A decreased expression of angiopoietin-like 3 is protective against atherosclerosis in apoE-deficient mice

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Abstract **KK/Snk mice (previously KK/San) possessing a recessive mutation (*hypl*) of the angiopoietin-like 3 (*Angptl3*) gene homozygously exhibit a marked reduction of VLDL due to the decreased *Angptl3* expression. Recently, we proposed that Angptl3 is a new class of lipid metabolism modulator regulating VLDL triglyceride (TG) levels through the inhibition of lipoprotein lipase (LPL) activity. In this study, to elucidate the role of Angptl3 in atherogenesis, we investigated the effects of *hypl* mutation against hyperlipidemia and atherosclerosis in apolipoprotein E knockout (apoEKO) mice. ApoEKO mice with *hypl* mutation (apoEKO-*hypl*) exhibited a significant reduction of VLDL TG, VLDL cholesterol, and plasma apoB levels compared with apoEKO mice. Hepatic VLDL TG secretion was comparable between both apoE-deficient mice. Turnover studies revealed that the clearance of both [^{3}H]TG-labeled and ^{125}I-labeled VLDL was significantly enhanced in apoEKO-*hypl* mice. Postprandial plasma TG levels also decreased in apoEKO-*hypl* mice. Both LPL and hepatic lipase activities in the postheparin plasma increased significantly in apoEKO-*hypl* mice, explaining the enhanced lipid metabolism. Furthermore, apoEKO-*hypl* mice developed 3-fold smaller atherogenic lesions in the aortic sinus compared with apoEKO mice. Taken together, the reduction of *Angptl3* expression is protective against hyperlipidemia and atherosclerosis, even in the absence of apoE, owing to the enhanced catabolism and clearance of TG-rich lipoproteins.**—Ando, Y., T. Shimizugawa, S. Takeshita, M. Ono, M. Shimamura, R. Koishi, and H. Furukawa. **A decreased expression of angiopoietin-like 3 is protective against atherosclerosis in apoE-deficient mice.** ***J. Lipid Res.*** **2003.** 44: **1216–1223.**

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The accrued evidence that lipid-lowering therapy limits the progression of atherosclerosis and reduces the events of coronary artery diseases is overwhelming (1, 2). The focus has been on the reduction of LDL cholesterol (3, 4). Recent studies have also pointed out the importance of reducing triglyceride (TG)-rich lipoproteins such as chylomicrons, VLDL, and their remnants, and of raising HDL cholesterol (5, 6). In addition, postprandial hypertriglyceridemia is mentioned as an independent risk factor for atherogenesis (7, 8).

In a colony of KK mice with mild obesity, hyperlipidemia, and diabetes, we found mutant mice (KK/Snk, previously KK/San) that were characterized by a significant decrease in plasma lipid levels, mainly due to the reduction of TG-rich lipoproteins, despite their obesity and diabetes. Genetic studies for the mutation, named *hypolipidemia* (*hypl*), in KK/Snk mice identified a 4 bp nucleotide insertion in exon 6 of a gene encoding angiopoietin-like 3 (*Angptl3*), which causes a premature stop codon after a frameshift (9). Angptl3 is a secretory protein of 70 kDa expressed predominantly in the liver, and has a signal sequence, coiled-coil domain, and fibrinogen-like domain similar to those of other angiopoietin families (10, 11). In KK/Snk mice with the homozygous *hypl* mutation, *Angptl3* expression was markedly decreased, probably due to the instability of mutant mRNA, resulting in a hypolipidemic trait (9). In contrast, adenovirus-mediated overexpression of *Angptl3* or intravenous injection of the purified protein elicited a marked elevation in circulating plasma lipid levels (9). We also investigated the regulatory mechanism of Angptl3 on the metabolism of TG-rich lipoproteins (12). VLDL turnover studies revealed that KK/Snk mice exhibited enhanced VLDL TG clearance compared with wild-type KK mice. Moreover, addition of recombinant human ANGPTL3 protein directly inhibited lipoprotein lipase (LPL) and hepatic lipase (HL) activities in in vitro studies

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Abbreviations: Angptl3, angiopoietin-like 3; apoEKO, homozygous for apoE gene knockout allele; apoEKO-*hypl*, homozygous for both apoE gene knockout and *hypl* allele; HL, hepatic lipase; *hypl*, a recessive mutation in *Angptl3* gene causing hypolipidemia.

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(12). Taken together, we consider that Angptl3 is a new class of metabolic modulator affecting lipid homeostasis.

