Original Article

Endothelial Lipase Exerts its Anti-Atherogenic Effect through Increased Catabolism of β-VLDLs

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Aim: Endothelial lipase (EL) plays an important role in lipoprotein metabolism. Our recent study showed that increased hepatic expression of EL attenuates diet-induced hypercholesterolemia, thus subsequently reducing atherosclerosis in transgenic (Tg) rabbits. However, it is yet to be determined whether increased EL activity itself per se is anti-atherogenic or whether the anti-atherogenic effect of EL is exclusively dependent on its lipid-lowering effect.

Methods: To determine the mechanisms underlying EL-mediated anti-atherogenic effect, we fed Tg and non-Tg rabbits diets containing different amounts of cholesterol to make their plasma cholesterol levels similarly high. Sixteen weeks later, we examined their lipoprotein profiles and compared their susceptibility to atherosclerosis.

Results: With Tg and non-Tg rabbits having hypercholesterolemia, the plasma lipids and lipoprotein profiles were observed to be similar, while pathological examinations revealed that lesion areas of both aortic and coronary atherosclerosis of Tg rabbits were not significantly different from non-Tg rabbits. Moreover, Tg rabbits exhibited faster clearance of Dil-labeled β-VLDLs than non-Tg rabbits.

Conclusion: The results of our study suggest that the enhancement of β-VLDL catabolism is the major mechanism for atheroprotective effects of EL in Tg rabbits.

Key words: Endothelial lipase, Hypercholesterolemia, VLDL metabolism, Atherosclerosis, Transgenic rabbits

Introduction

Endothelial lipase (EL) belongs to a triglyceride lipase family, which also includes lipoprotein lipase (LPL) and hepatic lipase (HL)¹. EL was originally cloned from endothelial cells², ³, but it is also expressed in the liver, lung, thyroid, and kidney⁴, ⁵. Although EL, LPL, and HL hydrolyze both triglycerides (TG) and phospholipids (PL) in the lipoproteins, they show different substrate selectivities. EL exhibits high phospholipase activity in high-density lipoprotein (HDL) but low triglyceride lipase activity, whereas LPL and HL mainly exercise triglyceride lipase activity⁶. Previous studies on mice², ⁷ and rabbits⁸ have
shown that overexpression of EL reduces HDL cholesterol (HDL-C) levels. On the contrary, plasma HDL-C levels are increased in mice with EL gene deficiency\(^7,9\) and EL-specific antibody injection\(^10\).

Furthermore, human studies showed that EL mass or activity was inversely associated with plasma HDL-C levels\(^11,12\). All these reports support the notion that EL is an important modulator of HDL metabolism. In addition to HDL, EL may be involved in the metabolism of apolipoprotein (apo)B-containing lipoproteins such as very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL). For example, EL was reported to hydrolyze VLDL-TG and -PL in vitro\(^6\). Overexpression of human EL reduces apoB-containing lipoproteins in apoE-deficient, LDL receptor-deficient, and human apoB transgenic (Tg) mice\(^13\) as well as cholesterol-fed Tg rabbits\(^8\). Besides, EL has been shown to exert non-catalytic function where it serves as a bridging molecule between plasma lipoproteins and vascular cells. Through this process, EL mediates the binding and uptake of HDLs and apoB-containing lipoproteins\(^14\).\(^16\). Although inhibition of EL was considered a new therapeutic strategy for the treatment and prevention of atherosclerosis through increasing plasma HDL-C\(^17\), it is still unclear what precise roles EL plays in the development of atherosclerosis. Studies using EL-deficient mice generated conflicting results regarding EL functions in atherosclerosis. Ishida et al.\(^18\) demonstrated that EL inactivation attenuated atherosclerosis in apoE knockout mice, whereas Ko et al.\(^19\) reported no effect on atherosclerosis in both apoE and LDL receptor knockout mice. In human studies, the relationship between EL and HDL-C and coronary artery disease (CAD) risk remains unclear. Loss-of-function EL variant exhibited higher plasma HDL-C levels but did not show any association with reduced risk for CAD\(^20\). On the other hand, plasma EL concentrations or activity was correlated with the risks for CAD\(^12,21\).

