Characterization of the lipolytic activity of endothelial lipase

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Abstract **Endothelial lipase (EL) is a new member of the triglyceride lipase gene family previously reported to have phospholipase activity. Using radiolabeled lipid substrates, we characterized the lipolytic activity of this enzyme in comparison to lipoprotein lipase (LPL) and hepatic lipase (HL) using conditioned medium from cells infected with recombinant adenoviruses encoding each of the enzymes. In the absence of serum, EL had clearly detectable triglyceride lipase activity. Both the triglyceride lipase and phospholipase activities of EL were inhibited in a dose-dependent fashion by the addition of serum. The ratio of triglyceride lipase to phospholipase activity of EL was 0.65, compared with ratios of 24.1 for HL and 139.9 for LPL, placing EL at the opposite end of the lipolytic spectrum from LPL. Neither lipase activity of EL was influenced by the addition of apolipoprotein C-II (apoC-II), indicating that EL, like HL, does not require apoC-II for activation. Like LPL but not HL, both lipase activities of EL were inhibited by 1 M NaCl. The relative ability of EL, versus HL and LPL, to hydrolyze lipids in isolated lipoprotein fractions was also examined using generation of FFAs as an end point. As expected, based on the relative triglyceride lipase activities of the three enzymes, the triglyceride-rich lipoproteins, chylomicrons, VLDL, and IDL, were efficiently hydrolyzed by LPL and HL. EL hydrolyzed HDL more efficiently than the other lipoprotein fractions, and LDL was a poor substrate for all of the enzymes.**—McCoy, M. G., G-S. Sun, D. Marchadier, C. Maugeais, J. M. Glick, and D. J. Rader. **Characterization of the lipolytic activity of endothelial lipase.** ***J. Lipid Res.*** **2002.** 43: **921–929.**

Supplementary key words lipoprotein lipase • hepatic lipase • triglyceride lipase family • phospholipase • apoC-II • heparin

The triglyceride lipase gene family plays a central role in dietary fat absorption, energy homeostasis, and plasma lipoprotein metabolism (1, 2). Its members include pancreatic lipase, lipoprotein lipase (LPL), and hepatic lipase (HL). The latter two enzymes function in the plasma compartment and are critical to the metabolism of lipids carried on plasma lipoproteins (3). We recently reported the cloning of a new member of this gene family (4) that we termed endothelial lipase (EL) due to the fact that it is synthesized in endothelial cells, a characteristic that distinguishes it from the other members of the family. This same gene was independently cloned by another group through differential display using endothelial cells undergoing microtubule formation (5). Like LPL and HL, EL also functions in the plasma compartment, and our initial report showed that overexpression of EL in mice profoundly altered plasma lipoprotein levels. The putative heparin-binding (6–8) and hydrophobic regions implicated in lipid or lipoprotein binding (9–12) that are present in LPL and HL are highly conserved in EL, suggesting that EL, like LPL and HL, is anchored to the luminal endothelial surface via heparan sulfate proteoglycans where it can interact directly with lipoproteins. Initial reports indicated that EL had phospholipase activity (4, 5) but the enzymatic activity was not characterized further.

The purpose of the present study was to systematically assess the lipolytic activity of EL toward a variety of synthetic and lipoprotein substrates to compare it directly to LPL and HL. Because we have not been able to purify EL in an active form, conditioned media from cells expressing each of the lipases were used as the source of enzymes. We demonstrated that EL has clearly measurable triglyceride lipase activity; however, the ratio of triglyceride lipase to phospholipase activity of EL is markedly less than both LPL and HL. EL does not require apolipoprotein C-II (apoC-II) for activity and, in fact, is inhibited by the addition of serum. Like LPL and unlike HL, the lipase activity of EL is inhibited by 1 M NaCl. An evaluation of the lipolytic activity of EL against isolated lipoprotein fractions showed that the preferred substrate for EL is the HDL fraction.

Abbreviations: AdGFP, adenovirus encoding GFP; AdhEL, adenovirus encoding human endothelial lipase; AdhHL, adenovirus encoding human hepatic lipase; AdhLPL, adenovirus encoding human LPL; DPPC, dipalmitoylphosphatidylcholine; chylo, chylomicrons; EL, endothelial lipase; EC, esterified cholesterol; FC, free cholesterol; GFP, green fluorescent protein; HL, hepatic lipase; LPPC, lysopalmitoylphosphatidylcholine; PL, phospholipid; PLA2, phospholipase A2.

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METHODS

Expression of enzymes

EL, LPL, and HL were expressed using recombinant adenoviruses. Recombinant adenoviruses encoding either human EL (AdhEL), human HL (AdhHL), or GFP (AdGFP; used as a negative control in all experiments) were constructed as previously described (13). A recombinant adenovirus encoding human LPL (AdhLPL) was a generous gift from Dr. Nicolas Duverger (Aventis Pharmceuticals). Subconfluent COS cells, plated in 100 mm plates, were exposed to recombinant adenoviruses in 2 ml serum-free medium at a multiplicity of infection of 3,000 particles/cell. Two hours later, 8 ml of additional serum-free medium was added to the cells. After 48 h, heparin was added to a final concentration of 10 U/ml. The plates were swirled gently to mix the media and returned to the incubator for an additional 30 min. The media were then harvested and immediately aliquotted and frozen at −80°C. Analysis of the stability of the activity in the culture medium established that EL activity is stable at 4°C for at least 3 days and at −80°C for at least 6 months. HL and LPL activities were also stable at −80°C for at least 6 months. There was variation in the level of enzyme activity obtained in different preparations of conditioned media; however, direct comparisons of activities against different substrates were always done on the same preparation. Expression of enzymes was confirmed by Western blotting using standard methods. All antibodies were rabbit polyclonal antibodies; that against human EL, which was previously described (4), was generated to a peptide in the N-terminal region of EL. Antibody to rat HL that cross reacts with human HL was also previously described (14). A polyclonal antibody to human LPL was a generous gift from Dr. Ira Goldberg.

