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Adiponectin deficiency suppresses ABCA1 expression and ApoA-I synthesis in the liver

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Abstract **Plasma high density lipoprotein (HDL)-cholesterol levels are inversely correlated with the incidence of cardiovascular diseases. HDL is mainly assembled in the liver through the ATP-binding cassette transporter (ABCA1) pathway. In humans, plasma HDL-cholesterol levels are positively correlated with plasma adiponectin (APN) concentrations. Recently, we reported that APN enhanced apolipoprotein A-I (apoA-I) secretion and ABCA1 expression in HepG2 cells. In the present study, we investigated HDL assembly in APN-knockout (KO) mice. The apoA-I protein levels in plasma and liver were reduced in APN-KO mice compared with wild-type-mice. The ABCA1 expression in liver was also decreased in APN-KO mice. APN deficiency might cause the impaired HDL assembly by decreasing ABCA1 expression and apoA-I synthesis in the liver.**
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Keywords: ABCA1; Adiponectin; ApoA-I; ApoB-100; HDL; Reverse cholesterol transport

1. Introduction

Plasma high density lipoprotein (HDL)-cholesterol levels are negatively correlated with the incidence of coronary artery disease (CAD). It is thought that HDL prevents the development of atherosclerosis by removing excess cholesterol from atheroma and transporting it back to the liver in the protective system, so-called "reverse cholesterol transport" (RCT) [1].

The ATP-binding cassette transporters (ABCA1 and ABCG1), which are expressed in the liver, small intestine and peripheral tissues, are thought to be rate-limiting factors for HDL assembly in RCT system [2,3]. ABCA1, the responsible gene for familial HDL deficiency including Tangier disease [4–6], promotes apoA-I-mediated cholesterol efflux, which is the initial step in RCT system, decreasing cholesterol accumulation in macrophages and initiating HDL formation in the liver [2]. ABCG1 also stimulates cholesterol efflux to mature HDL in macrophages [3].

Adiponectin (APN), a bioactive peptide secreted from adipocytes is one of the important molecules to inhibit the development of atherosclerosis. Several clinical studies have demonstrated that plasma levels of APN are extremely low in patients with the metabolic syndrome which clusters risk factors for CAD such as visceral obesity, dyslipidemia, impaired glucose tolerance and hypertension. Plasma APN concentrations are positively correlated with plasma HDL-cholesterol levels [7–11]. Although these findings suggest that APN might have an ability to prevent the development of atherosclerosis by the acceleration of RCT system, the underlying mechanisms for it has not been clarified yet. Recently, we reported that human recombinant APN enhanced the expression of ABCA1 and accelerated the synthesis of apoA-I in a human liver cell line, HepG2 cells, suggesting that APN might increase HDL assembly in the liver [12]. Therefore, in the present study, we investigated the HDL assembly in APN knockout (APN-KO) mice.

2. Materials and methods

2.1. Animals

Adiponectin-knockout (APN-KO) mice were generated as described previously and backcrossed to wild-type (WT) C57BL/6J mice [13]. Both APN-KO and WT mice (male) were housed in temperature and humidity controlled facility with a 12-h light/dark cycle and fed a normal chow diet (MF, OrientalBio Laboratories, Chiba, Japan) and sacrificed for analysis at the age of 8–10 weeks old. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

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Abbreviations: ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; APN, adiponectin; apoA-I, apolipoprotein A-I; apoB-100, apolipoprotein B-100; CAD, coronary artery disease; CM, chylomicron; FC, free cholesterol; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; KO, knockout; LDL, low density lipoprotein; MTP, microsomal triglyceride transfer protein; PL, phospholipids; RCT, reverse cholesterol transport; TC, total cholesterol; TG, triglyceride; VLDL, very low density lipoprotein; WT, wild-type

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2.2. Lipid profile by high performance liquid chromatography (HPLC) analysis

