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Farnesoid X receptor up-regulates expression of Lipid transfer inhibitor protein in liver cells and mice

Liangpeng Li [a,1], Hong Liu [b,1], Jiahe Peng [a], Yongchao Wang [a], Yan Zhang [a], Jinyu Dong [a], Xiaohua Liu [a], Dongmei Guo [a], Yu Jiang [a,*]

[a] Department of Biochemistry and Molecular Biology, College of Basic Medical Science, Third Military Medical University, Chongqing 400038, China
[b] Department of Hematology, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, China

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ABSTRACT

Apolipoprotein F is a component protein mainly secreted by liver and resides on several lipoprotein classes. It can inhibit lipids transfer between different lipoproteins. FXR is a member of the nuclear receptor superfamily which is also highly expressed in the liver. It modulates bile acids synthesis and lipids metabolism by transcriptional regulation. We aimed to determine whether apoF can be regulated by FXR. The FXR agonist Chenodeoxycholic acid (CDCA) and GW4064 both can activate the expression of apoF in liver cell lines and in C57/BL6 mouse liver. This is dependent on the binding of FXR to the FXR element ER1 (−2904 to −2892 bp) in the apoF gene promoter. Taken together, we have identified apoF as likely another target gene of FXR.

1. Introduction

Apolipoprotein F (apoF), also known as Lipid transfer inhibitor protein (LTIP), is an apolipoprotein mainly located in HDL [1,2]; a small amount also has been found in LDL. It can inhibit Cholesteryl Ester Transfer Protein (CETP) activity. In plasma, CETP mediates the net transfer of Cholesteryl Ester (CE) from LDL and HDL to VLDL in return for triglyceride (TG) [3,4]. This lipid transfer event is known to reduce HDL level in plasma, thus apoF is thought to be related to higher HDL levels in plasma due to its natural CETP inhibition function. ApoF decreases the net flux of CE from HDL to VLDL. Because there is a relatively short plasma half-life for VLDL, this exchange is proposed to facilitate the clearance of HDL-derived CE, thus apoF can promote reverse cholesterol transport [2]. Previous research indeed shows that apolipoprotein F may participate in altering the plasma TG and HDL levels via its inhibition activity on CETP. In a study with 397 Japanese healthy and 221 hypertriglyceridemic subjects enrolled, serum apoF concentration was correlated positively with HDL cholesterol and apo A-I levels in males but not in females, negatively with apoB and triglyceride levels. In hypertriglyceridemic patients, apoF concentration in plasma was lower than that in healthy controls [5].

Farnesoid X receptor is one of the members of the nuclear receptor super family. It participates in the regulations of bile acids, lipid and glucose metabolisms, with CDCA serving as its most suitable native ligand [6]. The FXR knock-out animal model shows a decrease in the clearance of serum HDL [7,8]. FXR activation also promotes the clearance of circulating TG [9,10]. In vivo treatment with the FXR agonist GW4064 significantly improves hypercholesterolemia in both ob/ob mice and db/db mice [10].

FXR and apoF both have highly expression in the liver, and both of them participate in the lipid metabolism and its homeostasis. So we sought to examine if FXR plays a role in human Hepatoma cell line HepG2 with respect to the regulation of the expression of apoF. To test this supposition, we explored the relationship between apoF and FXR. This may contribute to the notion that FXR could be a potential molecular target in lipid metabolism.

2. Methods and materials

2.1. Animals

All experiments were approved by the Animal Ethics Board of The Third Military Medical University on animal welfare. C57/BL6

Abbreviations: FXR, farnesoid X receptor; CETP, cholesterol ester transfer protein; apoF, apolipoprotein F.

* Corresponding author. Address: Department of Biochemistry and Molecular Biology, College of Basic Medical Science, Third Military Medical University, No. 30 St. Gao Tan Yan, Shapingba District, Chongqing 400038, China.
E-mail address: yujiang61@gmail.com (Y. Jiang).

[1] Both author contributed equally to this work.

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mice (Experimental Animal Center of The Third Military Medical University) were kept under standard laboratory conditions (food and water given ad libitum, 21 ± 2 °C environment temperature and a 12 h light/dark cycle). Male mice at 8 week were used on this study.

