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Synthetic phospholipids as specific substrates for plasma endothelial lipase

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ABSTRACT

We designed and prepared synthetic phospholipids that generate lyso-phosphatidylcholine products with a unique mass for convenient detection by LC–MS in complex biological matrices. We demonstrated that compound **4**, formulated either as a Triton X-100 emulsion or incorporated in synthetic HDL particles can serve as a substrate for plasma EL with useful specificity.

Low levels of plasma high density lipoprotein cholesterol (HDL-C) are a risk factor for atherosclerotic cardiovascular disease, independent of the benefit statins provide by reducing low density lipoprotein cholesterol (LDL-C) levels.[1] Endothelial lipase (EL), a member of the triglyceride lipase gene family that includes hepatic lipase (HL) and lipoprotein lipase (LPL), has been shown to be a negative regulator of plasma HDL-C levels in both mice and humans,[2] and multiple lines of evidence suggest that pharmacological inhibition of EL enzymatic activity might be beneficial for the treatment of atherosclerosis.[3] In order to evaluate the efficacy of EL inhibitors in vivo, the ability to correlate plasma EL enzymatic activity with plasma lipoprotein levels is desirable. While EL, LPL and HL have different lipoprotein substrate preferences, they are all competent to hydrolyze phospholipids (PL), as well as triglycerides.[4] Moreover, their lipolysis products, free fatty acids (FFA) and lyso-phosphatidylcholines (lysoPC or LPC) are abundant in plasma. Therefore, developing a sensitive and specific assay to measure EL activity with minimal processing of plasma samples to support characterization of EL inhibitors represents a significant challenge. Although multiple screening assays using fluorophore-labeled phospholipid substrates have been reported,[5] there are few reports addressing the specific measure of plasma EL activity. Radiometric assays using [1,2-^{14}C]-dipalmitoylphosphatidylcholine (DPPC) have historically been used to measure the general phospholipase activities in plasma or cell culture samples.[6] This method requires large volume of samples, laborious multiple-step processing of radioactive material, but more importantly, DPPC is not a specific substrate for the phospholipases. The application of more phospholipase A1 (PL-A1) specific fluorescent substrates in plasma has been the object of several reports.[7] We envisioned that using synthetic phospholipids as substrates to determine EL activity in plasma samples should have two advantages: they could be optimized to be specific substrates for EL, and the lysoPC product could be chosen so that its mass was distinct from endogenous lipids, and could be readily detected by LC–MS. Progress towards this goal is reported herein.

EL is a PL-A1, i.e., it catalyzes the hydrolysis of the ester on the first carbon of the glycerol molecule (denoted sn-1) preferentially over the ester on the second carbon of the glycerol molecule (denoted sn-2). We surmised that the majority of background hydrolysis could be suppressed by replacing the sn-2 ester with functionalities that would not be readily hydrolyzed. As shown in Table 1, we designed ethers (**1**–**3**) and carbamates (**4**–**5**) whose lysoPC mass would be distinct from circulating phospholipids.

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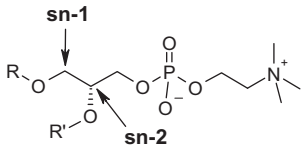
Table 1
Synthetic phospholipids investigated in these studies

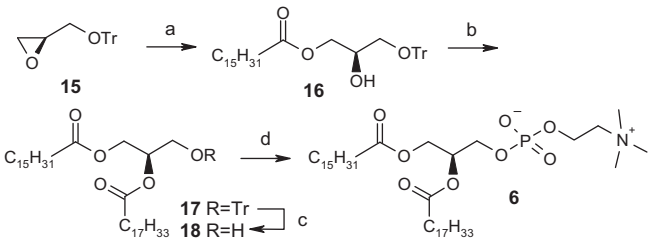


	R	R′	Formula	Exact mass	
				PC	LPC
1	Myristate	$-C_{12}H_{25}$	$C_{34}H_{70}NO_7P$	635.5	425.3
2	Palmitate	$-C_{12}H_{25}$	$C_{36}H_{74}NO_7P$	663.5	425.3
3	Myristate	$-C_6H_{13}$	$C_{28}H_{58}NO_7P$	551.4	341.2
4	Palmitate	$-C(O)NHC_{14}H_{29}$	$C_{39}H_{79}N_2O_8P$	734.5	496.3
5	Myristate	$-C(O)NHC_{14}H_{29}$	$C_{37}H_{75}N_2O_8P$	706.5	496.3
6	Palmitate	Oleate[a]	$C_{42}H_{83}NO_8P$	760.5	522.3

[a] Inverse configuration at sn-2.