One hundred microliter of blood from anesthetized mice (at the age of 8–10 weeks) were drawn from retro-orbital plexus *ad libitum* and plasma was immediately isolated from the collected blood by centrifugation at 4 °C. The lipid profile of plasma was analyzed by an online dual enzymatic method using high performance liquid chromatography (HPLC) at Skylight Biotech Inc. (Akita, Japan), according to the procedure as described by Usui et al. [14]. The plasma concentrations of total cholesterol (TC), triglyceride (TG), free cholesterol (FC) and phospholipids (PL) of four fractioned groups [chylomicron (CM): lipoprotein particle size >80 nm, very low density lipoprotein (VLDL): 30 < particle size <80 nm, low density lipoprotein (LDL): 16 < particle size <30 nm and HDL: 8 < particle size < 16 nm] were determined by using enzymatic reagents (Kyowa Medex, Tokyo, Japan).

2.3. Western blot analysis

Mice plasma or proteins isolated from the liver were separated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-Rad, Germany). Incubations of antibodies with the membranes were performed in TBS including 0.1% Tween 20 and 2% skim milk at 4 °C overnight. Detection of the immune complexes was carried out by ECL Advance Western Blot Detection System (Amersham Biosciences, UK). Anti-mouse apoA-I antibody (Biodesign, USA), anti-mouse apolipoprotein B48/100 (apoB) antibody (Biodesign, USA), anti-mouse ABCA1 antibody (Novus, USA) and anti-mouse ABCG1 antibody (Santa Cruz, USA) were used for the assay.

2.4. cDNA synthesis and quantitative PCR

One microgram of total RNA isolated from tissues was primed with 50 pmol of oligo (dT) 20 and reverse-transcribed with SuperScript III (Invitrogen, USA) for first strand cDNA synthesis, according to the protocol of the manufacturer. Real-time quantitative PCR was performed according to the protocol of DyNamo HS SYBR Green quantitative PCR kit. Relative gene expression was quantified using GAPDH as an internal control.

2.5. Primers used in this study

The primes for mouse ABCA1 were ABCA1-forward: 5′-TGGGAACTCCTGCTAAAAT-3′ and ABCA1-reverse: 5′-CCATGTGGTGTGTAGACA-3′, for mouse apoA-I, apoA-I-forward: 5′-GTGGCTCTGGTCTTCCTGAC-3′ and apoA-I-reverse: 5′-ACGGTTGAACCCAGAGTGTC-3′, for mouse apoB, apoB-forward: 5′-TGGGATTCCTCTGCCATCTCGAG-3′ and apoB-reverse: 5′-GTAGAGATCCATCACAGGACAATG-3′, for mouse GAPDH, GAPDH-forward: 5′-ACTCCACTCACGGCAAATTC-3′ and GAPDH-reverse: 5′-TCTCCATGGTGGTGAAGACA-3′.

2.6. Statistical analysis

Values were expressed as means ± S.D. Statistical significance was assessed by Student's *t*-test for paired values and set at $P < 0.05$.

3. Results

3.1. Plasma VLDL-TG levels were increased in APN-KO mice, while there was no significant difference in plasma levels of HDL-cholesterol between WT and APN-KO mice

Blood samples from anesthetized APN-KO ($n = 6$) and WT ($n = 6$) mice at the age of 8–10 weeks were drawn from retro-orbital plexus ad libitum and plasma was immediately isolated by centrifugation at 4 °C. The lipid profile of plasma was analyzed by automatic HPLC and enzymatic methods. The plasma levels of TG, in particular VLDL-TG, were significantly increased in APN-KO mice compared with WT mice (Fig. 1). However, there was no significant difference in plasma TC, HDL-cholesterol and PL levels between APN-KO and WT mice.

