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Reduced high density lipoprotein cholesterol in severe hypertriglyceridemic ApoCIII transgenic mice via lowered hepatic ApoAI synthesis

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ABSTRACT

Clinical and epidemiological investigations confirm that patients with loss-of-function mutations (R19X, etc.) in Apolipoprotein CIII (ApoCIII) showed beneficial lipid profile including decreased plasma triglyceride and increased high density lipoprotein (HDL) levels. However, whether HDL level would be reduced in hypertriglyceridemia (HTG) induced by high ApoCIII expression has not been demonstrated yet. Here we showed, ApoCIII transgenic mice (ApoCIIItg) displayed severe HTG and had significantly lower HDL level. Analysis of apolipoproteins in lipoprotein fractions by SDS-PAGE revealed marked decrease of apolipoprotein AI (ApoAI) in HDL in transgenic mice compared with the wild type mice (WT) as controls. Further examination demonstrated that hepatic but not intestinal ApoAI mRNA was significantly reduced. Therefore, the decreased ApoAI synthesis might be accounted for the lower plasma HDL level in ApoCIII transgenic mice.

1. Introduction

In the past few decades, the relationship between high density lipoprotein (HDL) and cardiovascular disease (CVD) has been widely studied in clinic and in animal experiments [1,2]. These studies have shown that HDL may be a new target for CVD that increasing HDL level could reduce the incidence of CVD and related mortality. However, in large-scale clinical trials, although effective HDL increasing strategies, including cholesterol ester transfer protein (CETP) inhibitor, niacin and so on, have been carried out in practice, so far, there is no evidence showing they can reduce the incidence of clinical cardiovascular events [3,4,5]. Further studies have shown that protective effect of HDL may be not only generally determined by its quantity, but the function of HDL also should be considered [2].

Apolipoproteins in HDL mainly include ApoAI, ApoAII, ApoAIV, ApoE and ApoCIII [6]. About 13% HDL particles contain ApoCIII. With the emerging role of ApoCIII in atherosclerosis [7,8], the effect of ApoCIII on HDL has been paid more and more attention. ApoCIII is mainly distributed in ApoB containing lipoproteins and HDL, accounting for about 50% of protein components in VLDL and 2% in HDL, and it quickly shifts between these particles [9]. During the process of VLDL hydrolysis mediated by lipoprotein lipase (LPL), ApoCIII distributes from VLDL to HDL, and then transfers back to the newly synthesized TRLs [10]. In healthy subjects, ApoCIII mainly exists in HDL [11], and in HTG patients, ApoCIII is mainly distributed in TRLs [12].

In clinic, patients with hyperlipidemia and diabetes show elevated plasma ApoCIII level and decreased HDL level [13]. Moreover, the loss-of-function mutations (R19X, etc.) are associated with lower levels of triglycerides and increased HDL, reflecting protection of cardiovascular diseases [14,15,16]. Recently, ApoCIII antisense oligonucleotide (ASO) showed promising effect to improve HTG and increase HDL in phase II clinical test [17]. However, in ApoCIII gene modified mice, there are limited researches focusing on HDL and ApoAI. So we studied plasma HDL and ApoAI level and related mechanism in ApoCIII transgenic (ApoCIIItg) mice in this project.

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2. Materials and methods

2.1. Animal procedures

Wt, ApoCIIItg and Ldlr−/− mice were purchased from the Jackson laboratory (USA). ApoCIIItgLdlr−/− mice were crossed by ApoCIIItg and Ldlr−/− mice. Animals were housed and allowed free access to tap water and standard laboratory chow diet. The 'Principles of Laboratory Animal Care' (NIH publication No. 85–23, revised 1996) were followed and the experimental protocol was approved by the Animal Care Committee, Peking University Health Science Center. High fat (HF) diet contained 20% lard and 0.5% cholesterol.

2.2. Plasma lipids assay

Total cholesterol (TC) and triglycerides (TG) in plasma were determined enzymatically using commercially available kits (Sigma). HDL cholesterol (HDL-c) was measured with the same kit after ApoB containing lipoproteins had been precipitated with 20% polyethylene glycol solution.

