Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2016 September ; 36(9): 1772–1781. doi:10.1161/ATVBAHA.116.307790.

Loss of transcription factor CREBH accelerates diet-induced atherosclerosis in *Ldlr*$^{-/-}$ mice

Jong-Gil Park, **Xu Xu**, **Sungyun Cho**, and **Ann-Hwee Lee**[1]

Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, NY 10065

Abstract

Objective—Liver-enriched transcription factor CREBH regulates plasma triglyceride clearance by inducing lipoprotein lipase (LPL) co-factors such as apolipoprotein A-IV (apoA-IV), apoA-V, and apoC-II. CREBH also regulates apoA-I transcription. This study aims to determine whether CREBH has a role in lipoprotein metabolism and development of atherosclerosis.

Approach and Results—CREBH-deficient *Creb3l3*$^{-/-}$ mice were bred with *Ldlr*$^{-/-}$ mice creating *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ double knockout mice. Mice were fed on a high-fat and high-sucrose western diet (WD) for 20 weeks. We showed that CREBH deletion in *Ldlr*$^{-/-}$ mice increased VLDL-associated TG and cholesterol levels, consistent with the impairment of LPL-mediated TG clearance in these mice. In contrast, HDL cholesterol levels were decreased in CREBH-deficient mice, which was associated with decreased production of apoA-I from the liver. The results indicate that CREBH directly activated *Apoa1* gene transcription. Accompanied by the worsened atherogenic lipid profile, *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ mice developed significantly more atherosclerotic lesions in the aortas than *Ldlr*$^{-/-}$ mice.

Conclusions—We identified CREBH as an important regulator of lipoprotein metabolism, and suggest that increasing hepatic CREBH activity may be a novel strategy for prevention and treatment of atherosclerosis.

Introduction

Atherogenic dyslipidemia characterized by high levels of triglycerides (TG), low-density lipoprotein (LDL), and low level of high-density lipoprotein (HDL) are major risk factors for atherosclerosis and cardiovascular diseases (CVD). Correcting dyslipidemia is an effective strategy to prevent and treat CVD [1–3].

Elevated plasma TG is considered an independent risk factor for CVD, and is becoming increasingly important in association with obesity epidemics [4, 5]. Lipoprotein lipase (LPL) is the key enzyme involved in the clearance of TG from the circulation [6–8]. LPL is bound to the luminal surface of capillary endothelial cells, and hydrolyzes TG to initiate transport of

[1]To whom correspondence should be addressed: Department of Pathology and Laboratory Medicine, Weill Cornell Medical College, 1300 York Avenue, New York, NY 10065, USA. anl2042@med.cornell.edu, Tel: 1-212-746-9087.

Disclosures

All authors report no conflicts.

fatty acids to peripheral tissues. Although homozygous LPL genetic mutations do not cause atherosclerosis in hypertriglyceridemia patients [9–11], it has been shown that individuals with reduced LPL activity due to missense genetic mutations in LPL are prone to premature atherosclerosis, suggesting that LPL has atheroprotective functions [12–16]. A recent study showed that loss of LPL activity due to combined homozygous mutations in glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1) gene was found in patients with hypertriglyceridemia and severe CVD, suggesting the complexity of the involvement of LPL-mediated TG clearance in atherosclerosis [17]. LPL activity is regulated by a number of activators and repressors that are secreted from the liver and intestine. Deficiencies of LPL inhibitors, such as apoC-III or angiopoietin-like proteins, conferred protective effects against atherosclerosis [18, 19]. In contrast, it has been shown that the loss of apoC-II LPL activator increased the risk of CVD in parallel with plasma TG and cholesterol levels [20, 21].

Liver-enriched cyclic AMP-responsive element-binding protein H (CREBH, encoded by *Creb3l3*) is a bZiP transcription factor, which plays important roles in glucose and lipid metabolism [22–25]. We have previously shown that CREBH knockout mice exhibit hypertriglyceridemia in association with decreased production of apoA-IV, apoA-V, and apoC-II apolipoproteins [23]. In addition, hepatic CREBH was activated in mice fed with atherogenic Paigen diet or a high-fat and high-cholesterol western diet [26, 27]. We also showed that hepatic apoA-I mRNA level was reduced in CREBH knockout mice, and increased by CREBH overexpression in primary mouse hepatocytes, suggesting the possibility that CREBH might play a role in HDL metabolism [26]. In the present study, we investigated the role of CREBH in lipoprotein metabolism and atherosclerosis in $Ldlr^{-/-}$ mice. We report that the compound mutant mice lacking both LDL receptor and CREBH ($Ldlr^{-/-}$ $Creb3l3^{-/-}$) have increased VLDL-TG, VLDL-C, decreased HDL-C, and increased atherosclerotic plaques compared with $Ldlr^{-/-}$ mice.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

CREBH deficiency increased plasma apoB/apoA-I ratio

The apoB/apoA-I ratio represents the balance between atherogenic and anti-atherogenic lipoprotein particles, and hence represents a strong risk factor for CVD [28–30]. To determine the importance of CREBH in apoA-I and apoB production, we measured the abundance of plasma apolipoproteins in CREBH-deficient $Creb3l3^{-/-}$ mice by immunoblotting. Plasma apoB and apoE protein levels were comparable between wild type (WT) and $Creb3l3^{-/-}$ mice (Figure 1A). We have previously shown that hepatic apoA-I and apoA-IV mRNA levels were reduced in CREBH-deficient mice [26]. Consistent with this, plasma apoA-I and apoA-IV protein levels were decreased by 2.3-and 3.8-fold, respectively, in $Creb3l3^{-/-}$ compared with WT littermates, resulting in a 2.2-fold increase in apoB/apoA-I ratio in $Creb3l3^{-/-}$ mice (Figure 1A). The decreased abundance of plasma apoA-I (2.0-fold) and

apoA-IV (5.0-fold) and increased apoB/apoA-I ratio (2.8-fold) in CREBH-deficient mice were maintained during 2 weeks of WD feeding (Figure 1B).

CREBH deficiency accelerates atherosclerosis in Ldlr knockout mice

To determine the role of CREBH in lipoprotein metabolism and the development of atherosclerosis, we generated *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ double knockout mice. *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ mice were viable and appeared indistinguishable from *Ldlr*$^{-/-}$ mice. We fed *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ double knockout mice WD for 20 weeks, and analyzed atherosclerotic plaques in the aortic sinus and whole aorta. Food consumption and body weights did not differ between *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ mice (Figure I in the online-only Data Supplement). *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ mice contained significantly more atherosclerotic plaques in the aortas compared with *Ldlr*$^{-/-}$ mice (Figure 2A, 2B). Similarly, plaque size in the aortic sinus was about 25% larger in *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ than in *Ldlr*$^{-/-}$ mice (Figure 2C). CD45 immunofluorescence staining showed comparable density of CD45-positive leukocytes within the plaque areas of the two groups, reflecting a proportional increase in leukocyte infiltration to the expanded plaque areas of *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ mice (Figure 2D, 2E). Similarly, the relative dimensions of acellular necrotic areas to the plaque areas were comparable between *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ and *Ldlr*$^{-/-}$ mice (Figure 2D, 2F). Collectively, these data suggest that the loss of CREBH worsened atherosclerosis in LDL receptor-deficient mice.

