Synthesis and secretion of the pancreatic-type carboxyl ester lipase by human endothelial cells

Feng LI and David Y. HUI[1]

Department of Pathology and Laboratory Medicine, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, OH 45267-0529, U.S.A.

Human aortic extracts contain significant cholesteryl ester hydrolytic activity. The enzymic activity was shown to be activated by trihydroxylated bile salt, but not by dihydroxylated bile salt. Monospecific antibodies prepared against rat pancreatic carboxyl ester lipase (CEL, cholesterol esterase) immunoprecipitated cholesteryl ester hydrolytic activity from human aorta, demonstrating that the neutral CEL in aorta is highly similar to and probably identical with the pancreatic enzyme. Reverse transcriptase PCR amplification of mRNA from human aortic endothelial cells revealed *de novo* synthesis of the pancreatic-type CEL by these cells. Preincubating human aortic endothelial cells with oxidized or native low-density lipoprotein resulted in an 8- and 3-fold increase in CEL activity secreted into the culture medium respectively. A potential physiological role for the endothelial CEL was demonstrated by studies showing its ability to confer partial protection against the cytotoxic effects of lysophosphatidylcholine. The protective effect of CEL is related to its bile-salt-independent lysophospholipase activity. However, CEL hydrolysis of lysophosphatidylcholine can be inhibited by excess cholesterol. Taken together, these results indicate that pancreatic-type CEL is synthesized by cells lining the vessel wall. Moreover, vascular CEL may interact with cholesterol and oxidized lipoproteins to modulate the progression of atherosclerosis.

INTRODUCTION

Carboxyl ester lipase (CEL), also called bile-salt-stimulated lipase and pancreatic cholesterol esterase, is a lipolytic enzyme with a wide substrate specificity capable of hydrolysing cholesteryl esters, triacylglycerol, phospholipids and lysophospholipids [1]. It is synthesized primarily by the pancreas and is found as a component of pancreatic juice. This enzyme is also found in the milk of numerous species [2]. Milk CEL is postulated to substitute for the pancreatic enzyme in the neonatal gastrointestinal tract before maturation of the pancreas [3]. One physiological function of CEL is the hydrolysis of dietary cholesteryl esters and lysophospholipids, facilitating their transport and absorption [4]. The enzyme is also responsible for the digestion and absorption of fat-soluble vitamin esters [5], and may act in concert with pancreatic lipase for fat digestion and absorption [6].

An identical CEL has also been found to be synthesized by the liver [7,8]. It is present in plasma [9,10], where it may catalyse the transfer of cholesteryl esters from high-density lipoprotein to the liver [11]. High plasma CEL has been shown to correlate positively with plasma cholesterol and low-density lipoprotein (LDL) levels, suggesting a possible role for CEL in formation and accumulation of atherogenic lipoproteins [9]. Recently, a protein with enzymic and immunological properties similar to those of CEL was shown to be present in human aortic extracts [12]. Shamir et al. postulated that CEL in the aorta may protect against atherosclerosis by decreasing the lysophospholipid content of oxidized LDL [12]. However, whether aortic CEL is identical with the pancreatic enzyme remains unclear. Moreover, the cellular origin of aortic CEL has not been identified. Therefore it is unclear whether the CEL detected in aortic extracts is derived from the circulation or by local synthesis. The purpose of the present study was to ascertain whether CEL can be synthesized by endothelial cells lining the vessel wall.