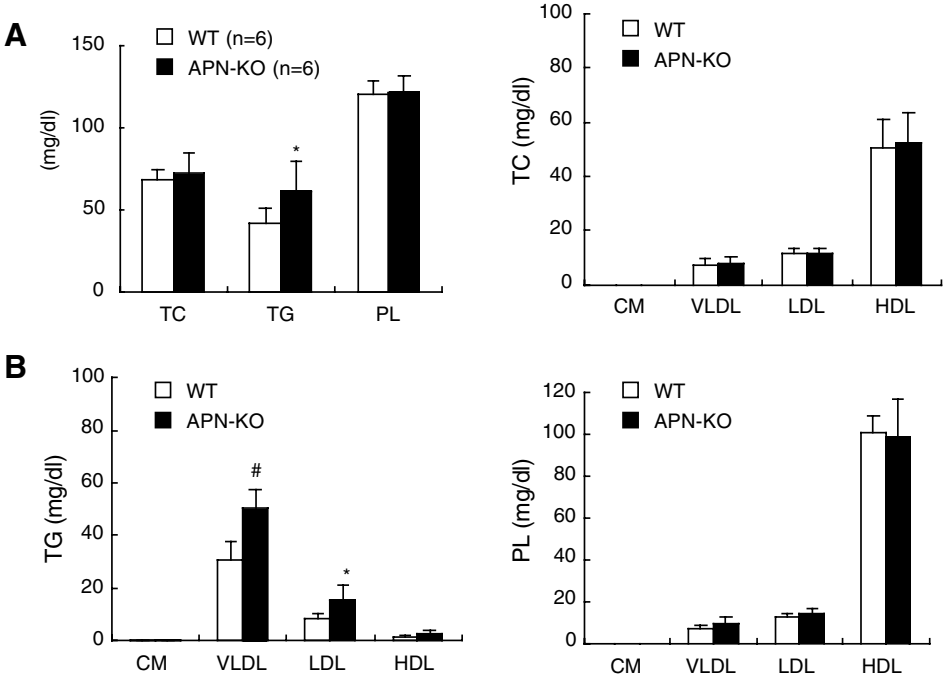

Fig. 1. Plasma lipid profile of APN-KO mice. Blood samples from anesthetized APN-KO ($n = 6$) and WT mice ($n = 6$) at the age of 8–10 weeks were drawn from retro-orbital plexus *ad libitum*. (A) Plasma TC, TG and PL levels. Plasma TG levels of APN-KO mice were significantly higher than those of WT mice. (B) Lipid composition of lipoproteins (CM, VLDL, LDL, and HDL). VLDL-TG concentrations were significantly increased in APN-KO mice compared with WT mice. Values are expressed as means ± S.D. $^{*}P < 0.05$, $^{\#}P < 0.01$, vs. WT mice.

3.2. ApoA-I levels in plasma and the liver were decreased in APN-KO mice

Recently, we reported that APN increased the secretion of apoA-I and decreased the release of apolipoprotein B-100 (apoB-100) from HepG2 cells. Therefore, first, plasma levels of apolipoproteins (apoA-I and apoB-100) in APN-KO mice were investigated by Western blot. As shown in Fig. 2A, plasma levels of apoA-I were slightly decreased in APN-KO mice compared with WT mice, while plasma concentrations of apoB-100 were increased in APN-KO mice. Furthermore, both the mRNA and protein levels of apoA-I in the liver were definitely reduced in APN-KO mice compared to WT mice (Fig. 2B and C). However, there was no significant difference in the mRNA levels of apoB-100 in the liver between APN-KO and WT mice.

3.3. ABCA1 expressions were reduced in APN-KO mice

Finally, we investigated in APN-KO mice the expression levels of ABC transporters (ABCA1 and ABCG1) to generate HDL in the liver. Both the protein (Fig. 3A) and mRNA (Fig. 3B) levels of ABCA1 were significantly decreased in the liver of APN-KO mice compared with WT mice. However, there was no significant difference in the levels of ABCG1 protein between APN-KO and WT mice (Fig. 3A).

