Apolipoprotein E and Lipoprotein Lipase Increase Triglyceride-Rich Particle Binding but Decrease Particle Penetration in Arterial Wall

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Objective—Liver-derived apolipoprotein E (apoE) decreases atherosclerosis without altering the circulating concentrations of plasma lipoproteins. We evaluated the effects of apoE and lipoprotein lipase (LpL) on the interactions of triglyceride-rich particles (TGRPs) in the arterial wall.

Methods and Results—Quantitative fluorescence microscopy was used to study the interactions of TGRPs (25- to 35-nm diameter) in the arterial wall. Carotid arteries were harvested from rats, placed in a perfusion chamber, and perfused with fluorescently labeled TGRPs. In the absence of apoE or LpL, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-TGRP (100 μg neutral lipid/mL) was poorly retained in the arterial wall. The addition of either apoE (10 μg/mL) or LpL (10 μg/mL) increased TGRP accumulation 220% and 100%, respectively. This effect was attenuated by heparin (10.0 IU/mL). Histological analyses of cross sections from these vessels demonstrate that in the absence of apoE or LpL, there is deep penetration of lipid into the arterial wall. With the addition of either apoE or LpL, arterial wall penetration of TGRP is blocked.

Conclusions—These results demonstrate that although apoE and LpL increase arterial wall accumulation of TGRPs, these proteins also reduce the penetration of TGRPs into the arterial wall. We postulate that this may represent a novel antiatherogenic property of apoE and LpL. (Arterioscler Thromb Vasc Biol. 2002;22:2080-2085.)

Key Words: apolipoprotein E ■ lipoprotein lipase ■ quantitative fluorescence microscopy ■ arterial wall ■ atherosclerosis

Complications from atherosclerosis represent a major cause of morbidity and mortality in Western society. ApoE is a key component of most lipoproteins and facilitates their delivery to tissues. A defect in apoE can lead to type III hyperlipoproteinemia (dysbetalipoproteinemia), a condition characterized by elevated blood triglyceride (TG) and cholesterol levels and premature atherosclerosis. Aside from this human disorder, studies in mice have related apoE to cardiovascular disease. Genetic deletion of apoE in mice leads to hyperlipidemia and the development of premature atherosclerosis, whereas apoE overexpression leads to reduced atherosclerosis.

It appears that apoE has other actions that modulate the progression of atherosclerosis. Overexpression of apoE, at levels that do not alter plasma lipoprotein concentrations, reduces atherosclerosis. Various authors have suggested that this is due to the ability of apoE to facilitate cellular cholesterol efflux. ApoE is a multifunctional protein exhibiting high binding affinity toward cell surface heparan sulfate proteoglycans (HSPGs). This interaction is thought to be critical in apoE-mediated lipoprotein clearance in the liver.

The arterial wall effects of apoE on atherogenesis remain controversial. For example, apoE expression in macrophages appears to be antiatherogenic; interactions of apoE with components of the vascular wall might be the reason for this. Paradoxically, apoE increases lipoprotein association with HSPGs; this process should increase lipoprotein retention within arteries and promote atherogenesis. The association of apoE with lipoproteins such as LDL could be antiatherogenic, whereas the association of apoE with HDL might be atherogenic (increased binding and retention of HDL in the arterial wall) or atheroprotective (facilitating removal of cholesterol ester from cells).

Lipoprotein lipase (LpL) is the enzyme that hydrolyzes circulating TG-rich lipoproteins. LpL has enzymatic and lipoprotein binding functions that occur while LpL is bound to vascular HSPGs. Like apoE, LpL is a constituent of the arterial wall, but recent studies have demonstrated increased atherogenicity when LpL is present in the arterial wall. In support of this concept are studies demonstrating that transplantation of LpL-deficient macrophages leads to reduced atherosclerosis. Macrophage-derived LpL could be atherogenic through several mechanisms: LpL has been...
shown to mediate lipoprotein retention, the creation of remnants from TG-rich lipoproteins, the production of toxic lipolysis products, or alterations in macrophage function.\(^9\)\(^{,}\)\(^{25}\)\(^{,}\)\(^{26}\) Thus, although LpL is a critical enzyme in normal lipid metabolism, its presence in the arterial wall may promote atherogenesis.