2.2. Cell culture

Hepatoma cell line HepG2 and fetal liver cell line L02 were purchased from ATCC, cultured in DMEM or RPMI 1640 media with 10% fetal bovine serine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C in 5% CO_2 atmosphere.

2.3. RT-PCR

HepG2 or L02 cells (10^6) were treated with CDCA or GW4064 respectively for 24 h. Cells were starved for 2 h before the addition of ligand, then the cells were harvested by Tripure regent and the total RNA were extracted. The toal RNA were reverse transcript to cDNA by M-MLV reverse transcript kit (Promega) RT-PCR were performed with the Taq polymerase kit (Takara) to test the mRNA level of LTIP in human liver cell lines. Primers used in this experiment were included in Table S1 (Supplementary Material).

2.4. Real time quantitative PCR

HepG2 or L02 cell lines (about 10^6) were treated with CDCA in different concentrations for 24 h. Cells were starved for 2 h before the addition of ligand, then the total RNA were extracted and reverse transcript into cDNA with M-MLV reverse transcript kit (Promega). Real time quantitative PCR were performed with EvaGreen Ssofast supermix (Bio-Rad) on IQ5 real time PCR cycler. (Bio-Rad) to assay the mRNA level of apoF in HepG2 and L02 cells when treated with CDCA. Primers used in this experiment were included in Table S1 (Supplementary Material).

2.5. Western blotting

HepG2 or L02 cells (about 10^6) were treated with CDCA or GW4064 respectively in different concentrations for 24 h. Cells were starved for 2 h before the addition of ligand, then the cultured cells were harvested and the total protein was extracted by RIPA Lysis Buffer (Beyotime). Western Blotting were performed to tested the protein level of LTIP in cultured liver cells when treated with CDCA or GW4064, anti LTIP antibody was purchased from Santa Cruz.

2.6. Luciferase reporter assay

Through bioinformatic analysis by online nuclear binding site search tool (www.nubiscan.unibas.ch), we have found that the 5′ flanking region of the apoF gene promoter may contain FXRE binding site ER1 (−2904 to −2892 bp). Genomic DNA were extracted from HepG2 cells, using PCR amplification and plasmids reconstruction, the (−3289 to +22 bp) and (−1872 to +36 bp) region of apoF gene were reconstructed into the PGL3-basic plasmid which contain luciferase reporter gene, acquired reconstructed plasmids pGL3/apoF(−3289 to +22 bp)and pGL3/apoF(−1872 to +36 bp). The reconstructed plasmids were co-transfected into the HepG2 cell with FXR expression plasmid vp-FXR. By Luciferase reporter assay, the fluorescence was read on a spectrophotometer (Thermo Scientific), the activities of the different regions of the apoF promoter were analyzed. Primers used in this experiment were included in Table S1 (Supplementary Material).

2.7. ChIP

Specific primer (sequences were included in Supplementary Table 1) which amplified the −2955 to −2759 bp of the apoF promoter region which contains the ER1 (evert repeat separated by 1 base pair) binding site were designed to performed in the ChIP assay. HepG2 cells (10^6) were treated with 100 μmol/L CDCA for 24 h. Then the cells were washed by PBS for twice, scratched, and transferred into a new sterilized EP tube, then centrifuged to collect the cell pallets. 200 μl SDS lysis buffer were added into the cells, cells were then resuspended, placed on ice for 10 min, sonicated by ultrasound using a Palmer ultrasound equipment. The genomic DNA was sheared into 200–1000 base pairs. The ChIP assay was performed with the ChIP kit (Millipore 17–295) following the instruction. Anti FXR antibody (Santa-Cruz, sc-1204c) and the goat IgG were used as treatment and control, respectively. The DNA fragments which interact with FXR were collected, the result were tested by PCR using the primers mentioned. Primers used in this experiment were included in Table S1 (Supplementary Material).

2.8. Animal model experiment

C57BL/6 mice were used as animal model, male C57BL/6 (4 weeks old) were divided random into three groups treated with different doze of CDCA (control, 10 mg/kg, 50 mg/kg) respectively by intragastric administration for a week, the liver was separated and the total RNA and total protein were extracted. RT-PCR and Western Blotting were performed to test the mRNA and protein levels of the liver tissue of C57BL/6 when treated with CDCA for 1 week. Primers used in this experiment were included in Table S1 (Supplementary Material).