Over the past several years, significant advances have been made in our understanding of new, alternative mechanisms by which LPL and HL modulate lipoprotein metabolism and the development of atherosclerosis (13, 14). Studies using transgenic and knockout animal models have shown that plasma LPL and HL are involved in the susceptibility to atherosclerosis in addition to regulating plasma lipid levels (15–18). In our previous in vitro study, it was also predicted that the *hypl* mutation, which markedly reduces *Angptl3* expression, would increase the plasma LPL and HL activities (12). Thus, in the present study, to elucidate the role of Angptl3 in atherogenesis, we investigated the effects of the *hypl* mutation on hyperlipidemia and atherosclerosis, which are developed due to the accumulation of TG-rich lipoproteins in the circulation in apolipoprotein (apo) E-deficient mice (19). The *hypl* mutation enhanced lipolysis of TG-rich lipoproteins and their clearance in the liver, and resulted in a marked reduction of plasma lipid and apoB levels in apoE-deficient mice. Both LPL and HL activities in the postheparin plasma were increased significantly by the *hypl* mutation, explaining such an enhanced lipid metabolism. Atherogenic lesions in the aortic valves observed in the absence of apoE were also significantly decreased in mice carrying the *hypl* mutation. These findings revealed that a reduction in *Angptl3* expression has protective effects against hyperlipidemia and atherosclerosis even in the absence of apoE. Therefore, it was considered that Angptl3 plays an important role in atherogenesis and that Angptl3 might be a useful target in the development of new treatments for atherosclerosis.

MATERIALS AND METHODS

Mice

Hypolipidemic KK/Snk mice from Nagoya University were bred in our laboratory. ApoE knockout (apoEKO) mice (B6;129-*Apoe*^{tm1Unc}) were obtained from Jackson Laboratory. C57BL/6J mice were obtained from Hamamatsu University School of Medicine. KK/Snk mice were backcrossed to C57BL/6J mice for ten generations by selecting heterozygous mice that possessed KK-type alleles for the *D4Mit15* and *D4Mit219* loci mapped to 3.2 cM proximal and 1.5 cM distal of the *Angptl3* locus, respectively (9), and then crossbred with apoEKO mice. Wild-type (*Apoe*$^{+/+}$ and *Angptl3*$^{+/+}$), *hypl* (*Apoe*$^{+/+}$ and *Angptl3*$^{hypl/hypl}$), homozygous for the apoEKO allele (*Apoe*$^{-/-}$ and *Angptl3*$^{+/+}$), and homozygous for both the apoEKO and the *hypl* allele (apoEKO-*hypl*) (*Apoe*$^{-/-}$ and *Angptl3*$^{hypl/hypl}$) mice were selected by genotyping with PCR using specific primers. For the *Apoe* locus, two sets of primers for the apoE gene (sense: 5′-TCCCAAGTCACACAAGAACTGAC-3′, antisense: 5′-CATCCAGAAGGCTAAAGAAGGCA-3′, GenBank accession number D00466) and the neomycin-resistant gene (Neo1285: 5′-AGGATCTCGTCGTGACCCATGGCGA-3′, Neo1485: 5′-GAGCGGCGATACCGTAAAGCACGAGG-3′) (20) were used. For the *Angptl3* locus, a set of primers distinguishing the wild-type from the *hypl* allele (sense: 5′-GGCTAAATAGTAAAACCC-TGGCG-3′, antisense: 5′-GTGCTTGCTGTCTTTCCAGTCTT-3′) was used. Of these four groups of mice used in this study, 75% and 25% of the genetic background was derived from C57BL/6 and 129P2 strain, respectively. All mice were housed under a controlled temperature (23 ± 1°C) with free access to water and mouse chow (CMF; Oriental Yeast), and male littermates were used at 6 to 31 weeks of age for this study. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Sankyo Co., Ltd.

Real-time PCR quantification of RNA

Total RNA was extracted from the liver using Trizol Reagent (Invitrogen) and subjected to reverse transcription and amplification according to the protocol supplied with the TaqMan Gold RT-PCR kit (Perkin-Elmer) for *Angptl3* and *36B4* gene. Primers and probes were designed as follows: *Angptl3* primers (sense: 5′-ACAT-GTGGCTGAGATTGCTGG-3′, antisense: 5′-CCTTTGCTCTGT-GATTCCATGTAG-3′), *Angptl3* probe (5′-CCTCCCAGAGCACA-CAGACCTGATGTTT-3′), *36B4* primers (sense: 5′-GCTCCAAG-CAGATGCAGCA-3′, antisense: 5′-CCGGATGTGAGGCAGCAG-3′), and *36B4* probe (5′-CAAGAACACCATGATGCGCAAGGC-5′). The amount of *Angptl3* mRNA was corrected by dividing the amount of *36B4* mRNA in each sample.

Plasma lipid and lipoprotein analysis

Plasma lipids were measured enzymatically using assay kits [Wako Pure Chemical Industries for TG, total cholesterol, and nonesterified fatty acids (NEFAs); Azwell, Inc. for phospholipids]. To determine the plasma lipoprotein distribution, 50 μl of pooled plasma was analyzed by fast protein liquid chromatography on a Superose 6 PC 3.2/30 column (SMART system; Amersham Biosciences), and eluted at a constant flow rate of 50 μl/min with PBS (pH 7.4, containing 1 mM EDTA). Fractions of 25 μl were collected and assayed for total cholesterol and TG levels as described above.

Immunoblot analysis

Plasma samples (1 μl per lane) were separated on 2–15% gradient gels (Daiichi Pure Chemicals), and the proteins were transferred onto nitrocellulose membranes (Bio-Rad). The membranes were incubated with goat anti-mouse apoB antibody (Santa Cruz Biotechnology). Horseradish peroxidase-labeled anti-goat immunoglobulin G (Chemicon) was used as a secondary antibody, and apoB bands were detected by enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences). The intensity of the bands was estimated by an imaging analyzer.

