National Cholesterol Awareness Month Article
Basic Sciences

Loss of Transcription Factor CREBH Accelerates Diet-Induced Atherosclerosis in \(Ldlr^{−/−}\) Mice

Jong-Gil Park, Xu Xu, Sungyun Cho, Ann-Hwee Lee

Objective—Liver-enriched transcription factor cAMP-responsive element-binding protein H (CREBH) regulates plasma triglyceride clearance by inducing lipoprotein lipase cofactors, 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 for 20 weeks. We showed that CREBH deletion in \(Ldlr^{−/−}\) mice increased very low-density lipoprotein–associated triglyceride and cholesterol levels, consistent with the impairment of lipoprotein lipase–mediated triglyceride clearance in these mice. In contrast, high-density lipoprotein 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 atherosclerotic lesion 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. (Arterioscler Thromb Vasc Biol. 2016;36:1772-1781. DOI: 10.1161/ATVBAHA.116.307790.)

Key Words: apolipoprotein • atherosclerosis • cholesterol • transcription factors • triglycerides

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 fatty acids to peripheral tissues. Although homozygous LPL genetic mutations do not cause atherosclerosis in hypertriglyceridemia patients,8-11 it has been shown that individuals with reduced LPL activity because of 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 because of combined homozygous mutations in glycosylphosphatidylinositol-anchored high-density lipoprotein–binding protein 1 (GPIHPBP1) 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 several activators and repressors that are secreted from the liver and intestine. Deficiencies of LPL inhibitors, such as apolipoprotein C (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.20,23

Liver-enriched cAMP-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.21 In addition, hepatic CREBH was activated in mice fed with atherogenic Paigen diet or a high-fat and high-cholesterol Western diet (WD).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.28 In this 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 very low–density lipoprotein...
(VLDL)-TG, VLDL-C, decreased HDL-C, and increased atherosclerotic plaques compared with \( \text{Ldlr}^{-/-} \) 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 antiatherogenic lipoprotein particles and hence represents a strong risk factor for CVD. To determine the importance of CREBH in apoA-I and apoB production, we measured the abundance of plasma apolipoproteins in CREBH-deficient \( \text{Creb3l3}^{-/-} \) mice by immunoblotting. Plasma apoB and apoE protein levels were comparable between wild-type and \( \text{Creb3l3}^{-/-} \) mice (Figure 1A). We have previously shown that hepatic apoA-I and apoA-1V mRNA levels were reduced in CREBH-deficient mice. Consistent with this, plasma apoA-I and apoA-1V protein levels were decreased by 2.3- and 3.8-fold, respectively, in \( \text{Creb3l3}^{-/-} \) compared with wild-type littermates, resulting in a 2.2-fold increase in apoB/apoA-I ratio in \( \text{Creb3l3}^{-/-} \) mice (Figure 1A). The decreased abundance of plasma apoA-I (2.0-fold) and apoA-1V (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 \( \text{Ldlr}^{-/-} \) \( \text{Creb3l3}^{-/-} \) double knockout mice. \( \text{Ldlr}^{-/-} \) \( \text{Creb3l3}^{-/-} \) mice were viable and appeared indistinguishable from \( \text{Ldlr}^{-/-} \) mice. We fed \( \text{Ldlr}^{-/-} \) and \( \text{Ldlr}^{-/-} \) \( \text{Creb3l3}^{-/-} \) 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 \( \text{Ldlr}^{-/-} \) and \( \text{Ldlr}^{-/-} \) \( \text{Creb3l3}^{-/-} \) mice (Figure 1 in the online-only Data Supplement). \( \text{Ldlr}^{-/-} \) \( \text{Creb3l3}^{-/-} \) mice contained significantly more atherosclerotic plaques in the aortas compared with \( \text{Ldlr}^{-/-} \) mice (Figure 2A and 2B). Similarly, plaque size in the aortic sinus was \( \approx \) 25% larger in \( \text{Ldlr}^{-/-} \) \( \text{Creb3l3}^{-/-} \) than in \( \text{Ldlr}^{-/-} \) mice (Figure 2C).