3. Results

3.1. FXR agonist induces the LTIP expression in liver cell lines

We used two cell lines, HepG2 and L02 as cell model to study the relationship between apoF and FXR. Cells were treated with CDCA or GW4064, and then the expression of apoF was measured. Since SHP is recognized as a typical FXR target gene and can be obviously induced by FXR agonist, we also measured the SHP mRNA level to monitor the activation of FXR. When treated with CDCA or GW4064 for 24 h, the SHP mRNA levels increased significantly, indicating that CDCA and GW4064 effectively activate FXR (Fig. S1). The apoF mRNA and protein levels were also increased by CDCA or GW4064 treatment in a dose-dependent manner (Figs. 1 and 2). These results suggested that FXR activation up-regulates apoF expression in liver cells.

3.2. The induced up-regulation of LTIP is depended on its promoter region containing FXRE

To illustrate whether the FXR induced up-regulation of apoF is dependent on its promoter, we have reconstructed two plasmids on the basis of reporter plasmid pGL3. One of the reconstructed plasmids pGL3/apoF(−3289 to +22 bp) contains the full length of the scanned promoter of humans, another reconstructed plasmids pGL3/apoF(−1872 to +36 bp) contains the truncated or proximal human apoF promoter. The two reconstructed plasmids and FXR expression plasmid vp-FXR were transfected into the HEK 293 cells, and the transfected cells were treated with or without CDCA for 24 h. The fluorescence was read on a spectrophotometer (Thermo Scientific). In the presence of pGL3/apoF(−3289 to +22 bp), the reporter activity increased about 10 times with the presence of vp-FXR compared with the absence of vp-FXR, while the reconstructed plasmid pGL3/apoF(−1872 to +36 bp) shows a slight reporter activity in the presence of vp-FXR versus in the