MATERIALS AND METHODS

Materials

Human umbilical-vein endothelial cells (HUVECs) and endothelial growth medium with supplements were purchased from Clonetics (San Diego, CA, U.S.A.). Human aortic endothelial cells (HAECs) and culture medium 200 with low serum supplements were obtained from Cascade Biologics Inc. (Portland, OR, U.S.A.). Human plasma was obtained from Hoxworth Blood Center (Cincinnati, OH, U.S.A.). Ultrafree-4 centrifugal filter device was purchased from Millipore Corp. (Bedford, MA, U.S.A.). Lysophosphatidylcholine (lyso-PtdCho) and rabbit anti-(pancreatic amylase) IgG were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Reverse transcriptase and *Taq* DNA polymerase were from Gibco–BRL (Grand Island, NY, U.S.A.). Cholesteryl [^{14}C]oleate and 1-[1-^{14}C]palmitoyl-L-lyso-3-phosphatidylcholine were obtained from DuPont–New England Nuclear Research Products (Boston, MA, U.S.A.). RNA Stat-60 was purchased from Tel-Test "B" Inc. (Friendswood, TX, U.S.A.). Other high-quality reagents and chemicals were obtained from either Sigma or Fisher Chemical Co. (Cincinnati, OH, U.S.A.). Prepacked ProteinA–agarose columns were purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.).

Preparation of aortic extract

Human aorta was obtained at autopsy according to guidelines of the Institutional Review Board. The sample was frozen im-

Abbreviations used: CEL, carboxyl ester lipase (also called cholesterol esterase); HAEC, human aortic endothelial cell; HUVEC, human umbilical-vein endothelial cell; LDL, low-density lipoprotein; lyso-PtdCho, lysophosphatidylcholine; RT-PCR, reverse transcriptase PCR.

[1] To whom correspondence should be addressed.

mediately in liquid nitrogen and stored at −70 °C until use. The sample was thawed on ice and rinsed several times with a solution containing 150 mM NaCl and 1 mM EDTA. The aorta was cut into small pieces and then homogenized in buffer containing 10 mM sodium phosphate, pH 6.2, 0.1 M NaCl, 0.02 % NaN_3, 1 mM EDTA and 1.5 % glycerol. The homogenate was centrifuged at 500 ***g*** for 5 min and the resulting supernatant was stored at −70 °C until use. Protein concentration of the aortic homogenate was determined by the method of Lowry et al. [13].

Immunoprecipitation of proteins

Antibodies against rat pancreatic CEL were produced by immunizing a New Zealand White rabbit with 250 μg of purified rat CEL mixed with complete (first injection) or incomplete (subsequent injections) adjuvant at 2-week intervals, as described previously [14]. Monospecific rabbit anti-(rat pancreatic CEL) IgG was isolated by affinity chromatography on a Protein A–agarose column according to the protocol described by the manufacturer. The specificity of the anti-CEL IgG was demonstrated by its reactivity with a single band with molecular mass corresponding to purified CEL on immunoblots [11]. The IgG was dialysed against PBS before use. A 1 ml portion of the human aortic homogenate was mixed in an end-to-end manner for 24 h at 23 °C with various concentrations of either anti-(pancreatic CEL) or anti-(pancreatic amylase) IgG. At the end of this initial incubation period, 20 μl of a 50 % suspension of Protein A–agarose beads was added and the incubation was continued for an additional 3 h. The sample was then centrifuged at 5000 ***g*** for 15 min. The supernatant was collected and dialysed against homogenization buffer before use.

Cell culture

Stock cultures of HAECs and HUVECs were grown in endothelial growth medium containing 2 % fetal bovine serum, 10 ng/ml human epidermal growth factor and 12 μg/ml bovine brain extract. The cells were maintained in 0.1 % gelatin-coated 75 cm² tissue culture flasks at 37 °C in a humidified incubator with 5 % CO_2. For experiments, HAECs at no greater than passage 8 were plated in 100 mm² tissue culture dishes and were incubated for 24 h with M199 medium in the presence or absence of lipoproteins.