2.3. Plasma lipid fractions analysis

Blood was drawn in the morning with the animals having free access to food. VLDL, LDL, and HDL fractions were separated by sequential ultracentrifugation. VLDL, LDL and HDL cholesterol, and triglyceride concentrations were measured enzymatically using commercial kits (Sigma). Protein concentrations in the VLDL, LDL and HDL fractions were measured using a commercial kit with BSA as the standard (Bio-Rad Laboratories, Richmond, CA).

2.4. Apolipoprotein analysis

VLDL, LDL and HDL from 50 μl plasma were delipidated with acetone-ethanol (1:1). Apolipoproteins in VLDL, LDL and HDL were identified by SDS-PAGE. After stained in 45% methanol/10% acetic acid/0. 1% Coomassie Blue R and destained in 30% methanol/10% acetic acid, the gels were scanned using an UltroScan XL Laser Densitometer (LKB Instruments, Inc., Gaithersburg, MD).

2.5. RNA isolation and quantitative real-time RT-PCR

Total RNA from the liver and intestine were extracted using Tri reagent (Molecular Research Center, USA) and first-strand cDNA was generated by using an RT kit (Invitrogen, USA). Quantitative real-time PCR was performed using following primer sets: ApoAI (5′-GT GTCCCAGTTTGAATCCTC-3′, 5′-CTCCAGGTTATCCCAGAAGT-3′); Lpl (5′-GTGGCCGAGAGCGAGAACAT-3′, 5′-GCTTTCACTCGGATCCTCTC-3′); Lrp-1 (5′-TGCCAATGAGACCGTATG-3′, 5′-TCCTGTCGTCAATGTCGT-3′); Srb1 (5′-CCGCACAGTTGGTGAGAT-3′, 5′-CTTCGTTGGGTGG GTAGA-3′); Abca1 (5′-CGTTTCCGGGAAGTGTCCTA-3′, 5′-GCTAGAG ATGACAAGGAGGATGGA-3′); Abcg1 (5′-CACCAGTGGCCTGGACAGCG-3′, 5′-CTCTTCAGCAGGCCGGTGCC-3′). Amplifications were performed in 35 cycles using an Opticon continuous fluorescence detection system (MJ Research) with SYBR green fluorescence (Molecular Probes, Eugene, USA). Each cycle consisted of heating denaturation for 45 s at 94 °C, annealing for 45 s at 56 °C, and extension for 60 s at 72 °C. All samples were quantitated by using the comparative CT method for relative quantitation of gene expression and normalized to β-actin.

2.6. Western blot analysis

Delipidated VLDL, LDL and HDL from 20 μl plasma were subjected to electrophoresis on 12% SDS–Page and transferred to nitrocellulose membranes (Sigma, MO, USA). After blocked with 5% bovine serum albumin for 2 h, the membrane was probed with 1:1000 goat anti-human ApoCIII polyclonal antibody (Biodesign, USA) and 1:500 rabbit anti-mouse ApoCIII polyclonal antibody (Santa Cruz, USA), followed by horseradish peroxidase-conjugated secondary antibody (rabbit anti -goat or goat anti-rabbit, 1:5000). The reaction was detected by chemiluminescence and exposed to Kodak X-Omat film (Kodak, Rochester, USA).

2.7. Triglyceride clearance test

Mice were fasted for 14 h, then 30% intralipid (Sigma) was i.v. injected from the tail vein in the amount of 10 folds of bodyweight. Blood samples were collected at 0, 3, 15, 30, 60, 120 and 180 min after injection. Triglyceride was measured by the kit mentioned above.

2.8. Fast-protein liquid chromatography fractionation of lipoproteins (FPLC)

Plasma aliquots (250 μL) were pooled from a group of mice and applied to Tricorn high-performance Superose S-6 10/300 GL columns using a fast-protein liquid chromatography system (Amersham Biosciences), followed by elution with PBS at a constant flow rate of 0.25 mL/min. Eluted fractions (500 μL) were assayed for triglyceride and cholesterol concentrations using the TG and cholesterol kits (BioSino, China).

2.9. Statistical analysis

The results are expressed as means ±SD. Statistical significance was determined by analysis of the Student t test and P value < 0.05 was regarded as significant.