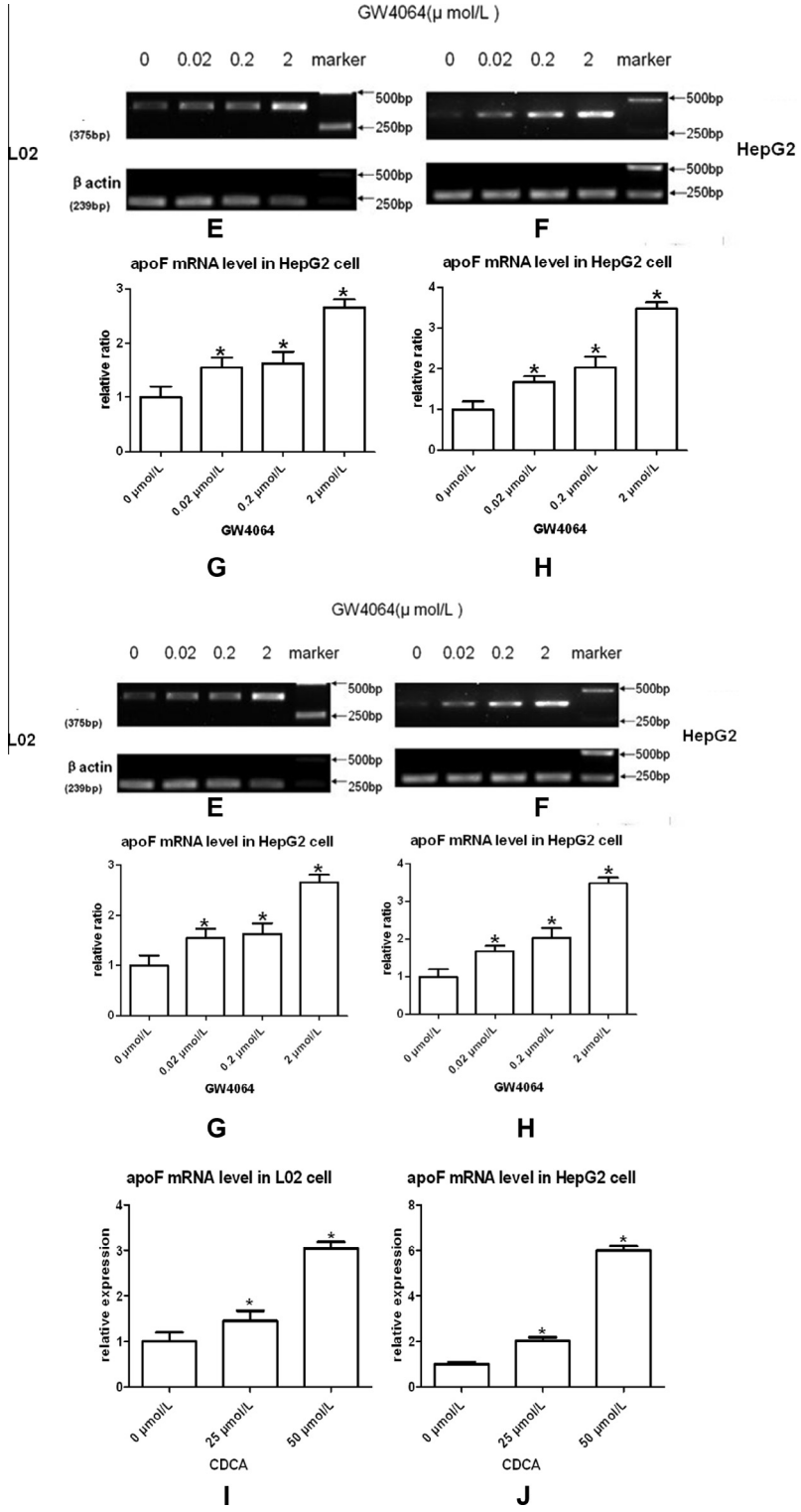

Fig. 1. FXR agonist CDCA and GW4064up-regulate mRNA level of apoF in L02 and HepG2 cell line. L02 and HepG2 cell were treated with CDCA (0 μmol/L, 25 μmol/L, 50 μmol/L, 100 μmol/L) and GW4064 (0 μmol/L, 0.02 μmol/L, 0.2 μmol/L, 2 μmol/L) for 24 h in a condition medium with 2% FBS. apoF mRNA level were assessed by RT-PCR. A, B: L02 and HepG2 treated with CDCA, E, F: L02 and HepG2 treated with gw4064: C, D, G, H: optic density rate of apoF and βactin; I, J: apoF mRNA level assessed by real time PCR in L02 and HepG2 cell line after treated with CDCA at indicated concentration. *: compared with control, $P < 0.05$.

absence of vp-FXR (no statistic significant) (Fig. 3A). This data suggest that it is the apoF promoter region(−3289 to −1872 bp) that is responsible for the FXR induced up-regulation, and support that the predicted FXRE located in the −2904 to −2892 bp could be of vital for the FXR binding and the sequential transcriptional up-regulation.

3.3. FXR interact with the FXRE in LTIP promoter region

Luciferse reporter assays suggested that the predicted FXRE located in −2904 to −2892 bp bp in apoF gene promoter was highly suspected to binding with FXR. However whether this event occurs in cells needs further clarification. To illustrate this, ChIP (Chroma-