Recently, we generated Tg rabbits expressing the human EL in the liver and showed that overexpression of EL attenuated diet-induced hypercholesterolemia and inhibited atherosclerosis\(^8\). Tg rabbits exhibited lower plasma HDL-C levels as well as remnant lipoproteins than non-Tg rabbits. However, it is unknown whether increased EL activity itself is anti-atherogenic or whether the anti-atherogenic effect of EL is dependent upon its lipid-lowering effect in Tg rabbits. To address this issue, we performed the following study using Tg rabbits which developed similar “high” hypercholesterolemia to non-Tg rabbits. Our studies revealed that anti-atherogenic effect in EL Tg rabbits mainly depends on its enhanced remnant lipoprotein clearance.

### Methods

#### Rabbits

EL Tg rabbits along with sex- and age-matched littermates were used as reported previously\(^8\). In this experiment, male Tg and non-Tg rabbits aged 3 months were utilized. Rabbits were housed with a 12-hour light/12-hour dark cycle and fed a normal standard diet (CR-3M; CLEA Japan, Tokyo, Japan). The rabbits were allowed access to diets and water *ad libitum*. All animal experiments were conducted in accordance with the Animal Care Committee of the Universities of Yamanashi and Saga and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

#### Induction of Similar Hypercholesterolemia

In the previous study, we found that Tg rabbits showed lower levels of plasma lipids than non-Tg rabbits on both normal and cholesterol-rich diets\(^8\). In order to make Tg and non-Tg rabbits to develop similarly “high” hypercholesterolemia, we fed Tg rabbits with a diet containing relatively high contents of cholesterol (≈1%), whereas non-Tg rabbits were fed a diet containing relatively low cholesterol (0.3%). To make this goal possible, we measured their plasma levels of total cholesterol (TC) weekly and then adjusted accordingly\(^22\). Six weeks later, plasma TC levels of both Tg and non-Tg rabbits reached about 800 mg/dL (arbitrary atherogenic levels in cholesterol-fed rabbits) and remained at such level until they were sacrificed at 16 weeks. Blood was collected from an auricular artery of fasted rabbits for analysis of plasma lipids weekly. Plasma TC, TG, PL, HDL-C, and HDL-PL were measured using enzymatic colorimetry assay kits (Wako Pure Chemical Industries, Osaka, Japan).

#### Plasma Lipoprotein Analysis

Plasma lipoproteins were isolated from rabbits fed with either a normal standard diet or a cholesterol-rich diet at 8 and 16 weeks using sequential ultracentrifugation with a Beckman Optima MAX-TL ultracentrifuge with a TLA120.2 rotor (Beckman, Brea, CA), as described previously\(^23\). Isolated individual density fractions contained the following lipoproteins: the d<1.006 g/mL fraction contains VLDL or β-VLDL from cholesterol-fed rabbits; the d=1.02 g/mL fraction contains intermediate-density lipoprotein (IDL); the d=1.04 g/mL fraction contains large LDL; the d=1.06 g/mL fraction contains both LDL and HDL1; the d=1.08 and 1.10 g/mL fractions are those of HDL2; while the d=1.21 g/mL fraction contains HDL3. TC, TG, and PL contents in each fraction...
were measured using Wako enzymatic colorimetry assay kits.

Lipoprotein Lipase Activity

Lipoprotein lipase (LPL) activity in post-heparinized plasma was measured using the method described previously24, 25. Briefly, blood was withdrawn from rabbits after heparin injection, and LPL activity in the post-heparin plasma was analyzed through an automated colorimetric method using the natural long-chain fatty acid 2-diglyceride as a substrate (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan).

Pathological Analysis of Atherosclerosis

At the end of the experiment, all rabbits were sacrificed for evaluation of atherosclerosis. The aortic gross and microscopic lesions were analyzed as described previously26. Briefly, aortas were stained with Sudan IV to evaluate the gross lesion size using an automated colorimetric method using the natural long-chain fatty acid 2-diglyceride as a substrate (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan).

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Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with SPSS software (IBM Co., Chicago, IL). T-test was used for comparison between two groups. A repeated measures ANOVA was used for comparing the time series of TC and HDL-C levels. P<0.05 was considered statistically significant.