Severe hyperlipidemia in Ldlr$^{-/-}$ Creb3l3$^{-/-}$ mice

We have previously shown that *Creb3l3*$^{-/-}$ mice have hypertriglyceridemia due to a defect in VLDL-TG clearance, which is associated with the decreased expression of LPL coactivators, such as apoA-IV, apoA-V and apoC-II [23]. Consistent with this, hepatic and intestinal mRNA levels of these CREBH target genes were decreased in *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ mice compared with *Ldlr*$^{-/-}$ mice (Figure 3A and 3B). Similarly, plasma TG levels were about three times higher in *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ than in *Ldlr*$^{-/-}$ mice throughout the course of WD feeding (Figure 3C). As expected, heat-inactivated serum from *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ exhibited reduced activity to augment LPL-mediated lipolysis compared with *Ldlr*$^{-/-}$ serum (Figure IIA in the online-only Data Supplement), confirming that loss of CREBH impaired TG clearance, causing hypertriglyceridemia [23]. Unexpectedly, *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ mice also exhibited a 42% increase in VLDL secretion rate compared with *Ldlr*$^{-/-}$ mice (Figure IIB in the online-only Data Supplement), contrasting with normal VLDL secretion in *Creb3l3*$^{-/-}$ mice fed normal chow [23]. The precise mechanism for the increased VLDL secretion by the loss of CREBH in *Ldlr*$^{-/-}$ mice remains to be further explored. We speculate that loss of CREBH increased non-esterified fatty acid (NEFA) flow to the liver leading to increased TG synthesis in hepatocytes and consequently increased VLDL production, which might be aggravated by the concomitant loss of CREBH and LDL receptor. Supporting this hypothesis, plasma NEFA level was markedly higher in *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ than in *Ldlr*$^{-/-}$ mice fed WD (Figure 3D), which is likely ascribed, in part, to impaired fibroblast growth factor 21 (FGF21) production in the absence of CREBH (Figure IIC in the online-only Data Supplement), considering the recently described function of FGF21 to reduce plasma NEFA concentration and VLDL secretion [31]. Expression of lipogenic transcription factor, sterol regulatory element binding protein-1 (SREBP-1) (*Srebf1*) and its target genes involved in

lipogenesis were not induced in *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ mice, excluding the possibility of increased lipogenesis in these mice (Figure IIC in the online-only Data Supplement). Interestingly, plasma cholesterol levels were also 20% higher in *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ than in *Ldlr*$^{-/-}$ mice when measured after 20 weeks of WD feeding (Figure 3E). Analysis of lipoprotein profile by fast protein liquid chromatography revealed that TG is mainly present in VLDL fractions of *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ mice (Figure 3F). VLDL-associated TG and cholesterol content were markedly higher in *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ than in *Ldlr*$^{-/-}$ mice (Figure 3F and 3G). Interestingly, CREBH deficiency decreased HDL-associated cholesterol content (Figure 3G), which correlated well with the decreases in hepatic and intestinal apoA-I mRNA levels by 3.6- and 1.4-fold, respectively (Figure 3A and 3B), and 40% decrease in plasma apoA-I protein in *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ mice compared with *Ldlr*$^{-/-}$ mice (Figure 3H). The abundance of apoA-I protein in plasma and liver lysates was also decreased by 1.6- and 1.4-fold, respectively in *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ mice compared with *Ldlr*$^{-/-}$ mice (Figure 3I and IID in the online-only Data Supplement). On the other hand, plasma apoB and apoE levels were comparable between *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ mice (Figure 3H and 3I), although hepatic apoB mRNA level was slightly lower in the latter group (Figure 3A). Taken together, CREBH deficiency in *Ldlr*$^{-/-}$ mice increased atherogenic VLDL-associated TG and cholesterol content, and decreased apoA-I and HDL cholesterol levels.

Transcriptional regulation of apoA-I by CREBH

To further investigate how CREBH regulates *Apoa1* gene expression, we overexpressed constitutively active CREBH(N) in mouse primary hepatocytes and human Huh7 cells using recombinant adenovirus vector. CREBH(N) strongly induced apoA-I mRNA as well as other known CREBH targets in both primary hepatocytes (Figure 4A) and Huh7 cells (Figure 4B), indicating that CREBH is sufficient to activate apoA-I expression in both species. Notably, CREBH(N) overexpression did not Western blotting analysis confirmed that CREBH(N) overexpression increased intracellular and secreted apoA-I protein levels by 2.1- and 6.2-fold, respectively (Figure 4C). As expected, apoA-IV protein expression was also dramatically increased by CREBH(N) overexpression (Figure 4C).

To determine whether *Apoa1* is regulated directly by CREBH, we constructed luciferase reporter vectors containing human *APOA1* (−954/+16) or mouse *Apoa1* (−999/+74) promoter fragments, and performed transient transfection assays in Huh7 cells. Both human and mouse *Apoa1* reporters were induced by the co-transfected CREBH(N) (Figure 4D). We next performed chromatin immunoprecipitation assays to determine whether CREBH binds to the *APOA1* promoter. Chromatin immunoprecipitation assays using mouse liver samples were not successful in demonstrating CREBH binding to any known CREBH target genes, including *Apoa4*, despite the fact that the antibody detected endogenous CREBH protein well. We therefore overexpressed HA-tagged CREBH(N) in Huh7 cells, and performed chromatin immunoprecipitation assays using an anti-HA antibody and a PCR primer pair extending from −167 to −89 from the transcription start site of *APOA1* gene. The results showed that CREBH(N) immunoprecipitation enriched *APOA1* promoter sequences by 7.2-folds, but not sequences of an irrelevant target HSPA5, an ER stress-inducible gene regulated by other bZIP transcription factors [32] (Figure 4E). *APOC2* and *APOA4* promoter sequences were also enriched by CREBH(N) immunoprecipitation. Although these data

have the caveat of being obtained in the setting of CREBH(N) overexpression focusing on select target genes, they suggest that CREBH(N) can directly bind to these promoters to activate transcription. Genome-wide analysis of CREBH binding sites should reveal the DNA binding specificity of CREBH, and how CREBH activates transcription of its target genes. Interestingly, we found that CREBH(N) induced *Apoa1* reporter in Huh7, but not in non-liver derived HEK293T cells (Figure 4F), suggesting that CREBH might require additional hepatocyte-expressed factor(s) to activate *Apoa1* promoter. Similarly, *Apoc2* reporter was induced by CREBH(N) in Huh7, but not in HEK293T cells (Figure 4G). In stark contrast, *Apoa4* reporter was strongly induced by the co-transfected CREBH(N) in both Huh7 and HEK293T cells (Figure 4H), indicating disparate regulation of these promoters by CREBH.

We next sought to identify the factor(s) that might synergize with CREBH(N) to stimulate *Apoa1* transcription. In a survey of liver-expressed transcription factors and coactivators, we found that hepatocyte nuclear factor (HNF)-4α synergized with CREBH(N) for *Apoa1* promoter activation in 293T cells (Figure III in the online-only Data Supplement). HNF-4α was abundantly expressed in Huh7 and HepG2 cells, but not in HEK293T cells (Figure 5A). Either CREBH(N) or HNF-4α alone had no effect on *Apoa1* and *Apoc2* promoter activities in 293T cells (Figure 5B). However, co-transfection of these two transcription factors strongly activated *Apoa1* and *Apoc2* promoters, increasing luciferase activities driven by these promoters by 38- and 116-fold, respectively (Figure 5B). In contrast, HNF-4α had minimal effect on *Apoa4*-luciferase reporter containing −900/+53 promoter fragment (Figure 5B). Furthermore, co-transfection of CREBH(N) and HNF-4α plasmids into HEK293T cells dramatically increased the expression of endogenous *APOA1* and *APOC2* genes by >1000- and 4-fold, respectively, although each transcription factor transfected individually had minimal effect on these genes (Figure 5C). ApoA-IV mRNA was markedly induced by CREBH(N) alone, and further increased by HNF-4α co-transfection. CREBH(N) and HNF-4α protein levels in the co-transfected cells were comparable to those in singly transfected cells, indicating that the synergism is not related to the expression level of each protein (Figure 5D). Taken together, these data suggest that CREBH requires HNF-4α to induce apoA-I and apoC-II, but not apoA-IV.

