Measurement of the phospholipase activity of endothelial lipase in mouse plasma

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Abstract Endothelial lipase (EL) is a major negative regulator of plasma HDL levels in mice, rabbits, and most probably, humans. Although this regulatory function is critically dependent on EL's hydrolysis of HDL phospholipids, as yet there is no phospholipase assay specific for EL in plasma. We developed such an assay for the mouse enzyme using a commercially available phospholipid-like fluorescent substrate in combination with an EL neutralizing antibody. The specificity of the assay was established using EL knockout mice and its utility demonstrated by detection of an increase in plasma EL phospholipase activity following exposure of wild-type mice to lipopolysaccharide. The assay revealed that murine pre-heparin plasma does not contain measurable EL activity, indicating that the hydrolysis of HDL phospholipids by EL in vivo likely occurs on the cell surface.—Basu, D., X. Lei, J. Josekutty, M. M. Hussain, and W. Jin. **Measurement of the phospholipase activity of endothelial lipase in mouse plasma. *J. Lipid Res.* 2013.** 54: **282–289.**

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Endothelial lipase (EL), which hydrolyzes HDL phospholipids and thus promotes the catabolism of HDL via scavenger receptor class B member 1 (1), is one of the major determinants of plasma HDL cholesterol (HDL-c) and apo A-I concentrations in mice (2–4) and rabbits (5). EL lowers HDL-c levels by hydrolyzing HDL-associated phospholipids (3). In humans, genome wide association studies have revealed that a common EL variant at the *LIPG* locus is significantly associated with plasma HDL-c levels as are several other EL gene polymorphisms (6–12). These observations indicate that EL also regulates HDL-c levels in humans. Furthermore, EL is upregulated in inflammatory states in humans, including atherosclerosis and metabolic syndrome (13, 14), and likely contributes to the low HDL-c levels seen in these conditions. Thus, EL is an attractive target for the development of inhibitors that would raise HDL-c levels.

In evaluating plasma EL function, measurement of EL enzymatic activity is more informative than quantification of EL concentration by immunoassay because the latter does not differentiate between active noncovalent homodimer and inactive monomer (15), active full-length enzyme and inactive proteolytically cleaved enzyme (16, 17), and the presence or absence of the endogenous plasma inhibitor of EL, angiopoietin-like protein-3 (17, 18).

Since the discovery of EL in 1999 (19, 20), substantial efforts have been devoted to developing an EL phospholipase A1 assay. Of those currently used, the nonradiolabeled assays (21–23) are suitable for screening inhibitors of EL in the test tube. However, there is no published data regarding their use for assaying EL in plasma. Radiometric assays using ^{3}H-trioleoyl glycerol or ^{14}C-labeled phosphatidylcholine as substrates in emulsions have been extensively used for determining plasma EL activity and studying EL biology (24). These assays, which require separation of the released labeled fatty acids, are time consuming and have a low throughput. Furthermore, they are not specific for EL due to the presence in plasma of HL and LPL. All three lipases belong to the triacylglycerol (TG) lipase family and can hydrolyze both TGs and phospholipids although with very different preferences for the two types of substrates (25, 26). For these reasons, there is a need for an easy-to-use assay specific for plasma EL activity. Of note, the major activity of purified EL in the test tube against substrates in emulsions is as a phospholipase A1 (PLA1) (23). This activity is much greater than its activity against the TGs in emulsions. Therefore, a phospholipase A1 assay for EL acting in plasma (i.e., not purified) will be more sensitive than a TG lipase assay.

Here, we describe development of a novel homogeneous PLA1 assay for plasma EL, which employs a commercially-available fluorogenic phospholipid analog, the PED-A1 substrate. The changes in both EL protein levels and PLA1 activity in plasma, with time after heparin injection, were

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Abbreviations: BODIPY-C5, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoic acid; EL, endothelial lipase; HDL-c, HDL cholesterol; KO, knockout; LPS, lipopolysaccharide; PLA1, phospholipase A1, TG, triacylglycerol.

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studied. In combination with use of an EL neutralizing antibody, the EL PLA1 activities of mouse postheparin plasma were compared under physiological conditions and in a lipopolysaccharide (LPS)-induced inflammatory state.

MATERIALS AND METHODS

Materials and reagents

The EnzChek Phospholipase A1 Assay Kit containing the PED-A1 substrate and the fluorescent standard 4,4-Difluoro-5,7-Dimethyl-4-Bora-3a,4a-Diaza-*s*-Indacene-3-Pentanoic Acid, abbreviated BODIPY-C5) were purchased from Invitrogen. The liposomes, which were prepared according to the kit's instructions, contained dioleoylphosphatidylcholine:dioleoylphosphatidylglycerol:PED-A1 in the ratio 5:5:1. The liposomes were stable for at least 3 months. Other reagents, including heparin (Cat. No. H-1027) and LPS (Cat. No. L4391), were obtained from Sigma-Aldrich, St. Louis, MO unless otherwise stated. Precast 4-20% polyacrylamide gels were obtained from Pierce (Rockford, IL). The anti-EL polyclonal and control antibodies were generated and IgG was purified using a protein A affinity column as previously reported (3).

PLA1 assay

A typical assay was carried out at 37°C for 30 min in a 96-well round bottom clear plate (Costar) with 0.5 μl of mouse plasma or indicated amounts of a stated enzyme and 16 μm PED-A1, which was either formulated in liposomes with the total assay volume adjusted to 100 μl with water or solubilized in DMSO to a total assay volume of 100 μl. The Spectramax M2 plate reader from MDS Analytical Technologies was used for fluorescent measurements. The software Softmax Pro 5 was used for data collection and analysis. The maximum excitation/emission wavelengths for PED-A1 were determined to be 485/538 nm with a 515 nm filter cutoff. The upper and lower detection limits for BODIPY-C5 using this assay and instrument are 2.4 μmole/well and 0.1 pmole/well, respectively.

