Plasma Activity of Endothelial Lipase Impacts High-Density Lipoprotein Metabolism and Coronary Risk Factors in Humans

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**Aim:** Endothelial lipase (EL) is a determinant of plasma levels of high-density lipoprotein cholesterol (HDL-C). However, little is known about the impact of EL activity on plasma lipid profile. We aimed to establish a new method to evaluate EL-specific phospholipase activity in humans.

**Methods:** Plasma samples were obtained from 115 patients with coronary artery disease (CAD) and 154 patients without CAD. Plasma EL protein was immunoprecipitated using an anti-EL monoclonal antibody after plasma non-specific immunoglobulins were removed by incubation with ProteinA. The phospholipase activity of the immunoprecipitated samples was measured using a fluorogenic phospholipase substrate, Bis-BODIPY FL C₁₁-PC.

**Results:** The EL-specific phospholipase assay revealed that plasma EL activity was inversely correlated with HDL-C levels ($R = -0.3088, p < 0.0001$). In addition, the EL activity was associated with cigarette smoking. Furthermore, EL activity in CAD patients was significantly higher than that in non-CAD patients. Concomitantly, the HDL-C level in CAD patients were significantly lower than that in non-CAD patients.

**Conclusion:** We have established a method for human plasma EL-specific phospholipase activity by combination of EL immunoprecipitation and a fluorogenic phospholipid substrate. Plasma EL activity was associated with not only plasma HDL-C levels but also the risks for CAD.


**Key words:** Endothelial lipase, High-density lipoprotein, Cholesterol, Coronary artery disease, Phospholipase

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**Introduction**

Low plasma levels of high-density lipoprotein cholesterol (HDL-C) are associated with the risk of coronary artery disease (CAD)¹. This relationship is independent of the effects of therapy in lowering the low-density lipoprotein cholesterol (LDL-C) level¹⁻³. Therefore, raising the HDL-C level has emerged as a key strategy for reducing the residual CAD risk in individuals optimally treated for elevated LDL-C³.

Endothelial lipase (EL) is a member of the triglyceride lipase family that exhibits a substantial phospholipase A₁ activity⁴⁻⁶. EL shows high substrate specificity to HDL and hydrolyzes phospholipids on HDL particles⁷. As a result, EL promotes the catabolism and remodeling of HDL particles and has a major influence on HDL metabolism, both in humans and mice⁵⁻¹⁰. Moreover, the EL concentrations are increased in patients with metabolic syndrome and inflammation and are associated with the development of coronary atherosclerosis⁹⁻¹². On the other hand,
statins increase the HDL-C levels partly by reducing the EL mass via the inhibition of RhoA). Because the inhibition of EL results in an increase in HDL particles with anti-inflammatory properties, EL is considered to be an attractive molecular target in HDL-C-raising pharmacological therapy.

Cell culture and animal studies suggest that the alteration of the EL expression is proportionally correlated with that of the EL activity. However, previous studies have also reported that the EL activity is regulated by a variety of factors. Moreover, it has been postulated that endogenous EL inhibitor(s) exist in human plasma. Based on this line of evidence, more detailed investigations are required regarding the measurement of EL-specific enzymatic activities in human plasma. There are multiple enzymes with lipase activity in the plasma, and it is difficult to discriminate the enzymatic activity of EL from that of other lipases. In the present study, we therefore aimed to establish a method to measure the EL-specific phospholipase activity in the plasma and investigate the correlations between the plasma EL activity and lipid profiles in human subjects. We herein documented associations between the EL activity and the plasma HDL-C level as well as various cardiovascular risk factors.

**Materials and Methods**

**Preparation of the Plasma Samples**

A total of 269 Japanese patients with cardiovascular diseases (191 men, age range: 22-88 (mean 64 ± 13 years) admitted to Kobe University Hospital between April 2008 and August 2009 were eligible for this study. Among them, 115 patients with coronary lesions exhibiting ≥ 75% angiographically narrowing of the coronary luminal diameter who underwent coronary intervention within the past six months were categorized as CAD patients. In addition, 154 patients with arrhythmias, valvular disease, non-ischemic cardiomyopathy, pulmonary hypertension or non-ischemic heart failure were categorized as non-CAD patients. The patient characteristics are shown in Table 1. Hypertension was diagnosed in patients with a systolic blood pressure of >140 mmHg or a diastolic blood pressure of >90 mmHg and in those treated with antihypertensive drugs. Diabetes mellitus was diagnosed in patients with a fasting serum glucose level of >126 mg/dL or a hemoglobin A1c value of >6.5% (NGSP), according to the clinical guidelines of the Japan Diabetes Society. A diagnosis of diabetes was also recorded in patients treated with antidiabetic drugs. Dyslipidemia was diagnosed in patients with a high serum LDL-C concentration, according to the Japan Atherosclerosis Society Guidelines for the Prevention of Atherosclerotic Cardiovascular Diseases. A diagnosis of dyslipidemia was also recorded in patients treated with antihyperlipidemic drugs. Patients with renal failure (i.e. a serum creatinine level of >2.0 mg/dL), cancer, active inflammatory disease (a C-reactive protein level of >1.0 mg/dL) or emergent admission were excluded. All patients provided their written informed consent, and the clinical study was approved by the Institutional Review Board of Kobe University Graduate School of Medicine. The investigation conformed to the principles outlined in the Declaration of Helsinki.

