methods

Fluorogenic substrates for high-throughput measurements of endothelial lipase activity

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Abstract Endothelial lipase (EL) has been shown to be a critical determinant for high density lipoprotein cholesterol levels in vivo; therefore, assays that measure EL activity have become important for the discovery of small molecule inhibitors that specifically target EL. Here, we describe fluorescent Bodipy-labeled substrates that can be used in homogeneous, ultra-high-throughput kinetic assays that measure EL phospholipase or triglyceride lipase activities. Triton X-100 detergent micelles and synthetic HDL particles containing Bodipy-labeled phospholipid or Bodipy-labeled triglyceride substrates were shown to be catalytic substrates for EL, LPL, and HL. More importantly, only synthetic HDL particles containing Bodipy-labeled triglyceride were ideal substrates for EL, LPL, and HL in the presence of high concentrations of human or mouse serum. These data suggest that substrate presentation is a critical factor when determining EL activity in the presence of serum.—Mitnaul, L. J., J. Tian, C. Burton, M-H. Lam, Y. Zhu, S. H. Olson, J. E. Schneeweis, P. Zuck, S. Pandit, M. Anderson, M. M. Maletic, S. T. Waddell, S. D. Wright, C. P. Sparrow, and E. G. Lund. **Fluorogenic substrates for high-throughput measurements of endothelial lipase activity. *J. Lipid Res.* 2007.** 48: **472–482.**

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Endothelial lipase (EL) belongs to the vascular heparan sulfate-associated family of lipases (for review, see Refs. 1–3). Members of this family include LPL, HL, and pancreatic lipase. Like LPL and HL, EL is believed to function mainly in the plasma compartment, where it hydrolyzes phospholipids, particularly in HDL. Although predominantly a phospholipase, EL also hydrolyzes triglycerides in lipoprotein particles (4). Genetic deletion of EL, or administration of inhibitory antibodies to EL, resulted in significant increases in high density lipoprotein cholesterol (HDL-C) levels in mice (5–7). The significance of this increase in HDL-C can be seen in one study in which EL and apolipoprotein E (apoE) double deficient mice had a considerable improvement in atherosclerosis, as measured by lesion area (8). However, in a more recent study, EL was shown to modulate HDL but led to no significant improvements in atherosclerosis in EL and apoE double deficient mice (9). If the positive effects of EL are translated to humans, these data suggest that small molecule inhibitors of EL may be beneficial to patients with coronary artery disease.

To rapidly screen for small molecule inhibitors of EL, high-throughput homogeneous assays that measure its lipolytic activity are preferred. Several lipase assays have been described (10), and most of these assays use radiolabeled lipid substrates emulsified in glycerol (containing either radiolabeled phospholipid or triglyceride). Although extensive biology has been learned using emulsified glycerol substrates, more native, endogenous EL substrates (such as HDL particles) are needed to understand inhibitor-EL particle interactions. For this reason, some lipase assays have made use of more sensitive, fluorogenic substrates (10, 11). For instance, large fluorescent moieties such as Bodipy have been added to lipid substrates, which did not obstruct cleavage of the conjugated natural substrates (12). Because of the fluorescence readout, these types of assays have allowed lipase activities to be tested in high-throughput, low-volume, sensitive assays (13).

Lipases are known to function on the surface of vascular endothelial cells, where they are in contact with plasma. Surprisingly, in the presence of 5% animal serum, emulsified radiolabeled lipid is a poor substrate with which to

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Abbreviations: apoE, apolipoprotein E; apo HDL, purified protein component of human high density lipoprotein; BD-ffa, Bodipy-labeled free fatty acid; bis-BD-PC, bis-labeled-Bodipy-phosphatidycholine; EL, endothelial lipase; HDL-C, high density lipoprotein cholesterol; HUVEC, human umbilical vein endothelial cell; HUVEC-EL, human umbilical vein endothelial cell-derived endothelial lipase; mono-BD-TG, mono-labeled-Bodipy-triglyceride; TNF-α, tumor necrosis factor-α.

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observe EL lipolytic activity in vitro (4, 14, 15). In contrast, LPL and HL were not inhibited by the addition of serum. It was suggested that animal serum may contain an inhibitory factor or factors that regulate EL activity, possibly similar to how apoC-II activates LPL (16). For instance, serum may contain a negative regulator of EL activity. Alternatively, the substrates used to measure EL activity in the presence of serum may not be optimal for observing EL catalysis in that reaction environment.

Here, we describe fluorescent Bodipy-labeled lipid substrates that can be used in homogeneous, high-throughput, kinetic assays that measure the phospholipase and triglyceride lipase activities of EL. Triton X-100 detergent micelles containing either bis-Bodipy-labeled C11-phosphatidylcholine (bis-BD-PC) or mono-Bodipy-labeled triglyceride (mono-BD-TG) are rapid, reliable, sensitive substrates that directly measure EL hydrolysis. Synthetically prepared fluorescent HDL particles containing bis-BD-PC or mono-BD-TG were also catalytic substrates, and more importantly, mono-BD-TG particles were ideal for measuring lipase activity in the presence of high concentrations (60%) of human or mouse serum. These data suggest that substrate presentation is critical when determining EL activity in the presence of serum. In addition, dual-labeled HDL particles containing radiolabeled PC and mono-BD-TG were ideal substrates for simultaneously observing EL phospholipase and triglyceride lipase activities in the same substrate.

MATERIALS AND METHODS

Materials and reagents

Bis-BD-PC and Bodipy (C11) fatty acid (BD-ffa standard) were purchased from Molecular Probes; Triton X-100, lipoprotein-deficient serum, heparin, dioleoyl L-α phosphatidylcholine, fatty-acid free BSA, and triolein were purchased from Sigma. Human HDL was purchased from Intracel (Frederick, MD). Human embryonic kidney (HEK293) cells and human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection and Cambrex Bio Science (Walkersville, MD), respectively. ApoC-II was purchased from BioDesign International (Saco, ME). Human serum was purchased from Biological Specialty Corp. (Colmar, PA), and mouse serum was purchased from Bioreclamation, Inc. (Hicksville, NY); both sera were heat-inactivated at 56°C for 1 h before use. Anti-human EL monoclonal antibody was a generous gift of Daniel Rader (University of Pennsylvania).

Chemical synthesis of mono-Bodipy triglyceride substrate

Unlike bis-BD-PC, mono-BD-TG is not commercially available. Thus, *sn*-1- and *sn*-2-labeled triglyceride molecules were synthesized. The *sn*-1-labeled mono-BD-TG (formula weight = 1,077.416) was synthesized by adding dicyclohexylcarbodiimide (5.0 mg, 2.8 eq), 1,3-diolein (5.4 mg, 1.03 eq; Sigma), and 4-(dimethylamino)pyridine (1.2 mg, 1.0 eq) to a flame-dried vial and then dissolving the components in dichloromethane (0.5 ml). Bodipy 558/568 C12 [4,4′-difloro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoic acid; 4.0 mg 1.0 eq] in dichloromethane (0.7 ml) was added to the reaction vial and stirred under nitrogen at room temperature. The *sn*-2-labeled mono-BD-TG (formula weight = 1,075.4) was synthesized by adding dicyclohexylcarbodiimide (6.2 mg, 2.0 eq), 1,2-diolein (10.0 mg, 1.1 eq; Sigma), and 4-(dimethylamino)pyridine (1.8 mg, 1.0 eq) to a flame-dried vial and dissolving the components in dichloromethane (1.5 ml). Bodipy 558/568 C12 [4,4′-difloro-5-(2-thienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoic acid; 7.0 mg 1.0 eq] in dichloromethane (0.5 ml) was added to the reaction vial and stirred under nitrogen at room temperature. After 15 h, TLC was performed on each of the products to confirm that the reaction was complete. The mixture was loaded on a silica gel column and eluted with dichloromethane. The fractions containing the desired product were concentrated to give 4.1–4.5 mg (28–45% yield) of the desired product. The structures were confirmed by NMR and HPLC/MS.