EL PLA1 activity

PLA1 activity was measured using 0.5 μl of plasma incubated with control IgG antibody (final concentration 30 μg/ml) or anti-EL inhibitory antibody (final concentration 30 μg/ml) for 5 min on ice prior to the PLA1 assay. At this concentration, the neutralizing antibody showed saturation. The EL PLA1 activity was calculated as the difference between the two measurements.

TLC

Plasma samples (1 μl) or 1 unit of snake venom PLA1 enzyme were incubated with liposomes for 30 min at 37°C and lipids were extracted using a total volume of 750 μl of chloroform and methanol (2:1), dried down and dissolved in 50 μl of chloroform. Samples were run on a silica gel plate for 10 min with a solvent system comprising chloroform: methanol:20% ammonium hydroxide in the ratio 14:6:1. Fluorescent bands on the plate were detected using a DyNA Light™ UV transilluminator (302 nm, Fisher Scientific, Pittsburgh, PA).

Adenoviruses

A recombinant adenoviral vector expressing murine EL and a control adenovirus expressing green fluorescent protein (Ad-GFP) were prepared as described previously (3).

Animal experiments

Age- and gender-matched C57BL/6 mice were used for the following experiments. All procedures were conducted in conformity with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the State University of New York Downstate Medical Center. Adenovirus was administered intravenously (iv) at a dose of 10^{11} viral particles diluted in PBS on day 0 of the each experiment. The plasma collected 5 min after iv heparin administration on day 3 posttransduction with adenoviral vector expressing murine EL is referred to as "EL plasma" (see Results). The EL plasma had a stable EL activity for at least 1 year when stored at −70°C.

For murine EL overexpression studies, mice were prebled, heparin was injected iv at 300 units heparin/kg body weight and the mice were bled again at indicated intervals. For LPS studies, 20 μg (1 mg/kg of body weight) of LPS or PBS were injected per mouse intraperitoneally (ip) and postheparin plasma was obtained after 16 h.

Mouse plasma

Blood was drawn in heparinized microcapillary tubes from the retro-orbital plexus at indicated time points before and after iv or ip injection of 300 units heparin/kg body weight. Blood was centrifuged at 10,000 *g* for 10 min at 4°C and plasma was separated and used for the analysis and/or stored at −70°C. Pre- and postheparin plasma from EL knockout mice were kindly provided by Dr. Daniel J. Rader, University of Pennsylvania.

Western blotting

SDS-PAGE and immunoblotting analysis of murine EL were carried out as described previously (16). One microliter of mouse plasma was added to sample loading buffer and β-mercaptoethanol and held at 70°C for 10 min before electrophoresis. The anti-EL serum was used at 1:2500 dilution, the secondary HRP-conjugated goat anti-rabbit IgG (Jackson Immunoresearch) at 1:5000 dilution, and detection was carried out using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Data analysis

The relative fluorescence intensity of the unquenched BODIPY-C5 fatty acid standard was used to calculate the initial velocity of enzymatic reactions producing this molecule as nmole/min/ml plasma. The initial velocities were calculated based on the linear increase in product over the time in this PLA1 assay, usually 0–15 min at the 16 μM of PED-A1 substrate concentration. The rate of hydrolysis as determined from the continuous increase in fluorescence intensity was equal to the rate of separation of the *sn*-1 fatty acid product from the PED-A1 substrate. Rates were presented as the mean ± SD of three determinations. The results from two sets of conditions were analyzed by either paired or unpaired Student's *t*-tests; $P < 0.05$ was considered to be statistically significant.

RESULTS

Optimizing conditions for the use of the PED-A1 substrate in the assay of EL in plasma

A new fluorogenic phospholipid substrate, PED-A1 (Invitrogen), was recently reported for the measurement of the PLA1 activity of purified EL, or of EL in conditioned medium (23). The glycerophosphoethanolamine substrate has a BODIPY fluor added at the *sn*-1 position, an alkyl chain with an ether bond that is resistant to esterase activity at the *sn*-2 position and a quencher, Dabcyl, at the

sn-3 position (23). It is thus a PLA1-specific substrate, producing a continuous fluorescence increase as the modified fatty acid at the sn-1 position is separated from the quencher at sn-3 position (23). The published PED-A1 assay cannot measure EL activity in the presence of plasma (23). We hypothesized that proper presentation of the PED-A1substrate to the EL enzyme is required for determining EL activity in the mouse plasma.

Because the active dimer of EL is attached to the endothelial cell surface via reversible binding to heparan sulfate proteoglycans (27), a bolus of heparin administered intravenously was used to release EL into the plasma compartment; this method is routine for LPL and HL activity measurements (28). The 5 min postheparin plasma from murine EL-overexpressing mice (referred to as EL plasma) was collected and used for optimization of assay conditions. The presence of elevated full-length murine EL protein in the EL plasma was confirmed by Western blotting using an antibody specific for murine EL (**Fig. 1A**). In a TG assay (29), the EL plasma had higher activity than wild-type (WT) postheparin plasma (51 ± 0.5 versus 25 ± 0.3 μmole/min/ml plasma), confirming the presence of active EL in the EL plasma.

PED-A1 and its fluorescent hydrolysis product were separated by TLC and visualized using a UV transilluminator (Fig. 1B). The intact substrate is shown in lane 1. When hydrolyzed by a positive control enzyme, commercially available snake venom PLA1, most of the PED-A1 substrate was converted to a slower moving band (Lane 5), the location of which matches that of the *sn*-1 BODIPY fatty acid standard (lane 2). Postheparin EL plasma also acted on the substrate to generate a product at this location (lane 4). Thus, EL plasma contains a PLA1 activity, which hydrolyzes the PED-A1 substrate.

Because EL acts at the water/lipoprotein surface of HDL, we reasoned that presenting the PED-A1 substrate incorporated into liposomes would mimic the phospholipid presentation in HDL particles. The ability of EL in the EL plasma to hydrolyze PED-A1 was compared in two conditions: in the presence of DMSO, used in test tube assays by Darrow et al. (23), and in a liposome format. As shown in Fig. 1C, the EL plasma hydrolyzed PED-A1 with a greater initial velocity and a linear response that extended over a longer time in liposomes than in DMSO, indicating that PED-A1 is a better substrate presented in liposomes. PED-A1 formulated in liposomes was used in all subsequent assays.