Lipase assays

For phospholipase and triglyceride lipase assays, conditioned medium containing LPL, HL, or EL was used as the enzyme source. Medium obtained from cells expressing GFP was always included as a control, and no lipase activity was detected in the control medium. When appropriate, heat-inactivated serum was added at concentrations indicated in the text. The amount of culture medium added was adjusted so that similar amounts of product were generated from each enzyme source and the amount of substrate hydrolyzed was <5% of the available substrate. The incubation times, which differ for the various substrates, were in the linear range for each assay. All enzyme activities are reported as specific activity based on volume of medium, namely as nmol FFA liberated/ml/h. Purified apoC-II (15) was a gift of Dr. Sissel Lund-Katz.

Triglyceride lipase activity was measured according to a modification of the method of Nilsson-Ehle and Schotz (16), which utilizes a glycerol-stabilized emulsion of triolein and egg phosphatidylcholine containing glycerol-tri[9,10(n)-^{3}H]oleate (3.30 mCi/mmol). The assay tubes contained, in a total volume of 0.3 ml, 0.05 M Tris-HCl, pH 8.0, 0.75% BSA, 3.4 mM triolein, approximately 250 μM phosphatidylcholine, NaCl at either 0.15 M or 1 M as indicated, and culture medium containing one of the various enzymes. Samples were incubated for 1 h at 37°C. Reactions were stopped and products were extracted by the method of Belfrage and Vaughan (17). The liberated fatty acids were quantitated by scintillation counting a 0.5 ml aliquot of the aqueous phase. Under these conditions, the partition coefficient of the oleic acid was approximately 0.4. Esterase activity against tributyrin was measured using the method of Shirai et al. (18).

Phospholipase activity was measured using a newly developed glycerol-stabilized emulsion, similar to that described above, that contained radiolabeled phospholipid and cholesteryl oleate as the neutral lipid core. Pilot experiments demonstrated that cholesteryl oleate is not a substrate for LPL, HL, or EL (data not shown). The emulsion of cholesteryl oleate (150 mg) and dipalmitoylphosphatidylcholine (DPPC) (8.88 mg unlabeled and 17.15 μCi [1,2-^{14}C]DPPC, 110 mCi/mmol) was prepared by sonication in 2.5 ml glycerol. The assay tubes contained, in a total volume of 0.3 ml, 0.05 M Tris-HCl, pH 8.0, 0.75% BSA, 4.6 mM cholesteryl oleate, 245 μM DPPC, 0.15 M NaCl, and culture medium as the enzyme source. Samples were incubated for 15 min at 37°C. For some experiments, a double-labeled substrate was prepared to monitor both phospholipase and triglyceride lipase activity in the same reaction. This substrate was a modification of the glycerol-stabilized triolein substrate described above, wherein DPPC was substituted for egg phosphatidylcholine, and radiolabeled [1,2-^{14}C]DPPC was included to provide a DPPC specific activity of 1.5 μCi/μmol. Reactions were stopped and products were extracted by the method of Belfrage and Vaughan (17) except that 100 μg lysopalmitoylphosphatidylcholine (LPPC) per ml was included as a carrier in the organic extraction mix. Each lipolytic event produces one molecule each of [^{14}C]palmitic acid and [^{14}C]LPPC of equal specific activity, and pilot studies have demonstrated that LPPC is not a substrate for these enzymes. Under these conditions, palmitic acid and LPPC partitioned into the aqueous phase with partition coefficients of 0.37 and 0.73, respectively. Products were quantitated by scintillation counting of a 0.5 ml aliquot of the aqueous phase. The mean of the individual partition coefficients (0.55) reflects the average partition of the two products and is used to calculate the moles of total product generated (FFA plus lysophosphatidylcholine). The amount of FFA produced is calculated by dividing the moles of product by two.

Fractionation of human serum

Human serum was heat-inactivated for 30 min at 56°C and subjected to ultracentrifugation in KBr at a density of 1.21 g/ml for 30 h at 4°C at 90,000 rpm in a Beckman TLA 100.2 rotor. The floating lipoprotein fraction was separated from the infranatant lipoprotein-deficient serum by tube slicing, and the two fractions were dialyzed extensively against PBS using dialysis tubing that retains molecules larger than approximately 3,500 Da.