Induction of EL from cultured HUVECs

HUVECs were purchased from Cambrex and cultured with the EGM-2 BulletKit, until they reached 90% confluence. Cells were then washed with PBS and incubated with serum-free medium containing 10 ng/ml tumor necrosis factor-α (TNF-α; R&D Systems) for 24 h at 37°C. To release heparan sulfate-associated EL, 10 U/ml heparin was added directly to the culture medium and incubated for 30 min at 37°C, and then the supernatant was collected and concentrated ~200-fold with an iCON™ concentrator (Pierce). The EL produced this way is referred to as HUVEC-EL (for human umbilical vein endothelial cell-derived endothelial lipase). Glycerol was added to a final concentration of 15% (v/v), and the HUVEC-EL was placed into aliquots, frozen on dry ice, and stored at −80°C. To ensure EL expression, immunoblot analysis of HUVEC cultured medium using a 1:6,000 dilution of rabbit anti-human EL antibody was performed as outlined by Rader and colleagues (17).

Generation of human LPL and HL from cultured HEK293 cells

To produce human LPL and HL, HEK293 cells were transiently transfected with either pcDNA3.1-LPL or pcDNA3.1-HL using LipofectAmine (Gibco BRL). HEK293 cells were plated in a TC-175 flask 18 h before transfection. Approximately 15 μg of DNA was then transfected into the cells according to the manufacturer's protocol. After 48 h, cells were rinsed three times with serum-free medium, and 10 U/ml heparin was added directly to the cells (in serum-free medium) to release LPL or HL. The medium containing the lipases was stored at 4°C until used and was stable for several months. Nontransfected HEK293 cells, which did not yield lipase activity, were used as a source of a negative control.

Generation of fluorogenic micelle substrates

To prepare fluorogenic phospholipid or triglyceride micelle substrates, a working solution was first prepared by mixing an equal volume of either 1.5 mM mono-BD-TG (dissolved in benzene) or 0.5 mg/ml bis-BD-PC (dissolved in ethanol) with 1% Triton X-100 (in chloroform) to make a final Triton concentration of 0.1% (v/v). The samples were mixed by vortexing and then dried to completion under argon with mild heat (37°C). The samples were then resuspended in PBS and mixed well. The substrates were stored at 4°C until used and were stable for several months.

Generation of fluorogenic synthetic HDL particles

Synthetic HDL particles were prepared according to Pittman et al. (18), with minor modifications. Briefly, the protein components of HDL were isolated by lyophilization of purified human HDL, followed by three rounds of lipid extraction of the pellet

using chloroform-methanol (2:1). After the third extraction, the protein pellet [referred to as apo HDL (for purified protein component of human high density lipoprotein)] was lyophilized and resuspended in 50 mM Tris (pH 8.6), 150 mM NaCl, and 1 mM EDTA by sonication. The apo HDL was stored at 4°C and was stable for at least 1 year. To prepare 30 ml of mono-BD-TG HDL particles, 20 mg of dioleoyl L-α phosphatidylcholine and 12.3 mg of mono-BD-TG were combined in a chloroform-resistant polypropylene tube and dried to completion under nitrogen. The pellet was then washed once with ethanol (100%) and dried again. Apo HDL (20 mg) was added, and the sample was resuspended by vortexing. The sample was then sonicated (three cycles of 10 min/cycle) with a microtip sonicator (Branson Sonicator 250) to create the synthetic particles. Synthetic HDL particles were then isolated in the density range of 1.063–1.21 g/ml by sequential flotation ultracentrifugation according to standard methods. The prepared particles were dialyzed against 50 mM Tris (pH 7.4), 100 mM NaCl, and 1 mM EDTA at 4°C. Bis-BD-PC particles were prepared using the same protocol except that dioleoyl L-α phosphatidylcholine and mono-BD-TG were replaced with bis-BD-PC (Molecular Probes) and triolein, respectively. HDL particles containing both radiolabeled PC and fluorescent triglyceride were produced exactly like the mono-BD-TG particles, except that 5 mg of *sn*-1-labeled [^{14}C]PC (Amersham-GE Healthcare) was added in addition to 15 mg of dioleoyl L-α phosphatidylcholine. Total protein recovery was typically ~60–70%, as judged by bicinchoninic acid protein analysis (Pierce). Synthetic HDL particles were stable at 4°C for several months.

Fluorogenic lipase assays

To determine EL, LPL, and HL activities using Triton X-100 micelle substrates, EL, LPL, and HL were added to 96-well black-coated plates (Costar) in PBS. Fluorescent micelle substrate was then added to the assay plate to begin the reaction. For these assays, the final bis-BD-PC and mono-BD-TG concentrations were ~1 μM and 20 μM, respectively. All assays using mono-BD-TG included 0.5% fatty acid-free BSA. In LPL assays, 125 ng of apoC-II was added to activate LPL (16). After the addition of substrate, assay plates were immediately monitored at 25°C at excitation at 490 nm/emission at 520 nm (bis-BD-PC substrates) or excitation at 538 nm/emission at 568 nm (mono-BD-TG substrates). HDL synthetic particles were assayed under identical conditions, except that fluorogenic HDL particles were used as substrates. The total volume of the fluorescence assays ranged from 100 to 200 μl per well. Because of modest differences between different preparations of synthetic particles, each new batch of synthetic particles was titrated as substrate to identify a consistent window of lipase activity, thereby allowing comparisons with previous assays. In assays involving inhibitors (1 M NaCl) or human and mouse serum, lipase and inhibitor/serum were incubated for ~10 min before the addition of substrate. When dual-labeled (radiolabeled PC and fluorescent triglyceride) substrates were used, kinetic monitoring of fluorescence was first conducted for 2 h, followed by TLC separation of the final reaction products.

Ultra-high-throughput (3,456 nanoplate) lipase assays

A Discovery Island articulated arm-based screening platform (Aurora Discovery, San Diego, CA) was used in a fully automated format. This platform consists of a Mitsubishi anthropomorphic arm on a 2 m rail. The 3,456-well plate assay was developed in nanowell plates produced by Greiner Bio-One. The plates were black with clear bottoms and had plasma tissue culture treatment. DMSO was preplated into assay plates using Aurora's Piesoelectric distribution robot. All reagents for the enzymatic assay were dispensed using the flying reagent dispenser. Incubations were carried out at 25°C in a humidified Cytomat incubator from Thermo. For the enzymatic reaction, 0.4 μl of 2.5 μM bis-BD-PC Triton X-100 substrate was added to plates that had 0.6 μl of a 1:40 dilution of the concentrated HUVEC-EL. The reactions proceeded for 30 min at 25°C in a humidified incubator. The plates were then read using the topology-compensated plate reader from Aurora Discovery.

