Angiopoietin-Like Protein3 Regulates Plasma HDL Cholesterol Through Suppression of Endothelial Lipase


Objectives—A low level of high-density lipoprotein (HDL) in plasma has been recognized as an aspect of metabolic syndrome and as a crucial risk factor of cardiovascular events. However, the physiological regulation of plasma HDL levels has not been completely defined. Current studies aim to reveal the contribution of angiopoietin-like protein3 (angptl3), previously known as a plasma suppressor of lipoprotein lipase, to HDL metabolism.

Methods and Results—Angptl3-deficient mice showed low plasma HDL cholesterol and HDL phospholipid (PL), and which were increased by ANGPTL3 supplementation via adenovirus. In vitro, ANGPTL3 inhibited the phospholipase activity of endothelial lipase (EL), which hydrolyzes HDL-PL and hence decreases plasma HDL levels, through a putative heparin-binding site in the N-terminal domain of ANGPTL3. Post-heparin plasma in Angptl3-knockout mice had higher phospholipase activity than did that in wild-type mice, suggesting that the activity of endogenous EL is elevated in Angptl3-deficient mice. Furthermore, we established an ELISA system for human ANGPTL3 and found that plasma ANGPTL3 levels significantly correlated with plasma HDL cholesterol and HDL-PL levels in human subjects.

Conclusions—Angptl3 acts as an inhibitor of EL and may be involved in the regulation of plasma HDL cholesterol and HDL-PL levels in humans and rodents. (Arterioscler Thromb Vasc Biol. 2007;27:366-372.)

Key Words: angptl3 ■ high density lipoprotein ■ endothelial lipase ■ phospholipase ■ triglyceride

Plasma concentrations of high-density lipoprotein (HDL) cholesterol are inversely correlated with the risk of atherosclerotic cardiovascular disease.1 HDL cholesterol levels are low in patients with metabolic disorders, such as obesity, insulin resistance, and diabetes.2,3 However, the genetic and metabolic factors that regulate HDL metabolism remain to be elucidated. Recently, endothelial lipase (EL) has been recognized as one factor that influences HDL metabolism. EL was originally discovered as a member of the family of triglyceride (TG)-lipases together with lipoprotein lipase (LPL) and hepatic lipase (HL). In contrast to LPL or HL, EL has relatively lower triglyceride lipase activity and substantially higher phospholipid lipase activity and can hydrolyze HDL phospholipids (PL).4 Overexpression of EL in mice resulted in reduced plasma HDL levels and EL knockout mice showed significant increase in HDL levels,5-7 indicating that EL regulates HDL metabolism.

In the colony of KK mice, characterized by obesity, diabetes mellitus, and hypertriglyceridemia, we recently identified a mutant subgroup of KK/Snk mice with low plasma TG levels despite maintaining the phenotype of obesity and diabetes. Genetic mapping and positional cloning identified the gene of angiopoietin-like protein 3 (Angptl3), which was mutated in the KK/Snk mice. The Angptl3 gene in KK/Snk mice contained a 4-bp nucleotide insertion in exon 6, which caused a premature stop codon attributable to a frameshift, leading to a lack of production of the protein.8 Angptl3 mRNA is expressed exclusively in the livers of humans and mice. ANGPTL3 protein contains a signal sequence of 18 amino acids at the N terminus, followed by a coiled-coil domain and a fibrinogen-like domain at the C-terminal side.8,9 Treatment with recombinant ANGPTL3 or adenovirus-mediated overproduction of ANGPTL3 significantly elevated plasma levels of TG, nonesterified fatty acids (NEFA), and total cholesterol in mice.8 In subsequent studies, we revealed that ANGPTL3 increased very low density lipoprotein (VLDL)-TG levels by inhibiting LPL activity via the putative heparin-binding motif in the N-terminal re-
gion. In another study, we also found that ANGPTL3 was able to bind to adipocytes and increase the release of NEFA through activating lipolysis. Thus, the molecular mechanisms of ANGPTL3-mediated increase in plasma TG and NEFA have been investigated. However, the effects of ANGPTL3 on plasma total cholesterol, especially on plasma HDL, which is the major lipoprotein carrying cholesterol in mice, have not yet been investigated.