Isolation of lipoprotein fractions

Human lipoproteins were isolated from pooled plasma samples from non-fasting, hypertriglyceridemic subjects as follows: Chylomicrons were isolated by centrifugation at d = 1.006 g/ml for 30 min at 20,000 rpm. VLDL (d > 1.006 g/ml), IDL (1.006 < d < 1.019 g/ml), LDL (1.019 < d < 1.063 g/ml), HDL (1.063 < d < 1.21 g/ml) were isolated by sequential ultracentrifugation at 39,000 rpm for 16 h in a Ti 70.1 Beckman rotor at 10°C. Lipoprotein fractions from fasting, normolipemic donors were isolated similarly. Insufficient chylomicrons or IDL were obtained from these donors for use in these experiments. Recovered fractions were dialyzed against PBS and stored at 4°C.

Lipase modification of fractionated lipoproteins

Isolated lipoprotein fractions were incubated with media containing lipases using a modification of the method of Bamberger et al. (19), and snake venom phospholipase A2 was used as a positive control. Each reaction tube contained 1.25 mM lipoprotein phospholipid, 40 μl conditioned medium containing one of the various enzymes, 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 8 mM $CaCl_2$, and 1% BSA, in a final volume of 100 μl. Tubes were incubated for 4 h at 37°C. Pilot studies indicated that hydrolysis was complete by 4 h (data not shown). At the end of 4 h, EDTA was added to a final concentration of 25 mM. Although this was done

for all assay tubes for the sake of uniformity, it was necessary only for stopping the snake venom phospholipase reaction, as the other enzymes do not depend on calcium. The released FFAs were measured enzymatically using a commercially available kit (NEFA C, Wako Pure Chemical Industries, Osaka, Japan). Data are presented as nmol of FFA produced in each reaction tube.

Protein and lipid analyses

Protein concentrations were determined with the BCA reagent according to the manufacturer's instructions (Pierce, Rockford, IL). Lipid concentrations (free and total cholesterol, triglyceride, and phospholipid) were measured using commercially available assay kits (Wako Pure Chemical Industries).

RESULTS

Endothelial lipase has triglyceride lipase activity

The initial publications on EL (4, 5) suggested that it is primarily a phospholipase. Our data indicated that EL had a very low level of triglyceride lipase activity (4), and another group reported that EL had no triglyceride lipase activity (5). Both laboratories had used triglyceride lipase assays that were developed for the assay of LPL activity and thus contained serum as a source of apoC-II (16, 20). We reexamined the ability of conditioned medium containing EL to hydrolyze triglycerides using two different triglyceride substrates, tributyrin and triolein, both in the absence of serum. The data in **Fig. 1**, shown as nanomoles of FFA produced per milliliter of conditioned medium, indicate that the activity of EL toward the two substrates is quite similar. The similarity of the data is not due to low signal. The actual counts obtained in the two assays were substantial. Signal above background was 6,001 ± 248 cpm for the triolein assay and 1,375 ± 101 cpm for the tributyrin assay. The triolein assay, which provides the better signal to noise ratio, offers a number of advantages as a routine assay. The substrate is convenient to prepare, stable for a period of weeks, and considerably less expensive than tributyrin or phospholipase substrates. This has led us to adopt the triolein-based assay in the absence of serum as a routine method of assaying this activity in culture medium for in vitro studies.

Fig. 1. Demonstration of the triglyceridase activity of endothelial lipase (EL). Aliquots of the same preparation of culture medium containing EL were assayed with either a tributyrin or triolein-based substrate as described in Methods. Data are the mean ± SD of triplicate assays and are expressed as nmol FFA/ml/h.

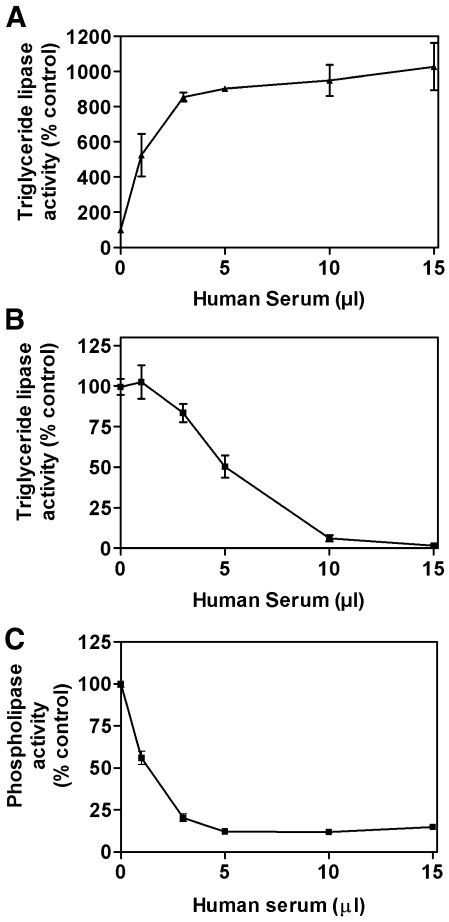

Fig. 2. Effect of human serum on triglyceride lipase activity of lipoprotein lipase (LPL) and EL and on the phospholipase activity of EL. Culture media containing LPL or EL were obtained from COS cell cultures infected with either adenovirus encoding human LPL (AdhLPL) or adenovirus encoding human endothelial lipase (AdhEL) as described in Methods. Triglyceride lipase activity of the two media and the phospholipase activity of culture medium containing EL were determined in the presence of increasing amounts of heat-inactivated human serum. Data for the triglyceride lipase activity of LPL (A), the triglyceride lipase activity of EL (B), and the phospholipase activity of EL (C), are expressed as the percentage of the activity measured in the absence of serum (control). Control triglyceride lipase activity of the LPL medium was 695 ± 40 nmol/ml/h and that of the EL medium was 373 ± 51 nmol/ml/h. Control phospholipase activity of the EL was 821 ± 26 nmol/ml/h.