Analysis of reaction products by TLC

Products were analyzed by TLC by first terminating the reactions by the addition of equal volumes of 0.2 N HCl. Lipids were then extracted with 3:2 hexane-isopropanol (v/v), and the upper phase was removed and dried under argon. The samples were then reconstituted in 3:2 hexane-isopropanol (v/v) and separated by silica gel TLC using hexane-diethyl ether-acetic acid (70:29:1). Products were analyzed on a Typhoon 9400 phospho imaging system (Amersham-GE Healthcare).

RESULTS

To create fluorogenic, real-time kinetic assays for EL, we focused on Bodipy-labeled substrates, both for their favorable fluorescent properties and because of previously published success with Bodipy substrates for phospholipases (12). **Figure 1** shows the chemical structures of the specific substrates used in this work: bis-BD-PC, labeled with Bodipy on both the *sn*-1 and *sn*-2 free fatty acid positions, as the fluorescent phospholipid substrate, and mono-BD-TG, labeled on either the *sn*-1 or *sn*-2 free fatty acid position, as the fluorescent triglyceride substrate (Fig. 1A). Because EL is known to possess phospholipase and triglyceride lipase activities (4), detergent micelles and synthetic HDL particles were prepared using each substrate (see Materials and Methods). The principle of the assays relies on the fact that when BD substrates are packed into micelles or particles, they self-quench the fluorescence of neighboring BD moieties in close proximity; therefore, there will be low initial background fluorescence (Fig. 1B). Fluorescence is revealed only after lipase hydrolysis of BD-PC or BD-TG and the resulting BD-labeled product is released. Therefore, fluorescence intensity over time is a direct kinetic readout of lipase hydrolysis.

Cellular expression of EL correlates with fluorescence activity

Jin et al. (17) and Hirata et al. (19) have shown that TNF-α specifically induces EL expression in cultured HUVECs and that the lipase activity in postheparin medium obtained after such stimulation was contributed solely by EL. To obtain EL for our studies, HUVECs were also stimulated with 10 ng/ml TNF-α for 24 h, and then cells were treated with 10 U/ml heparin for 30 min (37°C) to release heparin sulfate-associated EL. Western blot analysis of the postheparin medium using a monoclonal antibody to EL (17) is shown in **Fig. 2**. Although cultured HUVECs express EL (Fig. 2A, −TNF-α), TNF-α treatment significantly increased EL protein expression (Fig. 2A, +TNF-α). The induced EL protein migrated at 68 kDa in denaturing conditions, consistent with published data (17).

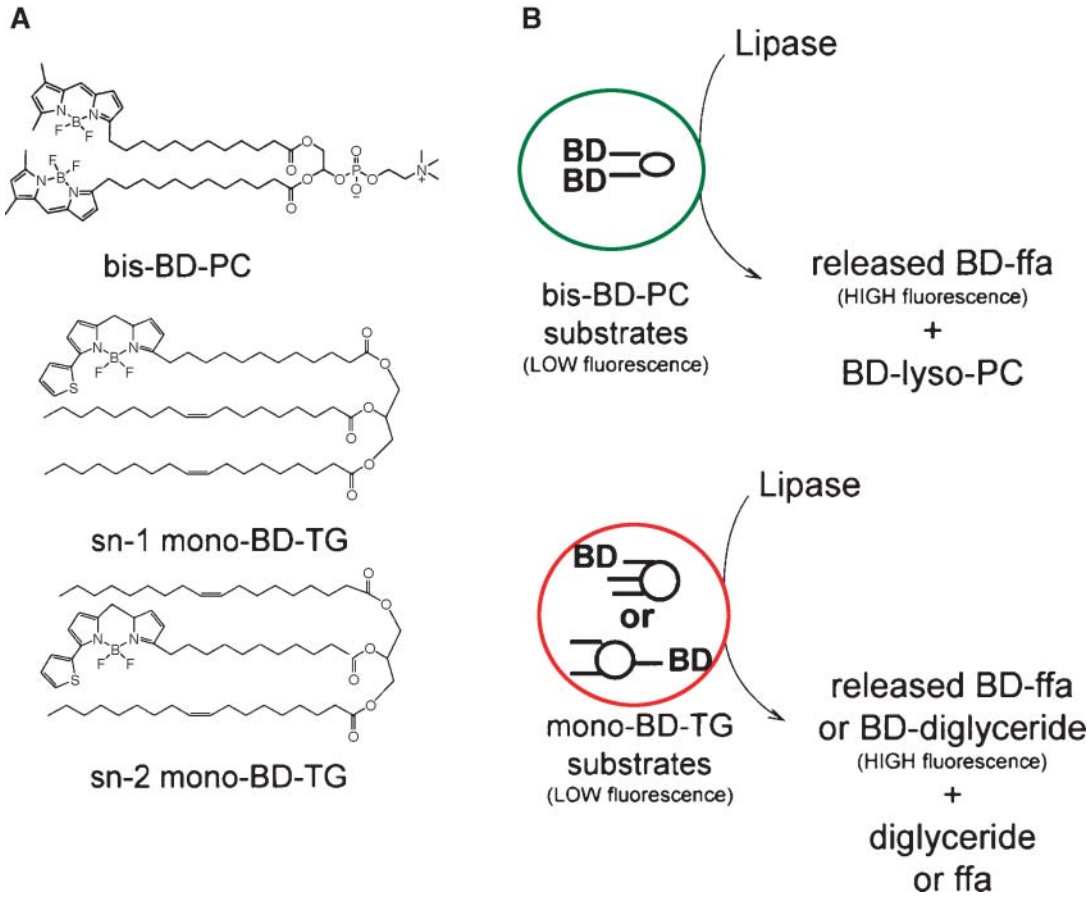

Fig. 1. Structures of fluorescent substrates and schematic diagram of lipase assays. A: Structures of fluorescent bis-Bodipy-C11-phosphatidylcholine [bis-BD-PC (Molecular Probes)] and mono-labeled-Bodipy-triglyceride (mono-BD-TG) (synthesis described in Materials and Methods) substrates. Bis-BD-PC is dual-labeled in the *sn*-1 and *sn*-2 positions, and mono-BD-TG is labeled in either the *sn*-1 or *sn*-2 position. B: Assay schemes. The assay relies on BD-lipid substrates being packed into micelles or synthetic HDL particles, which then self-quench the fluorescence of neighboring BD moieties from other BD-lipids in close proximity, thus exhibiting low initial background fluorescence. Fluorescence is thus revealed only after release of Bodipy-labeled free fatty acid (BD-ffa) or BD-diglyceride moieties by lipase hydrolysis of bis-BD-PC or mono-BD-TG substrates. In addition, lyso-BD-PC could also contribute to the observed fluorescence. Therefore, fluorescence intensity is a direct readout of lipase hydrolysis and can be kinetically observed over time.