To characterize the EL activity with the PED-A1 substrate, initial velocity was determined using a fixed amount of EL plasma (0.01 μl per well) and increasing amounts of PED-A1 (Fig. 1D). The rates were proportional to substrate concentration under the conditions used. No appreciable PLA1 activity was detected using the same amount (0.01 μl) of postheparin plasma from control WT mice, indicating that endogenous PLA1 activity is very low compared with that of overexpressed EL. The intra-assay coefficiency variation was 4.7%.

To determine the upper limit of the enzyme concentration range where the initial rate was proportional to enzyme concentration, initial velocity was determined using 16 μM PED-A1 and increasing amounts of the EL plasma (Fig. 1E). With this substrate concentration, increase in the initial velocity was linear to 0.04 μl of EL plasma per 100 μl assay solution (Fig. 1E).

An early report noted that the fluorescent phospholipid analog PED6 was a poor substrate for EL in the test tube (23). Consistent with this, EL plasma showed much less activity with this analog, whether or not it was presented in the liposome format (Fig. 1F). Thus, of the two substrates, PED-A1 is preferred for assaying mouse plasma EL enzymatic activity. It has the additional advantage that it does not allow complications from the hydrolytic activity of plasma PLA2 enzymes (30).

Use of the assay to study the fate of EL in mouse plasma after heparin administration

The same mouse EL overexpression system was used to study the fate of EL released into the bloodstream by heparin. There was no difference in PLA1 activity in the pre-heparin plasma samples from control mice and in mice where EL was overexpressed in the liver by adenoviral-mediated transduction (2.1 vs. 2.2 nmole/min/ml, $P > 0.05$). This was the case despite Western blotting detection of a doublet of 40 kDa bands from the cleaved N-terminal region of the EL protein in pre-heparin plasma of EL-transduced mice that is missing in mice transduced with GFP (**Fig. 2A**, compare lanes 1 and 2). Therefore, very little active EL is released into the plasma in this model in the absence of heparin and the N-terminal domain is not active in vivo.

Five minutes after the heparin injection, the EL activity in the plasma of EL-transduced mice increased to 4.7-fold the pre-heparin level. This activity then slowly decreased and was still above baseline level 30 min after injection (Fig. 2B). The amount of the full-length EL protein in these mice increased and decreased in parallel (Fig. 2A).

Ip heparin administration is much easier than iv injection in mice and has been used to release endothelial bound lipases (31). We therefore compared its effectiveness to that of iv injection. Plasma EL activity increased more slowly, to 2.9-fold the basal level, at 30 min after ip administration, and remained above baseline level at 60 min (Fig. 2D). As with the iv injections, plasma changes in EL full-length protein amounts followed changes in activity (Fig. 2C).

Of note, in both Fig. 2A and C, immediately after heparin administration, compared with pre-heparin samples, there were visible increased amounts of the closely-spaced N-terminal domain cleavage products of EL, indicating a rapid cleavage of the full-length EL once released by heparin into the plasma. This is in agreement with our previous observation that EL is cleaved in the extracellular compartment (16).

In summary, these data confirm that iv injection of heparin is more effective in releasing EL from the cell surface than ip injection and that plasma collected at 5 min after iv heparin injection contains the highest EL activity.