3. Results

3.1. HDL level decreased in ApoCIII transgenic mice

In previous experiments with combined hyperlipidemia mouse model to study atherosclerosis, we had noticed that compared with Ldlr−/− mice, ApoCIIItgLdlr−/− mice showed severe HTG and high total cholesterol (TC) level (Fig. 1A) which have been proved in other experiments [18,19,20]. HDL-c and the ratio of HDL-c and TC significantly decreased (Fig. 1B). The data of FPLC confirmed these results that the HDL peak strikingly decreased compared with Ldlr−/− mice after 12 week HF diet. To investigate whether and how ApoCIII affected plasma HDL, we used Wt and ApoCIIItg mice to study the mechanism. Consistent with ApoCIIItgLdlr−/− mice, ApoCIIItg mice also showed increased total triglyceride (TG) and TC level (Fig. 2A) and decreased HDL-c level and the ratio of HDL-c and TC (Fig. 2B). FPLC also showed a significant decrease in HDL peak of ApoCIIItg mice (Fig. 2C).

3.2. ApoAI decreased in ApoCIIItg mice

ApoAI is the main apolipoprotein in HDL particles, accounting for about 75% of total protein of HDL [21]. And the quantity of ApoAI in HDL is an indicator for HDL content. By sequential ultracentrifugation we separated VLDL, LDL and HDL fractions from plasma. Western blotting showed ApoCIII mainly existed in VLDL and HDL fractions, but transferred and accumulated in triglyceride-rich lipoproteins (VLDL) in ApoCIIItg mice (Fig. 3A). After being delipided, VLDL, LDL and HDL were examined by SDS-PAGE. Consistent with ApoCIII western blotting results, coomassie blue staining also presented accumulation of ApoCIII and ApoB in VLDL fraction in