Postprandial TG response

Mice were fasted for 16 h. After taking a basal blood sample by tail bleeding at t = 0, animals received an intragastric load of 400 μl of olive oil. Additional blood samples were drawn at 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h after the oral administration of olive oil. Plasma TG levels were measured at the time points as described above.

In vivo hepatic VLDL TG production

Mice were fasted for 16 h, and injected intravenously via the tail vein with Triton WR1339 (400 mg/kg body weight) using 20% (w/v) Triton solution in 0.9% NaCl. Blood samples were drawn from the tail vein at 0 min, 30 min, 60 min, and 120 min after the Triton injection and analyzed for TG as described above.

In vivo turnover studies using [^{3}H]TG-labeled VLDL

In vivo [^{3}H]TG-labeled VLDL turnover studies were based on a previously described method (21). [^{3}H]palmitic acid (Amersham Biosciences) in toluene was evaporated under nitrogen gas and redissolved in 0.9% NaCl containing 2 mg/ml BSA to a final

concentration of 1 mCi/ml. The apoEKO mice were injected intravenously via the tail vein with 100 μCi of the prepared [^{3}H]palmitate and bled from the abdominal aorta 25 min after injection. Radiolabeled VLDL to be analyzed in the clearance studies was isolated from the plasma of 20 mice by ultracentrifugation ($d < 1.006$ g/ml). To study the in vivo clearance of labeled VLDL TG, apoEKO, and apoEKO-*hypl*, mice were injected intravenously with 150,000 dpm of [^{3}H]TG-labeled VLDL. The disappearance rate of the radiolabeled VLDL was determined in 70 μl blood samples of mice drawn at the indicated time points after the injection. Total plasma radioactivity was used to represent VLDL TG radioactivity.

Labeling and removal of ^{125}I-labeled VLDL in vivo

Blood was collected from 19 apoEKO mice. Plasma samples were pooled, and VLDL ($d < 1.006$ g/ml) was obtained by ultracentrifugation. VLDL was labeled with ^{125}I by the ICl method (22). The specific radioactivity of ^{125}I-VLDL was ~86.8 cpm/ng of protein. After iodination, the VLDL samples were dialyzed extensively against a buffer containing 0.15 M NaCl and 0.3 mM EDTA (pH 7.4). ^{125}I-labeled VLDL (10 μg of tracer in 200 μl of 0.9% NaCl containing 2 mg/ml of BSA) was injected into the tail vein of the apoEKO and apoEKO-*hypl* mice. Blood samples of 70 μl were collected from the retro-orbital plexus at the indicated time points after the injection. The plasma content of ^{125}I-labeled apoB was determined by measuring the ^{125}I content in the pellet after propan-2-ol precipitation (23, 24).

Assay of LPL and HL enzyme activities

Postheparin plasma was prepared from blood taken 10 min after intravenous injection of heparin at a dose of 100 U/kg body weight into male mice fasted for 5 h. LPL and HL activities in postheparin plasma were determined on 5 μl of plasma. LPL activity assays were based on the method of Nilsson-Ehle and Schotz (25). The assays were carried out in a total volume of 0.2 ml with 0.1 ml of assay substrate and 0.1 ml of enzyme source. The assay substrate solution contained 2 mM glycerol-tri [9,10 (n)-^{3}H]oleate, 189 ng/ml L-α-phosphatidylcholine, 14 mg/ml BSA, 140 mM Tris-HCl (pH 8.0), 15% glycerol, and 10% FBS. The mixture was incubated at 37°C for 120 min and the enzyme reaction was terminated by the addition of 1 ml of 0.1 M potassium carbonate-borate buffer (pH 10.5) and 3.25 ml of methanol-chloroform-hexane, 1.41:1.25:1 (v/v/v). The mixture was vortexed vigorously for 15 s and centrifuged at 3,000 *g* for 15 min. Then, radioactivity in 1 ml of the supernatant was counted using a scintillation counter. HL assay was performed in the same manner as the LPL assay except that the NaCl concentration used was 1 M. LPL activity was calculated by the subtraction of HL activity from the lipase activity in the absence of 1 M NaCl. LPL and HL activities were expressed as micromoles of FFA/h/ml.

Pathological analysis

The cross-sectional lesion area was evaluated according to a modified method of Paigen et al. (26). In brief, the heart, including aorta, was perfused with saline containing 4% formalin, and fixed for more than 48 h in the same solution. The basal half of the hearts was embedded in paraffin, and 5 μm thick serial sections were obtained from the aortic sinus. Ten sections, sliced 50 μm apart, from each mouse were subjected to Elastica Masson staining, and the sum of the stained lesion areas was calculated using the IPAP-WIN system (Sumika Technoservice Corporation, Japan).

Statistics

Student's *t*-test was used to compare mean values between the wild-type and *hypl* mice and between the apoEKO and apoEKO-*hypl* mice.