Moreover, the amino acid sequence of EL is 44% identical to that of LPL, and in particular, the clusters of positively charged residues involved in heparin binding are conserved between EL and LPL, suggesting that ANGPTL3 might affect EL activity, because it inhibits LPL activity. In the current study, we investigated the potential involvement of ANGPTL3 in HDL metabolism and its effects on EL activity.

Methods

Animals
Studies were conducted in 15- to 19-week-old male wild-type KK and Angptl3-deficient KK/Snk mice. To obtain a congenic strain, KK/Snk mice were backcrossed to C57BL/6J mice for 10 generations, and designated as C57BL/6J Angptl3<sup>−/−</sup> mice.14 Angptl3-knock out mice were made as described previously.15 Experiments were conducted when the mice (males) were between 8 and 9 weeks of age. The mice were housed in a room under controlled temperature (23±1°C) with free access to water and mouse chow (Oriental Chemical Industries). Briefly, to determine HDL-cholesterol, total cholesterol, and TG concentrations, and designated as C57BL/6J Angptl3<sup>−/−</sup> mice, we injected 1 or 2×10<sup>5</sup> pfu of each recombinant adenovirus intravenously into C57BL/6J Angptl3<sup>−/−</sup> mice.14

Lipoprotein Analysis
Plasma lipoproteins were analyzed by an upgraded high performance liquid chromatography (HPLC) analysis according to the procedure described by Usui et al (Skylight Biotech Inc).

Recombinant ANGPTL3 Protein
Human recombinant ANGPTL3 protein was prepared as described previously, and it was confirmed to inhibit LPL in vitro and to increase plasma TG concentrations in mice.15 Recombinant proteins of truncated and/or mutated human ANGPTL3 were prepared as described previously.11

Phospholipase Activity
To obtain EL protein, we constructed human EL cDNA adding an in-frame DNA sequence, as described previously.13 Human expression constructs were transfected into HEK293 cells with Lipofectamine2000 (Life Technologies), and then a stable transfectant was obtained by G418 selection. The stable transfectant cells were incubated with Opti-MEM I (Invitrogen). After 48 hours, the conditioned (heparin-washed) media were harvested as the enzyme solution, and phospholipase activities were measured with recombinant ANGPTL3 proteins as described in supplemental Methods (available online at http://atvb.ahajournals.org). For the quantification of phospholipase activity in mouse plasma, studies were conducted in 11- to 13-week-old male C57BL/6J and C57BL/6J Angptl3-knockout mice. Plasma was collected into tubes, using a heparin-coated glass capillary, before and 10 minutes after the heparin (200 μg/kg) injection into the jugular vein. 20 μL of mouse plasma was used as an enzyme solution, and phospholipase activities were measured as described in supplemental Methods.

ELISA for Plasma ANGPTL3 in Humans
Two ANGPTL3 mouse antibodies were produced using the recombinant human ANGPTL3 as the antigen, and were introduced in a double-antibody sandwich enzyme immunoassay system (ELISA) to detect human ANGPTL3.8,10,14,16 45B1 mouse monoclonal antibody was fixed on the 96-well plates. 16-fold diluted plasma samples were immobilized on the 96-well plates at 4°C for 16 hours. Then, we washed the plates with PBS containing 0.1% Tween20 (PBST) and added horseradish peroxidase (HRP)-conjugated No.1 rabbit polyclonal antibody to these plates. After 1 hour incubation at 37°C, we washed the plates with PBST and added the detection reagent for HRP. Thirty minutes later, we stopped the reaction by the addition of an equal volume of 1N H<sub>2</sub>SO<sub>4</sub> and measured at 450 nm absorbance.

Western Blotting
Western blotting of recombinant human ANGPTL3 protein was conducted as described previously.13 The plasma protein bound to the ELISA plate fixed with 45B1 mouse monoclonal antibody was subjected to western blotting with HRP-conjugated No.1 rabbit polyclonal antibody.