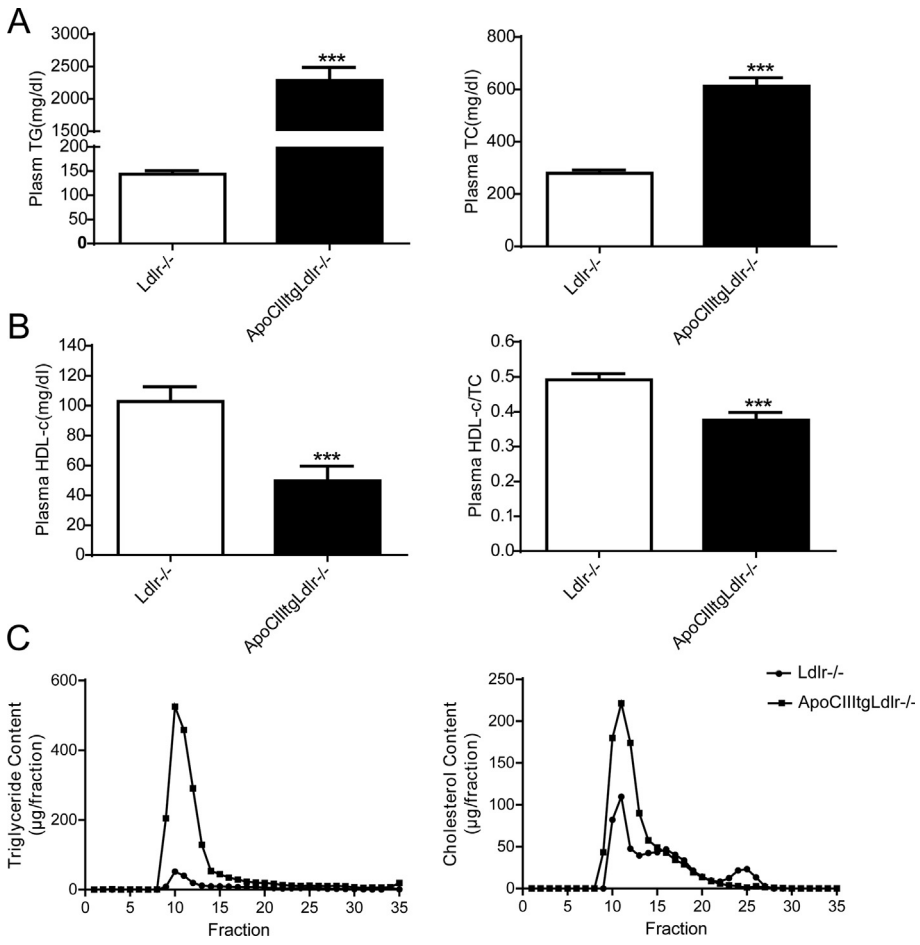

Fig. 1. Lipoprotein profile of Ldlr−/− and ApoCIIItgLdlr−/− mice. Total triglyceride, total cholesterol (A), HDL-c and the ratio of HDL-c/TC (B) in Ldlr−/− and ApoCIIItgLdlr−/− mice (n = 11). Lipoprotein triglyceride (left) and cholesterol (right) by FPLC of pooled fasted plasma of Ldlr−/− and ApoCIIItgLdlr−/− mice after 12 week HF diet (n = 6).Values are expressed as mean±SEM, ***p<0.001 vs. Ldlr−/− mice.

ApoCIIItg mice. Importantly, a significant decrease appeared in HDL-ApoAI in ApoCIIItg mice compared with Wt mice (Fig. 3B). We also investigated the triglyceride, cholesterol and protein content in VLDL, LDL and HDL fractions. Consistent with FPLC and SDS-PAGE results, the data showed ApoCIIItg mice had significantly increased triglyceride, cholesterol and protein content in VLDL fraction. And cholesterol and protein level declined to a large extent compared with Wt mice (Fig. 3C). All these data suggested decreased HDL-ApoAI may be one of the reasons for lower HDL-c level in ApoCIII transgenic mice.

3.3. *Liver ApoCIII synthesis decreased in ApoCIIItg mice*

Since there was a significant decline of HDL-ApoAI in ApoCIIItg mice, we tried to investigate the mechanism. ApoAI is mainly synthesized by liver and to a less extent by small intestine [6]. Therefore, we detected the genes expression related to HDL and ApoAI synthesis in liver and small intestine. Real-time PCR data showed liver ApoAI mRNA level markedly decreased, and there was no other difference in intestine or other genes related to HDL synthesis between Wt and ApoCIIItg mice. So decreased liver ApoAI synthesis may be the reason for lower HDL-ApoAI in ApoCIIItg mice. In addition, LPL deficient mice also showed significant decreased HDL which is explained by a rapid absorption of HDL components into TG-rich lipoproteins at an early stage of HDL maturation [22]. As ApoCIII being the LPL activity inhibitor [18], ApoCIIItg mice also showed much slower clearance of triglyceride after a fat load compared with Wt mice (Fig. 4C). Like LPL knockout mice, the accumulated TG-rich lipoproteins in plasma may be also contributed to the decreased HDL-c level in ApoCIIItg mice.

4. Discussion

Type I hyperlipoproteinemia caused by lipoprotein lipase (LPL) defect is an autosomal recessive genetic disease, showing extremely elevated plasma triglyceride (TG), low plasma total cholesterol (TC) and decreased high density lipoprotein cholesterol (HDL-c) level [23]. The decreased HDL-c is due to the lack of TG lipolysis function mediated by LPL, which damages the formation of HDL precursor and HDL maturation [22]. Moreover, in the patients with hypertriglyceridemia, switch of cholesterol esters from HDL to VLDL increases, further reducing the level of HDL-c.

Although there are many differences in lipid metabolism between human being and mouse, LPL knockout and GPIHBP1 knockout mice, both of which have damaged LPL activity, also show the same manifestation as patients in lipid profile including HTG and low HDL level [24,25]. ApoCIII is a strong inhibitor of LPL activity, and recent studies have proved that ApoCIII can displace the LPL from TRLs and deactivate it through the process mediated by angiopoietin like protein 4 [26]. So the reduced HDL-c level could be partially explained by the decreased LPL activity in ApoCIII transgenic mice (Fig. 4C).