**Severe Hyperlipidemia in Ldlr−/− Creb3l3−/− Mice**

We have previously shown that \( \text{Creb3l3}^{-/-} \) mice have hypertriacylglyceridemia because of 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. Consistent with this, hepatic and intestinal mRNA levels of these CREBH target genes were decreased in \( \text{Ldlr}^{-/-} \) \( \text{Creb3l3}^{-/-} \) mice compared with \( \text{Ldlr}^{-/-} \) mice (Figure 3A and 3B). Similarly, plasma TG levels were \( \approx \) 3 times higher in \( \text{Ldlr}^{-/-} \) \( \text{Creb3l3}^{-/-} \) than in \( \text{Ldlr}^{-/-} \) mice throughout the course of WD feeding (Figure 3C). As expected, heat-inactivated serum from \( \text{Ldlr}^{-/-} \) \( \text{Creb3l3}^{-/-} \) exhibited reduced activity to augment LPL-mediated lipolysis compared with \( \text{Ldlr}^{-/-} \) serum (Figure 3A in the online-only Data Supplement), confirming that loss of CREBH impaired

**Figure 1.** Plasma apolipoprotein levels in \( \text{Creb3l3}^{-/-} \) mice. A and B, Plasma samples were collected from male mice fed normal chow diet (A) or Western diet (B). Plasma apolipoprotein (Apo) levels were determined by Western blotting using indicated antibodies. The bottom part 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.

CD45 immunofluorescence staining showed comparable density of CD45-positive leukocytes within the plaque areas of the 2 groups, reflecting a proportional increase in leukocyte infiltration to the expanded plaque areas of \( \text{Ldlr}^{-/-} \) \( \text{Creb3l3}^{-/-} \) mice (Figure 2D and 2E). Similarly, the relative dimensions of atherosclerotic plaques to the area were comparable between \( \text{Ldlr}^{-/-} \) \( \text{Creb3l3}^{-/-} \) and \( \text{Ldlr}^{-/-} \) mice (Figure 2D and 2F). Collectively, these data suggest that the loss of CREBH worsened atherosclerosis in LDL receptor–deficient mice.
TG clearance, causing hypertriglyceridemia. 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. 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 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 nonesterified fatty acid 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 production in the absence of CREBH (Figure IIC in the online-only Data Supplement), considering the recently described function of fibroblast growth factor 21 to reduce plasma nonesterified fatty acid concentration and VLDL secretion. 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 (FPLC) 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 (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 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; Figure IID in the online-only Data Supplement). On the contrary, 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-C levels.

Transcriptional Regulation of ApoA-I by CREBH

To further investigate how CREBH regulates ApoA-I gene expression, we overexpressed constitutively active CREBH(N) in mouse primary hepatocytes and human Huh7 cells using recombinant adenovirus vector. CREBH(N) strongly induced cAMP-responsive element-binding protein H (CREBH) deficiency accelerates atherosclerosis in Ldlr−/− mice. Ldlr−/− and Ldlr−/− Creb3l3−/− male mice were fed Western diet for 20 wk. 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 to 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 and 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.
apoA-I mRNA and 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. 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 Apoal 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 Apoal reporters were induced by the cotransfected 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 polymerase chain reaction 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 endoplasmic reticulum stress-inducible gene regulated by other bZIP transcription factors32 (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 cotransfected 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, cotransfection of these 2 transcription factors strongly activated ApoA1 and ApoC2 promoters, increasing luciferase activities driven by these promoters by 38- and 116-fold, respectively (Figure 5B). Furthermore, cotransfection 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 cotransfection. CREBH(N) and HNF-4α protein levels in the cotransfected 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 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 contrary, 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

![Figure 5. Synergistic activation of apolipoprotein a1 (Apoa1) promoter by cAMP-responsive element-binding protein H (CREBH) and hepatocyte nuclear factor (HNF-4α).](http://atvb.ahajournals.org/)

A. HNF-4α mRNA levels in each cell line determined by reverse transcription polymerase chain reaction (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 indicate CREBH(N) and Flag-HNF-4α, respectively. C. HEK293T cells were transfected with the indicated plasmids. Cells were harvested 48 h later for RT-PCR.