Exploration of the effect of serum on the activity of EL

We examined the effect on EL activity of varying the serum concentration in the triglyceride lipase assay using LPL conditioned media as a positive control. As expected, the addition of serum increased LPL triglyceride lipase activity in a dose-dependent manner (**Fig. 2A**). In contrast, EL had the greatest triglyceride lipase activity when assayed in the absence of serum, and increasing amounts of serum added to the assay progressively reduced the amount of triglyceride lipase activity (Fig. 2B). Of note, the amount of serum we (4) and Hirata et al. (5) had previously used in triglyceride lipase assays was 5% (15 µl in our assay), which almost completely inhibited the triglyc-

TABLE 1. Effect of various sera on EL triglyceride lipase activity

Serum Type	Activity	
	% control	
	Expt. 1	Expt. 2
Human	5 ± 8	11 ± 1
Fetal bovine	53 ± 9	34 ± 11
Rabbit	56 ± 8	40 ± 4
Rat	0	0
Mouse	0	0

Conditioned medium from COS cells infected with AdhEL was assayed in the absence (control) or presence of 5% heat-inactivated serum derived from the species indicated. Values are the mean ± SD of triplicate assays. Control activity in Experiment 1 was 334 ± 37 nmol/ml/h and in Experiment 2 was 334 ± 10 nmol/ml/h.

eride lipase activity in these experiments. We also found that the phospholipase activity of EL is inhibited by human serum in a dose-dependent fashion (Fig. 2C). Therefore, both EL triglyceride lipase and phospholipase activities are inhibited by the addition of serum to the assay. These results suggest that human serum contains an endogenous inhibitor of EL activity. We were not successful in examining the effect of serum in the tributyrin assay, as even heat-inactivated serum contains a very high level of esterase activity against tributyrin.

The published assay (16) that we used in our previous studies (4) calls for the inclusion of 5% rat serum; however, we had substituted human serum. To determine if the source of serum was responsible for the inhibition of EL due to some component unique to human serum, we compared various sera including human, rat, rabbit, fetal bovine, and mouse at a concentration of 5% in the assay mixtures. As shown in **Table 1**, mouse and rat sera completely inhibit EL activity, inhibition by human serum is nearly complete, and both rabbit and fetal bovine sera are less inhibitory.

We explored the possibility that the inhibition in the presence of serum was a result of the presence of serum lipoproteins, which might serve as an alternate substrate, acting as a competitive inhibitor of the radiolabeled substrates. To test this hypothesis, serum lipoproteins were prepared from heat-inactivated human serum by flotation. Aliquots of the lipoprotein fraction or the lipoprotein-deficient fraction were added to assay tubes at concentrations comparable to 10 μl of unfractionated human serum, which was used as a control. As shown in **Table 2**, the serum fractions both showed inhibitory activity. However, inhibition by the lipoprotein fraction (34%) was much less that the inhibition by whole serum (95%) or by lipoprotein-deficient serum (84%). Thus, the inhibitory activity in serum is probably not due to the presence of competing lipoproteins, but either resides in the non-lipoprotein fraction or distributes between the fractions upon ultracentrifugation. Because of the methods used to prepare the serum and isolated fractions, we can also conclude that the inhibitory factor is heat-stable and non-dialyzable.

TABLE 2. Effect of fractionated human serum on EL triglyceride lipase activity

Serum Fraction	% Control
Whole serum	5 ± 5
Lipoprotein fraction	66 ± 8
Lipoprotein-deficient serum	16 ± 13
Reconstituted fractions	12 ± 2

Conditioned medium from COS cells infected with AdhEL was assayed in the absence or presence of heat-inactivated human serum (3.33%) or a comparable concentration of the indicated serum fractions derived from the same human serum. Values are the mean ± SD of triplicate assays. Control activity was 340 ± 55 nmol/ml/h.

Comparison of the lipolytic activities of EL with LPL and HL

To compare the relative triglyceride lipase and phospholipase activities of EL to other members of this gene family, human EL, LPL, and HL were expressed using recombinant adenoviruses under identical conditions. Expression was confirmed by Western blotting of conditioned media. Aliquots of the same conditioned media were assayed using separate triglyceride lipase and phos-