RESULTS

Plasma lipid levels and lipoprotein profiles

Expression levels of *Angptl3* mRNA in the liver of the *hypl* and apoEKO-*hypl* mice were markedly lower than those in the wild-type and apoEKO mice, respectively (**Fig. 1A**, **B**). Plasma lipid levels in fasted *hypl* mice in the presence and absence of apoE are summarized in **Table 1**. The levels of all lipids in the *hypl* and apoEKO-*hypl* mice were significantly lower than those in the wild-type and apoEKO mice, respectively. In particular, in the apoEKO-*hypl* mice, the TG levels were markedly reduced, and the levels of TG and NEFA were comparable to those in the wild-type mice. The reduction in the plasma TG levels was primarily due to the scarcity of VLDL-sized particles with and without apoE (**Fig. 2A**, **B**). Plasma cholesterol was mainly found in HDL-sized particles with apoE, and the peak of HDL cholesterol in the *hypl* mice was reduced to 68.7% compared with that in the wild-type mice (Fig. 2A). In the absence of apoE, VLDL- and IDL/LDL-sized particles contained the largest amounts of plasma cholesterol, and the peak was reduced to 39.4% compared with that in the apoEKO mice (Fig. 2B). We also investigated the difference of apoB-100/apoB-48 composition in the lipoproteins of the apoEKO and apoEKO-*hypl* mice. Contents of apoB-100 and apoB-48 in the apoEKO-*hypl* mice were decreased by 53.6% and 36.7%, respectively, compared with those in the apoEKO mice (**Fig. 3**). These results indicate that the *hypl* mutation of the *Angptl3* gene resulted in a reduction of apoB-containing lipoproteins in the absence of apoE.

In vivo VLDL metabolism

To determine whether the *hypl* mutation affects the production of TG by the liver, we injected Triton WR1339 into mice and monitored the secretion of endogenous VLDL TG over time. As shown in **Fig. 4B**, the VLDL TG secretion rate was comparable between the apoEKO and apoEKO-*hypl* mice, although a 25% decrease was observed in the *hypl* mice compared with the wild-type mice (Fig.

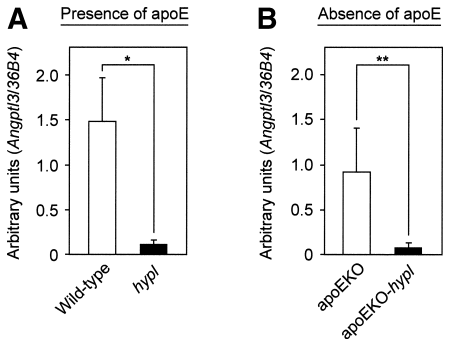

Fig. 1. Angiopoietin-like 3 (*Angptl3*) expression in the liver of mice with a recessive mutation in *Angptl3* gene causing hypolipidemia (*hypl*) in the presence (A) and absence (B) of apolipoprotein E (apoE). TaqMan analyses were performed in the liver of 24-week-old males (n = 4) fasted for 16 h. The amount of *Angptl3* mRNA was corrected by dividing it with that of *36B4* mRNA in each sample. Values are depicted as mean ± SD. $^*P < 0.05$, $^{**}P < 0.01$.

TABLE 1. Plasma lipid levels in *hypl* mutant mice in the presence and absence of apolipoprotein E

	Presence of ApoE		Absence of ApoE	
	Wild-type (n = 8)	*hypl* (n = 9)	apoEKO (n = 9)	apoEKO-*hypl* (n = 10)
Triglyceride (mg/dl)	160 ± 43.1	85 ± 18.7[a]	660.8 ± 216.5	132.3 ± 34.4[b]
Total cholesterol (mg/dl)	95 ± 15	58.3 ± 19.7[a]	1,308.8 ± 357.1	558.6 ± 100.6[b]
NEFA (mEq/l)	1.537 ± 0.167	1.147 ± 0.182[a]	2.549 ± 0.603	1.265 ± 0.138[b]
Phospholipid (mg/dl)	179.6 ± 19.4	134.3 ± 29.9[a]	720.3 ± 204.5	308.4 ± 48[b]
Body weight (g)	30.1 ± 1.9	29.3 ± 2.3	29 ± 2.6	28.8 ± 1.4

ApoEKO, homozygous for apoE gene knockout allele; apoEKO-*hypl*, homozygous for both apoE gene knockout and *hypl* allele; *hypl*, a recessive mutation in *Angptl3* gene causing hypolipidemia; NEFA, nonesterified fatty acids. Data were measured using plasma samples of 24- to 25-week-old male mice fasted for 16 h. Values are depicted as mean ± SD.

[a] $P < 0.01$, indicating the difference between the wild-type and *hypl* mice.

[b] $P < 0.001$, indicating the difference between apoEKO and apoEKO-*hypl* mice.

4A); however, there were no changes in weight or pathology of the liver between the wild-type and *hypl* mice or between the apoEKO and apoEKO-*hypl* mice (unpublished observations). Therefore, these results indicate that the *hypl* mutation has little effect on hepatic VLDL TG production, particularly in the absence of apoE.