We tested EL phospholipase activity in the postheparin cell medium by measuring the fluorogenic hydrolysis of bis-BD-PC. TNF-α treatment, which induced EL protein expression, led to a significant increase in phospholipase activity in two separate enzyme preparations (Fig. 2B). TLC of the final reaction mixture showed significant hydrolysis of the *sn*-1,*sn*-2-bis-BD-PC substrate by EL (Fig. 2C) and showed no significant hydrolysis when EL was tested against single-labeled, *sn*-2-BD-PC (data not shown). In addition, no activity was observed when the assay was performed in the presence of 1 M NaCl (data not shown), consistent with others (4) who demonstrated that high salt inhibits EL activity. Therefore, cellular EL protein expression correlates with the hydrolysis of fluorescent bis-BD-PC micelles, strongly supporting the notion that the activity measured is contributed solely by soluble EL. Such EL prepared from TNF-α-stimulated, postheparin HUVECs will subsequently be referred to as HUVEC-EL.

To determine whether the substrates and assays that we used accurately recapitulate the known substrate preferences of EL and lipoprotein lipase, we compared the hydrolytic activity of both of these enzymes toward both of the fluorogenic substrates. The results, shown in **Table 1**, confirmed that compared with LPL, HUVEC-EL is predominantly a phospholipase. When bis-BD-PC and mono-BD-TG micelle substrates were used, HUVEC-EL had 60 times more activity on bis-BD-PC substrates than on mono-BD-TG substrates. In contrast, LPL, which is known to be selective for triglycerides, had more activity on mono-BD-TG substrates. These data are in agreement with previous studies on EL and LPL (4).

Optimization of EL fluorescence assays for the 96-well plate format

To determine the optimal enzyme concentration for linear reaction kinetics, HUVEC-EL was titrated in assays using Triton X-100 bis-BD-PC micelles (**Fig. 3A**). There was a dose-dependent increase in fluorescence with increasing amounts of HUVEC-EL. At each HUVEC-EL concentration used, the activity yielded linear kinetics for the first 10–15 min, with 0.125× EL producing a 10-fold increase in fluorescence after 120 min (undiluted HUVEC concen-

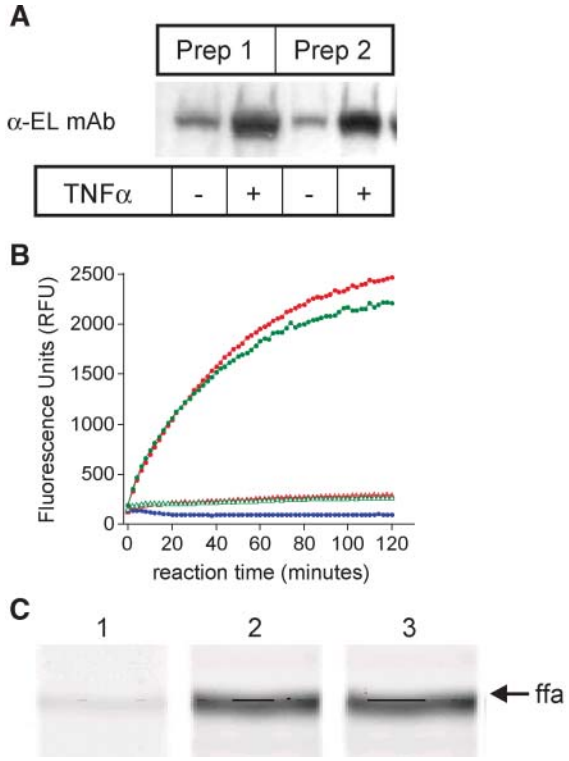

Fig. 2. Endothelial lipase (EL) protein expression correlates with measured fluorescence activity. A: Endogenous, cellular EL was induced by tumor necrosis factor-α (TNF-α) stimulation. EL was obtained from medium of cultured human umbilical vein endothelial cells (HUVECs) after stimulating cells with 10 ng/ml TNF-α for 24 h at 37°C. The addition of heparin (10 U/ml, 30 min at 37°C) released EL into the medium, which was collected, concentrated (200-fold), and stored at −20°C. Western blot analysis was performed on the postheparin medium using a monoclonal antibody (mAb) to human EL (17). Shown are two separate preparations of heparin-treated HUVEC medium, plus and minus TNF-α treatment. B: Human umbilical vein endothelial cell-derived endothelial lipase (HUVEC-EL) activity on micelles correlates with EL protein expression. HUVEC-EL phospholipase activity in postheparin medium was measured by incubating medium with bis-BD-PC Triton X-100 micelles. The increase in EL protein expression in medium after TNF-α treatment correlated with a significant increase in the 490/520 nm fluorescence. This assay represents one of three independent assays yielding identical results: bis-BD-PC micelles only (blue circles); micelles plus heparin-treated HUVEC medium (preparation 1, red triangles; preparation 2, green triangles); and micelles plus heparin-treated TNF-α-stimulated HUVEC medium (preparation 1, red circles; preparation 2, green circles). RFU, relative fluorescence units. C: HUVEC-EL produced BD-ffa. After incubating bis-BD-PC micelles with HUVEC-EL for 2 h at 25°C, TLC was performed on the reaction products using a hexane-diethyl ether-acetic acid (70:29:1) system that separates phospholipids and BD-ffas. Shown are assays using bis-BD-PC (*sn*-1 and *sn*-2 BD-labeled). Lane 1, bis-BD-PC micelles only; lane 2, micelles plus TNF-α-stimulated HUVEC medium (preparation 1); lane 3, micelles plus TNF-α-treated medium (preparation 2). All assays are representatives of at least two independent experiments.

trated medium equals 1×). To determine the optimal substrate concentration, bis-BD-PC was titrated into the assay using a constant HUVEC-EL concentration (0.125×). As more bis-BD-PC substrate was added, more 490/520 nm fluorescence was obtained, with similar initial rates for each concentration of substrate used (Fig. 3B). For the remaining studies, 1 μM bis-BD-PC and 0.125× HUVEC-EL were selected as the optimal concentrations for measuring EL phospholipase activity in Triton X-100 micelles. TLC confirmed that the increase in fluorescence directly correlated with the formation of BD-ffas (data not shown). Because EL also possesses triglyceride lipase activity (4), we tested whether mono-BD-TG micelles would serve as EL substrates. EL dose-dependently hydrolyzed mono-BD-TG (both *sn*-1- and *sn*-2-labeled) Triton X-100 micelles; however, 20 times more mono-BD-TG substrate (20 μM) was needed to obtain a similar increase in fluorescence to that with bis-BD-PC (data not shown). As described previously for EL (4), both phospholipase and triglyceride lipase activities of HUVEC-EL were inhibited by 1 M NaCl using both bis-BD-PC and mono-BD-TG substrates (data not shown). These rapid, sensitive, homogeneous HUVEC-EL assays are thus capable of kinetically measuring EL phospholipase and triglyceride lipase activities. With both BD-labeled lipids, initial background fluorescence was low, and upon addition of HUVEC-EL, each assay had a significant increase in fluorescence.