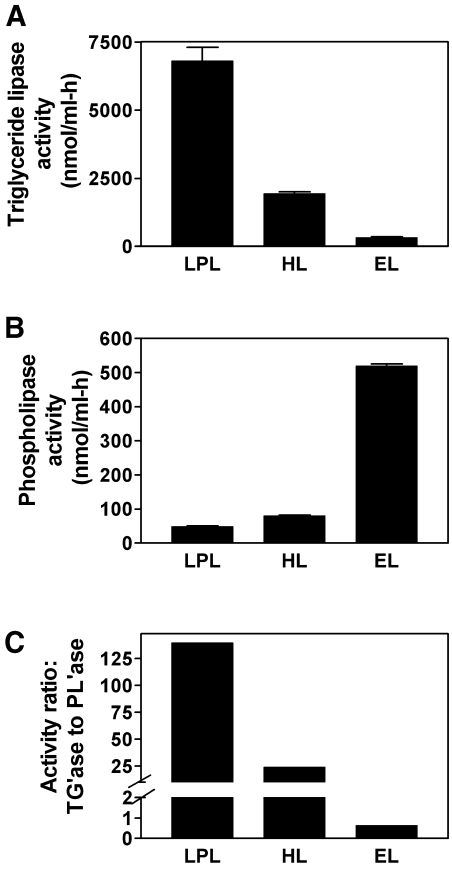

Fig. 3. Comparison of the triglyceride lipase and phospholipase activities of LPL, hepatic lipase (HL), and EL. Culture media containing LPL, HL, or EL were obtained from COS cells infected with adenoviruses containing the respective cDNAs as described in Methods. Aliquots of the same media were assayed for triglyceride lipase and phospholipase activity using the individual radiolabeled substrates as described in Methods. Triglyceride lipase activity is shown in A, and phospholipase activity is shown in B, and the ratios of triglyceride lipase to phospholipase are shown in C.

pholipase assays, and the activities were compared on the basis of specific activities based on volume of medium (μmol FFA liberated/ml/h). Conditioned medium from cells expressing GFP was included as a control. Assays for LPL contained serum as a source of apoC-II, but those for HL and EL did not. The data in **Fig. 3** show the activities of the three enzyme preparations against triolein and against DPPC using the respective glycerol-stabilized substrates. Medium containing LPL had the greatest triglyceride lipase activity, followed by HL, and then EL (Fig. 3A). In contrast, when phospholipase activity was assayed, medium containing EL had the greatest phospholipase activity, followed by HL, and then LPL (Fig. 3B). Comparison of the absolute activities should be viewed cautiously as the differences might reflect different amounts of expressed enzyme among the three preparations. However, the ratios of the two activities for each enzyme can be directly compared because the individual activities were determined on aliquots of the same conditioned medium. The ratio of triglyceride lipase to phospholipase activity was 139.9 for LPL, 24.1 for HL, and 0.65 for EL. We also compared the relative triglyceride lipase and phospholipase activities using a double-labeled emulsion substrate. The ratio of triglyceride lipase to phospholipase activity of LPL was 197, HL was 44, and EL was four. Although the absolute ratios are different, the rank order of the ratios for the three enzymes remains the same. These data demonstrate that, although all three enzymes have both triglyceride lipase and phospholipase activity, the dominant activity of the three enzymes differs dramatically; LPL is primarily a triglyceride lipase with low but detectable phospholipase activity, HL lies in the middle with substantially more triglyceride lipase than phospholipase activity, while EL has similar levels of phospholipase and triglyceride lipase activity.

Effects of apoC-II or high salt on endothelial lipase activity

A critical feature differentiating LPL and HL is the ability of apoC-II to serve as a cofactor for LPL but not HL. Because we had observed that serum is inhibitory in the assay of EL triglyceride lipase activity, we wished to determine whether purified apoC-II could activate EL in the absence of other serum factors or, alternatively, might be the inhibiting factor. Using the double-labeled substrate, we demonstrated that, as expected, apoC-II was extremely effective in increasing LPL triglyceride lipase and phospholipase activity; however it had no effect on either lipolytic activity of EL (**Fig. 4**). Therefore, apoC-II is not required for the lipolytic activity of EL, nor does it inhibit these activities. An additional feature that has been used to distinguish between LPL and HL is the inhibitory effect that 1 M NaCl has on LPL but not HL activity. We therefore examined the effect of 1 M NaCl on conditioned media containing each of the three enzymes. As shown in **Fig. 5**, we found that inclusion of 1 M NaCl in the assay inhibited the triglyceride lipase activity of EL even more effectively than that of LPL. High salt is an equally effective inhibitor of the phospholipase activity of EL (data not shown).

EL activity against isolated lipoprotein fractions

To evaluate the relative capacity of LPL, HL, or EL to hydrolyze lipids in isolated plasma lipoproteins, conditioned media containing each of the enzymes were incubated with chylomicrons, VLDL, IDL, LDL, or HDL at equivalent phospholipid concentrations in the assay tubes. Compositional data for representative preparations of the lipoprotein fractions are shown in **Tables 3** and **4**.

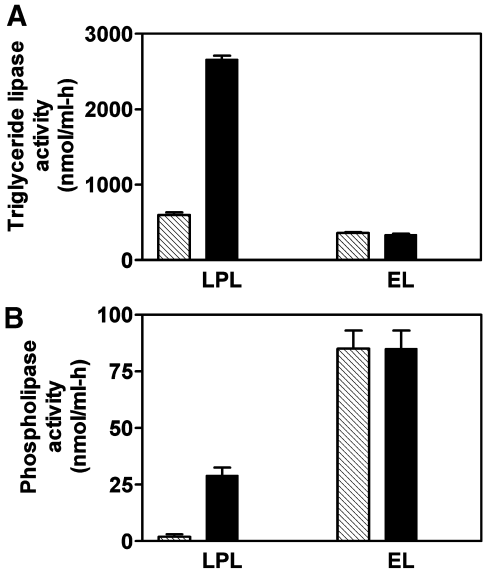

Fig. 4. Comparison of the effect of purified apoC-II on the activities of LPL and EL. Culture media containing LPL or EL were obtained from COS cell cultures infected with either AdhLPL or AdhEL as described in Methods. Triglyceride lipase and phospholipase activities of the two enzymes were determined using the double-labeled glycerol-stabilized substrate in the absence (hatched bars) and presence (filled bars) of purified apoC-II (10 μg/ml in the assay). Data are the mean ± SD of triplicate assays.