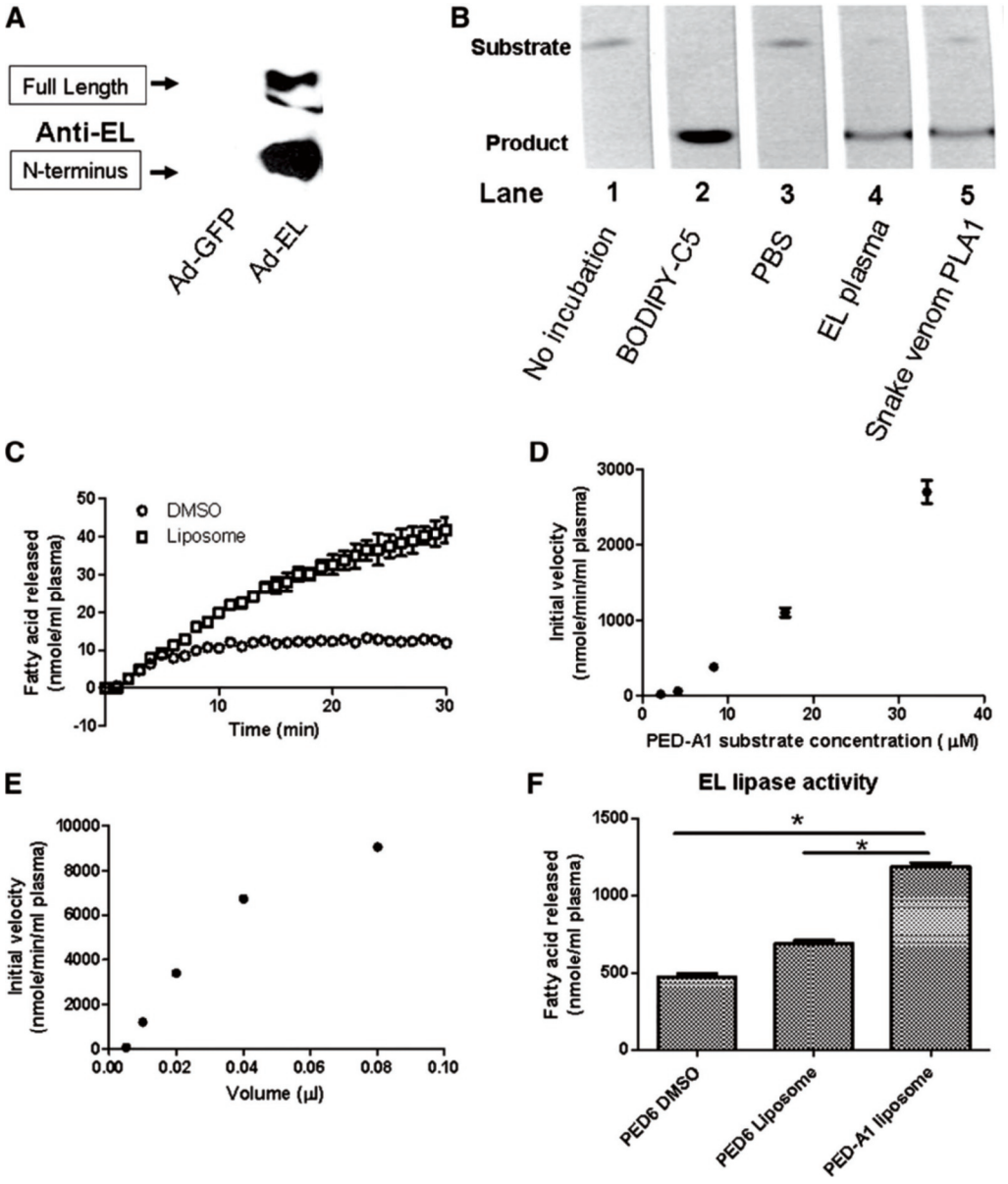

Fig. 1. Characterization of the PLA1 activity of EL in EL plasma with the fluorescent PED-A1 substrate: A: Immunoblot of mouse EL in EL plasma. B: A thin-layer chromatogram showing: lane 1, PED-A1 in liposomes; lane 2,the BODIPY-C5 standard; lane 3, PED-A1 in liposomes incubated with PBS as control for samples in lanes 4 and 5; lane 4, PED-A1 in liposomes hydrolyzed by post-heparin EL plasma; lane 5, PED-A1 in liposomes hydrolyzed by snake venom PLA1. C: Fluorescence increase with time was determined using a fixed amount of EL plasma (0.01 μl/well) with the substrate (16 μM) dissolved either directly in DMSO or presented in liposomes. The fluorescence at each point was converted to nmole fatty acids released/100 μl assay solution and expressed as the value that would be obtained if 1 μl of plasma was added to the assay. The error bars were too small to be seen. D: Initial rates of increase (initial velocity) obtained as in (C) using the liposome format were measured with increasing concentrations of the PED-A1 substrate. E: Velocities (nmole/min/100 μl assay solution) using the liposome format were obtained with increasing amounts of EL plasma. The error bars were too small to be seen. F: The phospholipase activity of EL in EL plasma with the fluorescent PED6 or PED-A1 substrate. Initial velocities were obtained using a fixed amount of EL plasma (0.01 μl/well) with the substrates (16 μM) dissolved either directly in DMSO or presented in liposomes. * $P < 0.05$ compared with PED-A1 liposome. In each figure each data point is the mean ± SD of triplicate determinations.

The endogenous EL activity in the postheparin plasma of WT mice

We carried out experiments in WT mice similar to those described above in EL-overexpressing mice to study total endogenous postheparin PLA1 activity (**Fig. 3A**, B). Significantly higher total PLA1 activity was observed in post-heparin relative to pre-heparin WT plasma after either intravenous or intraperitoneal heparin injection. The latter approach, using 30 min post-heparin plasma, yielded a smaller increase in PLA1 activity compared with