D. CREBH(N) and Flag-HNF-4α expression in the transfected cells were determined by Western blotting using anti-CREBH and anti-Flag antibodies.
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 and 6D). FPLC analysis revealed that VLDL cholesterol was markedly reduced by CREBH(N) overexpression, albeit HDL-C was also reduced (Figure 6E). Given that CREBH(N) overexpression dramatically decreased both plasma TG and cholesterol, we speculate that the reduction of HDL-C 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 Apoal and 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-C 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 antiatherogenic 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,43,34 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-C and high non–HDL-C.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 Apoai, Apoa4, Apoa5, and Apoc2.23 CREBH also regulates fibroblast growth factor 21,23 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.

Apo-A-I is produced from liver and small intestine and constitutes the predominant protein component of HDL.36 Apo-A-I interacts with ATP-binding cassette transporter A1 (ABCA1) and stimulates cholesterol efflux for reverse cholesterol transport.37–39 Loss of apo-A-I increases non–HDL-C and accelerates atherosclerosis in Ldlr−/− mice.38 In contrast, transgenic overexpression of apo-A-I or infusion of recombinant apo-A-I decreases plaque formation, with varying effects on the concentration of non–HDL-C.40 CREBH deficiency suppressed apo-A-I mRNA expression in both the liver and the intestine and accordingly reduced plasma apo-A-I protein and HDL-C levels, indicating that CREBH is an important transcriptional regulator of apo-A-I. In addition, CREBH(N) overexpression strongly induced apo-A-I mRNA in primary mouse hepatocytes and in human hepatoma cells and activated the luciferase reporters driven by Apoai promoter. Surprisingly, however, plasma apo-A-I protein and hepatic apo-A-I mRNA levels were not significantly increased by CREBH(N) overexpression in Ldlr−/− mice. We hypothesize that the endogenous CREBH is sufficient to promote apo-A-I transcription in vivo, and additional exogenous CREBH(N) has only marginal effects on Apoai 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.

Several nuclear hormone receptors directly bind to human APOAI promoter and regulate apo-A-I expression in the liver.44,45 HNF-4α is one of the transcription factors that activates hepatic APOAI 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.47,48,50 We find that CREBH requires HNF-4α to activate Apoai and Apoc2 promoters, implicating that CREBH and HNF-4α cooperatively regulate these genes. In contrast, HNF-4α was dispensable for Apo4 promoter activation by CREBH(N) in HEK293T, suggesting that CREBH regulates its target genes through diverse mechanisms.

Apo-A-IV expression is strictly dependent on CREBH in both liver and small intestine.26 Notably, previous studies have demonstrated that apo-A-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 apo-A-IV conferred protection against atherosclerosis in mice.55–57 Future studies are required to determine the impact of apo-A-IV downregulation in CREBH-deficient mice on atherosclerosis progression and whether apo-A-IV knock-out 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 apo-A-I compared with the wild-type 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 suggest 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.

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Disclosures
None.

References
O’Connell JR, Shuldiner AR. A null mutation in human APOC3 confers.


Kanazawa Y, Kawakami M. A case of apolipoprotein C-II deficiency with.


Seidell JC, Kromhout D, Lie KE, Kastelein J. A lipoprotein lipase muta-

tion (Asn291Ser) is associated with reduced HDL cholesterol levels in.


Hu Y, Liu W, Huang R, Zhang X. A systematic review and meta-anal-

alysis of the relationship between lipoprotein lipase Asn291Ser variant.


M600108-JLR200.


Apolipoprotein C-II mimetic peptide. Hypertriglyceridemia with an apoC-II mutant mice and correction of their.


Jemb.2012.09.008.

10.1007/bf02849767.