Human Studies
87 volunteers working at Sankyo Co. were enrolled in the study. All subjects gave informed consent. Several subjects with obesity, hypertriglyceridemia, hypertension, fatty liver, diabetes, kidney failure, low body weight, and detection of blood in the ura were excluded from the correlation analyses. Subjects taking drugs for hyperlipidemia also were excluded. Plasma samples were collected under overnight fasting conditions. Total cholesterol and TG concentrations were measured using an automatic analyzer from Wako Pure Chemical Industries. HDL cholesterol and HDL-PL concentrations were measured as described above.

Statistical Analysis and Ethical Considerations
The correlation coefficient (r) and probability (p) were calculated in human studies using Microsoft Excel 2003. All data were expressed as the means±SEM or SD. Differences between the groups were examined for statistical significance using a Student t test. A probability value less than 0.05 denoted the presence of a statistically significant difference. All study protocols described in this report were approved by the Human and Animal Experiments Ethics Review Committees of Sankyo.

Results

Low HDL lipids Were Observed in the Plasma of Angptl3-Deficient Mice
Figure 1A shows the plasma lipid concentrations in wild-type KK mice (n=5) and KK/Snk mice (n=5). Plasma Angptl3 was not detected in KK/Snk mice (29±4.9 mg/dL versus not detected, P<0.001, Figure 1A). The levels of plasma HDL cholesterol and HDL-PL were significantly lower in KK/Snk mice than in KK mice (41±4.1 versus 79±3.9 mg/dL, P<0.001, 105±13.7 versus 233±13.3 mg/dL, P<0.001,
ANGPTL3 Increased Plasma HDL Lipids in Angptl3-Deficient Mice

Next, we treated congenic C57BL/6J Angptl3<sup>hypl</sup> mice with adenosine expressing lacZ or human ANGPTL3. Plasma HDL cholesterol concentrations increased from day 4 (48±1.8 versus 32±1.3 mg/dL, *P*<0.001) and doubled on day 10 (69±3.0 versus 33±2.6 mg/dL, *P*<0.001) after treatment with adenosine producing ANGPTL3, compared with the control (Figure 2A). Plasma HDL–PL levels were also increased from day 4 and doubled on day 7 (399±5.6 versus 216±23 mg/dL, *P*<0.001) by the ANGPTL3 adenosine, compared with the control (Figure 2A). We also analyzed lipoprotein profiles of the pooled plasma collected from adenosine-treated congenic C57BL/6J Angptl3<sup>hypl</sup> mice on day 14 after adenosine injection, with high-resolution HPLC. Cholesterol and PL concentrations increased mainly in the HDL fraction of the mice treated with ANGPTL3 adenosine, compared with the control (Figure 2B). On the other hand, ANGPTL3 adenosine increased only the VLDL fraction of TG (Figure 2B), a finding consistent with our previous reports. These results suggest that ANGPTL3 does not only influence VLDL hydrolysis but also homeostasis of the HDL metabolism.

ANGPTL3 Inhibited EL Activity In Vitro

Next, we investigated whether EL might be a novel target of ANGPTL3, accounting for the association between ANGPTL3 and HDL levels in plasma. Both in vitro assays, using phosphatidylcholine (Figure 3A) and human HDL particles (Figure 3B) as substrates, revealed that recombinant ANGPTL3 protein markedly inhibited the activity of EL in a dose-dependent manner. HDL particles did not inhibit phospholipase activities of EL by themselves (data not shown). To determine the domain of ANGPTL3 responsible for inactivation of EL, we produced truncated and/or mutated ANGPTL3 proteins, as shown in Figure 3C. The N-terminal coiled-coiled region of ANGPTL3 (ANGPTL3-CC) protein suppressed EL activity in a manner similar to that by full-length ANGPTL3 protein (Figure 3C). This inhibitory effect was completely abolished when the region of the heparin-binding site was mutated (Figure 3C), suggesting that the putative heparin-binding site in the N-terminal region is important for ANGPTL3-induced suppression of EL activity.