4. Discussion

In the present study, we demonstrated for the first time that the expression levels of apoA-I in plasma and the liver were decreased in APN-KO mice as expected from our previous report [12]. Furthermore, we found that the ABCA1 expressions were also reduced in APN-KO mice. These data suggest that low plasma APN concentrations might suppress HDL assembly in the liver, suggesting that the subjects with low serum APN show low plasma HDL-cholesterol levels. However, there was no significant difference in plasma HDL-cholesterol levels between APN-KO and WT mice fed a normal chow diet despite the decrease of apoA-I levels in plasma and the liver of APN-KO mice. There might be some difference in the lipid composition of HDL particles, for example, in the apoA-I mass of HDL particles between APN-KO and WT mice. Abnormal HDL particles with low apoA-I concentrations might have a decreased ability in promoting cholesterol efflux to prevent against atherosclerosis.

Recently, Otabe et al. reported that overexpression of human APN in transgenic mice results in suppression of visceral fat accumulation and reduction of plasma fasting glucose, insulin and leptin levels compared with WT mice [15]. However, these differences were observed only when mice were

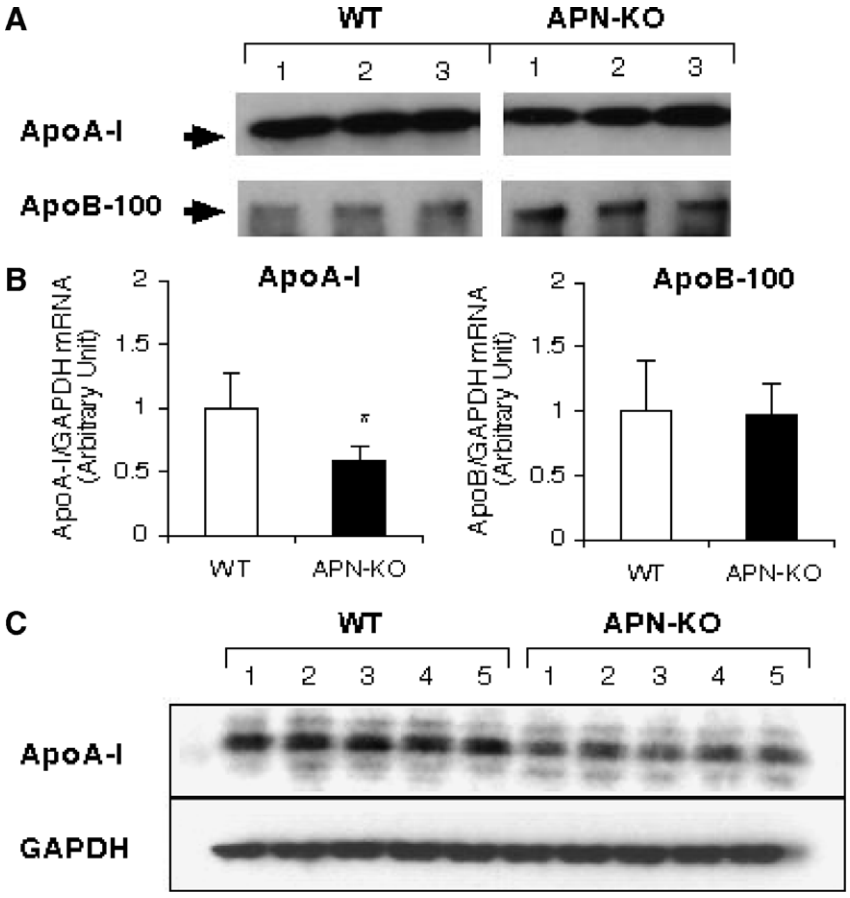

Fig. 2. ApoA-I levels in plasma and the liver were decreased in APN-KO mice. (A) Analysis of plasma apolipoprotein (apoA-I and apoB-100) levels by western blot. Plasma apoA-I levels were slightly decreased in APN-KO mice ($n = 3$) compared with WT mice ($n = 3$), while plasma apoB100 levels were increased in APN-KO mice. (B) The mRNA and (C) the protein expression levels of apoA-I or apoB-100 in the liver. Both the mRNA and protein of apoA-I levels were significantly reduced in the liver of APN-KO mice ($n = 5$) compared with WT mice ($n = 5$). Relative gene expression determined by quantitative PCR was quantified using GAPDH as an internal control. Values are expressed as means ± S.D. $^{*}P < 0.05$, vs. WT mice.