Results

Analysis of Plasma Lipids and Lipoproteins

Tg rabbits on a normal standard diet showed significantly lower plasma lipids than non-Tg rabbits, including 69% ↓ of TC, 85% ↓ of HDL-C, 51% ↓ of TG, 63% ↓ of PL, and 65% ↓ of HDL-PL (Fig. 1A and Supplementary Table 1). When challenged with a 0.3% cholesterol diet for 1 week, both Tg and non-Tg rabbits showed elevated plasma levels of TC, but Tg rabbits exhibited less response to a cholesterol diet because their plasma TC levels reached to a half value of those of non-Tg rabbits while HDL-C remained at lower levels (Fig. 1A). These results prompted us to investigate whether increased hepatic expression of EL affects VLDL catabolism in the liver in addition to hydrolyzing HDL-PL. To test this hypothesis, we compared the VLDL clearance capacity of Tg rabbits with non-Tg rabbits after injecting Dil-labeled β-VLDLs isolated from cholesterol-fed non-Tg rabbits. As shown in Fig. 1B, Dil-β-VLDLs were cleared faster in Tg rabbits than those in non-Tg rabbits after injection at 60 min. The fractional catabolic rate was measured but did not reach a significant difference (1.73 ± 0.08 in non-Tg vs 1.80 ± 0.09 pools/hour in Tg). We envisioned that increased EL expression led to enhancement of apoB-containing particle catabolism in the liver, thereby reducing atherogenic lipoproteins in the plasma.

To examine whether EL has any direct effect on the development of diet-induced atherosclerosis inde-
measured at 8 and 16 weeks (Supplementary Table 1).

Analysis of lipoproteins revealed that on a normal standard diet, Tg rabbits exhibited a marked decrease of TC, TG, and PL levels in all lipoproteins (VLDL, IDL, and HDL2–3) except LDLs (d=1.06 g/mL) (Fig. 3A). In contrast, under similarly “high” cholesterol levels were maintained within a range of 600–1,200 mg/dL until 16 weeks (Fig. 2). Plasma fluorescent intensity was measured by a fluorescent microplate reader. Data are normalized to 2 min fluorescent intensity levels at 100%. Data are expressed as mean ± SEM (n=4). *P<0.05 vs non-Tg rabbits by t-test.

Fig. 1. Plasma lipids and β-VLDL clearance

(A) Plasma total cholesterol (TC), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C) levels of transgenic (Tg) and non-Tg rabbits fed a normal standard and cholesterol-rich diet for 1 week. Data are expressed as mean ± SEM (n=8–12). *P<0.05, ***P<0.001 vs non-Tg rabbits by t-test. (B) β-very-low-density lipoprotein (β-VLDL) clearance. Tg and non-Tg rabbits were intravenously injected with fluorescent-labeled β-VLDLs (DiI-β-VLDL). Blood samples were withdrawn at 2, 5, 15, 30, and 60 min after DiI-β-VLDL administration. Plasma fluorescent intensity was measured by a fluorescent microplate reader. Data are normalized to 2 min fluorescent intensity levels at 100%. Data are expressed as mean ± SEM (n=4). *P<0.05 vs non-Tg rabbits by t-test.

dependent upon its lipid-lowering functions, we fed Tg rabbits with an adjusted cholesterol diet to match their plasma TC levels with those of non-Tg rabbits. After this manipulation, average plasma levels of TC and HDL-C of both Tg and non-Tg rabbits became close after 2–3 weeks (Fig. 2). This similarly “high” cholesterol levels were maintained within a range of 600–1,200 mg/dL until 16 weeks (Fig. 2). Plasma levels of TC remained at the same degree at 16 weeks because the area under the curve (AUC) of TC levels was almost identical between Tg and non-Tg rabbits (AUC 13,676 ± 774 in Tg vs. 12,593 ± 1,414 in non-Tg, P>0.05). In addition, plasma TG, PL, and HDL-PL levels were similar between Tg and non-Tg rabbits measured at 8 and 16 weeks (Supplementary Table 1).
Fig. 2. Plasma total cholesterol and HDL-C levels of rabbits after feeding a cholesterol diet for 16 weeks
Plasma total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) levels of transgenic (Tg) and non-Tg rabbits fed with an adjusted cholesterol-rich diet for 16 weeks. Data are expressed as mean ± SEM (n=8–12). *P<0.05 vs non-Tg rabbits by two-way repeated measure ANOVA.

Fig. 3. Plasma lipoprotein profiles of rabbits on a chow diet and a cholesterol diet
Plasma lipoproteins were separated by sequential gradient ultracentrifugation from plasma of rabbits on a normal (A) and an adjusted cholesterol diet for 8 weeks (B). Total cholesterol (TC), triglycerides (TG), and phospholipids (PL) in lipoproteins were measured. Data are expressed as mean ± SEM (n=6). *P<0.05, **P<0.01, ***P<0.001 vs non-Tg rabbits by t-test.
suggesting that increased EL did not affect lesion size including its cellular components. We also analyzed coronary atherosclerosis but did not find any difference between Tg and non-Tg rabbits (Fig. 6).