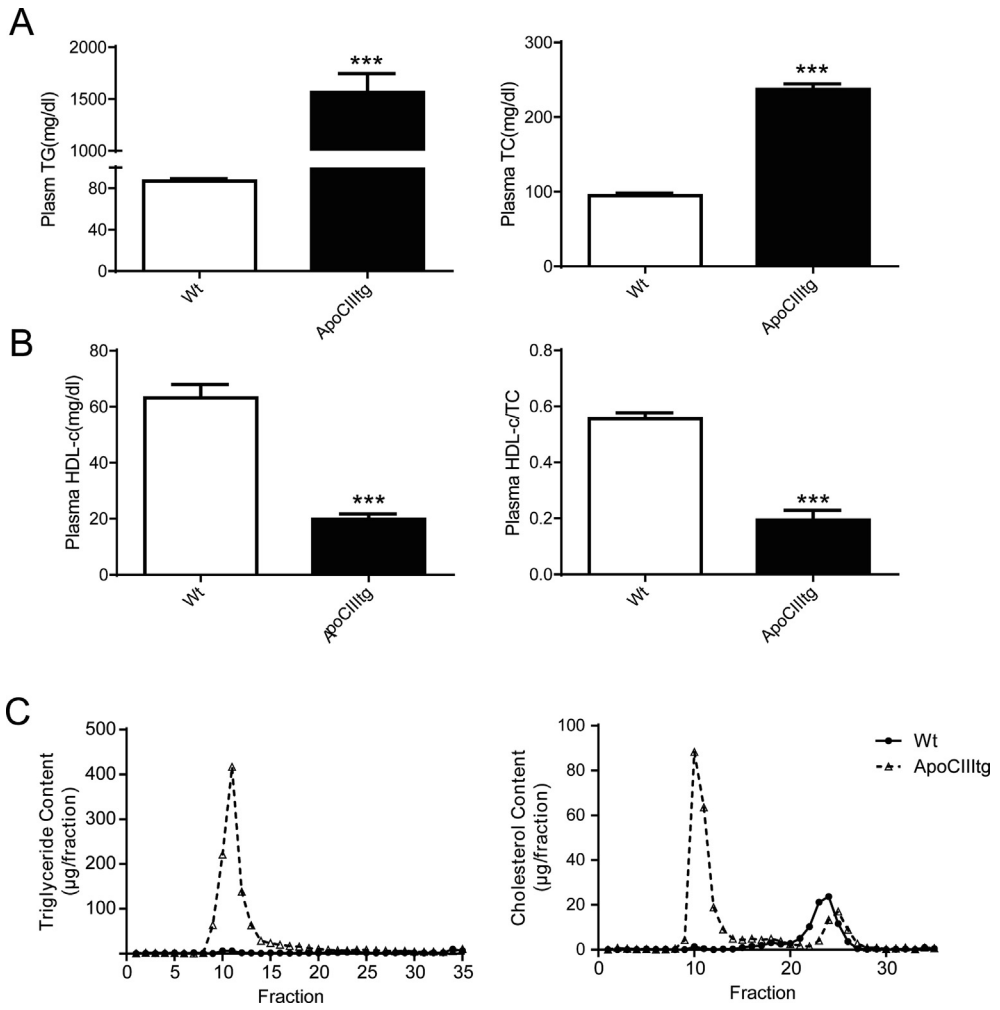

Fig. 2. Lipoprotein profile of Wt and ApoCIIItg mice. TG, TC (A), HDL-c, the ratio of HDL-c/TC (B) and lipoprotein triglyceride (left) and cholesterol (right) by FPLC of pooled fasted plasma (C) of Wt and ApoCIIItg mice (n = 11). Values are expressed as mean±SEM, ***p < 0.001 vs.Wt mice.

In clinic and epidemiology investigations, there is a lot of evidence showing the association between ApoCIII and HDL. Several important ApoCIII mutations in human populations were first described in isolated populations. For example, 5% Lancaster Amish were ApoCIII heterozygous carriers of R19X mutation. Compared with non-carriers, carriers of this mutation had decreased plasma triglyceride levels and elevated levels of HDL-c in fasting and postprandial state [27]. A recent study published in The New England Journal of Medicine sequenced 3734 people of European or Africa ancestry, and found four rare ApoCIII mutations associated with low levels of triglycerides: one nonsense mutation (R19X), two splice site mutations (IVS2+1G, A, T and IVS3+1G) and one missense mutation (A43T). In the carriers of mutations, triglyceride levels decreased by 39%, the content of ApoCIII in plasma decreased 46%, and HDL-c increased by 22% than non-carriers [15]. Authors of another article in The New England Journal of Medicine investigated the Denish Caucasian pedigrees, and found that 1/250 of the population carry IVS2+1G, R19X or A43T mutations, and their non-fasting triglyceride levels decreased. Carriers of IVS2+1G→ A and A43T mutations have increased plasma HDL-c and ApoAI level [14]. What is more, antisense oligonucleotide (ASO) drugs of ISIS Pharmaceuticals for ApoCIII have entered phase II trials which mean they can be a promising therapy for improving hypertriglyceridemia and increasing HDL level [28].