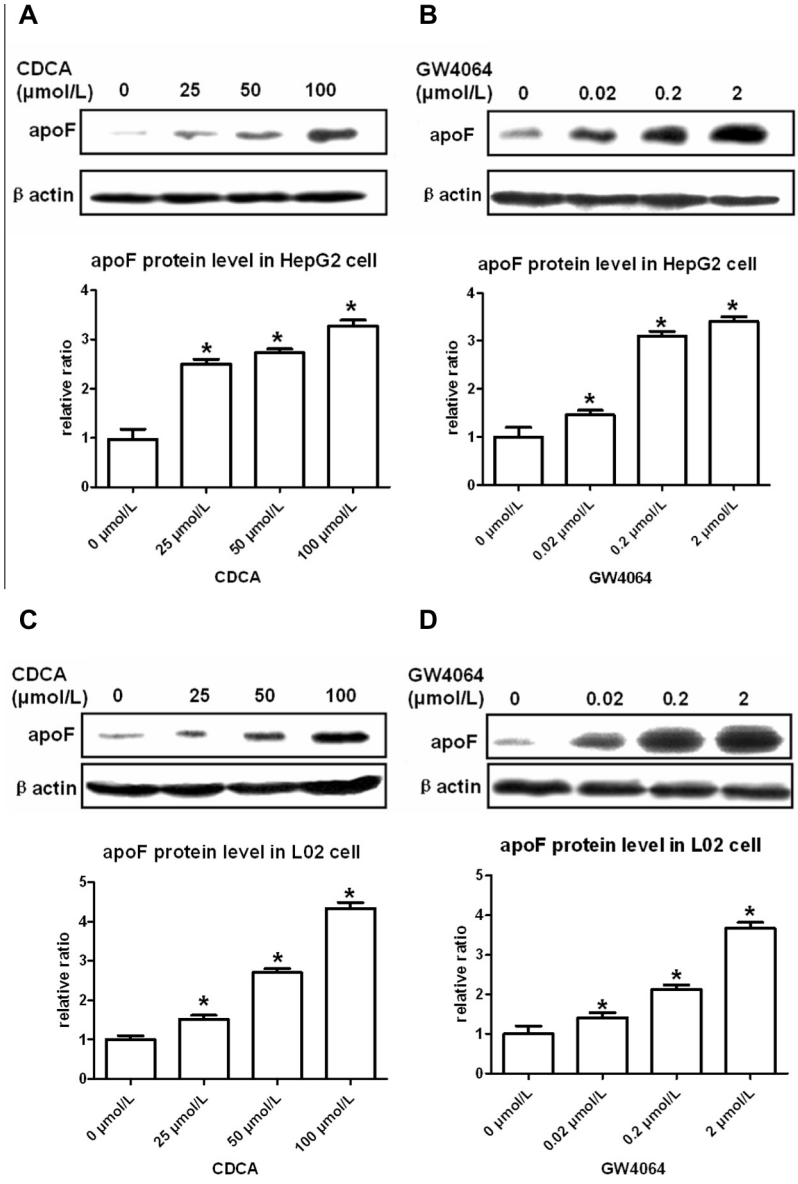

Fig. 2. FXR agonist CDCA and GW4064 up-regulate apoF protein level in HepG2 and L02 cell L02 and HepG2 cell were treated with CDCA (0 μmol/L, 25 μmol/L, 50 μmol/L, 100 μmol/L) and GW4064 (0 μmol/L, 0.02 μmol/L, 0.2 μmol/L, 2 μmol/L) for 24 h in a condition medium with 2% FBS. apoF mRNA level were assessed by RT-PCR. A: HepG2 cell treated with CDCA; B: HepG2 cell treated with GW4064; B: L02 cell treated with CDCA; D: L02 cell treated with GW4064. *: compared with control, $P < 0.05$.

tin Immunoprecipitation) assays were performed. Result suggested that there is an interaction between FXR and ER1 (−2904 to −2892 bp) in the human apoF promoter region, which is in consistence with the Luciferase reporter assay result. (Fig. 3B)

3.4. CDCA treatment induces LTIP expression in C57/BL6 mice livers

We have shown that FXR up-regulates apoF expression in HepG2 and L02 cells. To evaluate whether activation of FXR induce apoF expression in vivo, we tested the liver apoF expression when C57/BL6 mice treated with CDCA. Different concentrations (10 mg/kg, 50 mg/kg) of CDCA resolved in plant oil were delivered by intragastric administration to C57/BL6 male mice once a day for 1 week, the apoF mRNA and protein levels were accessed by RT-PCR and Western Blot respectively. The mRNA level of apoF in the liver increased to about 2.5-fold ($P < 0.05$) (Fig. 4A and B), and the proteins level of apoF in C57/BL6 mice liver increased to about 2.7 times compared with control ($P < 0.05$) (Fig. 4C and D). These data indicate that CDCA not only up-regulated apoF in liver cell cultures, but also in the mice liver.