Discussion

EL has been postulated to be involved in HDL metabolism through its phospholipase hydrolysis activity, yet its roles in apoB-containing lipoprotein metabolism and atherosclerosis have not been fully defined. Our previous study showed that on a 0.3% cholesterol diet for 16 weeks, Tg rabbits exhibited lower plasma levels of TC (48% decrease of AUC over non-Tg) and subsequently had 55% reduction of aortic atherosclerosis compared with non-Tg rabbits8). Although several mechanisms may be involved in EL-mediated anti-atherogenic functions, it is likely that EL may attenuate diet-induced hypercholesterolemia through enhanced hepatic catabolism of apoB-containing particle clearance.

Comparison of Aortic and Coronary Atherosclerosis

Because cholesterol diet feeding can completely abolish the lipid-lowering effect of EL, we were able to investigate whether EL can affect atherosclerosis without considering plasma lipid levels. Analysis of gross aortic lesion areas defined by Sudan IV staining revealed that there was no significant difference between Tg and non-Tg rabbits, although average aortic lesion areas of Tg rabbits were 21% less than those of non-Tg rabbits (n.s.) (Fig. 4). Histological examinations showed that the aortic lesions of both Tg and non-Tg rabbits were mainly composed of infiltrating macrophages with relatively less smooth muscle cells (Fig. 5). The intimal microscopic lesion sizes along with macrophages and smooth muscle cells were not significantly different between Tg and non-Tg rabbits, suggesting that increased EL did not affect lesion size including its cellular components. We also analyzed coronary atherosclerosis but did not find any difference between Tg and non-Tg rabbits (Fig. 6).

Fig. 4. Analysis of gross aortic lesions

Transgenic (Tg) and non-Tg rabbits were fed an adjusted cholesterol diet for 16 weeks, and then the aortic lesions were quantified. Representative pictures of aortas stained with Sudan IV are shown (left). The lesion area, defined by the sudanophilic area, was quantified (right). Each dot represents the lesion area of an individual animal (n=8–12). P>0.05 vs non-Tg rabbits by t-test.
Fig. 5. Microscopic analysis of aortic atherosclerosis

Representative micrographs of the aortic arch lesions from transgenic (Tg) and non-Tg rabbits are shown (top). Serial paraffin sections of the aortic arch were stained with hematoxylin-eosin (HE) and elastica van Gieson (EVG) or immunohistochemically stained with monoclonal antibodies against either macrophages or smooth muscle α-actin for smooth muscle cells (SMC). Intimal lesions on EVG stained sections and positively stained areas of macrophage and SMC were quantified (bottom). Data are expressed as mean ± SEM (n=8–12). P > 0.05 vs non-Tg rabbits by t-test.

Fig. 6. Analysis of coronary atherosclerosis

Representative micrographs of the coronary lesions stained with hematoxylin-eosin from transgenic (Tg) and non-Tg rabbits are shown (left). The lesion size, expressed as stenosis % of coronary arteries, was quantified (right). Data are expressed as mean ± SEM (n=7–11). P > 0.05 vs non-Tg rabbits by t-test.
in normal diet fed Tg and non-Tg rabbits in which hepatic LDL receptors along with other receptors are functional; however, it is currently unknown whether LDL receptor function is essentially required for EL-mediated clearance of \( \beta \)-VLDLs in the liver. To address this issue, we need to crossbreed Tg rabbits with WHHL rabbits in the future.

Since it is not known whether anti-atherogenic effect of EL is totally dependent upon its lipid-lowering effects, we conducted experiments using Tg rabbits fed with an adjusted cholesterol-rich diet to make them to similarly develop hypercholesterolemia with non-Tg rabbits. In this situation, plasma lipids, lipoprotein profiles, and apoB were almost identical between the two groups as EL lipid-lowering effect was totally overwhelmed by extra cholesterol feeding in Tg rabbits and it is possible to elucidate whether EL affects the development of aortic and coronary atherosclerosis without considering plasma atherogenic lipoproteins levels.