Adenoviral CREBH(N) overexpression improves lipoprotein profiles in hyperlipidemic mice

Given that CREBH deficiency exacerbated atherosclerosis in $Ldlr^{-/-}$ mice associated with worsened atherogenic lipoprotein profile: high VLDL cholesterol and TG, and low HDL, we hypothesized that active CREBH(N) might exert atheroprotective effects. To explore the effects of CREBH(N) overexpression on plasma lipids and lipoprotein profile in hyperlipidemic mice, we injected $Ldlr^{-/-}$ mice with Ad.CREBH(N) or control Ad.GFP adenoviruses. Similar to our results obtained from experiments using primary mouse hepatocytes and Huh7 cells, CREBH(N) overexpression increased the expression of *Apoa4*, *Apoa5* and *Apoc2* genes in the liver (Figure 6A). ApoA-I mRNA was only slightly induced by CREBH(N) overexpression, likely because basal apoA-I mRNA level was already very high. This small increase in apoA-I mRNA level by CREBH(N) overexpression was not translated into elevation of plasma apoA-I concentration (Figure 6B). On the other hand, plasma apoA-IV level was markedly increased by CREBH(N) overexpression, correlating

with a >500-fold increase in hepatic apoA-IV mRNA level. Other known CREBH targets such as *Fgf21* and *Cidec* were also induced by CREBH(N) adenovirus (Figure 6A). Importantly, CREBH(N) overexpression decreased plasma TG and cholesterol levels in *Ldlr*$^{-/-}$ mice by 3.0- and 2.1-fold, respectively (Figure 6C, 6D). Fast protein liquid chromatography analysis revealed that VLDL cholesterol was markedly reduced by CREBH(N) overexpression, albeit HDL cholesterol was also reduced (Figure 6E). Given that CREBH(N) overexpression dramatically decreased both plasma TG and cholesterol, we speculate that the reduction of HDL-cholesterol reflects an indirect consequence of decreased plasma lipids. Future studies are required to determine the effects of prolonged overexpression of CREBH(N) on the development of atherosclerosis.

We also examined the effects of CREBH(N) overexpression on plasma lipids and lipoprotein profile in C57BL/6 mice fed WD for 14 days. Similar to what we observed in *Ldlr*$^{-/-}$ mice, CREBH(N) adenovirus induced *Apoa1* as well as other known CREBH target genes, and reduced plasma TG and cholesterol levels (Figure IV in the online-only Data Supplement), suggesting that augmenting CREBH activity might be useful to treat dyslipidemia and exert atheroprotective effects.

Discussion

In this study, we demonstrated that loss of CREBH worsened atherogenic dyslipidemia (high plasma VLDL-TG and low HDL-cholesterol levels), and accelerated the development of atherosclerosis in *Ldlr*$^{-/-}$ mice. In contrast, overexpression of the constitutively active form of CREBH in the liver using recombinant adenoviral vector reduced plasma lipids, primarily those associated with VLDL. These data suggest that CREBH is an anti-atherogenic transcription factor, and augmenting CREBH activity may be a useful strategy to treat dyslipidemia and atherosclerosis.

Although plasma TG concentration shows a strong association with risk of CVD [4, 33, 34], it remains unclear whether hypertriglyceridemia is an independent CVD risk factor, and high TG levels contribute to atherosclerosis, because this association is reduced after adjusting for other risk factors such as low HDL-cholesterol and high non-HDL-cholesterol [35]. CREBH reduces plasma TG by facilitating LPL-mediated TG clearance, which is attributed, in part, to the transcriptional activation of apolipoprotein genes such as *Apoa1*, *Apoa4*, *Apoa5* and *Apoc2* [23]. CREBH also regulates FGF21 [23], which was recently shown to stimulate LPL-mediated TG clearance [31], decreased expression of which might have contributed to hypertriglyceridemia phenotype of CREBH-deficient mice. *Ldlr*$^{-/-}$ *Creb3l3*$^{-/-}$ mice fed WD for 20 weeks developed severe hypertriglyceridemia, which might have contributed to atherosclerosis in these mice. It would be interesting to test whether agents reducing plasma TG levels ameliorate atherosclerosis in CREBH-deficient mice.

ApoA-I is produced from liver and small intestine, and constitutes the predominant protein component of HDL [36]. ApoA-I interacts with ATP-binding cassette transporter A1 (ABCA1), and stimulates cholesterol efflux for reverse cholesterol transport [37–39]. Loss of apoA-I increases non-HDL-C, and accelerates atherosclerosis in *Ldlr*$^{-/-}$ mice [40]. In contrast, transgenic overexpression of apoA-I or infusion of recombinant apoA-I decreases plaque

formation, with varying effects on the concentration of non-HDL-C [41–43]. CREBH deficiency suppressed apoA-I mRNA expression in both the liver and the intestine, and accordingly reduced plasma apoA-I protein and HDL cholesterol levels, indicating that CREBH is an important transcriptional regulator of apoA-I. In addition, CREBH(N) overexpression strongly induced apoA-I mRNA in primary mouse hepatocytes as well as in human hepatoma cells, and activated the luciferase reporters driven by *Apoa1* promoter. Surprisingly, however, plasma apoA-I protein and hepatic apoA-I mRNA levels were not significantly increased by CREBH(N) overexpression in $Ldlr^{-/-}$ mice. We hypothesize that the endogenous CREBH is sufficient to promote apoA-I transcription *in vivo*, and additional exogenous CREBH(N) has only marginal effects on *Apoa1* gene expression in the liver. Consistent with this, CREBH mRNA is minimally expressed in cultured hepatocyte and hepatoma cells, contrary to the abundant expression in freshly-isolated mouse hepatocytes.

A number of nuclear hormone receptors directly bind to human *APOA1* promoter, and regulate apoA-I expression in the liver [4445]. HNF-4α is one of the transcription factors that activates hepatic *APOA1* gene expression by specifically binding to the proximal promoter and a distal *APOC2* enhancer [46–49]. HNF-4α is the master regulator of hepatocyte differentiation [47, 49, 50]. We find that CREBH requires HNF-4α to activate *Apoa1* and *Apoc2* promoters, implicating that CREBH and HNF-4α cooperatively regulate these genes. In contrast, HNF-4α was dispensable for *Apoa4* promoter activation by CREBH(N) in HEK293T, suggesting that CREBH regulates its target genes through diverse mechanisms.

ApoA-IV expression is strictly dependent on CREBH in both liver and small intestine [26]. Notably, previous studies have demonstrated that apoA-IV may participate in HDL metabolism by activating lecithin:cholesterol acyltransferase, a key enzyme in cholesterol transfer to newly synthesized HDL particles via conversion of free cholesterol into cholesteryl esters [51, 52], stimulating cholesterol efflux from macrophages [53], and activating receptor-mediated uptake of HDL by hepatocytes [54]. Furthermore, transgenic overexpression of human or mouse apoA-IV conferred protection against atherosclerosis in mice [55–57]. Future studies are required to determine the impact of apoA-IV down-regulation in CREBH-deficient mice on atherosclerosis progression, and whether apoA-IV knockout mice are also susceptible to atherosclerosis.