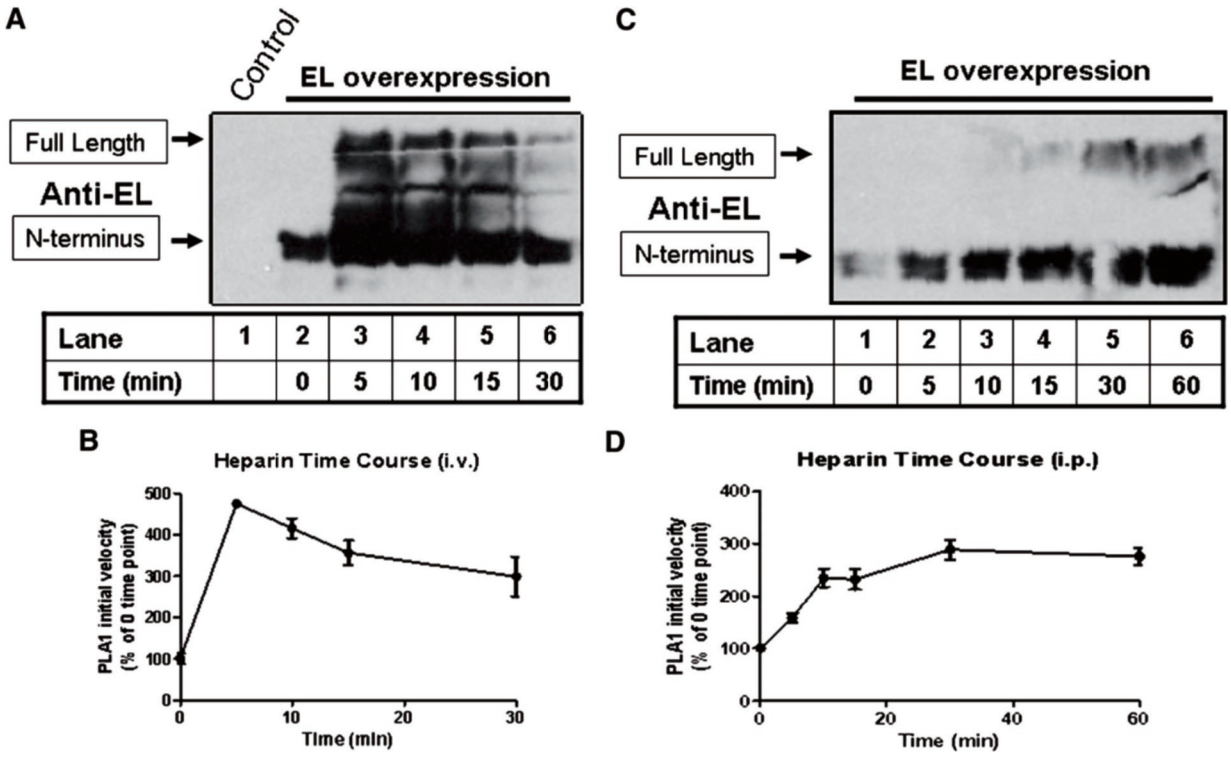

Fig. 2. Evaluation of EL activity and EL protein/fragments in plasma following heparin administration by different routes. A: Immunoblot of EL protein/fragments using the anti-EL antibody in plasma samples (1 μl/lane) taken at increasing times after iv administration of heparin (control pre-heparin plasma was obtained from mice transduced with the Ad-GFP virus). The EL-positive bands between the full-length protein and the N-terminus likely represent intermediates in EL degradation. B: PLA1 activity using the PED-A1 substrate in plasma samples (0.01 μl/well) collected at the indicated time points after injection of heparin intravenously. C: Immunoblot of EL protein/fragments in plasma samples (1 μl/lane) taken at increasing times after ip administration of heparin. D: PLA1 activity in plasma samples (0.01 μl/well) taken at the indicated times after ip injection of heparin. The PLA1 activity at 0 time point was 6.1 ± 0.26 nmole of fatty acid released/min/ml. Each data point is the mean ± SD of triplicate repeats on four animals.* $P < 0.05$.

iv injection using 5 min post-heparin plasma (Fig. 3A, B; 15% versus 30%). Thus, endogenous EL behaves similarly to ectopic EL in its response to these two methods of heparin administration. The intra-assay coefficiency variation was 4.0%.

Because active EL is released from the cell surface into the blood stream upon heparin treatment, we hypothesized that EL would be the major contributor to the difference in pre- and post-heparin total PLA1 activities that is apparent in Fig. 3A. To test this, the pre- and post-heparin plasma samples from EL knockout (KO) mice were collected and the total PLA1 activities measured. In support of this hypothesis, there was no significant difference in plasma PLA1 activity between the pre- and post-heparin plasma samples from EL KO mice (Fig. 3C).

Extending the assay for use in post-heparin plasma

To separate the EL PLA1 activity from the total PLA1 activity in a post-heparin plasma sample, an EL neutralizing antibody was added to post-heparin plasma prior to the assay to inhibit plasma EL activity (3). There was significant inhibition of post-heparin PLA1 activity using the neutralizing antibody compared with the activity using a control antibody (n = 5, $P < 0.05$, **Fig. 4A**), establishing the ability of the neutralizing antibody to inhibit EL phospholipase A1 activity in postheparin plasma samples. No EL PLA1 activity was detected in the pre-heparin plasma when the results with the two antibodies were compared (2.24 ± 0.11 vs. 2.28 ± 0.09 nmol/min/ml plasma), confirming that active endogenous EL is not present in the pre-heparin plasma. The antibody did not inhibit PLA1 activity in the post-heparin plasma samples from EL KO mice (Fig. 4B), validating the specificity of the anti-EL antibody.

Thus, measurement of the specific phospholipase activity of endogenous EL in mouse plasma following heparin treatment can be accomplished using a PLA1 assay with the substrate PED-A1 incorporated into liposomes and the assay carried out on a single post-heparin plasma sample in the presence and absence of the EL-neutralizing antibody.

LPS treatment and EL activity

Acute inflammation induced by LPS has been shown to increase EL mRNA and protein expression in various tissues in culture, including macrophages and endothelial cells from humans and mice (32, 33). We hypothesized that LPS treatment in vivo in mice would increase post-heparin plasma EL activity. We found, using the single post-heparin sample PLA1 assay with the neutralizing antibody, that overnight treatment of mice with LPS increased post-heparin EL activity about 2.0-fold (**Fig. 5**). This result supports a role for EL in the regulation of plasma HDL levels during the acute phase response (34).

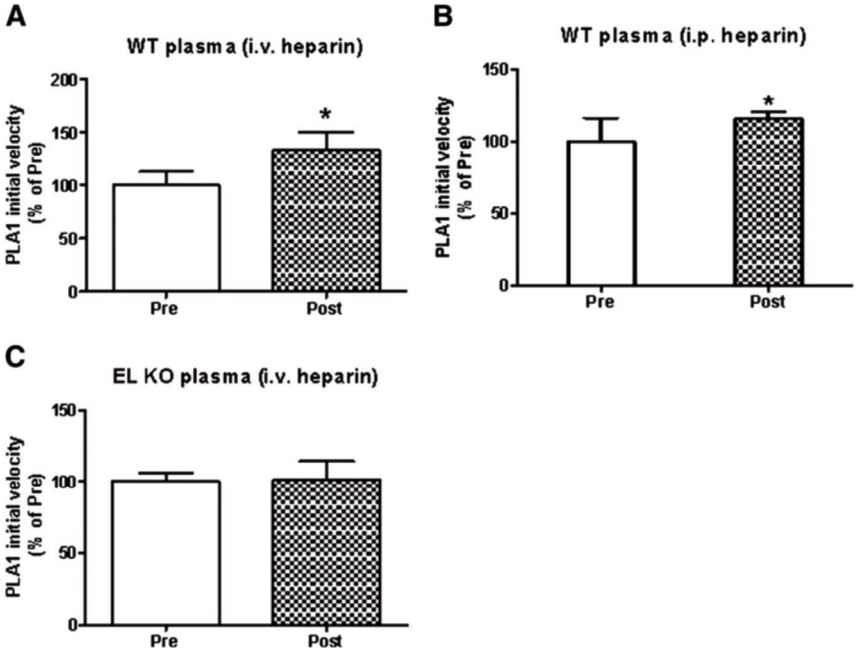

Fig. 3. Comparison of total PLA1 activity in pre and postheparin plasma in WT and EL KO mice. PLA1 activity was measured as described in Material and Methods. A: Plasma from WT mice was collected 5 min after intravenous injection of heparin. B: Plasma from WT mice was collected 30 min after intraperitoneal injection of heparin. C: Plasma of EL KO mice was collected 5 min after iv injection of heparin. Values are mean ± SD of determinations on five animals. * $P < 0.05$. N.S., not significant.