Heparin-Releasable Phospholipase Activity Was Elevated in Angptl3-Deficient Mice

EL is responsible for the bulk of heparin-releasable phospholipase activity in mice. To investigate whether Angptl3-deficiency leads to the elevation of EL activity in blood vessels, we measured the enzymatic activities of phospho-

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**Figure 1.** HDL cholesterol, HDL–PL, and triglyceride concentrations in Angptl3-deficient mice. A, Plasma levels of Angptl3, HDL cholesterol (HDL chol), HDL–PL, and triglyceride were measured in male wild-type KK (white bars, *n* = 5) and Angptl3-deficient KK/Snk (gray bars, *n* = 3) and Angptl3-knockout mice (KO, black bars, *n* = 4). Blood samples were taken under ad libitum conditions. Data are the means±SEM. **P*<0.001 vs wild-type KK mice; *P*<0.05 or versus KO; 29 mg/dL, *P*<0.01

**Table**: Comparison of plasma lipids and lipoprotein concentrations in wild-type C57BL/6J (WT, white bars, *n* = 5) and Angptl3-knockout mice (KO, black bars, *n* = 4). Blood samples were taken under ad libitum conditions. Data are the means±SEM. **P*<0.001 vs wild-type KK mice; *P*<0.05 or versus KO; 29 mg/dL, *P*<0.01

**Figure 2.** A, Plasma levels of Angptl3, HDL cholesterol (HDL chol), HDL–PL, and triglyceride were measured in male wild-type KK (white bars, *n* = 5) and Angptl3-knockout mice (KO, black bars, *n* = 4). Blood samples were taken under ad libitum conditions. Data are the means±SEM. **P*<0.001 vs wild-type KK mice; *P*<0.05 or versus KO; 29 mg/dL, *P*<0.01

**Figure 3.** ANGPTL3 inhibited EL activity in vitro in a dose-dependent manner. A, ANGPTL3 inhibited EL activity in a dose-dependent manner. B, ANGPTL3 inhibited EL activity in a dose-dependent manner. C, ANGPTL3 inhibited EL activity in a dose-dependent manner.
lipase in the plasma of C57BL/6J and Angptl3-deficient mice before and after a heparin injection. Plasma phospholipase activities were slightly elevated by heparin-injection in C57BL/6 mice (100±2 versus 108±3%, Figure 4). On the other hand, in Angptl3-knockout mice, the elevation of plasma phospholipase activities by heparin-injection was marked compared with C57BL/6J mice (103±4 versus 163±19%, Figure 4). These results indicate that circulating Angptl3 should contribute to the inhibition of the phospholipase activity of EL via the heparin-binding site in vivo.

Plasma HDL Cholesterol, HDL-PL, and ANGPTL3 Levels Correlated in Humans

To date, the physiological role of Angptl3 has only been assessed in rodents. To investigate the physiological and pathological roles of ANGPTL3 in humans, we constructed an ELISA system to measure ANGPTL3 concentration in human plasma. To construct the sandwich ELISA system, mouse monoclonal antibody (45B1) and rabbit polyclonal antibody (No.1) were raised against human ANGPTL3. These antibodies specifically detected recombinant human ANGPTL3 protein (please see supplemental materials). In the sandwich ELISA system, we used the 45B1 monoclonal antibody as the first antibody and detected ANGPTL3 with HRP-conjugated No.1 polyclonal antibody. We confirmed that this sandwich ELISA system specifically detect ANGPTL3 protein in human plasma by western blotting (please see supplemental materials). Using this sandwich ELISA system, we were able to generate a linear calibration curve using serial dilutions of the recombinant human ANGPTL3 protein (please see supplemental materials).

We found that the presence of other plasma proteins in the sample hindered quantitative analysis, especially when the plasma samples were directly subjected to ELISA. This was avoided by dilution of the plasma samples by more than 1/16. Neither ethylenediaminetetraacetic acid (EDTA) nor heparin, which are anticoagulants used for collecting plasma samples, had any effect on the above measurement (data not shown). The quantifiable range of the ANGPTL3 concentration in human plasma was 50 to 800 ng/mL using our system. Furthermore, ANGPTL3 concentrations of plasma samples were stable throughout five freeze-thaw cycles (data not shown).