Adenoviral overexpression of CREBH(N) decreased plasma TG and cholesterol in $Ldlr^{-/-}$ mice, which correlated well with the induction of apolipoprotein genes in the liver that have LPL-activating properties. Surprisingly, however, HDL-C was also decreased by CREBH(N) overexpression. Given that CREBH-deficient mice had normal HDL-C and rather lower apoA-I compared with the WT mice, it is unlikely that the reduction of HDL-C by CREBH(N) overexpression reflects the physiological function of CREBH in HDL metabolism. Alternatively, the reduction of HDL-C might be an indirect consequence of the decreased plasma lipids in CREBH(N) adenovirus-infected mice.

In conclusion, our data suggests that CREBH is an important transcriptional regulator that exerts beneficial effects on plasma lipids against atherosclerosis, and augmenting CREBH activity may be a useful strategy to treat dyslipidemia and atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. David Cohen for FPLC, David Hajjar and John Parks for critical reading of this manuscript.

Sources of Funding

This work is supported in part by NIH grant K99 R01DK089211 (A. Lee).

Abbreviations

apo	apolipoprotein
CREBH	cyclic AMP-responsive element-binding protein H
CVD	cardiovascular disease
FGF21	fibroblast growth factor 21
HDL	high-density lipoprotein
LDL	low-density lipoprotein
TG	triglyceride
WD	western diet
WT	wild type

References

1. Roger VL, Go AS, Lloyd-Jones DM, et al. Heart disease and stroke statistics--2012 update: A report from the american heart association. Circulation. 2012; 125:e2–e220. [PubMed: 22179539]
2. Wong ND. Epidemiological studies of chd and the evolution of preventive cardiology. Nat Rev Cardiol. 2014; 11:276–289. [PubMed: 24663092]
3. Bamba V, Rader DJ. Obesity and atherogenic dyslipidemia. Gastroenterology. 2007; 132:2181–2190. [PubMed: 17498511]
4. Hokanson JE, Austin MA. Plasma triglyceride level is a risk factor for cardiovascular disease independent of high-density lipoprotein cholesterol level: A meta-analysis of population-based prospective studies. Journal of cardiovascular risk. 1996; 3:213–219. [PubMed: 8836866]
5. Gryn SE, Hegele RA. Novel therapeutics in hypertriglyceridemia. Current opinion in lipidology. 2015; 26:484–491. [PubMed: 26780002]
6. Kersten S. Physiological regulation of lipoprotein lipase. Biochim Biophys Acta. 2014; 1841:919–933. [PubMed: 24721265]
7. Mead JR, Irvine SA, Ramji DP. Lipoprotein lipase: Structure, function, regulation, and role in disease. J Mol Med (Berl). 2002; 80:753–769. [PubMed: 12483461]
8. Wang H, Eckel RH. Lipoprotein lipase: From gene to obesity. Am J Physiol Endocrinol Metab. 2009; 297:E271–288. [PubMed: 19318514]
9. Ebara T, Okubo M, Horinishi A, Adachi M, Murase T, Hirano T. No evidence of accelerated atherosclerosis in a 66-yr-old chylomicronemia patient homozygous for the nonsense mutation (tyr61-->stop) in the lipoprotein lipase gene. Atherosclerosis. 2001; 159:375–379. [PubMed: 11730817]

10. Ebara T, Endo Y, Yoshiike S, Tsuji M, Taguchi S, Murase T, Okubo M. A 60-y-old chylomicronemia patient homozygous for missense mutation (g188e) in the lipoprotein lipase gene showed no accelerated atherosclerosis. Clin Chim Acta. 2007; 386:100–104. [PubMed: 17854791]
11. Kawashiri MA, Higashikata T, Mizuno M, Takata M, Katsuda S, Miwa K, Nozue T, Nohara A, Inazu A, Kobayashi J, Koizumi J, Mabuchi H. Long-term course of lipoprotein lipase (lpl) deficiency due to homozygous lpl(arita) in a patient with recurrent pancreatitis, retained glucose tolerance, and atherosclerosis. J Clin Endocrinol Metab. 2005; 90:6541–6544. [PubMed: 16174715]
12. Benlian P, De Gennes JL, Foubert L, Zhang H, Gagne SE, Hayden M. Premature atherosclerosis in patients with familial chylomicronemia caused by mutations in the lipoprotein lipase gene. N Engl J Med. 1996; 335:848–854. [PubMed: 8778602]
13. Saika Y, Sakai N, Takahashi M, Maruyama T, Kihara S, Ouchi N, Ishigami M, Hiraoka H, Nakamura T, Yamashita S, Matsuzawa Y. Novel lpl mutation (l303f) found in a patient associated with coronary artery disease and severe systemic atherosclerosis. Eur J Clin Invest. 2003; 33:216–222. [PubMed: 12641539]
14. Reymer PW, Gagne E, Groenemeyer BE, et al. A lipoprotein lipase mutation (asn291ser) is associated with reduced hdl cholesterol levels in premature atherosclerosis. Nat Genet. 1995; 10:28–34. [PubMed: 7647785]
15. Wittrup HH, Tybjaerg-Hansen A, Abildgaard S, Steffensen R, Schnohr P, Nordestgaard BG. A common substitution (asn291ser) in lipoprotein lipase is associated with increased risk of ischemic heart disease. J Clin Invest. 1997; 99:1606–1613. [PubMed: 9120004]
16. Hu Y, Liu W, Huang R, Zhang X. A systematic review and meta-analysis of the relationship between lipoprotein lipase asn291ser variant and diseases. J Lipid Res. 2006; 47:1908–1914. [PubMed: 16741292]
17. Yamamoto H, Onishi M, Miyamoto N, Oki R, Ueda H, Ishigami M, Hiraoka H, Matsuzawa Y, Kihara S. Novel combined gpihbp1 mutations in a patient with hypertriglyceridemia associated with cad. Journal of atherosclerosis and thrombosis. 2013; 20:777–784. [PubMed: 23831619]
18. Adachi H, Fujiwara Y, Kondo T, et al. Angptl 4 deficiency improves lipid metabolism, suppresses foam cell formation and protects against atherosclerosis. Biochem Biophys Res Commun. 2009; 379:806–811. [PubMed: 19094966]
19. Pollin TI, Damcott CM, Shen H, Ott SH, Shelton J, Horenstein RB, Post W, McLenithan JC, Bielak LF, Peyser PA, Mitchell BD, Miller M, O'Connell JR, Shuldiner AR. A null mutation in human apoc3 confers a favorable plasma lipid profile and apparent cardioprotection. Science. 2008; 322:1702–1705. [PubMed: 19074352]
20. Kawano M, Kodama K, Inadera H, Saito Y, Saito M, Yaginuma T, Kanazawa Y, Kawakami M. A case of apolipoprotein c-ii deficiency with coronary artery disease. Clin Exp Med. 2002; 2:29–31. [PubMed: 12049186]
21. Sakurai T, Sakurai A, Vaisman BL, Amar MJ, Liu C, Gordon SM, Drake SK, Pryor M, Sampson ML, Yang L, Freeman LA, Remaley AT. Creation of apolipoprotein c-ii (apoc-ii) mutant mice and correction of their hypertriglyceridemia with an apoc-ii mimetic peptide. J Pharmacol Exp Ther. 2016; 356:341–353. [PubMed: 26574515]
22. Lee AH. The role of creb-h transcription factor in triglyceride metabolism. Current opinion in lipidology. 2012; 23:141–146. [PubMed: 22262056]
23. Lee JH, Giannikopoulos P, Duncan SA, Wang J, Johansen CT, Brown JD, Plutzky J, Hegele RA, Glimcher LH, Lee AH. The transcription factor cyclic amp-responsive element-binding protein h regulates triglyceride metabolism. Nat Med. 2011; 17:812–815. [PubMed: 21666694]
24. Lee MW, Chanda D, Yang J, Oh H, Kim SS, Yoon YS, Hong S, Park KG, Lee IK, Choi CS, Hanson RW, Choi HS, Koo SH. Regulation of hepatic gluconeogenesis by an er-bound transcription factor, crebh. Cell metabolism. 2010; 11:331–339. [PubMed: 20374965]
25. Xu X, Park JG, So JS, Lee AH. Transcriptional activation of fsp27 by the liver-enriched transcription factor crebh promotes lipid droplet growth and hepatic steatosis. Hepatology. 2015; 61:857–869. [PubMed: 25125366]
26. Xu X, Park JG, So JS, Hur KY, Lee AH. Transcriptional regulation of apolipoprotein a-iv by the transcription factor crebh. J Lipid Res. 2014; 55:850–859. [PubMed: 24598141]