We also investigated inversion of configuration at the glycerol chiral center, as in **6**. Syntheses of **1**–**5** started from glycerophosphocholine (**7**), which was tritylated at the less hindered hydroxyl in good yields (Scheme 1). Alkylation of the secondary alcohol in **8** to give **9** and **10** was accomplished using dimsyl sodium, freshly prepared using sodium hydride in DMSO, and the appropriate iodoalkane. Carbamate **11** was prepared by microwave heating **8** with the corresponding isocyanate. TFA was employed to cleave the trityl group in **9**–**11**. Finally sn-1 ester formation was accomplished with DCC/DMAP and the appropriate acid to give **1**–**5**. In order to prepare a PC with inverted configuration, we started with enantiopure epoxide **15** (Scheme 2). Mixing **15** and palmitic acid with tetraethylammonium bromide and heating to 100 °C gave secondary alcohol **16** in 65% yield.[8] DCC/DMAP esterification, followed with TFA treatment afforded **18**. Conversion of **18** to PC **6** was achieved according to a published protocol.[9]

All PLs were formulated as Triton X-100 emulsions, based on a published protocol,[6] and after 60 min of incubation with the lipase preparations, lysoPC products were quantified by LC–MS. We found that all synthetic PLs could serve as substrates for recombinant human EL (Fig. 1, black bars). However, ethers **1**–**3** and ester **6** were relatively poor substrates, with only 7–20% lysoPC produced compared to DPPC. In contrast, the carbamates **4** and **5** were comparable to DPPC, with amounts of lysoPC generated as 52% and 58%



Scheme 2. Synthesis of ester **6**. Reagents and conditions: (a) palmitic acid, tetraethylammonium bromide, 100 °C, 3 h, 65%. (b) Oleic acid, DCC, DMAP, chloroform, rt, 2 days, 88%. (c) TFA, DCM, rt, 3 days, 42%. (d) **18**, 2-chloro-2-oxo-1,3,2-dioxaphospholane, TEA, toluene, 2 days, rt, then switch solvent to acetonitrile, trimethylamine, −78 °C to 65 °C, 36 h, 28%.

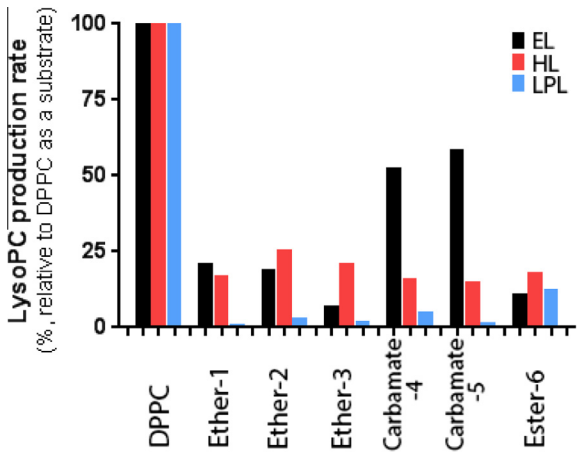
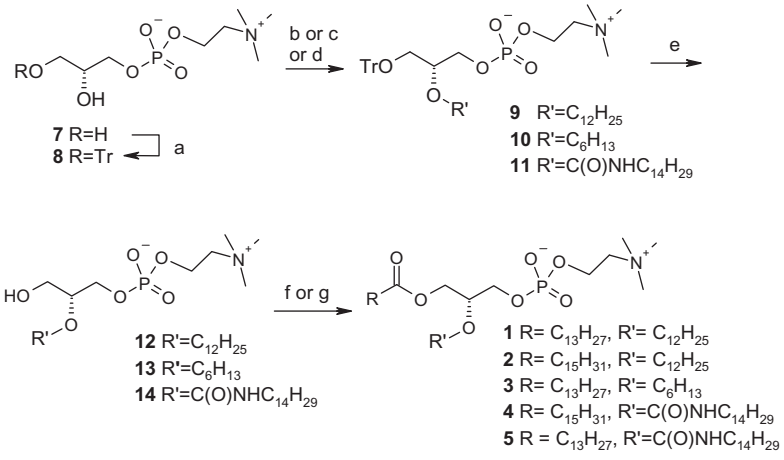


Figure 1. LysoPC production by recombinant EL, HL and purified LPL in assay buffer for PL **1**–**6** after a 60 min incubation, expressed as a percentage of lyso-DPPC control ($n = 2$).

that of DPPC, respectively. All six synthetic PLs were poor substrates for recombinant HL (Fig. 1, red bars), relative to DPPC, and in contrast to EL, HL showed no substrate preference. In the case of LPL, only ester **6** showed any significant hydrolysis. Overall, the two carbamate PLs **4** and **5**, while serving as relatively poor substrates for HL and LPL, retained fairly high lipolysis by EL, and **4** was selected for further study.



Scheme 1. Synthesis of ethers **1**–**3** and carbamate **4**–**5**. Reagents and conditions: (a) $ZnCl_2$, DMF, 4 °C, 30 min, then TrCl, 4 °C, overnight, 50%. (b) **8**, 1-iododecane, dimsyl sodium, DMSO, rt, 1 h, 3%. (c) **8**, 1-iodohexane, dimsyl sodium, DMSO, rt, 1 h, 2%. (d) **8**, 1-isocyanatotetradecane, DMSO, μW, 125 °C, 45 min, 5%. (e) TFA, chloroform, rt, 4 h, 87% (**12**), 71% (**13**), 15% (**14**). (f) Myristic acid, DCC, DMAP, chloroform, rt, overnight, 29% (**1**), 57% (**3**), 9% (**5**). (g) Palmitic acid, DCC, DMAP, chloroform, rt, overnight, 30% (**2**), 37% (**4**).