Wallidius G, Jungner I. Apolipoprotein B and apolipoprotein A-I: risk indi-


Thompson A, Danesh J. Associations between apolipoprotein B, apoli-


Lee AH, Ikawoshi NN, Glomich LH. XBP-1 regulates a subset of endo-


**Highlights**

- cAMP-responsive element-binding protein H (CREBH) induces genes involved in triglyceride clearance, and loss of CREBH increased very low-density lipoprotein–associated triglyceride and cholesterol levels.
- CREBH directly activates APOA1 promoter.
- Loss of CREBH exacerbates atherosclerosis in Ldlr⁻/⁻ mice.
Loss of Transcription Factor CREBH Accelerates Diet-Induced Atherosclerosis in Ldlr−/− Mice
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Loss of transcription factor CREBH accelerates diet-induced atherosclerosis in Ldlr<sup>−/−</sup> mice

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Materials and Methods

**Animal experiments** Creb3l3−/− mice were backcrossed onto a C57BL/6 background at least 10 times. Ldlr−/− mice (B6;129S7-Ldlr<sup>tm1Her</sup>/J) were obtained from Jackson laboratory, and crossed with Creb3l3−/− mice to produce Ldlr−/− Creb3l3−/− mice. Mice were housed in a specific pathogen free facility at the Weill Cornell Medical College on a 12h light/dark cycles and fed ad libitum standard chow (PicoLab Rodent diet 20, #5058, Lab diet) or western diet (21% fat wt/wt, 50% carbohydrate wt/wt, 0.21% cholesterol wt/wt; Research Diets # D12079B). Age-matched male mice were used for all animal experiments. Animal studies and experiments were approved and carried out according to Weill Cornell Medical College’s Standing Committee on Animals and National Institutes of Health guidelines for animal use and care.

**Plasma lipid analysis** Plasma TG, NEFA and cholesterol concentrations were measured using assay kits (Serum Triglyceride Determination Kit, Sigma; NEFA-HR(2) kit, Wako; Amplex® Red Cholesterol Assay Kit, Life technologies). Lipoprotein profile in pooled plasma was analyzed by fast performance liquid chromatography (FPLC) at the Metabolic Core Facility of Brigham and Women’s Hospital, as described previously.²

**Histological analysis** Hearts and aortas were isolated from euthanized mice after perfusion with phosphate-buffered saline (PBS) through the left ventricle, as described previously.² Aortas were dissected longitudinally, pinned on black silicone plates, and fixed in 10% (v/v) formaldehyde in PBS overnight for en face analysis. Fixed aortas were stained by oil red O for 4 h and digitally photographed. Total aortic areas and lesion areas were calculated by AxioVision software (Carl Zeiss, Jena, Germany). Hearts were embedded...
and frozen in OCT compound (Sakura, Tokyo, Japan). Embedded hearts were sectioned serially at 10 µm thickness starting from the appearance of the aortic valve, and the aortic sinus were stained with oil red O or hematoxylin and eosin (H&E). Eight sections per mouse were examined to calculate the lesion areas within 360 µm depth of proximal aorta using AxioVision software. Immunofluorescence staining was performed using an anti-CD45 antibody (R&D systems, AF114).

**RNA isolation, and real time PCR** Total RNAs were isolated using TRIZOL Reagents (Life Technologies). Synthesis of complementary DNA and real time PCR analysis were performed as described previously\(^3\). Primer sequences are:

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**Western blotting** Plasma apolipoproteins were resolved by SDS-PAGE, transferred to PVDF membranes, and probed with antibodies against apoA-I (Meridian Life Science, K23500R), apoA-IV (Santa Cruz, sc-19036), apoB (BBA, 1761, 182) and apoE
CREBH and Flag-tagged HNF4α expressed in HEK293T cells were detected using a rabbit polyclonal antibody raised against recombinant CREBH protein, and anti-DYKDDDDK Tag Antibody (Biolegend, #63730), respectively.

**Adenoviruses** Recombinant adenovirus expressing human CREBH(N) is described previously. Male c57BL/6 and Ldlr-/- mice were injected intravenously via tail vein with recombinant adenoviruses, and sacrificed 4 days later for gene expression and lipid analysis.