Consistent with the epidemiological and clinical evidence, our study showed that ApoCIII transgenic mice manifested HTG, lower HDL-c (Figs. 1 and 2) and ApoAI level (Fig. 3B). By checking gene expression in liver and intestine, we found that the decreased ApoAI level may be due to the lower ApoAI mRNA expression in liver (Fig. 4A). So our data also provided a clue to further study the increased HDL-c and ApoAI level in loss-of-function mutations in human being, which is that ApoAI expression in the liver should be considered. As we all know, ApoC-III is an exchangeable apolipoprotein located in the ApoAI/CIII/AIV gene cluster on chromosome 11q23 [29]. Whether higher or lower expression of ApoCIII could lead to complementary changes in ApoAI expression needs to be further studied.

In animal experiments, the association of ApoCIII and HDL are involved in multiple studies, but conclusions are not consistent. In 1990, Jan L Breslow etc. proved that the ApoCIII transgenic mice with high copy number had decreased plasma HDL level and ApoAI level [18].However, In 1997 Alan R. Tall etc. examined that there were no differences in HDL content between Ldlr−/− and ApoC-IIItgLdlr−/− mice under both chow and high fat diet [20]. The divergence among these data may be attributed to the different ApoCIII transgenic lines and HDL assay methods. In our study, sample numbers were quite large, and the results were reproducible. We confirmed that ApoCIII transgene leaded to a lower HDL-c