TABLE 1. EL preferentially cleaves bis-BD-PC and LPL cleaves mono-BD-TG substrates

Enzyme	Bis-BD-PC (490/520 nm)	Mono-BD-TG (538/568 nm)	PC/TG Ratio
EL	3,519 ± 350	573 ± 137	62
LPL	443 ± 59	4,093 ± 283	0.11
None	85 ± 22	187 ± 80	—

Bis-BD-PC, bis-labeled-Bodipy-phosphatidycholine; EL, endothelial lipase; HUVEC-EL, human umbilical vein endothelial cell-derived endothelial lipase; mono-BD-TG, mono-labeled-Bodipy-triglyceride. HUVEC-EL preferentially cleaves fluorescent phospholipid substrate and LPL fluorescent triglyceride substrate. Fluorescent phospholipid or triglyceride hydrolysis was monitored in EL assays containing either bis-BD-PC or mono-BD-TG substrate micelles. Each fluorogenic lipid was reconstituted into Triton-X-100 micelles and incubated at 25°C with either 30 μl of HUVEC-EL (0.1× HUVEC-EL for bis-BD-PC and 1× HUVEC-EL for mono-BD-TG) or 20 μl of LPL (1×) for 2 h, monitoring the 490/520 nm (PC) or 538/568 nm (TG) fluorescence every minute using a SpectraMax Genimi XS fluorescent plate reader (Molecular Probes). Values are averages of three separate experiments ± SD. PC/TG ratios were determined after normalizing the lipase activity per volume of enzyme used in both assays. None indicates assays in which no HUVEC-EL or LPL enzyme was present and thus represents the basal fluorescence of the substrates.

Optimization of HUVEC-EL fluorescence assays for the ultra-high-throughput (3,456-well) nanoplate format

Given that HDL-C levels appear to be influenced by EL activity, it is possible that inhibitors of EL might be novel therapeutics for increasing HDL-C. Therefore, we optimized our HUVEC-EL fluorescence assays for ultra-high-throughput screening formats. EL fluorescence assays were run in a total volume of 1 μl with 1 μM bis-BD-PC Triton X-100 micelles and HUVEC-EL (1× and 0.5×) on 3,456-well nanoplates for different amounts of time. Even at this low-volume, high-throughput format, HUVEC-EL has a reproducible 5-fold increase in fluorescence at 60 min (**Fig. 4A**). To mimic a real ultra-high-throughput

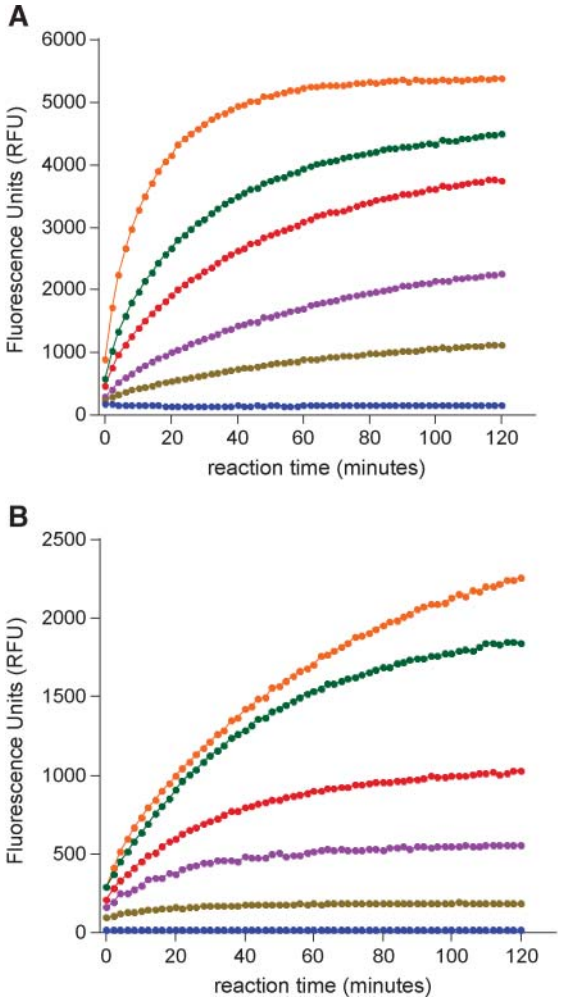

Fig. 3. Optimization of HUVEC-EL fluorescence assays for the 96-well plate format. A: Increasing enzyme volume increased observed fluorescence. HUVEC-EL was titrated in assays using Triton X-100 bis-BD-PC micelles (1 μM bis-BD-PC). A dose-dependent increase in fluorescence was observed with increasing amounts of HUVEC-EL, with linear kinetics for the first 10–15 min for each dilution of enzyme tested. A 0.125× dilution of HUVEC-EL medium yielded linear fluorescence up to 30 min and a 10-fold window after 2 h (undiluted TNF-α-stimulated HUVEC medium equals 1× HUVEC-EL). Dilution of heparin-treated cell medium is as follows: 30 μl of 1× (orange circles), 0.5× (green circles), 0.25× (red circles), 0.125× (purple circles), 0.063× (yellow circles), no medium (blue circles; bis-BD-PC micelles only). RFU, relative fluorescence units. B: Increasing fluorescent substrate increased fluorescence. Bis-BD-PC substrate concentration was titrated into a constant concentration of Triton X-100 micelles from 1 to 0.063 μM, then assays were performed using 0.125× HUVEC-EL. A concentration of 1 μM bis-BD-PC substrate was optimal for measuring EL phospholipase activity in Triton X-100 micelles, yielding linear activity between 0 and 15 min [1 μM, orange circles; 0.5 μM, green circles; 0.25 μM, red circles; 0.125 μM, purple circles; 0.063 μM, yellow circles; 1 μM substrate only, blue circles (no enzyme)].

screen, 3,456 nanoplates containing DMSO or 1× or 0.5× HUVEC-EL concentrations were tested in screening mode. The 0.5× HUVEC-EL concentrations were added here to resemble and observe 50% inhibition of EL activity. The overall results show a significant assay window, with a signal-to-baseline ratio of 9.3 and a confidence coefficient well suited for ultra-high-throughput screening ($Z' = 0.84$). These data clearly demonstrate the advantage and sensitivity of using fluorescent lipid substrates in miniaturized lipolytic assays.

Synthetic, fluorescent HDL particles are substrates for HUVEC-EL, even in the presence of serum

For drug discovery, native substrates are the preferred substrates to use; therefore, we reconstructed synthetic HDL particles containing fluorescent BD-lipids to closely resemble authentic human HDL. Synthetic human HDL particles were prepared by combining bis-BD-PC or mono-BD-TG with purified human apo HDL protein (see Materials and Methods). The resulting synthetic particles were purified by ultracentrifugation in the standard HDL density range (1.063–1.21 g/ml). Particles made with bis-BD-PC as the phospholipid were sensitive substrates for HUVEC-EL (**Fig. 5A**). There was a dose-dependent increase in fluorescence with increasing HUVEC-EL concentrations. Synthetic particles prepared with *sn*-1-labeled mono-BD-TG were also good substrates for HUVEC-EL, as shown in Fig. 5B. Although a dose-dependent increase in fluorescence was obtained with increasing HUVEC-EL concentrations, >10 times more HDL was required in assays using mono-BD-TG (4.3 μg of total protein) compared with assays performed with bis-BD-PC (0.4 μg total protein), consistent with the known substrate preference of this enzyme. TLC analysis showed that mono-BD-TG HDL particles resulted in HUVEC-EL-dependent hydrolysis of the fluorescent triglyceride, resulting in the formation of BD-ffas or BD-diglycerides attributable to hydrolysis at the *sn*-1 position (Fig. 5C). Mono-BD-TG HDL particles were also sensitive substrates for human and bovine LPL and human HL (data not shown).

