American Heart Association (09BGIA2060705, Southwest Affiliate) to S.M.R., (10BGIA458005 and 12 BGIA11380005, Southwest Affiliate) to M.M. and American Diabetes Association Mentored Post-Doctoral Fellowship to M.C.

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