Preparation of LDL

Human LDL ($d = 1.02$–1.063) was isolated from human plasma by density-gradient centrifugation for 18 h at 375000 ***g*** (59000 rev./min) in a 70 Ti rotor (Beckman Instruments, Palo Alto, CA, U.S.A.), as described [15]. The LDL supernatant was collected and washed by re-centrifugation with a KBr solution at $d = 1.05$ for 16 h at 375000 ***g***, dialysed extensively against 150 mM NaCl/1 mM EDTA, and stored at 4 °C under nitrogen until use. Oxidation of LDL was achieved by dialysing a stock solution of LDL against EDTA-free PBS, and then incubating with PBS supplemented with 10 μM copper sulphate for 18 h at 37 °C [16]. Acetylated LDL was prepared by sequential addition of acetic anhydride to the LDL stock as described previously [17]. The modified LDL was dialysed extensively against 150 mM NaCl/1 mM EDTA and characterized by agarose-gel electrophoresis.

Determination of CEL activity

Conditioned medium from HAECs incubated with or without 100 μg of oxidized LDL or native LDL was concentrated to 500 μl by centrifugation at 5000 ***g*** through ultrafree-4 centrifugal filters. An aliquot of each sample (usually 140 μl) was diluted into 60 μl of a solution to yield a final concentration of 50 mM Tris/HCl, pH 7.5, and 33 mM cholate. The samples were incubated at 37 °C for 15 min before the addition of 1.96 nmol of cholesteryl [^{14}C]oleate to initiate the reaction. The incubation was continued for 2 h at 37 °C and then terminated with 1.05 ml of 50 mM Na_2CO_3/50 mM sodium borate, pH 10, and 3.25 ml of methanol/chloroform/heptane (1.41:1.25:1.0, by vol.). The samples were centrifuged at 1500 ***g*** for 30 min, and 500 μl of the top aqueous phase was collected for liquid-scintillation counting. Enzyme activity was determined as pmol of cholesteryl [^{14}C]oleate hydrolysed/h per mg of cell protein, as described previously [7]. Cholesterol ester hydrolytic activity in human aortic extract was determined by the same procedure.

Reverse transcriptase PCR (RT-PCR) amplification of RNA

Total cellular RNA was isolated from HAECs using the single-step guanidinium thiocyanate/phenol/chloroform extraction method with RNA-Stat 60 [18]. The CEL oligonucleotide primers, synthesized on an Applied Biosystems DNA Synthesizer (Foster City, CA, U.S.A.), were based on sequences derived from exons 2 and 7 of the human pancreatic CEL gene [19–21]: 5′-AGCACCTACGGGGATGAAGA-3′ and 5′-GGCTCGGGG-ATCAGTAACCT-3′. A 1 μg portion of RNA was reverse-transcribed into cDNA with oligo(dT) primers. One-twentieth of the resulting cDNA in 1 μl was applied to PCR amplification. Thirty-five cycles of PCR amplification were performed with denaturation set at 95 °C for 30 s, annealing at 60 °C for 50 s, and extension at 72 °C for 90 s. The extension time was increased to 10 min for the final cycle. The PCR products were separated by electrophoresis on 1 % agarose gel.

Effect of lyso-PtdCho on endothelial cells

HUVECs were plated in 48-well dishes coated with 0.1 % gelatin. When the cells reached more than 90 % confluency, fresh medium was added before exposure of the cells for 24 h to different concentrations of lyso-PtdCho with or without the presence of 2.67 μg/ml CEL. The dead cells were removed by washing with PBS. Living cells were harvested with 1 M NaOH for protein determination using the Lowry method [13].

Lysophospholipase activity of human CEL

Human CEL was purified from pooled human milk samples using the procedure described previously for the purification of rat pancreatic CEL [14]. Homogeneity of the CEL preparation was verified by the presence of a single band with molecular mass 120 kDa on SDS/polyacrylamide gels. Purified human CEL (5 μg) was incubated at 37 °C for 30 min with 10 μM 1-[1-^{14}C]palmitoyl-L-lyso-3-phosphatidylcholine in 50 mM potassium phosphate, pH 7.0, in the presence or absence of increasing concentrations of cholesterol. At the end of the incubation period, the samples in 200 μl final volume were extracted by addition of 2.5 ml of Dole's mixture (propanol/heptane/0.05 M H_2SO_4, 40:10:1, by vol.), 100 mg of silica gel and 1.5 ml of water. The samples were vortex-mixed and centrifuged to separate the phases. The top aqueous phase, containing the hydrolysed product, [^{14}C]palmitate, was isolated and quantified by scintillation counting.