**Cell Culture and Transfection.** Primary hepatocytes were isolated from male mice at 8-10 weeks of age by using a collagenase perfusion method as described previously. Hepatocytes were seeded on Primaria 6-well plates (353846, Corning), and cultured in medium 199 (M4530, Sigma) supplemented with 10% fetal bovine serum. Huh7 and HEK293T cells were maintained in DMEM medium supplemented with 10% fetal bovine serum. For reporter gene assays, cells were transfected with 25 ng luciferase reporter, 25 ng of effector, and 5 ng of pRL/CMV Renilla plasmids using Lipofectamine 2000 (Invitrogen). Luciferase assays were performed using Dual-luciferase reporter assay kit (Promega). Transfection efficiency was normalized to the Renilla activity.

**Chromatin Immunoprecipitation (ChIP) Assay.** Huh7 cells were infected with Ad.GFP or Ad.HA-CREBH(N) adenoviruses. Forty eight hours later, cells were treated with 1% formaldehyde for 10 min, harvested, and sonicated using M220 Focused-ultrasonicator (Covaris) according to the manufacturer’s recommendation. Sheared chromatin samples were subjected to immunoprecipitation using anti-HA (F7, Santa Cruz) antibody or control
mouse IgG. Immunoprecipitated DNA was eluted using Chelex reagent as described previously \(^5\), and used as template for quantitative real-time PCR using following primers.

<table>
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<th>Gene</th>
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<th>Reverse primer</th>
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<td>HSPA5 ChIP</td>
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**Statistical analysis.** All data are presented as mean ± SEM, unless indicated otherwise. Comparisons between groups were made by unpaired two-tailed Student's t-test. \(P < 0.05\) was considered statistically significant.

**References**

**Supplementary Figure I.** (A) Food consumption (g/mouse/day) (n=6 per group) and (B) body weight (g) of Ldlr−/− and Ldlr−/−Creb3l3−/− mice fed WD (n=12-15 per group).
Supplementary Figure II. TG production and clearance in *Ldlr*−/−*Creb3l3*−/− mice. (A) Plasma collected thirty minutes after i.p injection of 200U sodium heparin into a male C57BL/6 mouse was used as LPL source. Plasma collected from *Ldlr*−/− and *Ldlr*−/−*Creb3l3*−/− mice, and heated to 56 °C for 1 hr was used apoc source. LPL substrate was prepared by sonicating 44 mg of triolein and 150 mg/ml gum arabic (Sigma) in 1.0 ml of 0.2 M Tris buffer (pH 8.2). The substrate was incubated with 2 µl LPL and 3 µl heat-inactivated serum for 30 min. NEFA concentration in the reaction mixture was measured. (B) Plasma TG levels measured after a 4-h fast followed by administration of Tyloxapol (0.5 g/kg body weight, i.p.). Values represent changes in TG concentration after Tyloxapol administration. N=5 male mice per group. *P < 0.05, **P < 0.01. (C) Quantitative RT-PCR analysis of liver samples of *Ldlr*−/− and *Ldlr*−/−*Creb3l3*−/− mice fed WD for 20 weeks. n=4 per male mice group. (D) ApoA-I protein level in liver lysates of mice fed WD for 20 weeks measured by western blotting. The graph on the right shows apoA-I quantitation.
Supplementary Figure III. HEK293T cells were cotransfected with Apoa1-luciferase reporter plasmid containing -999/+74 promoter fragment, CREBH(N) expression vector, and various transcription factors and coactivators as indicated. Luciferase assays were performed 24 hours after transfection.
Supplementary Figure IV. Effects of CREBH(N) overexpression on plasma lipids and lipoprotein profile in WD-fed C57BL/6 mice. Male C57BL/6 mice were fed WD for 10 days prior to i.v. injection with Ad.GFP or Ad.CREBH(N) adenoviruses. Mice were sacrificed 4 days later. (A) Hepatic mRNA levels of apolipoproteins and CREBH target genes. (B) Plasma apolipoproteins, (C) TG, and (D) cholesterol levels. (E) FPLC analysis of lipoprotein profile.
APOA4, APOA5, APOC2
CREBH
Stimulate TG clearance
APOA1
apoA-I
HDL

APOA4, APOA5, APOC2
CREBH

APOA1
VLDL
HDL
Atherosclerosis