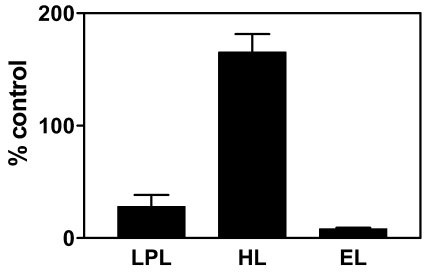

Fig. 5. Comparison of the effect of 1 M NaCl on the activities of LPL, HL, and EL. Culture media containing LPL, HL, or EL were obtained from COS cell cultures infected with adenoviruses encoding the enzymes as described in Methods. Triglyceride lipase activity of each enzyme was measured in the presence of 0.15 M NaCl (control) and in the presence of 1 M NaCl. In the case of LPL, purified apoC-II was added at a final concentration of 10 μg/ml in the assay. Data are presented as a percentage of the control activity. Values are mean ± SD of data from four experiments for LPL, five experiments for HL, and six experiments for EL.

TABLE 3. Compositional data from a representative isolation of lipoproteins from a single fasted, normolipemic donor

	% FC	% EC	% TG	% PL	% Protein
VLDL	4.8	12.3	51.3	22.6	9.0
LDL	7.9	49.4	2.9	22.3	17.5
HDL	2.6	25.5	3.1	24.4	44.4

Unlike the assays with the radiolabeled substrates, these are end-point assays, reflecting the maximal lipid hydrolysis achieved by a given enzyme. The data in **Fig. 6** compare the release of FFAs by the various enzymes from each lipoprotein preparation. The number of experiments contributing to the pooled data is shown below each data bar. There was very little lipolysis in tubes containing conditioned media from cells infected with AdGFP (3 ± 2 nmol FFA/reaction tube). Activity with snake venom phospholipase A2 was similar for each lipoprotein fraction (74 ± 6 nmol FFA/reaction tube). The control data are derived from four experiments with chylomicrons, eight with VLDL, four with IDL, seven with LDL, and nine with HDL. As expected, LPL has the highest level of lipase activity against the triglyceride-rich lipoproteins, HL has less, and EL has the least. EL has more activity against HDL than against any of the other lipoproteins, producing fatty acid equivalent to approximately 20% of the HDL phospholipids present in the reaction tube.

DISCUSSION

In these studies, we demonstrated that EL has the ability to hydrolyze triglycerides, both with short-chain fatty acyl groups (tributyrin) and long-chain fatty acyl groups (triolein) with similar levels of activity toward both types of substrates. This similarity is in contrast to what has been reported for two other members of the triglyceride lipase family, LPL and HL, where ratios of triolein lipase to tributyrin esterase activities were approximately 20 (12) and four (18) for LPL and HL, respectively. We further characterized the lipolytic activity of EL in comparison to these other two members of the triglyceride lipase family using artificial radiolabeled lipid substrates as well as isolated lipoproteins. Because our efforts to purify EL in an active form have not been successful, we made comparisons of the activities in conditioned media containing each of the three enzymes that were produced under identical conditions. Although Western blotting established the presence of substantial amounts of each of the enzymes in the culture media, a quantitative assay for EL protein mass is not currently available. Therefore, it is not yet possible to make a direct comparison of the specific activities of the enzymes with respect to either lipase activity. However, the relative response to varying components in the assays and the ratios of activities with different substrates can be directly compared.