To investigate the significance of ANGPTL3 in lipid homeostasis in humans, we analyzed plasma lipids and ANGPTL3 concentration of Japanese healthy volunteers [n=87, mean age, 33.6±8.4 years (±SD, range, 21 to 57), male/female: 45/42] (Figure 5). This study revealed that plasma ANGPTL3 concentrations (470±122 ng/mL) correlated strongly with plasma HDL cholesterol (62±14 mg/dL; r=0.500, P<0.001) and HDL-PL levels (92±25 mg/dL; r=0.286, P=0.007), but not with plasma total cholesterol (182±33 mg/dL; r=0.125, P=0.249) or TG level (77±54 mg/dL; r=0.169, P=0.117) or HDL-PL levels.

Discussion

A low level of plasma HDL has been recognized as an aspect of metabolic syndrome and is a crucial risk of cardiovascular events. Various factors have been demonstrated to influence plasma HDL-cholesterol level, including apoA1, ATP-binding cassette transporter (ABC) A1, lecithin:cholesterol acyltransferase (LCAT), PLTP, and cholesteryl ester transfer protein (CETP), etc.20 However, to date, pathophysiological regulation of the HDL level in plasma is not completely defined. Recently, lines of study have revealed that EL is a crucial factor in determining the plasma HDL level. Overexpression of EL in mice resulted in reduced plasma HDL levels, and EL knockout mice showed significant increase of HDL levels.5-7 In another study, injection of a neutralizing

Figure 2. Alterations of plasma lipid profiles by supplementation of ANGPTL3 via adenovirus in Angptl3-deficient mice. A, Angptl3-deficient congenic C57BL/6J Angptl3hypl/lacZ mice were treated with recombinant adenoviruses carrying β-galactosidase (Ad/lacZ, circles) or human ANGPTL3 (Ad/ANGPTL3, squares). On the indicated days after the viral injection, HDL cholesterol, HDL-PL, and triglyceride concentrations in plasma were measured as described in Methods. The indicated fractions are CM, chylomicron; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein. B, Plasma samples were collected on day 14 from mice injected with Ad/LacZ (dotted line) or Ad/ANGPTL3 (bold line). Pooled plasma samples from each group were subjected to highly-sensitive HPLC. Cholesterol, phospholipid, and triglyceride profiles in lipoprotein fractions were determined as described in Methods. The indicated fractions are CM, chylomicron; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.
antibody against EL increased plasma HDL in mice. Human genetic analysis showed that a single nucleotide polymorphism (584C/T) in EL cDNA, causing one amino acid replacement (T111I), was significantly associated with plasma HDL concentrations, but not with plasma total cholesterol or TG. However, the mechanism which regulates EL activity in vivo has not been clarified yet. In the present study, we showed that ANGPTL3, a hepatic secretory factor, significantly inhibited the activity of recombinant EL protein. We also found that the N-terminal domain, especially the putative heparin-binding region, is crucial for ANGPTL3-mediated suppression of EL activity. Furthermore, in Angptl3-deficiency, the phospholipase activity of post-heparin plasma was significantly elevated in vivo. Besides EL, LPL and HL also have phospholipase activity. However, McCoy et al previously demonstrated that the phospholipase activity of LPL and HL was extremely low compared with EL, whereas they had relatively high levels of triglyceride-lipase activity. Moreover, the loss of EL in the homozygous knockout mice resulted in a significant decrease in the post-heparin augmentation of phospholipase activity. These data clearly point to EL as a major contributor to heparin-releasable phospholipase activity in mice. Based on this previous evidence and our in vitro data, we assume that the elevation of heparin-releasable phospholipase activity in Angptl3-null mice should be explained by the lack of inhibitory effect of Angptl3 on EL. However, further analyses, eg, with double knockout mice of Angptl3 and EL, are still required to provide definitive evidence.