Blood was obtained after an overnight fast without the administration of heparin. The plasma levels of total cholesterol (Tcho), triglycerides (TG), HDL-C, glucose and hemoglobin A1c were measured using a standard assay at the Clinical Laboratory of Kobe University Hospital. The LDL-C level was calculated using the Friedewald formula. The homeostasis model assessment insulin resistance index (HOMA-IR) was calculated as fasting plasma glucose×immunoreactive insulin/405, after excluding patients with a fasting plasma glucose level of >126 mg/dL and/or those treated for diabetes. The plasma levels of interleukin-6 and adiponectin were measured using a latex particle-enhanced turbidimetric immunoassay and a chemiluminescent enzyme immunoassay, respectively, at SRL, Inc. (Hachioji, Tokyo, Japan).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Non-CAD (n = 154)</th>
<th>CAD (n = 115)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, n (%)</td>
<td>99 (64.3)</td>
<td>91 (79.1)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>60.8 ± 14.3</td>
<td>67.6 ± 9.8</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.5 ± 3.6</td>
<td>24.6 ± 2.9</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>61 (39.6)</td>
<td>89 (77.0) *</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>25 (16.2)</td>
<td>53 (46.0) *</td>
</tr>
<tr>
<td>Dyslipidemia, n (%)</td>
<td>56 (36.3)</td>
<td>88 (76.5) *</td>
</tr>
<tr>
<td>Current alcohol consumption, n (%)</td>
<td>55 (35.7)</td>
<td>51 (44.3)</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoked, n (%)</td>
<td>64 (41.5)</td>
<td>39 (33.9) *</td>
</tr>
<tr>
<td>Former smoker, n (%)</td>
<td>31 (20.1)</td>
<td>54 (47.0) *</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>48 (31.1)</td>
<td>22 (19.1) *</td>
</tr>
<tr>
<td>Statin</td>
<td>24 (15.6)</td>
<td>71 (61.7) *</td>
</tr>
<tr>
<td>Fibrate</td>
<td>1 (0.6)</td>
<td>2 (1.7)</td>
</tr>
</tbody>
</table>

The values are expressed as the mean ± SD or frequencies (%). * p <0.05 vs. non-CAD. Former smokers had not smoked for ≥ 1 year. CAD, coronary artery disease.
Preparation of Recombinant Human EL Protein

Recombinant human EL protein (rhEL) was purified from COS7 cells that stably overexpress c-myc epitope tagged-hEL (hEL-COS7)⁴. The hEL-purified from COS7 cells that stably overexpressed rhEL was used as a working standard for the EL activity assay.

Plasma IgG Removal and Immunoprecipitation

To measure the EL-specific phospholipase activity, we immunoprecipitated EL proteins in the plasma with an anti-EL antibody and Protein A. However, because Protein A binds nonspecifically to the heavy chain domain of IgG, it was expected that Protein A may bind not only to the sample of EL-immunoprecipitation (EL-IP), but also native IgG in the plasma. Therefore, plasma native IgG was pulled-down using Protein A, prior to EL-IP. In the pilot experiment, we confirmed with SDS-PAGE that plasma IgG was completely removed by pretreatment with 80 μL of Protein A, when less than 20 μL of plasma was used (data not shown). Therefore, 20 μL of plasma was found to be optimal and chosen for the EL activity assay. Briefly, the 20-μL plasma samples were mixed with 2 μL of 10 mmol/L phenylmethanesulfonylfluoride and 80 μL of nProtein A Sepharose 4 Fast Flow (GE Healthcare, Sweden) that was washed three times with 1× IP buffer (20 mmol/L of Tris-HCl, pH 7.5, 50 mmol/L of NaCl). After one hour rotating at room temperature, 400 μL of 1× IP buffer was added, and the sample was incubated for four hours at 4°C rotating on a rotator. The samples were then centrifuged, and 300 μL of the supernatant was obtained as a conditioned plasma sample.