The recognition that EL indeed has triglyceride lipase

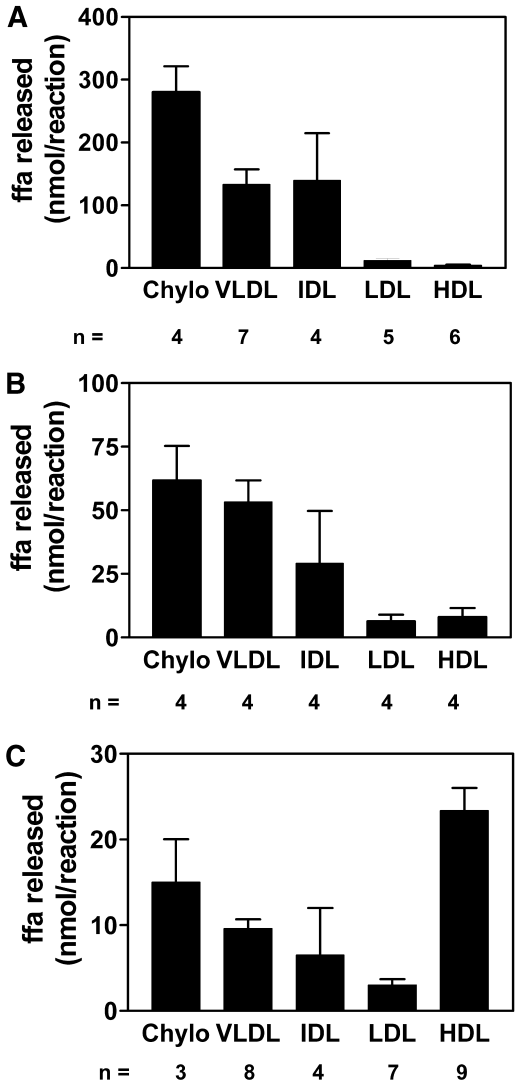

Fig. 6. Comparison of the extent of hydrolysis of isolated human lipoprotein fractions by conditioned media containing LPL, HL, or EL. Isolated lipoprotein fractions (1.25 mM lipoprotein phospholipid) were incubated with conditioned media containing LPL, HL, or EL as described in Methods. Media from cells infected with adenovirus encoding GFP (AdGFP) served as the negative control, and snake venom phospholipase A2 served as the positive control. FFAs were assayed as described in Methods in triplicate in each experiment. Data are expressed as nmol FFA released per reaction tube and represent the mean ± SD for the entire set of experiments for each enzyme and lipoprotein fraction. The number of experiments contributing to each data bar is shown beneath the bar. A: Shows data for LPL. B: Shows HL. C: Shows EL.

TABLE 4. Compositional data from a representative isolation of lipoproteins from pooled plasma from non-fasted, hyperlipemic subjects

	% FC	% EC	% TG	% PL	% Protein
Chylomicrons	5.7	9.5	65.0	14.7	5.0
VLDL	5.4	17.3	47.2	23.0	7.0
IDL	6.3	24.9	35.9	22.7	20.7
LDL	7.0	44.3	5.8	22.2	20.7
HDL	2.5	24.0	5.4	26.6	41.5

activity led us to systematically examine the relative triglyceride lipase to phospholipase activities for all three enzymes. Both LPL and HL have triglyceride lipase activity, and this activity forms the basis by which LPL and HL activity have traditionally been measured in post-heparin plasma and in cell culture studies. HL has been shown to have substantial phospholipase activity (21), whereas LPL has relatively low phospholipase activity (22). In most cases where the relative triglyceride lipase and phospholipase activities have been compared for LPL and HL, regardless of the substrate presentation, the relationship of the ratios is similar, with LPL having less phospholipase activity relative to triglyceride lipase activity (22–24).

Although absolute activities vary depending on the substrate presentation, a variety of emulsifying agents have been used to measure LPL and HL activities against triolein. These include the use of phospholipids alone, phospholipids and glycerol, gum arabic, or detergents. Because it is a convenient, reproducible substrate for LPL, HL, and EL, we continued to use the glycerol-stabilized triolein substrate (16) for these studies. The phospholipase activities of both HL and LPL have been measured, primarily using phosphatidylcholine substrates stabilized by Triton X-100 (25). Unlike the emulsions used for the presentation of triglycerides, which approximate lipoprotein structure, Triton-stabilized substrates are mixed micelles (26). In addition, double-labeled substrates have been used that incorporated both triglyceride and phosphatidylcholine into the same substrate preparation (23), similar to the double-labeled substrate used in the present studies. Pilot studies on EL using a micellar, Triton X-100-stabilized phospholipid substrate (25) were unsatisfactory in several ways. A fresh substrate preparation was required for every experiment. There was a lack of reproducibility between substrate preparations and an unacceptable level of variability within sets of replicates in the same experiment. To address these difficulties, the glycerol-stabilized phospholipid substrate described here was developed. Like the triglyceride substrate, it is an emulsion that more closely approximates a lipoprotein particle. Once prepared, the substrate is stable for several weeks and gives highly reproducible results. Therefore, for the purposes of this work, the glycerol-stabilized substrates were chosen because they allowed reliable, although perhaps not optimal, measurement of all the lipases we were examining. In the present study, the triglyceride lipase to phospholipase ratios that we observed for LPL and HL were similar to previously published data (23, 27). In comparison to the other two major members of this gene family, EL has the lowest triglyceride lipase to phospholipase ratio. Although the absolute values of the triglyceride lipase to phospholipase ratios differed between the singly labeled and doubly labeled substrates, the relationship of the ratios of the three enzymes was similar. Using the activities measured in the separate triglyceride lipase and phospholipase assays, EL appears to be more active as a phospholipase than a triglyceride lipase (triglyceride lipase to phospholipase ratio = 0.65). The triglyceride lipase to phospholipase ratio derived using the double-labeled substrate (4.0) might suggest that EL is more active as a triglyceride lipase than as a phospholipase, but this may be misleading. Triglyceride in the emulsion provides a competing substrate that is present in approximately 14-fold molar excess, which almost certainly leads to an underestimation of the phospholipase activity. The data also demonstrate that our estimate of triglyceride lipase activity using the single label is likely to be an underestimate, because that substrate also contains phospholipid. Therefore, we conclude that the triglyceride lipase and phospholipase activities of EL are roughly similar, in sharp contrast to LPL and HL.