In accordance with previous studies (4, 20), LPL activity on mono-BD-TG synthetic HDL particles was sensitive to 1 M NaCl and dependent on apoC-II (data not shown). We then tested the use of these particles in HUVEC-EL assays containing serum. Triton X-100 micelles and synthetic HDL particles were tested as substrates in the presence of 30% heat-inactivated mouse serum. Figure 5D shows the HUVEC-EL activity obtained from mono-BD-TG in micelles (red and blue circles) and in HDL particles (purple and green circles). Surprisingly, HUVEC-EL had significant catalytic activity in the presence of serum (green circles) when using the HDL particles but not when using the mono-BD-TG micelles. After 2 h, the total fluorescence change attributable to HUVEC-EL addition was 10-fold using HDL particles (purple vs. green circles). Thus, the presentation of mono-BD-TG in the HDL particle appears to be critical because HUVEC-EL had no activity on micelles containing the identical substrate in the presence of serum. These data contrast with the data of McCoy et al. (4), who used emulsions of triolein and egg phosphatidylcholine containing radiolabeled glycerol trioleate and found that 5% human (or mouse) serum completely inhibited EL triglyceride lipase activity. With the mono-BD-TG particles, a noticeable lag occurred during the first 5 min of the assay attributable to serum, followed by linear kinetics for ~40 min. For unknown reasons, HUVEC-EL had no activity on bis-BD-PC

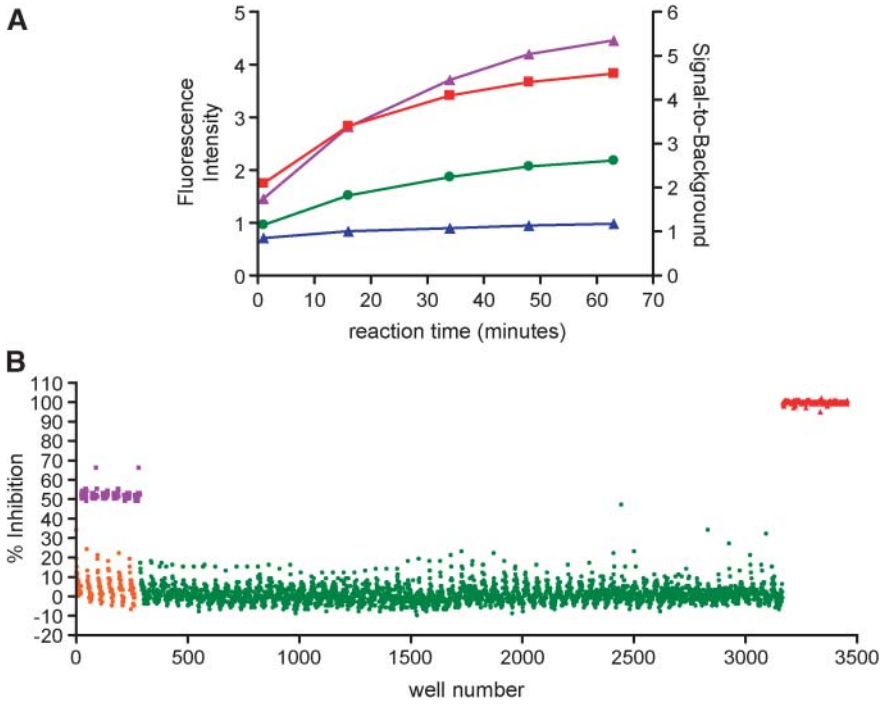

Fig. 4. Optimization of HUVEC-EL fluorescence assays for the ultra-high-throughput 3,456-well nanoplate screening format. A: On 3,456-well nanoplates, HUVEC-EL fluorescence assays were run with 1 μM bis-BD-PC Triton X-100 micelles. At different time points, the plates were read using a topology-compensated plate reader (Aurora Discovery). The 1× (red squares), 0.5× (green circles), and no enzyme (blue triangles) graphs are shown, along with the signal-to-background ratio (purple triangles) at 1× HUVEC-EL. A reproducible 5-fold increase in fluorescence (with small standard deviations) was obtained using 1× enzyme. B: The 3,456-well nanoplates containing DMSO or different HUVEC-EL concentrations were tested in ultra-high-throughput screening mode for 1 h. The results yielded significance and confidence for ultra-high-throughput screening of inhibitors to EL ($Z' = 0.84$, signal-to-baseline ratio = 9.3). Green circles represent the area of the plate where test samples were added (instead, this graph shows DMSO [0.5%]), yellow circles represent the area of the plate where the reaction negative control was added (no inhibitor), purple squares represent the area where 0.5× HUVEC-EL was added, and red triangles represent the area of the plate where no enzyme was added (100% inhibition).

HDL particles (data not shown). Overall, these data strongly suggest that how the substrate is presented to EL may be a critical factor when measuring EL activity in serum.

EL has significant lipolytic activity on mono-BD-TG HDL particles in high concentrations of animal serum

To test the effects of other animal sera on HUVEC-EL activity, human, mouse, and lipoprotein-deficient bovine sera were heat-inactivated (56°C, 1 h) and diluted to different concentrations. HUVEC-EL triglyceride lipase activity was then determined using *sn*-1-labeled mono-BD-TG HDL particles in the presence of increasing concentrations of serum. Although HUVEC-EL had significant activity at each serum concentration tested, serum had a dose-dependent decrease in triglyceride lipolysis over time, with human and lipoprotein-deficient serum having the greatest effects (**Fig. 6A, C**). Interestingly, mouse serum did not have as a dramatic effect on HUVEC-EL activity (Fig. 6B), and in high concentrations (60%), HUVEC-EL yielded a 5-fold increase in fluorescence after 2 h. High concentrations (60%) of human serum resulted in a 1.5-fold increase in fluorescence after 2 h (Fig. 6A), and extending the reaction time up to 18 h led to a 6-fold increase in fluorescence (data not shown). Assays in each serum resulted in a lag during the first 5 min of the assay. The lag, however, did not appear to be dependent on the concentration of serum; a lag in 10% serum was indistinguishable from that observed in 60% serum. The triglyceride lipase activities of human LPL and HL were also measurable in high concentrations of human and mouse serum using mono-BD-TG HDL particles (data not shown). To confirm the catalytic cleavage of fluorescent substrates by HUVEC-EL in human serum, TLC was performed on reaction mixtures after 2 h. Figure 6D shows that addition of HUVEC-EL to the reaction resulted in catalytic cleavage of the substrate and the formation of BD-ffas or BD-diglycerides, consistent with the observed increase in fluorescence (Fig. 6A, orange circles).