EL-IP was performed using a monoclonal IgG antibody against human full-length EL proteins raised in mice (clone 26A1, Immuno-Biological Laboratories, Fujioka, Gunma, Japan) that reacts with the amino terminus of EL proteins. 26A1 mouse IgG (10 μL, 1 μg) was crosslinked onto 40 μL of nProtein A and incubated overnight with the 300-μL conditioned plasma samples to immunoprecipitate EL. After several washes, the EL-IP samples were subjected to measurement of the EL activity.

EL Activity Measurement

The EL-IP samples (90 μL/well) were transferred to a 96-well plate (Nunc A/S, Roskilde, Denmark). The reaction was started by adding 10 μL/well of a fluorogenic crude phospholipase A substrate, BODIPY FL C₁₁-PC (B7701, Invitrogen, Carlsbad, CA) to a final working concentration of 0.02 mmol/L. After 10 minutes of pre-incubation at 37°C, the fluorescence intensity (FLI) was continuously monitored using the Fluoroskan Ascent FL (Thermo LabSystems, Cambridge, UK) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm for 21 minutes at 37°C. The data were analyzed and expressed as FLI (arbitrary unit)/min/mL. In each assay, a standard curve was constructed, and the EL activity was calculated and expressed as fluorescence units (see details in the Results section). The intra-assay CV and inter-assay CV for the phospholipase assay were 4.0% and 19.3%, respectively.

Statistical Analysis

The one-way ANOVA was used to compare continuous variables between groups, and the Chi-square test was used to compare categorical values between groups. Relationships between the EL activity and the levels of serum lipids, lipoproteins and other variables were examined using Pearson correlation coefficients. All statistical analyses were performed using the Stata 11.2 software package (Stata, Texas, USA). A value of P<0.05 was considered to be statistically significant.

Results

Validation of the EL Activity Assay

Fig. 1A shows the relationship between the level and activity of rhEL standard protein according to the FLI. The EL phospholipase activity was time- and dose-dependent; therefore, the EL activity was calculated as (FLI at 21 min - FLI at 1 min)/20 min and expressed as FLI/min. We created a standard curve using the protein dose (Fig. 1B) in each assay and defined the activity of 1 μg of rhEL standard protein as one Unit. The EL activity in the samples was calculated and expressed in Units according to the standard curve. When the activity of different volumes of the rhEL standard protein was assessed before and after IP, the EL activity exhibited a linear relationship with fluorescence consistently, both before and after IP (Fig. 1C), which indicates that all EL proteins were successfully pulled down by IP and the EL activity was not affected during the procedure.
Although a previous study reported that the plasma EL mass increases in response to heparin\textsuperscript{10}, no significant differences in the EL activity were observed between the pre- and post-heparin treatment plasma values (Fig. 1D). Therefore, in this study, the human plasma was collected without heparin treatment.

**Associations between the EL Activity and the Plasma Lipid Profiles and Coronary Risk Factors**

We measured the plasma EL activity in 269 patients with cardiovascular disease. As shown in Table 2, there were no significant associations between the EL activity and non-lipid parameters, such as age, BMI, waist circumference, systolic and diastolic blood pressure, fasting glucose, HOMA-IR, interleukin-6, and adiponectin.

**Table 2.** Associations between the EL activity and non-lipid cardiovascular risk factors

<table>
<thead>
<tr>
<th>Variable</th>
<th>R</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>−0.0252</td>
<td>0.6802</td>
</tr>
<tr>
<td>Body mass index</td>
<td>−0.0261</td>
<td>0.6966</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>−0.0190</td>
<td>0.7840</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>0.0066</td>
<td>0.9172</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>0.0382</td>
<td>0.5485</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>0.0463</td>
<td>0.4510</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>−0.0426</td>
<td>0.4884</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>0.0293</td>
<td>0.6332</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>0.0589</td>
<td>0.3374</td>
</tr>
</tbody>
</table>

HOMA-IR, homeostasis model assessment index-insulin resistance.
Plasma Endothelial Lipase Activity Assay

smokers, and the EL activity levels in the former smokers were higher than those observed in the current smokers. Interestingly, the plasma HDL-C levels in the non-smokers were significantly higher than those observed in the former or current smokers (Fig. 3A). Next, we compared the EL activity levels in the CAD- and non-CAD patients. As shown in Table 1, the CAD patients were older and had a higher prevalence of hypertension, diabetes, dyslipidemia and statin use than the non-CAD patients. Although the CAD patients included subjects with habitual cigarette smoking habits (i.e., former and current smokers), many were not current smokers (Table 1). Reflecting the associations between the EL activity and the risk factors, the plasma EL activity levels were modestly but significantly higher in the CAD patients than in the non-CAD patients (Fig. 3B). Moreover, the HDL-C levels in the CAD patients were 15% body mass index, waist circumference, blood pressure and the glucose, interleukin-6 and adiponectin levels. The associations between the plasma EL activity and lipid profiles are shown in Fig. 2. The plasma EL activity was inversely correlated with the HDL-C level (R = -0.3088, p < 0.0001); however, no relationships were observed with the Tcho (R = -0.0613, p = 0.3195), LDL-C (R = 0.0207, p = 0.7371) or TG levels (R = 0.0480, p = 0.4523).