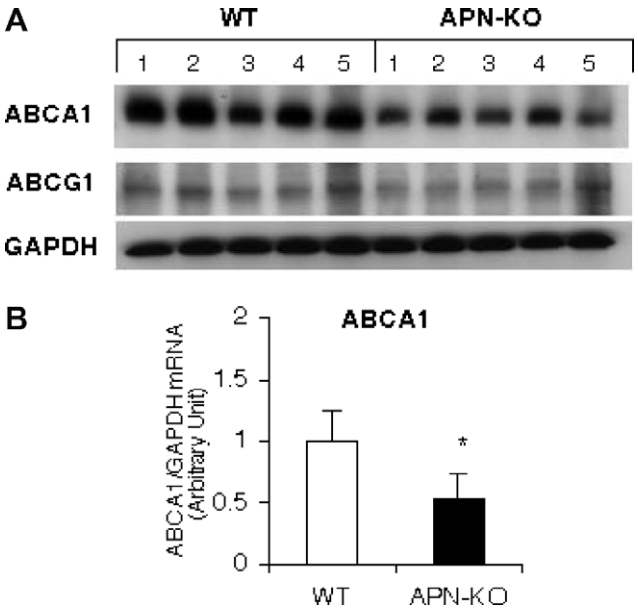

Fig. 3. ABCA1 expressions were reduced in the liver of APN-KO mice. Both the protein (in A) and mRNA (in B) levels of ABCA1 were significantly reduced in the liver of APN-KO mice ($n = 5$) compared with WT mice ($n = 5$). Relative gene expression determined by quantitative PCR was quantified using GAPDH as an internal control. Values are expressed as means ± S.D. $^{*}P < 0.05$, vs. WT mice.

fed a high fat/high sucrose diet, but not a normal chow diet. In our study, the lipid profiles of APN-KO mice were examined with feeding only a normal chow diet. The effect of APN on some parameters including HDL-cholesterol levels might be dependent on the nutritional condition. Therefore, possibly, low plasma HDL-cholesterol might be observed in APN-KO mice fed with over nutrition like a high cholesterol/high fat diet. These issues will be studied in the near future.

We found that apoB-100-containing lipoproteins (VLDL and LDL)-TG, in particular, VLDL-TG levels were increased in APN-KO mice. Recently, it is clinically focused that the accumulation of TG-rich lipoprotein like VLDL in plasma is also strongly linked to CAD as well as that of an atherogenic lipoprotein, LDL [16]. Therefore, plasma VLDL accumulation might be in part associated with the development of atherosclerosis in APN deficiency.

Although plasma VLDL-TG levels were significantly increased in APN-KO mice, there was no significant difference in the apoB-100 expression in the liver between APN-KO and WT mice. As shown in our previous and Neumeier's reports [12,17], in vitro, the apoB secretion from HepG2 cells or primary human hepatocytes was enhanced by recombinant APN, while the mRNA expression levels of apoB were not influenced by APN. Therefore, APN might be involved in the assembly or secretion of VLDL in the liver, but not in apoB-100 synthesis. Microsomal triglyceride transfer protein (MTP) is well known to be an intracellular lipid transfer protein, closely associated with VLDL output from the liver [18,19]. We need to examine the effect of APN on MTP expression (or activity) in the liver.

In summary, we clarified that apoA-I synthesis and ABCA1 expression in the liver were suppressed in APN-KO mice. APN might play an important role in preventing the development of atherosclerosis by the acceleration of HDL assembly in RCT system.

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