4. Discussion

The high level of plasma LDL and the low level of HDL are two crucial factors in atherogenesis. HDL is responsible for the reverse cholesterol transportation; it can take the cholesterol from the peripheral tissue to the liver to metabolize, so it is believed that HDL is capable of exerting positive effect on anti-atherosclerosis [11]. Recent study shows that the transfer effect mediated by CETP directly diminished the plasma HDL level, and increased the LDL level, which could attenuate the reverse cholesterol transportation [3]. So researchers propose that the inhibition of CETP could lead to the anti-atherosclerosis effect [4]. By now, there are three artificial CETP inhibitors that have been used in clinical experiments, some of these experiments have been stopped because of the severe hypertension side effect, however, the study of the CETP inhibitor

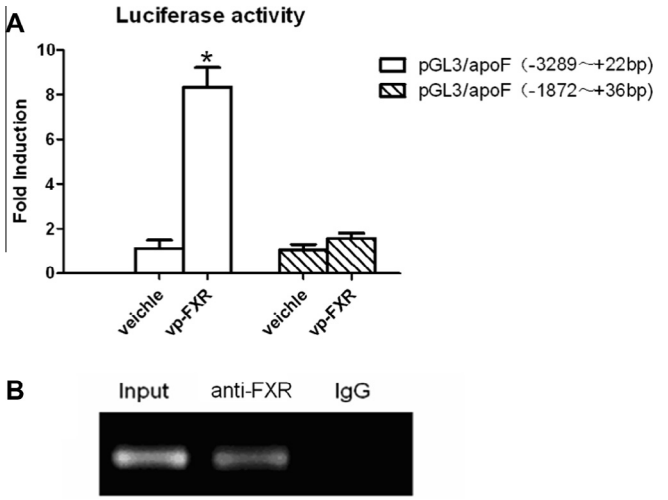

Fig. 3. Transcription activity of FXR on apoF is dependent on the FXRE in apoF promoter. A: pGL3/ApoF(−3289 to +22 bp)and pGL3/ApoF(−1872 to +36 bp)were transfected into the HepG2 cell with or without consistently active FXR expression plasmid vp-FXR, after 24 h the cells were harvested and the fluorescence was measured by a fluorescence photometric equipment. All values are normalized to control with pGL3 basic plasmid transfected. B: HepG2 cell (10^6) were treated with 100 μmol/L CDCA for 24 h, the aliquot of lysate were precipitate with anti-FXR antibody or IgG control, lysate without precipitation were loaded as Input. The acquired DNA were assessed by PCR using primers targeting the promoter region with predicted FXRE (−2904 to −2892 bp) included.

has not been terminated [12,13]. A kind of molecule which could inhibit CETP activity was separated from the plasma in 1982, and has been named as apolipoprotein F (apoF), also known as Lipid transfer inhibitor protein, LTIP [14]. It can inhibit the lipid transfer by repressing CETP ability, the following studies shows that this effect is through the mechanism that the apoF competes with lipoproteins for the binding site of CETP [2,15]. Notably, it has been found that the LTIP levels are inversely related to the triglyceride levels [14,16].

In the regulation of lipid metabolism, FXR has played a crucial role. The knowledge of the function of FXR focused on the participation of FXR in the bile acid metabolism, [17,18]. More and more genes participated in lipid metabolism have been found to be regulated by FXR. In the following studies, FXR also played important roles in the regulation of lipid metabolism. FXR could reduce the TG synthesis by inhibiting the SREBP-1c [19] and PPARγ [20], it also can promote the activity of the LPL by up-regulating apoC-II [9] and down-regulating apoC III [21]. LPL is a key enzyme which catalyzes the triglycerides hydrolization; it can result in the degradation of VLDL and CM, so as to reduce the plasma triglyceride concentration. Besides, apoE was also identified as a target gene of FXR [22], the FXR and apoE double deficiency mice exhibited an aggravation of atherogenesis versus apoE deficiency mice [23]. FXR target genes also have been involved in lipoprotein metabolism; it may play a role in high density lipoprotein metabolism via the regulation of PLTP gene expression, a lipid transfer protein responsible for phospholipid transfer from VLDL into HDL [24]. FXR activation was also reported to induce the scavenger receptor B-I (SR-BI) which is essential for HDL metabolism [10]. In all, FXR are believed to have anti-atherosclerosis effects.

To study whether apoF is regulated by FXR, we observed if apoF expression can be induced by FXR. Our data have shown that the mRNA and protein levels of apoF increased significantly in HepG2 and L02 cells treated with the native FXR agonist CDCA and the artificial agonist GW4064, respectively.