27. Zhang C, Wang G, Zheng Z, Maddipati KR, Zhang X, Dyson G, Williams P, Duncan SA, Kaufman RJ, Zhang K. Endoplasmic reticulum-tethered transcription factor camp responsive element-binding protein, hepatocyte specific, regulates hepatic lipogenesis, fatty acid oxidation, and lipolysis upon metabolic stress in mice. Hepatology. 2012; 55:1070–1082. [PubMed: 22095841]
28. Walldius G, Jungner I. Apolipoprotein b and apolipoprotein a-i: Risk indicators of coronary heart disease and targets for lipid-modifying therapy. J Intern Med. 2004; 255:188–205. [PubMed: 14746556]
29. Walldius G, Jungner I. The apob/apoa-i ratio: A strong, new risk factor for cardiovascular disease and a target for lipid-lowering therapy--a review of the evidence. J Intern Med. 2006; 259:493–519. [PubMed: 16629855]
30. Thompson A, Danesh J. Associations between apolipoprotein b, apolipoprotein ai, the apolipoprotein b/ai ratio and coronary heart disease: A literature-based meta-analysis of prospective studies. J Intern Med. 2006; 259:481–492. [PubMed: 16629854]
31. Schlein C, Talukdar S, Heine M, Fischer AW, Krott LM, Nilsson SK, Brenner MB, Heeren J, Scheja L. Fgf21 lowers plasma triglycerides by accelerating lipoprotein catabolism in white and brown adipose tissues. Cell metabolism. 2016; 23:441–453. [PubMed: 26853749]
32. Lee AH, Iwakoshi NN, Glimcher LH. Xbp-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. Molecular and cellular biology. 2003; 23:7448–7459. [PubMed: 14559994]
33. Sarwar N, Danesh J, Eiriksdottir G, Sigurdsson G, Wareham N, Bingham S, Boekholdt SM, Khaw KT, Gudnason V. Triglycerides and the risk of coronary heart disease: 10,158 incident cases among 262,525 participants in 29 western prospective studies. Circulation. 2007; 115:450–458. [PubMed: 17190864]
34. Freiberg MS, Arnold AM, Newman AB, Edwards MS, Kraemer KL, Kuller LH. Abdominal aortic aneurysms, increasing infrarenal aortic diameter, and risk of total mortality and incident cardiovascular disease events: 10-year follow-up data from the cardiovascular health study. Circulation. 2008; 117:1010–1017. [PubMed: 18268154]
35. Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, Wood AM, Lewington S, Sattar N, Packard CJ, Collins R, Thompson SG, Danesh J. Emerging Risk Factors, C. Major lipids, apolipoproteins, and risk of vascular disease. Jama. 2009; 302:1993–2000. [PubMed: 19903920]
36. Horowitz BS, Goldberg IJ, Merab J, Vanni TM, Ramakrishnan R, Ginsberg HN. Increased plasma and renal clearance of an exchangeable pool of apolipoprotein a-i in subjects with low levels of high density lipoprotein cholesterol. J Clin Invest. 1993; 91:1743–1752. [PubMed: 8473514]
37. Lee JY, Parks JS. Atp-binding cassette transporter ai and its role in hdl formation. Current opinion in lipidology. 2005; 16:19–25. [PubMed: 15650559]
38. Eisenberg S. High density lipoprotein metabolism. J Lipid Res. 1984; 25:1017–1058. [PubMed: 6392459]
39. Wang N, Silver DL, Costet P, Tall AR. Specific binding of apoa-i, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing abc1. J Biol Chem. 2000; 275:33053–33058. [PubMed: 10918065]
40. Moore RE, Kawashiri MA, Kitajima K, Secreto A, Millar JS, Pratico D, Rader DJ. Apolipoprotein a-i deficiency results in markedly increased atherosclerosis in mice lacking the ldl receptor. Arterioscler Thromb Vasc Biol. 2003; 23:1914–1920. [PubMed: 12933536]
41. Miyazaki A, Sakuma S, Morikawa W, et al. Intravenous injection of rabbit apolipoprotein a-i inhibits the progression of atherosclerosis in cholesterol-fed rabbits. Arterioscler Thromb Vasc Biol. 1995; 15:1882–1888. [PubMed: 7583568]
42. Plump AS, Scott CJ, Breslow JL. Human apolipoprotein a-i gene expression increases high density lipoprotein and suppresses atherosclerosis in the apolipoprotein e-deficient mouse. Proc Natl Acad Sci U S A. 1994; 91:9607–9611. [PubMed: 7937814]
43. Rubin EM, Krauss RM, Spangler EA, Verstuyft JG, Clift SM. Inhibition of early atherogenesis in transgenic mice by human apolipoprotein ai. Nature. 1991; 353:265–267. [PubMed: 1910153]