**Fig. 2.** Plasma EL activity and lipid profiles

The pre-heparin plasma EL-specific activity levels were determined in 269 patients with cardiovascular disease. The relationships between the EL activity and the plasma levels of HDL-cholesterol (HDL-C), total-cholesterol (Tcho), LDL-cholesterol (LDL-C) and triglycerides (TG) are shown. There was an inverse relationship between the EL activity and the HDL-C level (R = -0.3088, P < 0.00001).

**Associations between the EL Activity and Coronary Risk Factors**

Because habitual cigarette smoking is known to be a cause of a low HDL-C level, we analyzed the relationship between the plasma EL activity and cigarette smoking. As shown in Fig. 3A, the EL activity levels in the patients who did not smoke were significantly lower than those observed in the former or current smokers, and the EL activity levels in the former smokers were higher than those observed in the current smokers. Interestingly, the plasma HDL-C levels in the non-smokers were significantly higher than those observed in the former or current smokers (Fig. 3A).
lower than those observed in the non-CAD patients (Fig. 3B). These findings indicate that the EL activity is correlated with the risk of CAD.

**Discussion**

Since the identification of EL in 1999, the role of the plasma EL activity in lipoprotein metabolism has remained incompletely understood. Recently, Miksztowicz and colleagues measured the HL phospholipase activity in the presence of 1 mol/L of NaCl to inhibit the EL activity and calculated the EL activity as the difference between the total- and HL-specific phospholipase activities. In addition, they reported that the EL activity regulates the plasma HDL-C concentration in patients undergoing hemodialysis. The present study successfully established a direct method for assessing the plasma human EL-specific phospholipase activity using a combination of IP and a fluorogenic phospholipid substrate. The principle of this assay has recently been reported and validated for the measurement of the murine plasma EL activity. In the present study, furthermore, we depleted native IgG using pretreatment of plasma samples with Protein A prior to EL-IP, as plasma is abundant in native IgG and may interfere with the IP process. The catalytic triad (Ser-His-Asp), which determines the enzyme activity of lipase, exists in the amino terminal portion of EL, as is the case with LPL and HL. Given that the phospholipase activity was preserved after EL-IP, however, the IP step did not interfere with the EL enzymatic activity.

Interestingly, the EL phospholipase activity was inversely correlated with the plasma HDL-C level (Fig. 2). In addition, the EL activity was positively associated with cigarette smoking (Fig. 3A). A high EL activity and low HDL-C level were observed not only in current smokers, but also in former smokers (Fig. 3A), for unknown reasons. We speculate that this may be because the former smoker group included more CAD patients than the current smoker group (Table 1). Furthermore, the EL activity was elevated in the patients with CAD (Fig. 3B). Because the majority of CAD patients were treated with statins, which inhibit the EL expression and/or phospholipase activity, the high EL activity levels observed in the CAD patients were considered to be rather significant. These findings indicate that the EL activity not only regulates the plasma HDL-C level, but is also associated with the risk of CAD. Although previous animal studies suggest that the EL expression in vivo affects the plasma concentrations of apoB-containing lipoproteins, the present study clearly demonstrated that the EL activity is not associated with the plasma concentrations of LDL-C or triglycerides.

Several studies have documented a significant inverse correlation between the plasma EL mass and the HDL-C level in humans. However, the plasma EL mass in these studies showed a large amount (100 times) of variation, in contrast to the small amount of variation observed in the HDL-C level (25-100 mg/dL). This discrepancy can be explained, at least in part, by the variation in the EL catalytic activity in the plasma, as a variety of factors, including
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human plasma EL-specific phospholipase activity using a combination of IP and a fluorogenic phospholipid substrate. The plasma EL activity was found to be inversely associated with the plasma HDL-C level. In addition, the plasma EL activity levels were elevated in the CAD patients and smokers. These findings indicate that the plasma EL activity impacts the plasma HDL-C level and risk of cardiovascular disease.

Note
In a recent article by Singaraja R.R. et al., the EL phospholipase activity assay described in the present paper was cited in an abstract.

Acknowledgements
None.

Notice of Grant Support
This work was supported by Grants-In-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, grants from the SENSHIN Medical Research Foundation and a VRI Research Grant from AstraZeneca.

Conflicts of Interest
No authors have any financial relationships with a biotechnology manufacturer, pharmaceutical company or other commercial entity with an interest in the subject matter or materials discussed in this manuscript.

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