RESULTS

Extracts from human aorta were prepared for characterization of cholesterol ester hydrolytic activity in the vessel wall. Results showed that cholesteryl ester hydrolysis by enzymes present in

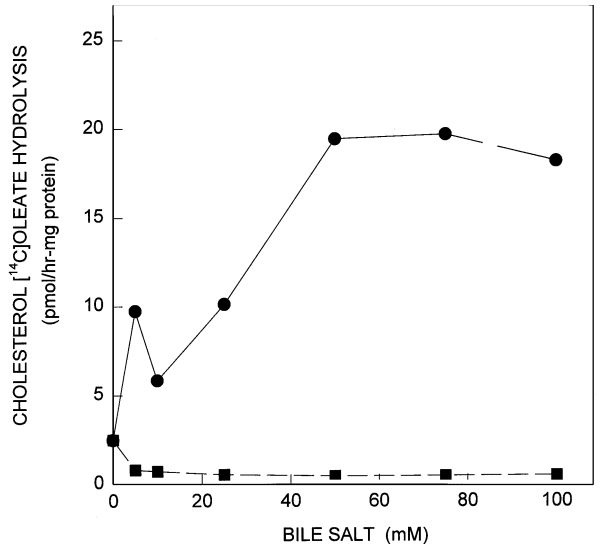

Figure 1 Bile-salt-dependent activation of cholesteryl ester hydrolytic activity in human aorta

Homogenates of human aorta (100 μg) were incubated for 2 h at 37 °C in buffer containing 50 mM Tris/HCl, pH 7.5, 1.96 nmol of cholesteryl [^{14}C]oleate and the indicated concentrations of sodium cholate (●) or sodium deoxycholate (■). Enzyme activity was determined as the amount of [^{14}C]oleate produced/h per mg of protein. Each data point represents the mean of triplicate determinations.

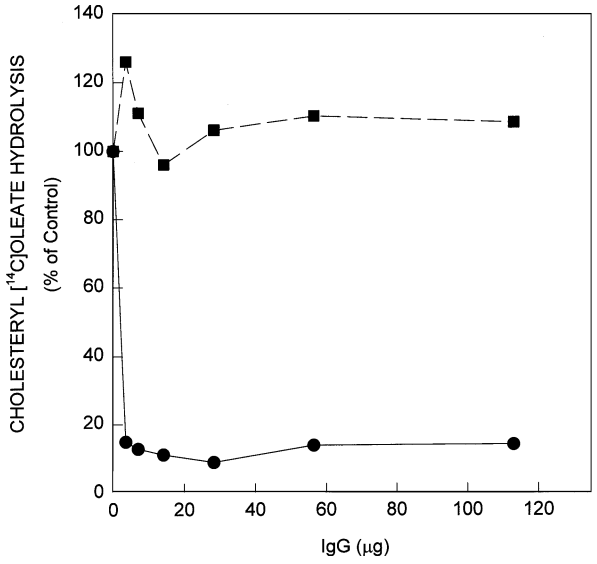

Figure 2 Immunoprecipitation of cholesteryl ester hydrolytic activity from human aorta

A 1 mg portion of protein from human aortic extracts, in a 1 ml volume, was incubated with rabbit anti-CEL (●) or anti-amylase (■) for 24 h at 23 °C. The immunocomplex was precipitated by addition of Protein A–agarose beads. The sample was then centrifuged at 5000 ***g*** for 15 min and the supernatant was used to measure cholesteryl ester hydrolytic activity. Each data point represents the mean of triplicate determinations.