44. Kardassis D, Gafencu A, Zannis VI, Davalos A. Regulation of hdl genes: Transcriptional, posttranscriptional, and posttranslational. Handb Exp Pharmacol. 2015; 224:113–179. [PubMed: 25522987]
45. Zannis VI, Kan HY, Kritis A, Zanni E, Kardassis D. Transcriptional regulation of the human apolipoprotein genes. Front Biosci. 2001; 6:D456–504. [PubMed: 11229886]
46. Tzameli I, Zannis VI. Binding specificity and modulation of the apoa-i promoter activity by homo- and heterodimers of nuclear receptors. J Biol Chem. 1996; 271:8402–8415. [PubMed: 8626539]
47. Sladek FM, Zhong WM, Lai E, Darnell JE Jr. Liver-enriched transcription factor hnf-4 is a novel member of the steroid hormone receptor superfamily. Genes Dev. 1990; 4:2353–2365. [PubMed: 2279702]
48. Harnish DC, Malik S, Karathanasis SK. Activation of apolipoprotein ai gene transcription by the liver-enriched factor hnf-3. J Biol Chem. 1994; 269:28220–28226. [PubMed: 7961760]
49. Li J, Ning G, Duncan SA. Mammalian hepatocyte differentiation requires the transcription factor hnf-4alpha. Genes Dev. 2000; 14:464–474. [PubMed: 10691738]
50. Bolotin E, Liao H, Ta TC, Yang C, Hwang-Verslues W, Evans JR, Jiang T, Sladek FM. Integrated approach for the identification of human hepatocyte nuclear factor 4alpha target genes using protein binding microarrays. Hepatology. 2010; 51:642–653. [PubMed: 20054869]
51. Chen CH, Albers JJ. Activation of lecithin: Cholesterol acyltransferase by apolipoproteins e-2, e-3, and a-iv isolated from human plasma. Biochim Biophys Acta. 1985; 836:279–285. [PubMed: 4041470]
52. Steinmetz A, Utermann G. Activation of lecithin: Cholesterol acyltransferase by human apolipoprotein a-iv. J Biol Chem. 1985; 260:2258–2264. [PubMed: 3918999]
53. Fournier N, Atger V, Paul JL, Sturm M, Duverger N, Rothblat GH, Moatti N. Human apoa-iv overexpression in transgenic mice induces camp-stimulated cholesterol efflux from j774 macrophages to whole serum. Arterioscler Thromb Vasc Biol. 2000; 20:1283–1292. [PubMed: 10807744]
54. Steinmetz A, Barbaras R, Ghalim N, Clavey V, Fruchart JC, Ailhaud G. Human apolipoprotein a-iv binds to apolipoprotein a-i/a-ii receptor sites and promotes cholesterol efflux from adipose cells. J Biol Chem. 1990; 265:7859–7863. [PubMed: 2159462]
55. Duverger N, Tremp G, Caillaud JM, Emmanuel F, Castro G, Fruchart JC, Steinmetz A, Denefle P. Protection against atherogenesis in mice mediated by human apolipoprotein a-iv. Science. 1996; 273:966–968. [PubMed: 8688083]
56. Cohen RD, Castellani LW, Qiao JH, Van Lenten BJ, Lusis AJ, Reue K. Reduced aortic lesions and elevated high density lipoprotein levels in transgenic mice overexpressing mouse apolipoprotein a-iv. J Clin Invest. 1997; 99:1906–1916. [PubMed: 9109435]
57. Ostos MA, Conconi M, Vergnes L, Baroukh N, Ribalta J, Girona J, Caillaud JM, Ochoa A, Zakin MM. Antioxidative and antiatherosclerotic effects of human apolipoprotein a-iv in apolipoprotein e-deficient mice. Arterioscler Thromb Vasc Biol. 2001; 21:1023–1028. [PubMed: 11397714]

Highlights

- CREBH induces genes involved in TG clearance, and loss of CREBH increased VLDL-associated TG and cholesterol levels.
- CREBH directly activates *APOA1* promoter.
- Loss of CREBH exacerbates atherosclerosis in $Ldlr^{-/-}$ mice.

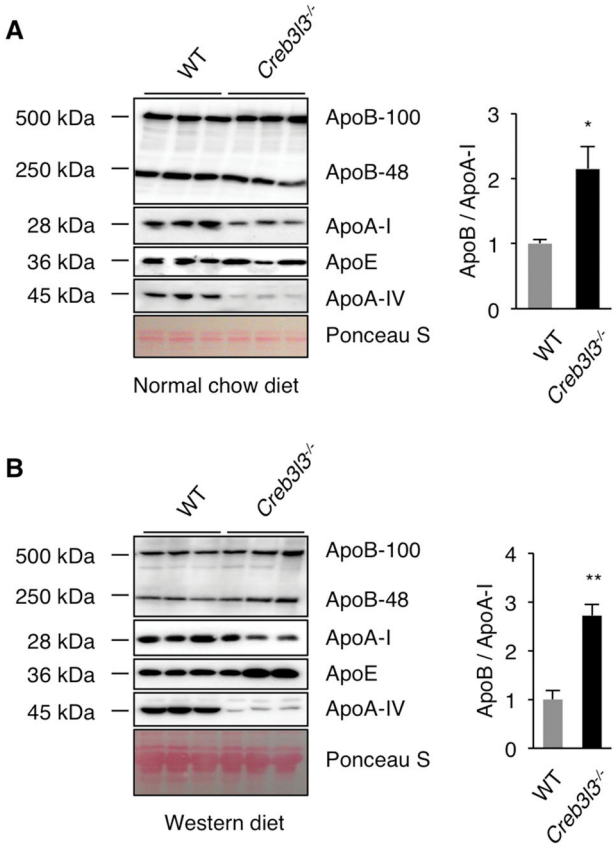

Figure 1. Plasma apolipoprotein levels in *Creb3l3*$^{-/-}$ mice

(A, B) Plasma samples were collected from male mice fed normal chow diet (A) or WD (B). Plasma apolipoprotein levels were determined by western blotting using indicated antibodies. The bottom panel shows Ponceau S staining of the membrane as a loading control. The blots shown are representative of 5 mice per group. Graphs on the right show relative ApoB/ApoA-I ratios. Data are shown as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

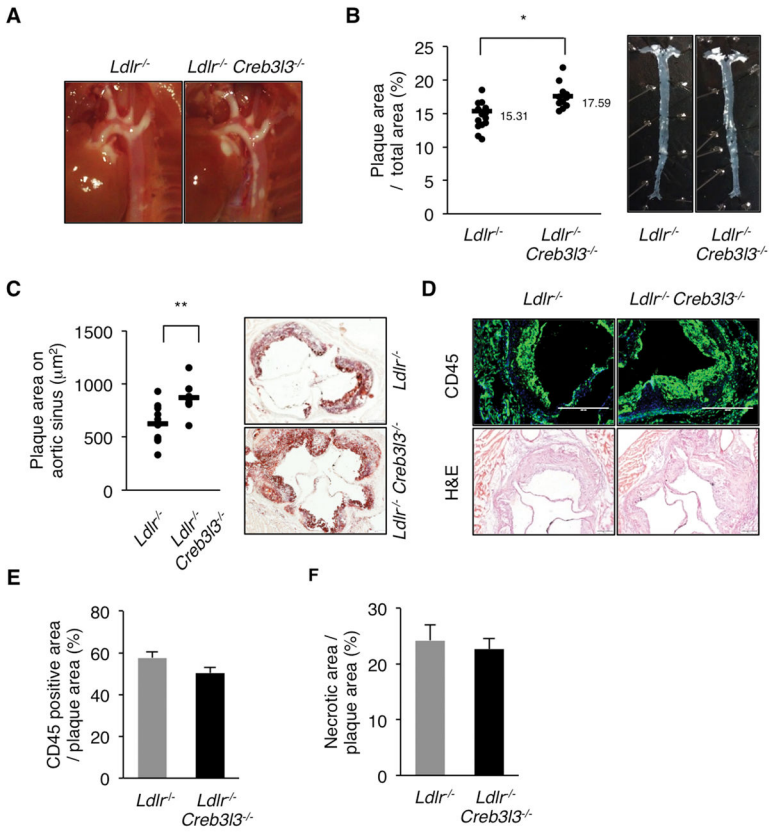

Figure 2. CREBH deficiency accelerates atherosclerosis in *Ldlr*$^{-/-}$ mice

Ldlr$^{-/-}$ and *Ldlr*$^{-/-}$*Creb3l3*$^{-/-}$ male mice were fed WD for 20 weeks. (A) Representative photographs of ascending and descending aortas. (B) Representative images of aortas. The graph shows quantification of Oil Red O-stained plaque area. n = 12–15 per group. (C) Representative images of Oil Red O-stained sections of aortic sinus. The graph shows quantification of plaque area. n = 10 per group. (D) Representative photomicrography of CD45 immunostaining (n = 5 per group) and hematoxylin & eosin (H&E) staining (n = 10 per group) of aortic sinus sections. CD45 positive area (E), and acellular necrotic area (F) in the aortic sinus were measured. Data are shown as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