Demonstrating that EL has triglyceride lipase activity rested on omitting serum from the assay. Previous work using conditioned media obtained from cells transfected with EL cDNA had revealed substantial phospholipase activity but little or no triglyceride lipase activity when a standard LPL assay, which included the addition of human serum as a source of apoC-II, was used (4, 5). The present studies indicate that heat-inactivated human serum inhibits both activities of EL in a dose-dependent manner, with nearly complete inhibition at the serum concentration that is standard in the LPL assay. However, the shapes of the inhibition curves for triglyceride lipase versus phospholipase activities are different. It is possible that factors in serum influence the surface pressure of the emulsion, affecting the access to the ester linkage in the phospholipid at lower surface pressures than for triglyceride.

A screen of sera from other species showed that the inhibitory effect was not uniquely conferred by human serum. All sera tested showed some degree of inhibition at the concentration tested, with mouse and human sera being the most potent inhibitors. The possibility that plasma lipoproteins present in the serum were inhibiting hydrolysis of the radiolabeled triglyceride substrate by acting as competing substrates was explored by fractionating the human serum into lipoprotein and lipoprotein-deficient fractions by ultracentrifugation. Although some inhibition was observed when the lipoprotein fraction was added to the assay, it was much less than that observed with the lipoprotein-deficient fraction, which, like the unfractionated whole serum, produced nearly complete inhibition of the EL activity. This suggests that the inhibitory factor is likely a protein (it was not removed in the dialysis following ultracentrifugation) and may be an apolipoprotein that distributes between the floating and bottom fraction during ultracentrifugation. In contrast to EL, neither LPL nor HL has been reported to be inhibited simply by the addition of whole serum to the assay at concentrations of 5% (standard for activating LPL). Other than the well characterized requirement of LPL for apoC-II as a cofactor, data on stimulatory or inhibitory effects of other apolipoproteins on LPL or HL in in vitro assays have been controversial, largely due to differences in outcome obtained with different substrate presentations (28). Further studies will be required to determine the nature of the EL inhibitory factor in human serum.

Although they have many similarities, LPL and HL differ in two additional characteristics: *1*) LPL requires apoC-II as a cofactor (29), whereas HL does not, and *2*) in

the presence of high salt, LPL activity is inhibited, whereas that of HL is not, relative to low salt conditions. A comparison of the effect of apoC-II in assays of LPL and EL showed definitively that, at a concentration of apoC-II that maximally stimulates LPL activity, there was no effect on EL activity. This demonstrates that EL has no requirement for apoC-II as a cofactor and also that apoC-II is not the factor in serum that is inhibiting EL. An examination of the salt sensitivity of the three enzymes showed that, of the three, EL is the most sensitive to the effects of high salt. This characteristic may prove useful in evaluating the relative amounts of HL and EL in plasma samples using a phospholipase assay.

The physiologic role of EL remains unclear. Whether the observed ratios of triglyceride lipase to phospholipase activities accurately reflect the in vivo activities of any of these enzymes has not been established. However, the ratios are consistent with the roles that LPL and HL are thought to play as important regulators of plasma lipoprotein metabolism. Although "bridging" effects that are independent of lipolytic activity have been shown to be important for both LPL and HL (30–32), the physiologic effects of both enzymes are also mediated by their lipolytic activities. For LPL, the primary activity is considered to be its triglyceride lipase activity. The triglyceride lipase activity of HL is also thought to be critical to its metabolic effects on both remnant lipoproteins as well as HDL; the contribution of its phospholipase activity to its metabolic effects is less certain (1, 28). Our previous work (4) demonstrated that expression of EL in mice using adenoviral gene transfer led to a depletion of plasma HDL, suggesting that HDL might be a physiologic substrate for EL. Thus, we examined the ability of conditioned media containing EL, LPL, or HL to hydrolyze lipids in isolated lipoprotein fractions. Consistent with the physiologic roles of LPL and HL and the relative activities measured on artificial substrates, LPL was active against all of the triglyceride-rich lipoproteins, as was HL. Of the three enzymes, only EL promoted significant hydrolysis of HDL lipid under these conditions, consistent with our in vivo observations (4). These results confirm that EL is active on lipids in isolated HDL. Given the molar excess of phospholipids relative to triglycerides in HDL, we conclude that EL modulates lipoprotein metabolism, at least in part, through its phospholipase activity. However, we cannot not rule out the possibility that the triglyceride lipase activity of EL could also have a physiologic role.

An interesting conundrum raised by these studies is how EL functions in the plasma compartment. The inhibition of both activities in vitro in the presence of 5% serum suggests that the activity of EL may actually be regulated in the plasma compartment by an as yet unknown regulator. This could be analogous to the case with pancreatic lipase, which functions in the intestine in a milieu rich in bile salts, but is inactive in the presence of bile salts (33). Relief of the inhibition by bile salts is accomplished by the binding of the activating cofactor, colipase (33). Further studies are required to elucidate the physiologic role of this new member of the lipase gene family.

This work was supported by grants HL55323 (D.J.R.) and HL55756 (J.M.G.) from the National Institutes of Health. We are indebted to Robert Hughes for valuable assistance. Cyrille Maugeais is supported by a grant from ARCOL (France) and from the American Heart Association, Pennsylvania Delaware Affiliate. D.J.R. is an established investigator of the American Heart Association and is a recipient of the Burroughs Wellcome Foundation Clinical Scientist Award in Translational Research.

Manuscript received 15 January 2002 and in revised form 28 February 2002.

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