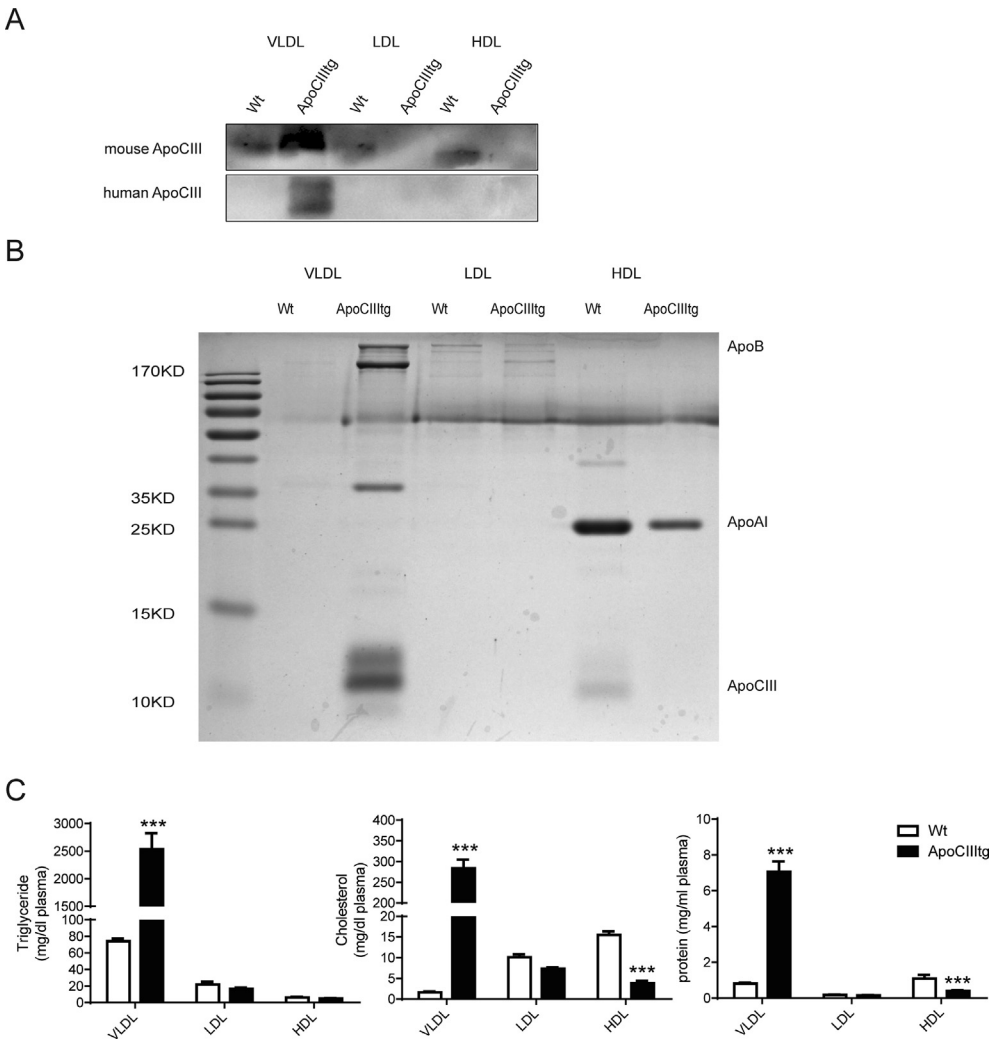

Fig. 3. ApoAI decreased in ApoCIIItg mice. Western blot image of human and mouse ApoCIII (A), SDS-PAGE analysis (B) and triglyceride, cholesterol and protein content (C) of VLDL, LDL, and HDL fractions (n = 4) in WT and ApoCIIItg mice. Western blot and SDS-Page experiments were repeated for three times, ***P<0.001 vs. Wt mice.

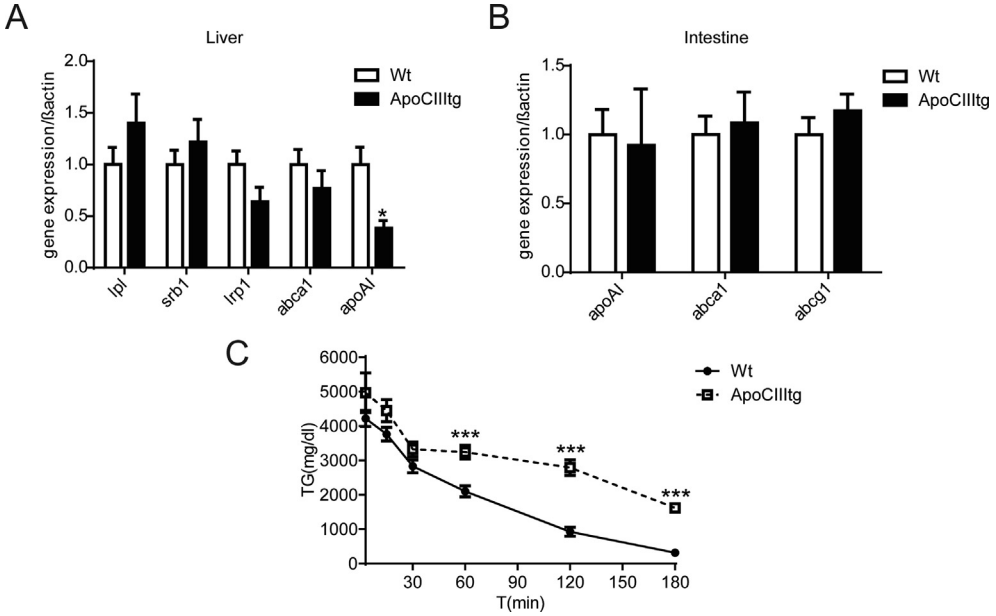

Fig. 4. Decreased ApoAI synthesis and triglyceride clearance in ApoCIIItg mice. HDL related gene expression in liver (A) and intestine (B) of Wt and ApoCIIItg mice (n = 5). Triglyceride clearance after i.v. injection of intralipid emulsion in Wt and ApoCIIItg mice (n = 9). Values are expressed as mean±SEM, *p < 0.5, ***p < 0.001 vs. Wt mice.

and HDL-ApoAI level, which was consistent with Alan R.Tall' s results.

We also should note that human lipid metabolism is rather different from mouse. Human beings exhibit low level of hepatic low-density lipoprotein (LDL) receptor activity, intestinal-only ApoB editing, high level of cholesteryl ester transport protein (CETP) which plays a crucial role in HDL maturation. All of these characters are not observed in mice. Therefore, other animal models, such as hamster [30]whose lipid profile is much more similar with human, should be used to further investigate the relationship between ApoCIII and HDL metabolism in our future study.

Conflict of interest

None.

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