DISCUSSION

Using a newly described phospholipid-like fluorescent substrate in combination with an EL neutralizing antibody, we determined EL PLA1 activity in the post-heparin plasma of mice. The specificity of this approach was validated using post-heparin samples from EL KO mice. The ability of the assay to measure plasma EL activity was demonstrated in measurements on plasma from WT mice treated with LPS, known to induce EL expression in endothelial cells, macrophages, and a variety of tissues. In addition, using the pre- and post-heparin samples approach, we provided a dynamic view of the turnover of EL in plasma after heparin treatment.

We found that pre-heparin plasma does not contain measurable EL activity, a property also exhibited by LPL (28). The absence of pre-heparin plasma EL activity could be due to the relatively high affinity of EL for heparan sulfate proteoglycans, fast inactivation of any released enzyme by proteolytical cleavage, and/or inhibition of activity by the inhibitor angiopoietin-like protein-3. Because there is no active EL enzyme in the nonheparinized plasma, the hydrolysis of HDL phospholipids by EL in vivo likely occurs on the cell surface in a manner similar to the interaction between LPL and TG-rich particles. This local interaction of EL and HDL may affect local cellular cholesterol and fatty acid uptake as well as signal transduction through peroxisome proliferator-activated receptors (35).

In contrast to our previous report that EL does not significantly contribute to the TG lipase activity present in the post-heparin plasma (29), despite exhibiting a low level of TG lipase activity when the purified enzyme is assayed in the test tube (much lower than an equivalent amount of LPL), our data (Fig. 4) demonstrate that EL does contribute to the significant PLA1 activity present in the post-heparin plasma. This provides direct evidence that EL in vivo functions as a phospholipase. The result is consistent with the elevated levels of plasma phospholipids but not plasma TGs in EL KO mice compared with WT controls (2, 4). Thus, in combination with use of the TG lipase assay (29), we are able to differentiate among the enzymatic activities of HL, EL, and LPL in post-heparin plasma of mice.

As indicated above, the increase in PLA1 activity in post-heparin versus pre-heparin plasma is most likely due to the release of active EL from the cell surface (Fig. 4). However, we still do not know which enzyme(s) besides HL are responsible for the PLA1 activity in the preheparin samples. This "pre-existing" PLA1 activity could be altered when heparin is added. For example, ANGPTL3 is released from the cell surface into plasma by heparin (36), and it is an endogenous inhibitor of HL (37). Also, additional HL is released by heparin in mice (38). Therefore, plasma EL activity cannot be reliably measured by calculating the difference between post- and pre-heparin activities (data not shown). Instead, comparison of the PLA1 activities of a single post-heparin plasma sample, which is pretreated with control antibody or anti-EL antibody, avoiding interference by HL and possibly other factors, is the preferred method of assessing plasma EL activity.

We recognize that our current assay has some limitations. The performance of the assay could be improved by increasing the substrate concentration. The assay can be

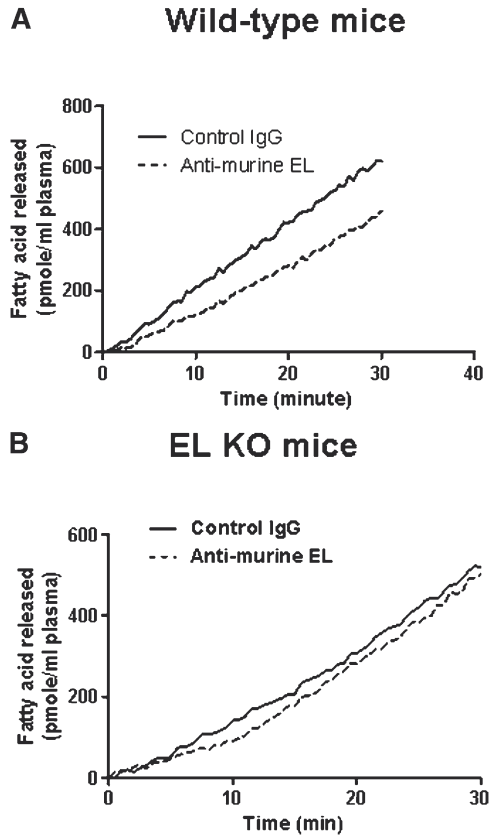

Fig. 4. Characterization of endogenous EL PLA1 activity in WT and EL KO mouse plasma as described for Figure 1C. A representative set of data was shown using a fixed amount of post-heparin plasma (0.5 μl/well) incubated with a control IgG antibody or an anti-EL inhibitory antibody for 5 min prior to analysis: (A) plasma from WT mice; (B) plasma from EL knockout mice. The final concentrations of each antibody were 30 μg/ml.

used only for EL activity in mouse plasma, not that of humans, because the inactivating antibody does not neutralize human EL activity. To differentiate EL phospholipase activity from that of other phospholipases in human post-heparin plasma, we are developing a neutralizing antibody for human EL activity.

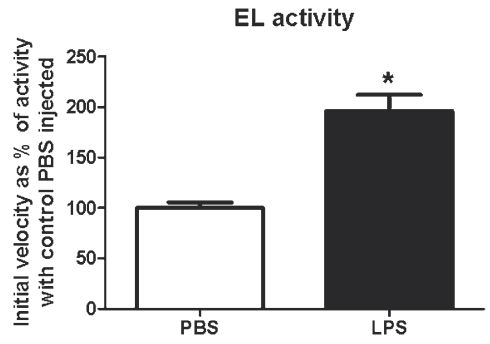

Fig. 5. Plasma EL PLA1 activity in WT mice 16 h after LPS or PBS treatment. EL PLA1 activity was measured using 0.5 μl of post-heparin plasma. EL activity was calculated as the difference in the PLA1 activities of post-heparin plasma sample preincubated with either control IgG or with anti-EL IgG and expressed as a percentage of the activity measured for the PBS group. Values are mean ± SD of measurements on five animals.* $P < 0.05$.