Through bioinformatics analysis of the −3000 bp flanking the 5′ side of promoter region of LTIP, we have found a FXR binding site

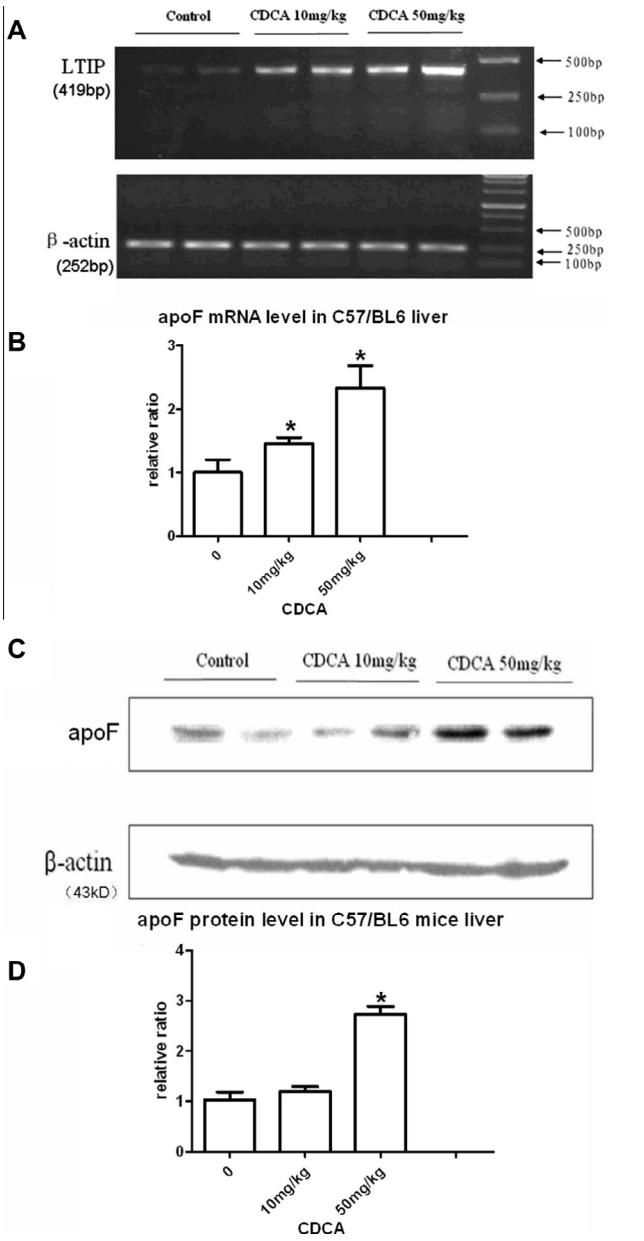

Fig. 4. CDCA treatment induce apoF expression in mice liver C57/BL6 mice at 4 weeks age were treated with CDCA at concentrations of 10 mg/kg, 50 mg/kg or vehicle control by intragastric administration once a day for a week, each group includes six subjects and the representative results were showed. apoF expression in mice liver were tested by RT-PCR and Western Blot. A, B: CDCA treatment increase apoF mRNA level in liver; C, D: CDCA treatment increase apoF protein level in liver. *: compared with vehicle control, $P < 0.05$.

ER1 in its promoter. Luciferase reporter assay and ChIP assay indicated that FXR up-regulated LTIP expression likely due to FXR directly bind to LTIP promoter by ER1 (−2904 to −2892 bp).

To test whether FXR activation also increases apoF expression in vivo, we delivered CDCA at 10 mg/kg and 50 mg/kg by intragastric administration to C57/BL6 mice for one week respectively, since apoF was mainly expressed in liver and then secreted into plasma, we then sacrificed the mice and extract the total RNA and protein of the mice liver. In accordance with the in vitro result, apoF mRNA and protein levels were both elevated significantly in mice livers. These data confirmed that FXR activation up -regulated apoF expression in C57/BL6 mice liver.

Our finding suggested that apoF may be likely another target gene of FXR, CETP inhibition is a promising therapy for lipid lowing

and atherosclerosis protection, as a naturally existing protein and a component of lipoprotein (mainly in HDL), which function to inhibit CETP activity, apoF may serve as another option for CETP inhibition and triglyceride lowering in plasma. More studies are required to investigate the potential of FXR ligand-based therapy in the treatment of dyslipidemia.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.156.

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