the aortic extract was stimulated by increasing concentrations of sodium cholate (Figure 1). Half-maximal activation of cholesteryl ester hydrolysis was observed at approx. 17 mM sodium cholate (Figure 1). In contrast, sodium deoxycholate was ineffective in

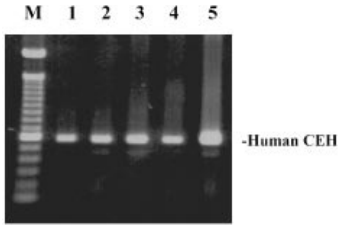

Figure 3 Detection of CEL mRNA in HAECs

Total RNA from HAECs was reverse-transcribed and amplified by PCR with oligonucleotide primers specific for human pancreatic CEL. The PCR products were subjected to 1% agarose-gel electrophoresis. The gel was stained with ethidium bromide to identify the reaction products. The expected 579 bp product was identified using RNA isolated from HepG2 cells as a positive control (lane 5). The RT-PCR products using RNA obtained from control HAECs (lane 1), and HAECs incubated with oxidized LDL (lane 2), acetylated LDL (lane 3) and native LDL (lane 4) are compared. Lane M shows the 100 bp molecular-size marker used as standard.

activating cholesteryl ester hydrolytic activity in human aorta (Figure 1). The specificity of activation by trihydroxylated bile salts instead of dihydroxylated bile salts indicated that the stimulation was independent of the detergent effect of the bile salt. This specificity in trihydroxylated-bile-salt stimulation of cholesteryl ester hydrolysis was reminiscent of the CEL activity in the pancreas. Thus additional experiments were performed to ascertain similarities and differences between aortic and pancreatic CELs. A monospecific antibody prepared against purified rat pancreatic CEL was shown to be effective in immunoprecipitating the cholesteryl ester hydrolytic activity of human aortic extract (Figure 2). An irrelevant antibody, prepared against pancreatic amylase, was unable to immunoprecipitate CEL activity from human aorta (Figure 2). These results, using a different preparation of anti-CEL, confirmed the results of Shamir et al. [12] and indicated that the enzyme responsible for cholesterol ester hydrolysis in human aorta is similar to and probably identical with pancreatic CEL.

The cellular origin of the CEL in human aorta was investigated by determining the possible synthesis of this enzyme by HAECs. Total cellular RNA isolated from cultured HAECs was reverse-transcribed and then amplified with specific oligonucleotide primers for the human CEL gene. An RT-PCR amplification product of 579 bp was observed with RNA isolated from control HAECs as well as cells incubated with oxidized, acetylated and native LDL (Figure 3). A similar RT-PCR product was also observed using RNA isolated from HepG2 cells, a human hepatoma cell line that has been shown recently to be able to synthesize pancreatic CEL [11]. Thus the identification of similar RT-PCR products indicated that endothelial cells are also capable of *de novo* CEL biosynthesis.

Previous studies have shown that the CEL synthesized by pancreas, liver and lactating mammary glands is secreted into the extracellular environment. Thus additional experiments were performed to ascertain whether its synthesis in HAECs also resulted in its secretion. Conditioned medium from HAECs was isolated to determine cholesteryl ester hydrolytic activity. The conditioned medium displayed significant trihydroxylated-bile-salt-dependent cholesteryl [^{14}C]oleate hydrolytic activity (Table 1). Interestingly, an approximately 8-fold increase in CEL activity was detected in the conditioned medium of endothelial cells after incubation with oxidized LDL, and that incubated with native LDL displayed a 3-fold increase (Table 1).

Consideration was given to the avid bile-salt-independent lysophospholipase activity of pancreatic CEL. Thus endothelial

Table 1 Effect of oxidized and native LDL on CEL synthesis and secretion by HAECs

HAECs were incubated for 18 h with 100 μg/ml oxidized LDL or unmodified LDL. The conditioned medium was collected and used to determine hydrolytic activity against cholesteryl [^{14}C]oleate in the presence of sodium cholate. Results are means $\pm$ S.E.M. from triplicate experimental determinations.