Dual-labeled (mono-BD-TG and [^{14}C]PC) synthetic HDL particles can be used to simultaneously observe HUVEC-EL phospholipase and triglyceride lipase activities

Because EL has phospholipase and triglyceride lipase activities, substrates containing phospholipids and tri-

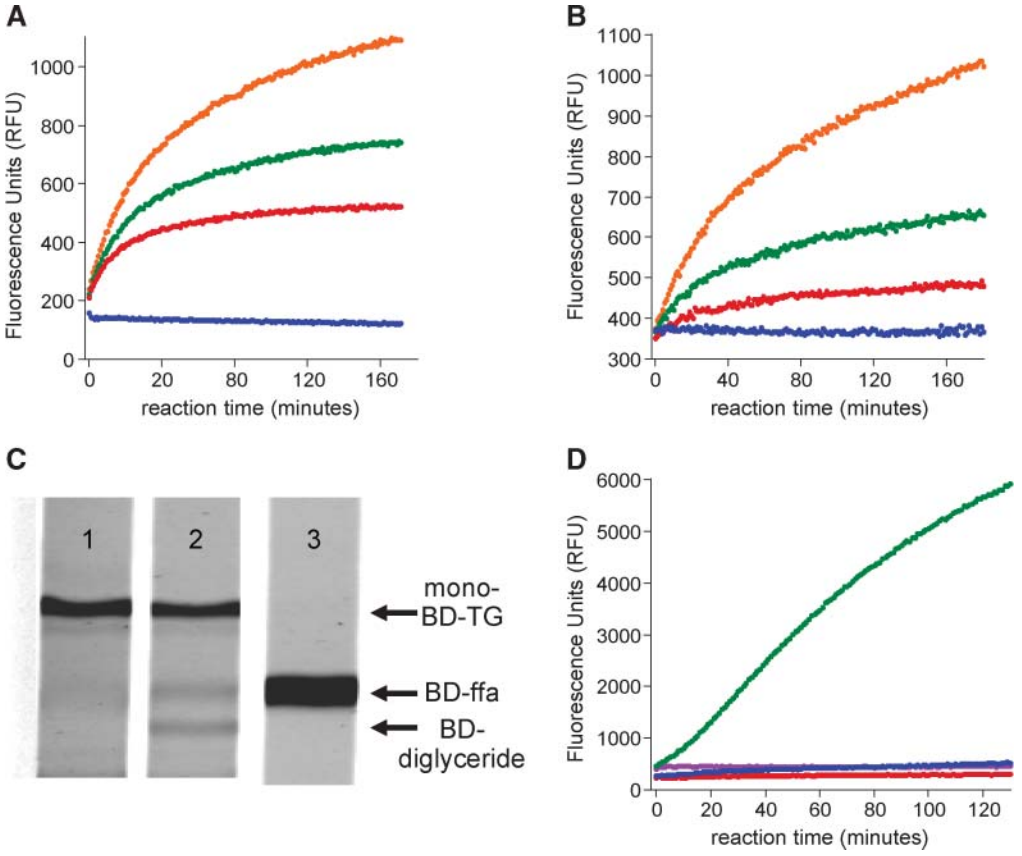

Fig. 5. Synthetic, fluorescent HDL particles are substrates for EL even in the presence of mouse serum. A, B: Synthetic HDL particles containing either bis-BD-PC or *sn*-1-labeled mono-BD-TG were generated as described in Materials and Methods and used in EL fluorescence assays. HUVEC-EL was titrated (based on volume added into the assay) on both synthetic HDL particles [0.4 μg of bis-BD-PC particles (A); 4.3 μg of mono-BD-TG particles (B)]. Although similar activities were obtained using both substrates, 10 times more mono-BD-TG particles (4.3 μg of protein) was needed than bis-BD-PC particles (0.4 μg of protein), reiterating the fact that EL prefers phospholipid substrates. Symbols are as follows: 20 μl (orange circles), 10 μl (green circles), and 5 μl (red circles) of 1× HUVEC-EL. RFU, relative fluorescence units. C: After 2.5 h at 25°C, TLC of the mono-TG synthetic HDL particles treated with HUVEC-EL was performed on the extracted reaction products using a hexane-diethyl ether-acetic acid (70:29:1) separation system. Lane 1, mono-BD-TG particles only (no enzyme); lane 2, mono-BD-TG particles plus 20 μl of 1× HUVEC-EL; lane 3, a standard C11-BD-free fatty acid. Arrows designate mono-BD-TG, BD-ffa, and BD-diglyceride moieties. D: Mono-BD-TG micelles and particles were tested in EL assays in the presence of freshly prepared 30% heat-inactivated (56°C, 1 h) C57BL/6 mouse serum. Mono-BD-TG Triton X-100 micelles incubated in 30% serum with (blue circles) and without (red circles) HUVEC-EL (50 μl of 1×) both yielded low fluorescence. Synthetic mono-BD-TG HDL particles were incubated in 30% mouse serum with (green circles) and without (purple circles) HUVEC-EL and yielded significantly different fluorescence. Although serum caused a short lag at the beginning of the assay, linear kinetics was obtained up to 1 h at 25°C, and a 14-fold increase in fluorescence was obtained at the end of the assay (2 h).

glycerides were created to observe simultaneous lipolysis in the same reaction. Synthetic HDL particles containing *sn*-2-labeled mono-BD-TG and [^{14}C]PC were generated as described in Materials and Methods. To observe HUVEC-EL triglyceride lipase activity, reactions were first monitored for fluorescence, which directly measured mono-BD-TG hydrolysis (**Fig. 7A**). As shown, HUVEC-EL caused a significant increase in fluorescence, yielding a 10-fold assay window. Then, to observe HUVEC-EL phospholipase activity, reaction products were extracted and separated by TLC. Two sequential scans of fluorescence (Fig. 7B, lanes 1, 2) and radioactivity (Fig. 7B, lanes 3, 4) were performed. The fluorescence scan shows HUVEC-EL-dependent formation of BD-diglycerides from *sn*-2-labeled mono-BD-TG substrates within the particles, which confirms the observed fluorescence measured kinetically (Fig. 7A). Because *sn*-2-labeled mono-BD-TG was used here, only BD-diglycerides are produced and no BD-ffas are produced. These data show that both *sn*-1- and *sn*-2-labeled substrates could be used to measure lipase activity. HUVEC-EL phospholipase activity was revealed in the radiolabeled scan of the TLC plates (Fig. 7B, lanes 3, 4), which shows HUVEC-EL-dependent formation of radiolabeled free fatty acids. Thus, using dual-labeled particle