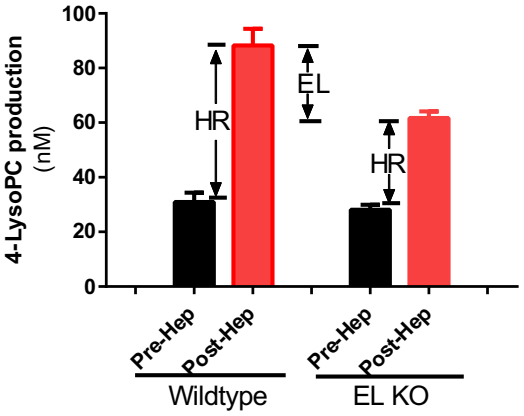


Figure 2. Amount of **4**-lysoPC produced by incubating a Triton X-100 emulsion of **4** with wild-type and EL KO plasma pre- and post-heparin for 60 min ($n = 3$).

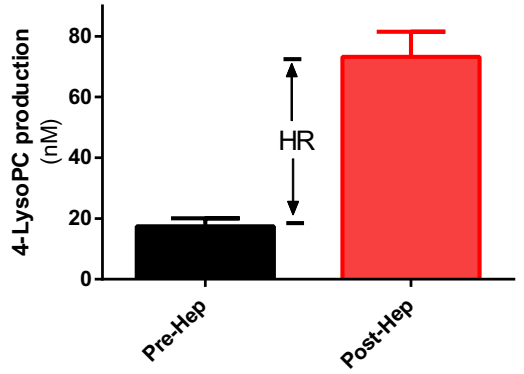


Figure 3. Production of **4**-lysoPC from **4**-rHDL incubated with pre- and post-heparin wild-type mouse plasma ($n = 3$).

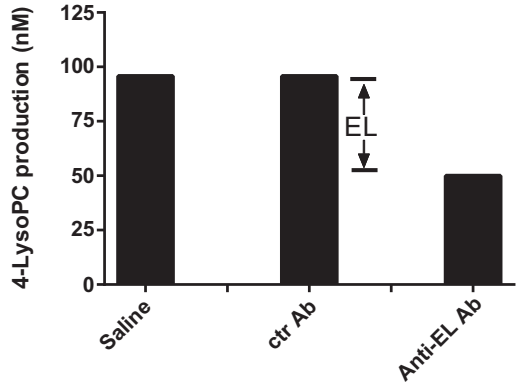


Figure 4. Production of **4**-lysoPC from **4**-rHDL incubated with post-heparin wild-type mouse plasma in the presence of a control Ab or anti-EL Ab ($n = 1$).

EL is a secretory protein which binds to the endothelial surface of blood/lymphatic vessels via cell-surface proteoglycans and is thus heparin-releasable.[10] Therefore, the hydrolytic activity of mouse plasma on PL **4** was evaluated in pre- and post-heparin samples, affording a measure of heparin-releasable (HR) activity. In order to evaluate the specific contribution of EL, comparison of activity was made between wild-type and EL knock-out (KO) plasma. The results for PL **4** formulated as a Triton X-100 emulsion are shown in Figure 2. Some hydrolysis of **4** occured in pre-heparin plasma, with no meaningful difference observed between wild-type and EL KO samples. Additional **4**-lysoPC formation was observed in both wild-type and KO post-heparin plasma compared to pre-heparin samples. As anticipated, the post-heparin over pre-heparin **4**-lysoPC ratio was greater in wild-type (2.8:1) versus EL KO (2:1). Although the hydrolysis of **4** in post-heparin EL KO plasma was substantial, the significant difference in HR activity between wild-type and EL KO plasma suggested a window to measure specific plasma EL activity.

We also evaluated **4** as a substrate with a more physiologically relevant formulation. Synthetic HDL particles (rHDL)[11] with HDL-like sizes (diameter = 7.6 nm) were produced using a 60:12:1 molar ratio of compound **4** to cholesterol oleate to apoA-I, respectively. Using this **4**-rHDL substrate, a 3.2:1 ratio of post-heparin to pre-heparin **4**-lysoPC was observed (Fig. 3), suggesting that **4** incorporated in synthetic HDL might offer better specificity compared to its Triton-X100 emulsion. The relative specificity of **4**-rHDL for plasma EL was further assessed by treating mouse plasma with anti-EL antibody. Using wild-type post-heparin mouse plasma, an antibody specific for mouse EL inhibited **4**-lysoPC production significantly compared to a control Ab (Fig. 4).

In conclusion, we designed and prepared synthetic phospholipids that generate lysoPC products with a unique mass for convenient detection by LC–MS in complex biological matrices. We demonstrated that compound **4**, formulated either as a Triton X-100 emulsion or incorporated in synthetic HDL particles can serve as a substrate for plasma EL with useful specificity.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.06.032.

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