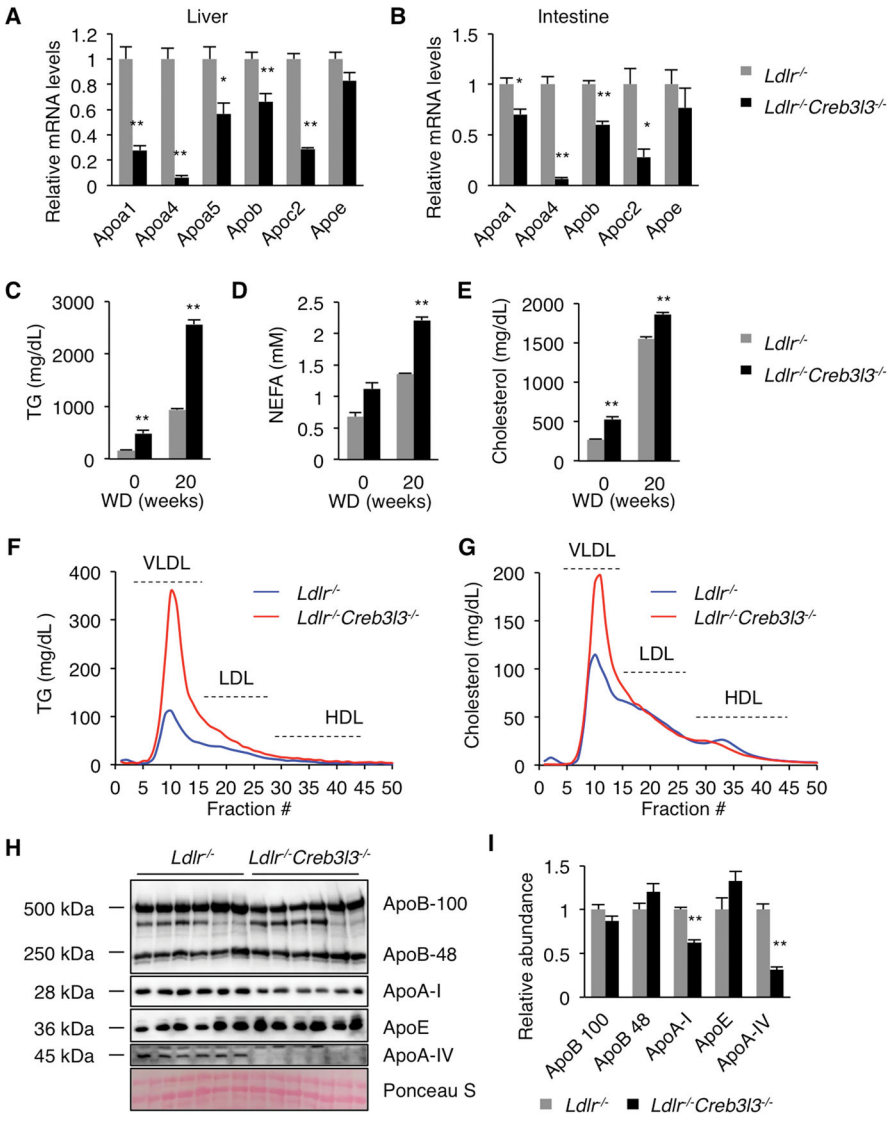

Figure 3. Hyperlipidemia and dyslipoproteinemia in *Ldlr*$^{-/-}$*Creb3l3*$^{-/-}$ mice

(A, B) Quantitative RT-PCR analysis of apolipoprotein genes expressed in liver (A) and intestine (B) of *Ldlr*$^{-/-}$ and *Ldlr*$^{-/-}$*Creb3l3*$^{-/-}$ mice fed WD for 20 weeks. n = 4 male mice per group. (C) Plasma TG, (D) NEFA, and (E) cholesterol levels in mice measured before and after 20 weeks of WD feeding. n = 10–16 male mice per group. (F, G) Pooled plasma samples were subjected to fast protein liquid chromatography for lipoprotein analysis. TG (F) and cholesterol (G) contents in each fraction were measured enzymatically. (H) Plasma samples were subjected to western blotting using indicated antibodies. Each lane represents individual mouse. (I) Quantification of the western blots in panel H. Data are shown as mean ± SEM. *$P < 0.05$, **$P < 0.01$.

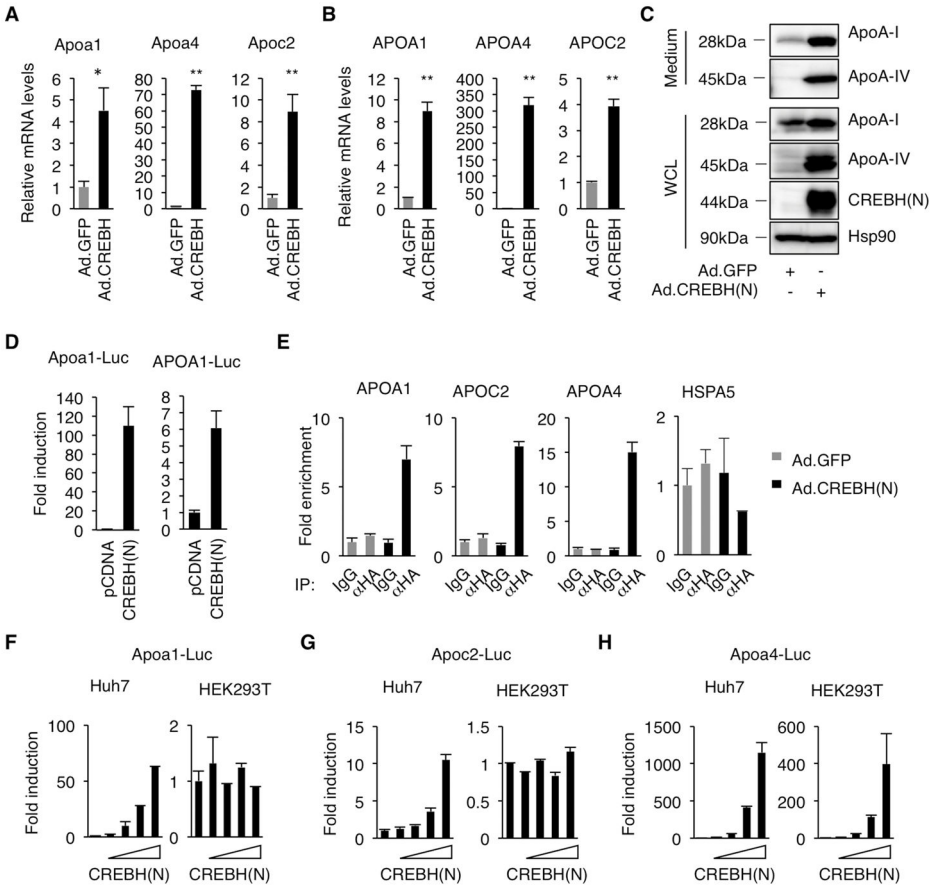

Figure 4. CREBH induces apoA-I in mouse primary hepatocytes and Huh7 cells

(A, B) Mouse primary hepatocytes (A), and Huh7 cells (B) were infected with Ad.GFP or Ad.CREBH(N) adenoviruses. Two days after viral transduction, mouse *Apoa1* or human *APOA1* mRNA levels were determined by RT-PCR. n = 4 per group. $*P < 0.05$, $**P < 0.01$. (C) Western blot analysis of adenovirus-infected mouse primary hepatocytes and culture supernatant using indicated antibodies. (D) Luciferase constructs containing mouse *Apoa1* or human *APOA1* promoter were co-transfected into Huh7 cells with CREBH(N) expression vector. Values represent fold induction of the luciferase activity by CREBH(N) co-transfection. Representative data from at least 4 independent experiments. (E) Huh7 cells infected with Ad.GFP or Ad.HA-tagged CREBH(N) adenoviruses were subjected to chromatin immunoprecipitation assays using anti-HA antibody. Quantitative RT-PCR was performed using immunoprecipitated chromatin samples and primer sets amplifying proximal promoter region of indicated gene. Data are shown as mean ± SE. The results are representative of more than three independent experiments. (F-H) Luciferase reporters for *Apoa1* (F), *Apoc2* (G), or *Apoa4* (H) promoter were co-transfected with increasing amount of CREBH(N) plasmid into Huh7 and HEK293T. Graphs show fold induction of luciferase activity by CREBH(N) co-transfection.