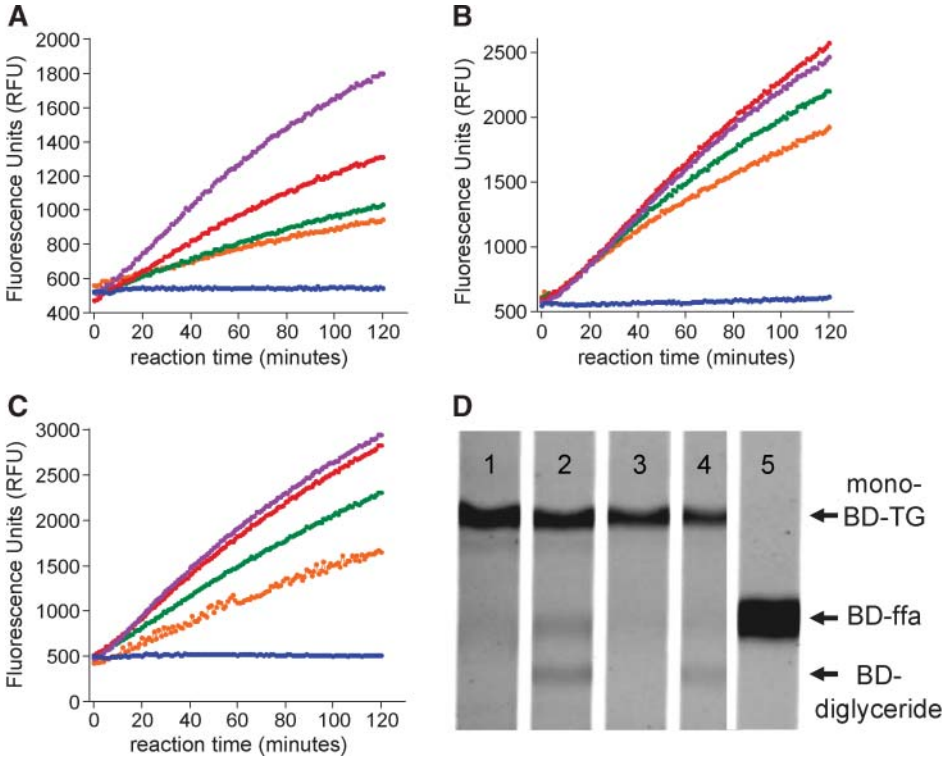

Fig. 6. HUVEC-EL has significant lipolytic activity on *sn*-1-labeled mono-BD-TG HDL particles in high concentrations of animal serum. A–C: HUVEC-EL fluorescence assays were performed in freshly prepared heat-inactivated (56°C, 1 h) human (A), mouse (B), and lipoprotein-deficient bovine (C) sera using mono-BD-TG particles as substrates at 25°C for the indicated times. HUVEC-EL yielded significant fluorescence activity at high concentrations of animal sera (60%). Animal sera concentrations in the final assay were 60% (purple circles), 40% (red circles), 20% (green circles), and 10% (orange circles); mono-BD-TG particles in 60% serum (no added HUVEC-EL) was included as a negative control (blue circles). RFU, relative fluorescence units. D: After 2 h, assay products from 40% human serum were extracted and observed by TLC using a hexane-diethyl ether-acetic acid (70:29:1) separation system. Arrows designate cleavage of the mono-BD-TG substrate into BD-ffas and BD-diglycerides. Lane 1, mono-BD-TG HDL particles only (no enzyme; no serum); lane 2, HDL particles plus 50 μl of 1× HUVEC-EL (no serum); lane 3, HDL particles plus 40% human serum (no enzyme); lane 4, HDL particles plus HUVEC-EL plus 40% human serum; lane 5, BD-ffa standard.

substrates, EL phospholipase and triglyceride lipase activities can be simultaneously measured in the same reaction. Replacing the [^{14}C]PC with a different fluorescence-PC substrate may allow an even more robust and high-throughput fluorescent assay of both EL activities. Such dual-labeled substrates may then allow a direct characterization of substrate-specific modulators of EL.

DISCUSSION

We describe fluorescent BD-labeled substrates that can be used in homogeneous, high-throughput kinetic assays that measure the phospholipase and triglyceride lipase activities of EL. The same assays can also be used to measure the hydrolytic activity of lipoprotein lipase. Triton X-100 detergent micelles containing either bis-BD-PC or mono-BD-TG are efficient EL substrates that yield a linear increase in fluorescence, resulting in a significant assay window. In addition, the bis-BD-TG micelle assay was so sensitive and robust that it could be miniaturized to an ultra-high-throughput (3,456-well) format (Fig. 4). Furthermore, the mono-BD-TG fluorogenic substrate could also be used to monitor lipase activity after incorporation into synthetic HDL particles.

Lipases are known to function on the surface of vascular endothelial cells in contact with the plasma compartment. However, it was reported previously that EL phospholipase and triglyceride lipase activity could not be measured in vitro in the presence of animal serum at concentrations of >5% (4). Therefore, it was suggested that serum may contain an inhibitory factor that regulates EL activity. An alternative explanation would be that the substrates, once mixed with serum, took on a physical state that did not allow catalysis to occur. It is well known that lipase enzyme activity is sensitive to substrate-enzyme interfaces (21, 22). Our data are consistent with this hypothesis; specifically, we found that EL activity could be detected in the presence of serum when the fluorogenic substrate mono-BD-TG was presented in synthetic HDL particles but not when it was presented in Triton X-100 micelles. Because mouse serum did not significantly inhibit EL activity

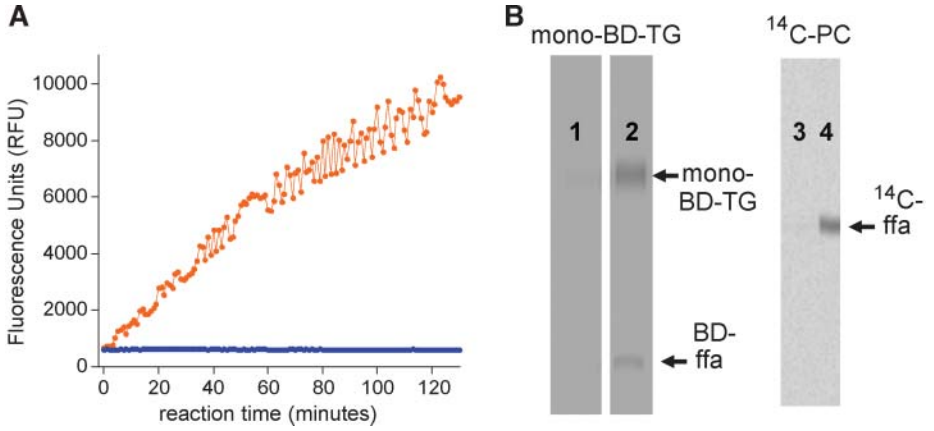

Fig. 7. Dual-labeled (mono-BD-TG and [^{14}C]PC) synthetic HDL particles were used to simultaneously observe EL phospholipase and triglyceride lipase activities. Synthetic HDL particles containing both *sn*-2-labeled mono-BD-TG and *sn*-1-labeled [^{14}C]PC were generated as described in Materials and Methods. A: Approximately 4.3 ng of HDL was incubated with 1× HUVEC-EL, the 538/568 nm fluorescence was observed for 2.5 h (measures fluorescent mono-BD-TG hydrolysis), and then TLC of the reaction products was performed using a hexane-diethyl ether-acetic acid (70:29:1) separation system. RFU, relative fluorescence units. B: Cleavage of both fluorescent (mono-BD-TG) and radiolabeled ([^{14}C]PC) substrates was observed by sequentially scanning the TLC plates for fluorescence and radioactivity. Arrows designate fluorescent and radiolabeled free fatty acids produced by HUVEC-EL. Fluorescence scans are shown in lanes 1 and 2, and radiolabeled scans are shown in lanes 3 and 4. Lane 1, dual-labeled particles only (no enzyme); lane 2, dual-labeled particles plus 1× HUVEC-EL; lane 3, dual-labeled particles only (no enzyme); lane 4, dual-labeled particles plus 1× HUVEC-EL.

(Fig. 6B), our data do not support the presence of serum inhibitory regulators of EL; rather, they suggest that substrate presentation is critical to obtaining EL activity. The assays we describe allow the detection of EL activity in the presence of serum, which may have many uses, including the determination of the serum dependence of inhibitors and the possible development of assays of EL activity in postheparin plasma.

EL activity is a critical determinant for HDL-C levels in vivo (1–3). We describe novel substrates that can be formatted into assays that can measure HUVEC-EL hydrolytic activity and aid in the discovery of small molecule inhibitors that target EL. Such inhibitors could then be tested to determine whether EL inhibition in humans is beneficial to patients with coronary artery disease. In addition, these novel substrates are convenient counterscreening substrates for LPL and HL activities.

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