Analysis of both aortic and coronary atherosclerosis showed that there were no differences in the lesion size and lesional cellular components between Tg and non-Tg rabbits, suggesting that increased EL expression cannot inhibit the development of atherosclerosis under the similar hypercholesterolemia. In other words, EL does not directly influence arterial wall cells during atherogenesis. This result further strengthened the contention that anti-atherogenic functions of EL are totally attributed to its lipid-lowering effect, namely, enhanced hepatic catabolism of apoB-containing particles.\(^2\), \(^13\). In this aspect, EL exhibits significant phospholipase activity with less triglyceride lipase activity, which is different from other lipases such as LPL because LPL mainly hydrolyzes TG in VLDLs and chylomicrons. Our previous studies have shown that overexpression of LPL reduced plasma lipids and atherosclerosis.\(^3\). However, under similar hypercholesterolemia, LPL, resembling hepatic lipase, enhances the conversion of VLDL/IDL particles to small LDLs, which are more atherogenic.\(^22\). Unlike LPL, EL does not confer a prominent function on the conversion of VLDLs/IDLs to LDLs. In addition, EL overexpression did not interfere with LPL activity because there was no difference in LPL activity between Tg and non-Tg rabbits (Supplementary Fig. 3). Takiguchi \( et \) al. recently reported that hepatic EL overexpression in mice maintained reverse cholesterol efflux despite of hypoalphalipoproteinemia.\(^32\). In the current study, HDL-C levels were also similar between Tg and non-Tg rabbits, but it is unlikely that reverse cholesterol efflux mediated by EL plays any role in atherosclerosis under similar hypercholesterolemia.

In summary, increased EL expression enhances \( \beta \)-VLDL catabolism, thereby inhibiting cholesterol diet-induced hypercholesterolemia and atherosclerosis in Tg rabbits. Abolishment of EL lipid-lowering function eliminates anti-atherogenic effects of EL. We concluded that overexpression of EL in the liver protects against diet-induced atherosclerosis via enhanced \( \beta \)-VLDL clearance.

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**Conflict of Interest**

The authors declare that they have no conflict of interest.

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Supplementary Table 1. Plasma TG, PL, and HDL-PL levels

|                | non-Tg | Tg           |
|----------------|--------|--------------|
| TG (mg/dL)     |        |              |
| 0 W            | 51 ± 4 | 25 ± 2***    |
| 8 W            | 51 ± 4 | 46 ± 3       |
| 16 W           | 55 ± 5 | 65 ± 14      |
| PL (mg/dL)     |        |              |
| 0 W            | 79 ± 3 | 29 ± 3***    |
| 8 W            | 306 ± 22 | 292 ± 29   |
| 16 W           | 363 ± 27 | 413 ± 64   |
| HDL-PL (mg/dL) |        |              |
| 0 W            | 58.7 ± 2.4 | 20.5 ± 1.7*** |
| 8 W            | 40.9 ± 1.6 | 40.3 ± 1.5       |
| 16 W           | 42.5 ± 1.6 | 41.3 ± 1.6       |

TG, triglycerides; PL, phospholipids; HDL-PL, high-density lipoprotein phospholipids. Data are expressed as mean ± SEM (n=8-12). *** P<0.001 vs non-Tg rabbits by t-test.

Supplementary Fig. 1. Validation of DiI distribution in lipoproteins

WT rabbits fed a normal diet were intravenously injected with fluorescent labeled β-VLDLs (DiI-β-VLDL). Blood samples were withdrawn at 0, 2, 30, 60 and 120 min after DiI-β-VLDL administration. Plasma lipoproteins were isolated using sequential ultracentrifugation. Density fractions contained following lipoproteins: VLDL (d<1.006 g/mL), LDL (d=1.006-1.063 g/ml), and HDL (d=1.063-1.21 g/ml). (A) Fluorescent intensity and (B) total cholesterol (TC) were measured. Two rabbits were evaluated for the validation of DiI-β-VLDL stability and representative data are shown.
**Supplementary Fig. 2.** Plasma lipoprotein and apolipoprotein analysis of rabbits on a cholesterol diet for 16 week

Plasma lipoproteins were separated by sequential gradient ultracentrifugation. (A) Total cholesterol (TC), triglycerides (TG) and phospholipids (PL) in the lipoprotein fractions were measured. Data are expressed as mean ± SEM (n=6). *P*<0.05 vs non-Tg rabbits by *t*-test. (B) Apolipoprotein distribution in the lipoprotein fractions were analyzed by 4-20% SDS-PAGE stained with Coomassie Brilliant Blue. Representative gel images from each group are shown.

**Supplementary Fig. 3.** Analysis of lipoprotein lipase activity

Post-heparinized plasma from non-Tg and Tg fed a cholesterol diet for 16 weeks were collected and lipoprotein lipase (LPL) activity was measured using a commercial colorimetric assay kit (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan). Data are expressed as mean ± SEM (n=8-12).