The experiments showing the changes in the protein and PLA1 activity of EL in plasma with time following release by heparin provided a snapshot of global EL activity but did not give information regarding tissue or cellular levels. Because the BODIPY labeled fatty acid, after lipase-mediated hydrolysis of the PED-A1 substrate, can enter cells and be incorporated into cellular lipids, the self-quenched PED-A1 substrate has been used for monitoring EL-mediated fatty acid uptake in cell culture (23). We plan to investigate whether PED-A1 can be used as a functional probe for tissue EL activity in vivo.

In summary, we have developed a homogeneous PLA1 assay employing the EL-neutralizing antibody for the determination of murine post-heparin plasma EL PLA1 activity. This is the first such assay that is effective for EL in plasma. Armed with this assay, researchers will be able to screen for the efficacy of new potential inhibitors of EL in mice and study proteins with EL-regulating properties.

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REFERENCES

1. Nijstad, N., H. Wiersma, T. Gautier, M. van der Giet, C. Maugeais, and U. J. Tietge. 2009. Scavenger receptor BI-mediated selective uptake is required for the remodeling of high density lipoprotein by endothelial lipase. *J. Biol. Chem.* **284:** 6093–6100.
2. Ishida, T., S. Choi, R. K. Kundu, K. Hirata, E. M. Rubin, A. D. Cooper, and T. Quertermous. 2003. Endothelial lipase is a major determinant of HDL level. *J. Clin. Invest.* **111:** 347–355.
3. Jin, W., J. S. Millar, U. Broedl, J. M. Glick, and D. J. Rader. 2003. Inhibition of endothelial lipase causes increased HDL cholesterol levels in vivo. *J. Clin. Invest.* **111:** 357–362.
4. Ma, K., M. Cilingiroglu, J. D. Otvos, C. M. Ballantyne, A. J. Marian, and L. Chan. 2003. Endothelial lipase is a major genetic determinant for high-density lipoprotein concentration, structure, and metabolism. *Proc. Natl. Acad. Sci. USA.* **100:** 2748–2753.
5. Zhang, J., Y. Yu, K. Nakamura, T. Koike, A. B. Waqar, X. Zhang, E. Liu, K. Nishijima, S. Kitajima, M. Shiomi, et al. 2012. Endothelial lipase mediates HDL levels in normal and hyperlipidemic rabbits. *J. Atheroscler. Thromb.* **19:** 213–226.
6. Bauer, R. C., I. M. Stylianou, and D. J. Rader. 2011. Functional validation of new pathways in lipoprotein metabolism identified by human genetics. *Curr. Opin. Lipidol.* **22:** 123–128.
7. Chasman, D. I., G. Pare, S. Mora, J. C. Hopewell, G. Peloso, R. Clarke, L. A. Cupples, A. Hamsten, S. Kathiresan, A. Malarstig, et al. 2009. Forty-three loci associated with plasma lipoprotein size, concentration, and cholesterol content in genome-wide analysis. *PLoS Genet.* **5:** e1000730.
8. Edmondson, A. C., P. S. Braund, I. M. Stylianou, A. V. Khera, C. P. Nelson, M. L. Wolfe, S. L. Derohannessian, B. J. Keating, L. Qu, J. He, et al. 2011. Dense genotyping of candidate gene loci identifies variants associated with high-density lipoprotein cholesterol. *Circ. Cardiovasc. Genet.* **4:** 145–155.
9. Edmondson, A. C., R. J. Brown, S. Kathiresan, L. A. Cupples, S. Demissie, A. K. Manning, M. K. Jensen, E. B. Rimm, J. Wang, A. Rodrigues, et al. 2009. Loss-of-function variants in endothelial lipase are a cause of elevated HDL cholesterol in humans. *J. Clin. Invest.* **119:** 1042–1050.
10. Heid, I. M., E. Boes, M. Muller, B. Kollerits, C. Lamina, S. Coassin, C. Gieger, A. Doring, N. Klopp, R. Frikke-Schmidt, et al. 2008. Genome-wide association analysis of high-density lipoprotein cholesterol in the population-based KORA study sheds new light on intergenic regions. *Circ. Cardiovasc. Genet.* **1:** 10–20.