Next, to investigate whether the plasma TG decrease in the apoEKO-*hypl* mice is due to enhanced TG clearance, the apoEKO and apoEKO-*hypl* mice were injected with [^{3}H]TG-labeled VLDL. As shown in **Fig. 5A**, the ^{3}H-labeled TGs were more rapidly cleared from the circulation in the apoEKO-*hypl* mice. To determine the metabolic pathway of the lipoproteins containing apoB, we also performed a turnover study using ^{125}I-labeled VLDL as a marker for whole-particle clearance. As a result, the injected ^{125}I-labeled VLDL apoB disappeared significantly faster from the plasma in the apoEKO-*hypl* mice compared with the apoEKO mice (Fig. 5B). These data indicate that the reduction of plasma lipid levels by *hypl* mutation in the absence of apoE is due to an enhancement of TG hydrolysis and whole-particle clearance of VLDLs and their remnants.

Postprandial TG response

To investigate the postprandial response of plasma TG levels, mice received an intragastric load of olive oil. Plasma TG levels after an intragastric fat load in the apoEKO-*hypl* mice were significantly lower than those in the apoEKO mice (**Fig. 6B**), and showed a gradual increase similar to that seen in wild-type mice (Fig. 6A). By contrast, no such postprandial response was observed in the *hypl* mice (Fig. 6A). These results indicate that postprandial hypertriglyceridemia was prevented from developing by the *hypl* mutation even in the absence of apoE.

LPL and HL activities of postheparin plasma

To elucidate whether the enhanced lipid metabolism by the *hypl* mutation results from the increase of lipase activity, we determined LPL and HL activities of postheparin plasma in the apoEKO and apoEKO-*hypl* mice. As shown in **Fig. 7A** and **B**, activities of both LPL and HL increased significantly in the apoEKO-*hypl* mice compared with the apoEKO mice. These results suggest that the reduction of

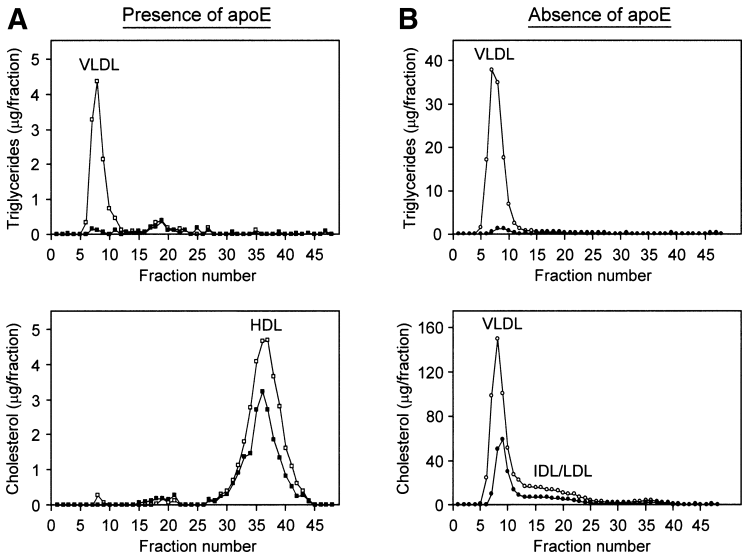

Fig. 2. Lipoprotein profiles in *hypl*-mutated mice in the presence and absence of apoE. A: Plasma of the wild-type (open squares) and *hypl* (closed squares) mice and (B) plasma of the homozygous mice for apoE gene knockout allele (apoEKO) (open circles) and homozygous mice for both apoE gene knockout and *hypl* allele (apoEKO-*hypl*) (closed circles) were obtained after 16 h of fasting. The pooled plasma samples (n = 5 per group) were fractionated by fast protein liquid chromatography. Total cholesterol and triglyceride (TG) contents in the individual fractions were determined enzymatically. The relative positions of VLDL, IDL/LDL, and HDL are indicated.

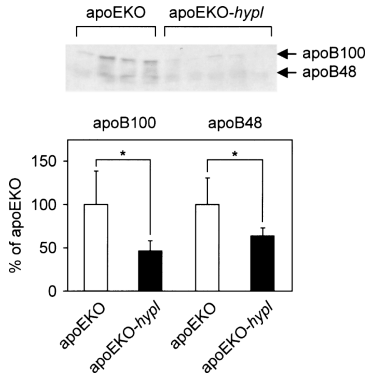

Fig. 3. Immunoblot analysis of plasma apoB in the apoEKO and apoEKO-*hypl* mice. Plasma was obtained from 17-week-old apoEKO (n = 4) and apoEKO-*hypl* (n = 5) mice fasted for 16 h. Plasma samples (1 μl per lane) were separated on 2–15% gradient gels and immunoblotted with the polyclonal antibody against mouse apoB. The intensity of the bands was estimated by an imaging analyzer, and the percentage to that of apoEKO was calculated. The values are depicted as means ± SD.

Angptl3 increases lipase activity in vivo, resulting in the enhancement of lipolysis and clearance of TG-rich lipoproteins in the *hypl* mutant mice.