Lipoproteins added (μg/ml)	Cholesteryl [^{14}C]oleate hydrolysis (pmol/h per mg of cell protein)
None	5.4 $\pm$ 0.6
Oxidized LDL	43.0 $\pm$ 10.1
Native LDL	16.8 $\pm$ 8.1

Table 2 CEL protection against cytotoxic effects of lyso-PtdCho

HUVECs were incubated for 24 h at 37 °C with lyso-PtdCho in the presence or absence of purified human CEL as indicated. Cell viability was determined as the amount of cellular protein remaining on the plates after incubation. The amount of cellular protein after incubation without lyso-PtdCho was taken as 100%. The data are means $\pm$ S.E.M. for duplicate determinations from triplicate experiments.

Lyso-PtdCho (μM)	CEL (μg/ml)	Endothelial cell protein (% of control)
0	0	100
0	2.6	100
25	0	81 $\pm$ 4
25	2.6	69 $\pm$ 4
50	0	17 $\pm$ 4
50	2.6	59 $\pm$ 5*
100	0	8 $\pm$ 2
100	2.6	22 $\pm$ 2*

* Significant difference from incubation without CEL; $P < 0.01$.

synthesis of the enzyme may have a protective role against cytotoxic damage by the atherogenic agent lyso-PtdCho. To examine this, HUVECs were incubated in culture medium for 24 h with lyso-PtdCho in the presence or absence of 2.6 μg/ml CEL. At the end of the incubation period, viable cells were harvested for protein determination. Lyso-PtdCho was found to be cytotoxic to the endothelial cells in a dose-dependent manner, with significant reduction in cell viability observed with concentrations of lyso-PtdCho as low as 50 μM (Table 2). However, inclusion of CEL in the incubation medium significantly reduced the cytotoxic effects of lyso-PtdCho (Table 2). This inhibition is probably mediated by CEL hydrolysis of the lyso-PtdCho. The ability of CEL to hydrolyse lysophospholipids was confirmed directly by incubating 1-[1-^{14}C]palmitoyl-L-lyso-3-phosphatidylcholine with purified human CEL and measuring the appearance of [^{14}C]palmitate. A specific activity of 9.2 $\pm$ 0.5 μmol of fatty acid released/min per mg of purified CEL was observed. Interestingly, CEL hydrolysis of lyso-PtdCho was inhibited by increasing concentrations of cholesterol in the incubation medium. An IC_{50} for lysophospholipid hydrolysis was observed at 18 μM cholesterol (Figure 4).

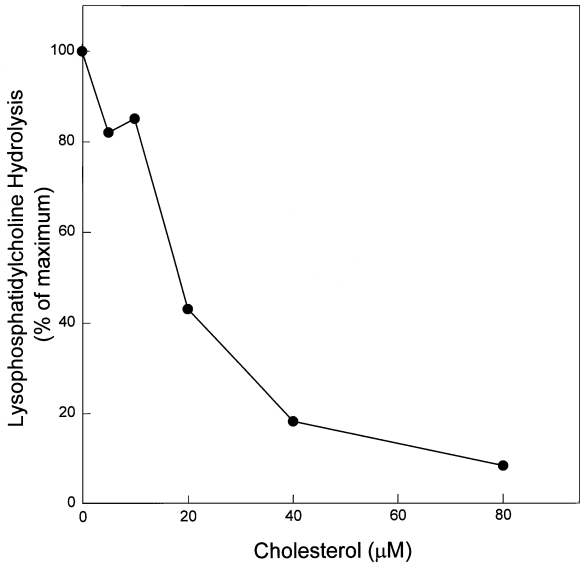

Figure 4 Cholesterol inhibition of CEL-mediated lyso-PtdCho hydrolysis

Purified human CEL (5 μg) was incubated for 30 min at 37 °C with 10 μM 1-[1-^{14}C]palmitoyl-L-lyso-3-phosphatidylcholine in 50 mM potassium phosphate, pH 7.0, in the presence or absence of unesterified cholesterol. Results are percentages of the maximum activity obtained in the absence of cholesterol. Each data point represents the mean of duplicate experiments.