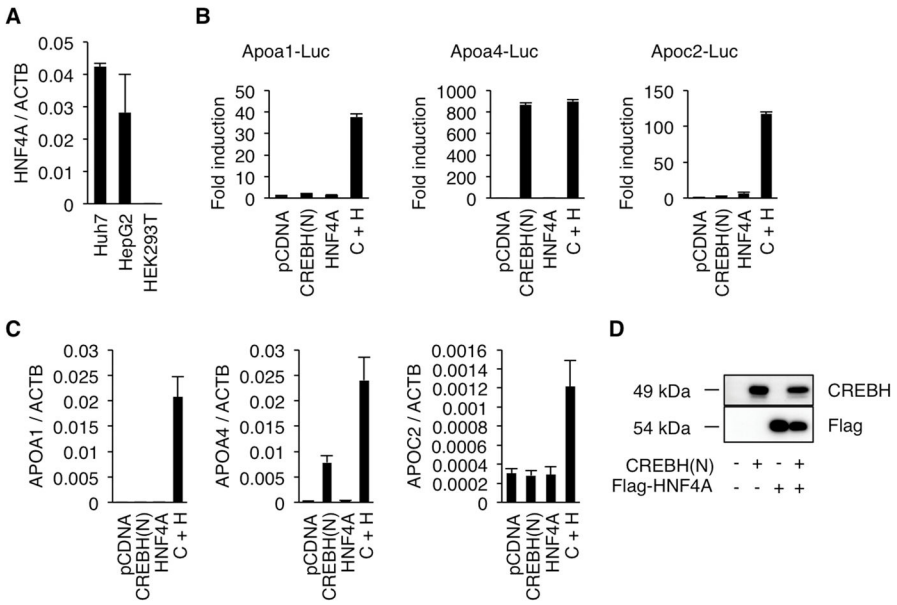

Figure 5. Synergistic activation of Apoa1 promoter by CREBH and HNF-4α

(A) HNF-4α mRNA levels in each cell line determined by RT-PCR. (B) CREBH(N) and HNF-4α synergistically activate *Apoa1* and *Apoc2*, but not *Apoa4* luciferase reporter in HEK293T cells. Equal amount of CREBH(N) and Flag-tagged HNF-4α plasmids were transfected into HEK293T cells as indicated. C and H represent CREBH(N) and Flag-HNF-4α, respectively. (C) HEK293T cells were transfected with the indicated plasmids. Cells were harvested 48 hours later for RT-PCR analysis. (D) CREBH(N) and Flag-HNF-4α expression in the transfected cells were determined by western blotting using anti-CREBH and anti-Flag antibodies.

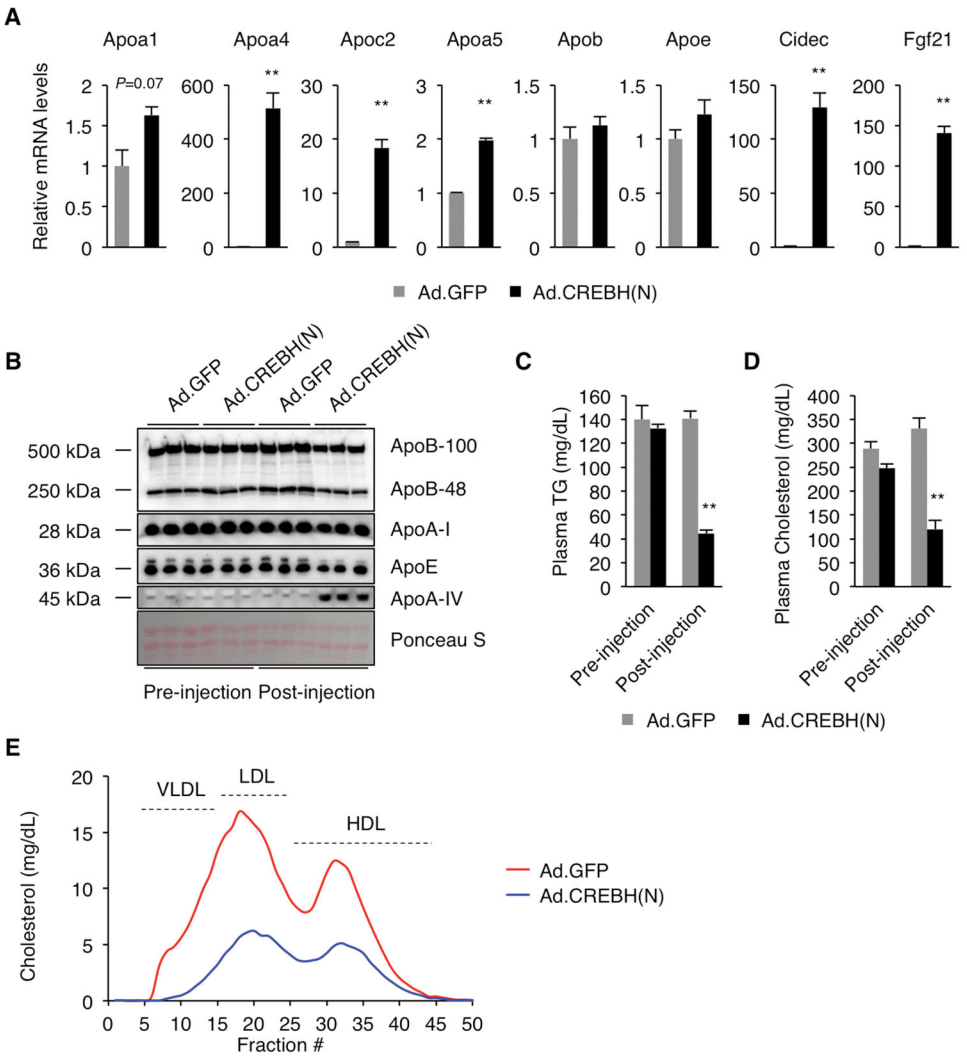

Figure 6. Effects of CREBH(N) overexpression on plasma lipids and lipoprotein profile in *Ldlr*$^{-/-}$ mice

(A) Hepatic mRNA levels of apolipoprotein and CREBH target genes in *Ldlr*$^{-/-}$ mice injected i.v. with Ad.GFP or Ad.CREBH(N). Eight-week-old male mice fed a standard chow diet were used. n = 4 per group. (B) Plasma apolipoproteins, (C) TG, and (D) cholesterol levels measured 4 days after adenovirus injection. (E) Plasma from adenovirus injected *Ldlr*$^{-/-}$ mice was pooled and subjected to fast protein liquid chromatography for lipoprotein analysis. Data are shown as mean ± SEM. **P* < 0.05, ***P* < 0.01