Atherosclerotic lesions in aortic valves

To examine the effect of the *hypl* mutation on atherogenesis in apoE-deficient mice, a histological study was conducted. As shown in **Fig. 8A**, the apoEKO mice exhibited typical fatty streak lesions with foam cells and cholesterol crystals in the aortic sinus, whereas the apoEKO-*hypl* mice had only small fatty streak lesions. The cross-sectional lesion area of the apoEKO-*hypl* mice was significantly smaller than that of the apoEKO mice by 69% ($3.608 \pm 1.921 \times 10^5$ μm^2 vs. $11.559 \pm 6.204 \times 10^5$ μm^2, $P < 0.01$) (Fig. 8B). These results indicate that the *hypl* mutation has protective effects against atherosclerosis even when associated with apoE deficiency.

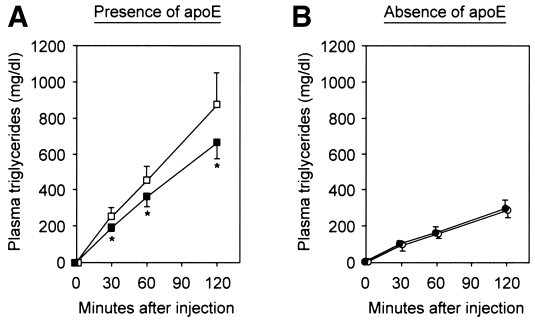

Fig. 4. Hepatic VLDL TG production in *hypl*-mutated mice in the presence and absence of apoE. Triton WR1339 (400 mg/kg body weight) was injected into 21- to 27-week-old wild-type (open squares) and *hypl* (closed squares) mice (A), and apoEKO (open circles) and apoEKO-*hypl* (closed circles) mice (B) after 16 h of fasting. Plasma TG levels were determined at the specified time points and corrected for the TG levels at the time of Triton injection (0 min). The values represent means ± SD of six mice per group. *$P <$ 0.05.

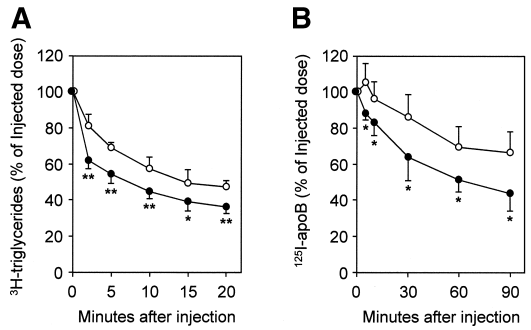

Fig. 5. In vivo metabolism of [^{3}H]TG and ^{125}I-labeled VLDL in apoEKO and apoEKO-*hypl* mice. [^{3}H]TG-labeled VLDL (d < 1.006) (A) or ^{125}I-labeled VLDL (d < 1.006) (B) was injected intravenously into 9-week-old apoEKO (open circles) and apoEKO-*hypl* (closed circles) mice. The radioactive decay of the respective labels was determined at the indicated time points. The values represent means ± SD of five mice per group. *$P <$ 0.05, **$P <$ 0.01.

DISCUSSION

In the present study, we clarified that the *hypl* mutation, which reduces *Angptl3* expression, enhanced lipolysis and the clearance of TG-rich lipoproteins, and prevented hypertriglyceridemia, hypercholesterolemia, and atherosclerosis, often observed in apoE-deficient mice, from developing.

We reported previously that adenovirus-mediated overexpression of *Angptl3* gene or intravenous injection of human ANGPTL3 protein increased the plasma TG levels in mice (9, 12). These findings suggest that the plasma TG level varies according to the plasma Angptl3 level. The *Angptl3* mRNA expression levels in hypertriglyceridemic apoEKO mice was 38% lower than that in wild-type mice, but was not statistically significant (Fig. 1A, B). The decreased *Angptl3* mRNA expression may be a reactive response, although the mechanism is not yet clear, because the TG levels in the apoEKO mice were four times higher due to the accumulation of TG-rich lipoproteins compared with those in the wild-type mice.

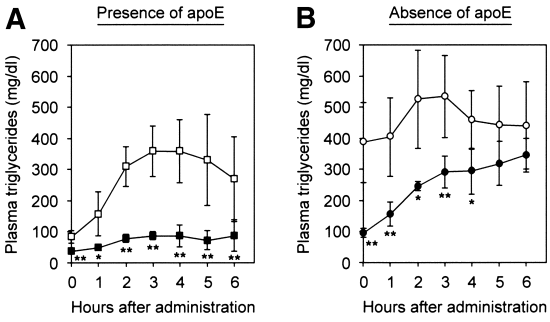

Fig. 6. Fat-loading test in *hypl*-mutated mice in the presence and absence of apoE. Wild-type (open squares) and *hypl* (closed squares) mice (A), and apoEKO (open circles) and apoEKO-*hypl* (closed circles) mice (B) were fasted for 16 h and received an intragastric fat load (400 μl of olive oil) at time point 0. Subsequently, plasma TG levels were determined at the indicated time points. The values are depicted as means ± SD of five mice (31-week-old) per group. *$P <$ 0.05, **$P <$ 0.01.