DISCUSSION

Both neutral and acid CEL activities have been reported to be present in arterial tissues [22]. Although previous studies have documented lysosomal acid lipase as the enzyme responsible for the arterial acid CEL activity [22], the identity of the neutral CEL in the vessel wall remains unknown. The results of the present study provide several lines of evidence to support the hypothesis that at least one of the arterial neutral CELs is identical with the pancreatic enzyme. First, we confirmed the results of Shamir et al. [12] and showed that activation of the neutral CEL activity in vessel walls is dependent on trihydroxylated bile salt, dihydroxylated bile salt being ineffective. This is a typical characteristic of pancreatic CEL [23]. The inhibition of the aortic CEL activity by monospecific antibodies against pancreatic CEL also revealed the high degree of similarity between the pancreatic and aortic enzymes. The ability of the anti-CEL IgG to precipitate more than 80% of the cholesteryl ester hydrolytic activity from human aortic extract further suggests that CEL is primarily responsible for the neutral CEL activity in this tissue. Finally, HAECs in culture were shown to possess pancreatic-type CEL mRNA and be capable of CEL synthesis and secretion.

The identification of pancreatic CEL mRNA in HAECs adds to a growing list of cell types capable of CEL biosynthesis. In addition to the pancreas and lactating mammary glands [24–27], the mRNA for pancreatic-type CEL has been shown to be present in liver [8,28], eosinophils [29] and human macrophages [30]. The wide tissue distribution of CEL suggests its importance in physiology and pathophysiology. For example, the abundance of CEL in the digestive tract indicates its possible importance in nutrient absorption [4]. The presence of CEL in plasma [9,10] and its synthesis by macrophages and endothelial cells, especially in an oxidized-LDL-stimulated manner ([30]; the present study), suggests that it may also play a crucial role in lipid metabolism and atherosclerosis.

The precise physiological function of the endothelial-derived CEL in atherosclerosis remains speculative. Although its stimulation by oxidized LDL suggests that it may promote atherogenesis, it is also possible that CEL synthesis is a protective response against the atherogenic effects of lyso-PtdCho. Lyso-PtdCho is a well-documented pro-atherogenic agent generated during LDL oxidation [31]. It serves as a chemoattractant for monocytes [32], induces monocyte adhesion to arterial endothelial cells [33], impairs endothelium-dependent arterial relaxation [34] and promotes macrophage cell proliferation [35]. The ability of CEL to protect against at least one of the effects of lyso-PtdCho, namely cytotoxic damage to the endothelium (Table 2), supports the suggestion of a protective role for vascular CEL. It is noteworthy that lyso-PtdCho levels increased 8-fold in areas of atherosclerotic lesion in comparison with lesion-free areas of the aorta [36]. This observation suggests that lyso-PtdCho is not hydrolysed in areas of severe atherosclerosis. Thus CEL is either absent from sites of atherosclerotic lesion or is rendered inactive during the atherosclerotic process. Although the exact concentration of CEL in lesion-free and lesion-prone areas is unknown at this time, the ability of cholesterol to inhibit lysophospholipid hydrolysis by CEL, either directly through enzyme inhibition or indirectly via alteration of the substrate, suggests that the enzyme becomes inactive during atherosclerosis when cholesterol accumulation is prominent. If this hypothesis is correct, then reduction of cholesterol accumulation will protect against oxidized LDL-induced atherosclerosis by decreasing lyso-PtdCho content in the vessel wall. A similar protective effect may also be accomplished by increasing CEL concentration in the vessel wall.

This research was supported by National Institutes of Health grants DK40917 and DK46405.

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Received 4 July 1997/4 September 1997; accepted 30 September 1997