11. Ma, L., J. Yang, H. B. Runesha, T. Tanaka, L. Ferrucci, S. Bandinelli, and Y. Da. 2010. Genome-wide association analysis of total cholesterol and high-density lipoprotein cholesterol levels using the Framingham heart study data. *BMC Med. Genet.* **11:** 55.
12. Sarzynski, M. A., P. Jacobson, T. Rankinen, B. Carlsson, L. Sjostrom, L. M. Carlsson, and C. Bouchard. 2011. Association of GWAS-based candidate genes with HDL-cholesterol levels before and after bariatric surgery in the Swedish obese subjects study. *J. Clin. Endocrinol. Metab.* **96:** E953–E957.
13. Lamarche, B., and M. E. Paradis. 2007. Endothelial lipase and the metabolic syndrome. *Curr. Opin. Lipidol.* **18:** 298–303.
14. Badellino, K. O., M. L. Wolfe, M. P. Reilly, and D. J. Rader. 2008. Endothelial lipase is increased in vivo by inflammation in humans. *Circulation.* **117:** 678–685.
15. Griffon, N., W. Jin, T. J. Petty, J. Millar, K. O. Badellino, J. G. Saven, D. H. Marchadier, E. S. Kempner, J. Billheimer, J. M. Glick, et al. 2009. Identification of the active form of endothelial lipase, a homodimer in a head-to-tail conformation. *J. Biol. Chem.* **284:** 23322–23330.
16. Jin, W., I. V. Fuki, N. G. Seidah, S. Benjannet, J. M. Glick, and D. J. Rader. 2005. Proprotein convertases [corrected] are responsible for proteolysis and inactivation of endothelial lipase. *J. Biol. Chem.* **280:** 36551–36559.
17. Jin, W., X. Wang, J. S. Millar, T. Quertermous, G. H. Rothblat, J. M. Glick, and D. J. Rader. 2007. Hepatic proprotein convertases modulate HDL metabolism. *Cell Metab.* **6:** 129–136.
18. Shimamura, M., M. Matsuda, H. Yasumo, M. Okazaki, K. Fujimoto, K. Kono, T. Shimizugawa, Y. Ando, R. Koishi, T. Kohama, et al. 2007. Angiopoietin-like protein3 regulates plasma HDL cholesterol through suppression of endothelial lipase. *Arterioscler. Thromb. Vasc. Biol.* **27:** 366–372.
19. Jaye, M., K. J. Lynch, J. Krawiec, D. Marchadier, C. Maugeais, K. Doan, V. South, D. Amin, M. Perrone, and D. J. Rader. 1999. A novel endothelial-derived lipase that modulates HDL metabolism. *Nat. Genet.* **21:** 424–428.
20. Hirata, K., H. L. Dichek, J. A. Cioffi, S. Y. Choi, N. J. Leeper, L. Quintana, G. S. Kronmal, A. D. Cooper, and T. Quertermous. 1999. Cloning of a unique lipase from endothelial cells extends the lipase gene family. *J. Biol. Chem.* **274:** 14170–14175.
21. Mitnaul, L. J., J. Tian, C. Burton, M. H. Lam, Y. Zhu, S. H. Olson, J. E. Schneeweis, P. Zuck, S. Pandit, M. Anderson, et al. 2007. Fluorogenic substrates for high-throughput measurements of endothelial lipase activity. *J. Lipid Res.* **48:** 472–482.
22. Keller, P. M., T. Rust, D. J. Murphy, R. Matico, J. J. Trill, J. A. Krawiec, A. Jurewicz, M. Jaye, M. Harpel, S. Thrall, et al. 2008. A high-throughput screen for endothelial lipase using HDL as substrate. *J. Biomol. Screen.* **13:** 468–475.
23. Darrow, A. L., M. W. Olson, H. Xin, S. L. Burke, C. Smith, C. Schalk-Hihi, R. Williams, S. S. Bayoumy, I. C. Deckman, M. J. Todd, et al. 2011. A novel fluorogenic substrate for the measurement of endothelial lipase activity. *J. Lipid Res.* **52:** 374–382.
24. McCoy, M. G., G. S. Sun, D. Marchadier, C. Maugeais, J. M. Glick, and D. J. Rader. 2002. Characterization of the lipolytic activity of endothelial lipase. *J. Lipid Res.* **43:** 921–929.
25. Rader, D. J., and M. Jaye. 2000. Endothelial lipase: a new member of the triglyceride lipase gene family. *Curr. Opin. Lipidol.* **11:** 141–147.
26. Yasuda, T., T. Ishida, and D. J. Rader. 2010. Update on the role of endothelial lipase in high-density lipoprotein metabolism, reverse cholesterol transport, and atherosclerosis. *Circ. J.* **74:** 2263–2270.
27. Broedl, U. C., C. Maugeais, D. Marchadier, J. M. Glick, and D. J. Rader. 2003. Effects of nonlipolytic ligand function of endothelial lipase on high density lipoprotein metabolism in vivo. *J. Biol. Chem.* **278:** 40688–40693.
28. Dallinga-Thie, G. M., A. J. Zonneveld-de Boer, L. C. van Vark-van der Zee, R. van Haperen, T. van Gent, H. Jansen, R. De Crom, and A. van Tol. 2007. Appraisal of hepatic lipase and lipoprotein lipase activities in mice. *J. Lipid Res.* **48:** 2788–2791.
29. Basu, D., J. Manjur, and W. Jin. 2011. Determination of lipoprotein lipase activity using a novel fluorescent lipase assay. *J. Lipid Res.* **52:** 826–832.
30. Murakami, M., and I. Kudo. 2003. New phospholipase A(2) isozymes with a potential role in atherosclerosis. *Curr. Opin. Lipidol.* **14:** 431–436.
31. Lavín-Palmieri, M. 1986. Effect of 6-hydroxydopamine on heart lipoprotein lipase activity in mice. *Cor Vasa.* **28:** 73–79.
32. Jin, W., G. S. Sun, D. Marchadier, E. Octtaviani, J. M. Glick, and D. J. Rader. 2003. Endothelial cells secrete triglyceride lipase and phospholipase activities in response to cytokines as a result of endothelial lipase. *Circ. Res.* **92:** 644–650.
33. Wang, X., W. Jin, and D. J. Rader. 2007. Upregulation of macrophage endothelial lipase by toll-like receptors 4 and 3 modulates macrophage interleukin-10 and -12 production. *Circ. Res.* **100:** 1008–1015.
34. Yasuda, T., K. Hirata, T. Ishida, Y. Kojima, H. Tanaka, T. Okada, T. Quertermous, and M. Yokoyama. 2007. Endothelial lipase is increased by inflammation and promotes LDL uptake in macrophages. *J. Atheroscler. Thromb.* **14:** 192–201.
35. Ahmed, W., G. Orasanu, V. Nehra, L. Asatryan, D. J. Rader, O. Ziouzenkova, and J. Plutzky. 2006. High-density lipoprotein hydrolysis by endothelial lipase activates PPARalpha: a candidate mechanism for high-density lipoprotein-mediated repression of leukocyte adhesion. *Circ. Res.* **98:** 490–498.
36. Robciuc, M. R., E. Tahvanainen, M. Jauhiainen, and C. Ehnholm. 2009. Quantitation of serum angiopoietin-like proteins 3 and 4 in a finnish population sample. *J. Lipid Res.* **51:** 824–831.
37. Ando, Y., T. Shimizugawa, S. Takeshita, M. Ono, M. Shimamura, R. Koishi, and H. Furukawa. 2003. A decreased expression of angiopoietin-like 3 is protective against atherosclerosis in apoE-deficient mice. *J. Lipid Res.* **44:** 1216–1223.
38. Peterson, J., G. Bengtsson-Olivecrona, and T. Olivecrona. 1986. Mouse preheparin plasma contains high levels of hepatic lipase with low affinity for heparin. *Biochim. Biophys. Acta.* **878:** 65–70.