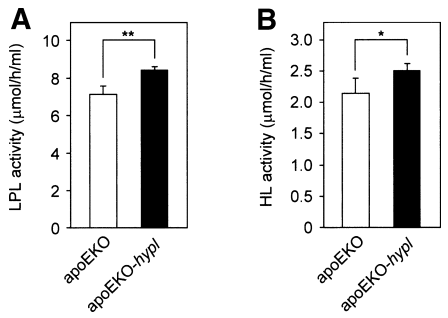

Fig. 7. Lipoprotein lipase (LPL) and hepatic lipase (HL) activities of postheparin plasma in apoEKO and apoEKO-*hypl* mice. LPL (A) and HL (B) activities were determined using postheparin plasma prepared from 6-week-old apoEKO and apoEKO-*hypl* mice. The values are depicted as means ± SD of four mice per group. $*P < 0.05$, $**P < 0.01$.

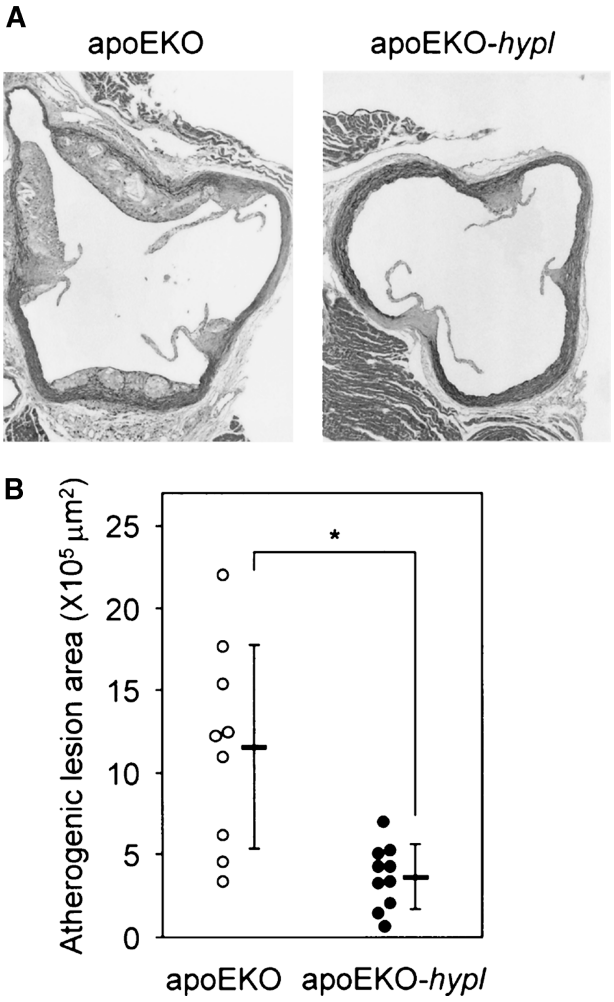

Fig. 8. Atherosclerotic lesions in the aortic valves of apoEKO and apoEKO-*hypl* mice. Cross-sections of the aortic valves in 24-week-old males fed a normal chow diet were prepared and stained with Elastica Masson staining. (A) Representative photographs obtained from apoEKO and apoEKO-*hypl* mice are shown. (B) The lesion areas in each of the apoEKO (n = 9) and apoEKO-*hypl* (n = 10) mice. Each spot is represented as the sum of 10 cross-sectional areas of the aortic valves, each separated by 50 μm, per mouse. The bold horizontal lines and error bars indicate means ± SD. $*P < 0.01$.

Plasma lipid levels are thought to be regulated by a balancing of their secretion and clearance. The rate of TG secretion from the liver was not affected by the *hypl* mutation in apoE-deficient mice, as shown by the high plasma TG levels (Fig. 4B), but was 25% lower in *hypl* mice with apoE compared with wild-type mice, exhibiting relatively low plasma TG levels (Fig. 4A). Our previous study also showed that the secretion rate in *hypl*-mutated KK/Snk mice was 15% lower or the same, compared with wild-type KK mice (12, 27), whose mean plasma TG level (273.9 ± 38.5 mg/dl) was between that of the apoEKO and wild-type mice used in this study (9). These data indicate that the *hypl* mutation, which induces a decrease in *Angptl3* expression, appears to reduce basal hepatic TG secretion, although the effect is not recognized in mice showing markedly high levels of plasma TG basally.

We analyzed the effect of Angptl3 on the metabolism of VLDL accumulated in the circulating blood in the absence of apoE. A metabolic experiment of VLDL containing ^{3}H-labeled TG revealed that VLDL TG was decreased significantly and more rapidly in the apoEKO-*hypl* mice compared with the apoEKO mice (Fig. 5A). We clarified that the activity of LPL increased in apoEKO-*hypl* mice, as shown in Fig. 7A. In contrast, in the previous report, we showed that human ANGPTL3 protein inhibited LPL activity dose-dependently in vitro (12). Yagyu et al. (15) also showed that an overexpression of LPL combined with apoE deficiency caused a decrease in plasma TG levels. Thus, these results suggest that the reduction in plasma TG levels in the apoEKO-*hypl* mice, as shown in Table 1 and Fig. 2B, is due to the increased TG hydrolytic activity of LPL, which was enhanced by the decrease in *Angptl3* expression.