Our previous and current studies demonstrated that ANGPTL3 suppressed the activities of two lipases, LPL and EL, in vitro, and Angptl3-deficiency led to a significant reduction of plasma TG and HDL levels, and supplementation of ANGPTL3 restored them in vivo. Furthermore, in the current study, we constructed an ELISA system for measuring ANGPTL3 concentrations in human plasma.
and revealed that the plasma ANGPTL3 level significantly correlated to the plasma HDL cholesterol, suggesting that ANGPTL3 should play an essential role as a regulatory factor of plasma HDL-cholesterol levels in humans, but not of plasma TG. Our previous studies showed that in mice, either the administration of ANGPTL3 protein or an injection of ANGPTL3-adenovirus promptly elevated the plasma TG level, but the elevated TG level started to decrease shortly afterward, in spite of the high level of ANGPTL3 protein or an injection of ANGPTL3-adenovirus.14,23 In a recent study, McCoy MG, Sun GS, Marchadier D, Maugeais C, Glick JM, Rader DJ. Characterization of the lipolytic activity of endothelial lipase. J Lipid Res. 2002;43:921–929.


Figure 5. Plasma lipids and ANGPTL3 levels in humans. Plasma concentrations of ANGPTL3, HDL cholesterol, HDL-PL, total (T-) cholesterol and triglyceride were determined under overnight fasting conditions in healthy Japanese subjects (n=87). The values of correlation and probabilities are shown in the figures of ANGPTL3 and HDL cholesterol, and HDL-PL.

In conclusion, ANGPTL3 may be involved in the regulation of plasma HDL cholesterol levels through the inhibition of EL activity. Our findings provide new insight into understanding the regulation of EL activity and HDL metabolism via angptl3. Further epidemiological studies will provide more information for understanding the complicated HDL metabolism in humans.

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Disclosures

None.

References


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Supplemental Methods

For the phospholipase activity assay, phosphatidylcholine (PC) emulsion was prepared by combining 1, 2 di [1-14C] oleyl-L-3-phophatidylcholine (Amersham Pharmacia Biotech, Piscataway, NJ) and triolein, with final concentrations of PC and TG in the reaction mixture of 1.14 μM and 106 μM, respectively. The mixture was evaporated under nitrogen. The dried lipid was reconstituted with 29 mM Tris, pH 8.0, 0.29% bovine serum albumin (BSA), 3.1% glycerol by sonication for 15 sec repeated five times. Reactions were performed in a mixture prepared with PC emulsion (100 μl), conditioned media (50 μl) and samples (50 μl). The reaction mixtures were incubated at 37°C for 120 min, and the reaction was terminated by the addition of 0.1 M K2CO3 borate buffer, pH 10.5 (1 ml) and extracted with methanol:chloroform: hexane solution (141:125:100; 3.2 ml). A sample (0.5 ml) of the upper hexane layer was collected and quantitated in a scintillation counter. Phospholipase activities were also determined using HDL particles as substrates, instead of a phosphatidylcholine (PC) emulsion.
Supplemental Figure I

(A) 0.1 and 1 (or 0.01 and 0.1) µg of recombinant human ANGPTL3 protein and 1.0 (or 0.1) µg of BSA were subjected to SDS-PAGE and western blots were conducted with 45B1 mouse monoclonal antibody or No.1 rabbit polyclonal antibody, both of which were used for the sandwich ELISA, as described in ‘Methods’. (B) The plasma protein bound to the ELISA plate fixed with 45B1 mouse monoclonal antibody was subjected to western blotting with HRP-conjugated No.1 rabbit polyclonal antibody. Arrow indicates ANGPTL3 protein. (C) A sandwich ELISA system for human ANGPTL3 was constructed using the above antibodies and the calibration curve was determined. Recombinant human ANGPTL3 protein at concentrations of 3.13, 6.26, 12.5, 25 and 50 ng/ml was subjected to the sandwich ELISA system and absorbance was measured at 450 nm.