On the other hand, a metabolic experiment of VLDL containing ^{125}I-labeled apoB revealed that apoB decreased more rapidly in the apoEKO-*hypl* mice compared with the apoEKO mice (Fig. 5B). This result indicates that VLDLs and their remnant particles are removed effectively from the blood in the apoEKO-*hypl* mice. Thus, we supposed that the effective removal of these lipoproteins caused the plasma cholesterol (Table 1, Fig. 2B) and apoB (Fig. 3) levels to decrease significantly in the apoEKO-*hypl* mice. It has been reported that VLDL hydrolyzed by LPL is rapidly cleared from the plasma, possibly through the receptor-mediated uptake by the liver (28, 29). Aviram et al. also showed that conformational changes of apoB-100 after the hydrolysis of VLDL by LPL may enhance the binding of VLDL to the LDL receptor (30). In addition, it is known that LPL enhances the binding of lipoproteins to the cell surface via heparan sulfate proteoglycans (31). Therefore, the low plasma apoB and cholesterol levels in apoEKO-*hypl* mice may be due to the enhanced uptake of the apoB-containing lipoproteins by LPL.

Some previous studies have shown that apoC-III effectively inhibits the LPL-mediated hydrolysis of VLDL TG and HL activity, and/or interferes with the binding of TG-rich lipoproteins to hepatic lipoprotein receptors (32–35). Maeda et al. (36) also reported that apoC-III-deficient mice exhibited hypotriglyceridemia and showed no sign of post-

prandial hypertriglyceridemia, a phenotype similar to that of *hypl*-mutated mice. These results suggest that Angptl3 may regulate VLDL metabolism through the inhibition of lipases in a manner similar to apoC-III. In addition, Jong et al. (37) reported that apoC-III deficiency accelerated the selective clearance of VLDL cholesteryl esters from the plasma and VLDL TG hydrolysis in the absence of apoE, although it did not enhance VLDL-apoB clearance. It is known that HL enhances the selective uptake of cholesteryl esters (18). We also demonstrated that the activity of HL increased in the *hypl*-mutated mice in this study (Fig. 7B), and previously reported that HL activity is slightly inhibited by Angptl3 (12). Therefore, the HL-mediated cholesteryl ester clearance may also contribute to the reduction of plasma cholesterol levels in the apoEKO-*hypl* mice.

The *hypl* mutation resulted in significantly inhibiting the increase of plasma TG levels in a fat-loading test of olive oil in both the presence and absence of apoE (Fig. 6A, B). This suggests that the metabolism of chylomicrons and their remnants, as well as VLDL, might be promoted by increased LPL activity due to the reduction of plasma Angptl3. Shimada et al. (38) also reported that an overexpression of LPL led to an enhanced catabolism and clearance of chylomicrons in their metabolic study using LPL transgenic mice. Therefore, the effective clearance of chylomicrons may be one of the reasons why the *hypl* mutation leads to the reduction of plasma lipids and apoB levels in this study.

We also found a significant decrease of NEFA in *hypl*-mutated mice in both the presence and absence of apoE (Table 1). In transgenic mice overexpressing LPL specifically in both the skeletal and cardiac muscles, Levak-Frank et al. (39) have demonstrated that LPL augments the uptake of NEFA in the muscles. Therefore, the increased LPL activity owing to a reduction in *Angptl3* expression may enhance uptake of NEFA in the peripheral tissues.

As shown in Fig. 8, the *hypl* mutation was found to have protective effects against atherosclerosis caused by apoE deficiency. It has been reported that the atherosclerosis formed by the apoE deficiency is suppressed due to a modification in some of the genes involved in lipid metabolism, causing, for example, an overexpression of LPL, a deficiency in the LPL inhibitor apoC-III, a deficiency of the phospholipid transfer protein involved in the secretion of lipoproteins containing apoB from the liver, or an overexpression of the LDL receptor (15, 37, 40, 41). These results also indicate that the irregular lipoprotein profile owing to apoE deficiency is improved by a reduction in TG-rich lipoproteins. The *hypl* mutation also enhanced the catabolism and clearance of VLDL in the absence of apoE (Fig. 5A, B) through the increase of lipase activity (Fig. 7A, B). Therefore, it is likely that the suppression of atherosclerosis in the apoEKO-*hypl* mice observed in this study is mainly due to this improvement of the lipoprotein profile. In addition, an overexpression of LPL leads to increased HDL cholesterol levels, which are antiatherogenic; however, HDL cholesterol was not increased in the *hypl* mice (Fig. 2A). Thus, it does not appear that a decreased *Angptl3* expression suppresses atherogenesis by means of increasing the levels of HDL. Further investigation is needed to elucidate whether Angptl3 has direct atherogenic effects on the arterial wall.

Increased circulating lipoproteins have been recognized as a major determinant of atherosclerosis and coronary artery disease. In the present study, we demonstrated that a decreased *Angptl3* expression suppressed the accumulation of TG-rich lipoproteins and atherosclerosis in apoE-deficient mice, which is an animal model for human type III hyperlipoproteinemia. Thus, Angptl3 may be a useful target in the treatment of atherosclerosis and other human diseases involving hyperlipidemia.

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