The goal of these experiments was to examine the effects of apoE and LpL on lipid accumulation in the arterial wall. ApoE and LpL bind to lipids and proteoglycans, such as HS PGs, and have been shown in the present study to increase the association of lipids within the arterial wall matrix. However, apoE and LpL limit the penetration of these particles deep into the arterial wall. We hypothesize that this inhibition of lipoprotein penetration into the vascular wall may represent a novel antiatherogenic role for these proteins.

Methods

Chemicals and Materials

Krebs-Henseleit buffer consisted of (mmol/L) NaCl 116, KCl 5, CaCl\(_2\), H\(_2\)O 2.4, MgCl\(_2\) 1.2, NH\(_4\)PO\(_4\) 1.2, and glucose 11. BSA (1% by weight in perfusate) was obtained from Sigma Chemical Co. LpL was isolated from bovine buttermilk. Recombinant apoE3 was produced in Escherichia coli by transforming cells with an expression vector containing the human apoE3 CDNA sequence encoding the entire mature apoE3.\(^{27}\) The fluorophore 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine (DiI) was obtained from Molecular Probes and was used to fluorescently label the TG-rich particles (TGRPs).

Animal Care

Sprague-Dawley (Crl:CD [SD] BR) rats (6 weeks old) were obtained from Charles River Laboratories (Wilmington, Mass). Animals received rat chow and water ad libitum and were kept on a 12/12-hour light/dark cycle. All protocols and animal care were approved by the Animal Use Committee at the University of California, Davis.

Preparation of TGRPs and TGRP-ApoE

TGRPs were generated from lipid emulsions consisting of triolein, cholesteryl oleate, and egg yolk phosphatidylcholine combined in a 1:1:2 weight ratio, as previously described.\(^{28}\) The fluorescence probe DiI was added to the lipid emulsions at 0.25 mol% of egg yolk phosphatidylcholine to generate fluorescent TGRPs with the following spectral characteristics: excitation maximum 540 nm, emission maximum 570 nm. Sonication and ultracentrifugation of emulsion TGRPs were generated from lipid emulsions consisting of triolein, cholesteryl oleate, and egg yolk phosphatidylcholine combined in a 1:1:2 weight ratio, as previously described.\(^{28}\) The fluorescence probe DiI was added to the lipid emulsions at 0.25 mol% of egg yolk phosphatidylcholine to generate fluorescent TGRPs with the following spectral characteristics: excitation maximum 540 nm, emission maximum 570 nm. Sonication and ultracentrifugation of emulsion complexes were formed (ratio of protein to NL 1:10) by incubating apoE with TGRP at room temperature for 20 minutes by gentle inversion.\(^{29}\) Experiments were performed with 100 μg NL/mL and 10 μg apoE/mL.

Measurement of Arterial Lipid Accumulation

Rats were anesthetized with pentobarbital (0.1 mL of 50 mg/mL sodium pentobarbital per 100 g body weight). The carotid arteries from the rats were dissected, cannulated, removed, and placed in a microscope-viewing chamber, as previously described.\(^{30}\) After the vessel was positioned under the microscope, the rate of fluorescently labeled TGRP accumulation was measured. Each trial consists of 10 minutes of fluorescently labeled TGRP perfusion, followed by 10 minutes of washout with the nonfluorescent buffer solution. Trials were performed in triplicate to measure the TGRP accumulation rate for each vessel. Both carotid arteries were examined in each animal.

During the entire experiment, the artery was continuously perfused with 1% BSA–Krebs-Henseleit buffer at a rate of 7 mL/min at 37°C and pH 7.4. Additionally, the artery was continuously superfused with Krebs-Henseleit buffer maintained at 37°C and pH 7.4. Distal resistance was adjusted to maintain 100 mm Hg hydrostatic pressure within the vessel at all times. The measurement of TGRP accumulation is performed during the washout phase (Figure 1). This estimation involves analyzing the washout data as 2 distinct processes, a rapid washout of the lumen filled with the fluorescent solution, followed by a slower arterial wall washout. Fluorescence intensity (I) accumulation was determined from the intersection of tangents drawn to approximate these 2 processes. The accumulation rate is calculated as the I, accumulation divided by the length of TGRP perfusion (10 minutes). This value is then converted from the units of millivolts per minute to nanograms lipid per minute per centimeter squared by using the correct conversion factors determined from the following: (1) surface area of vessel under the photometric window, (2) lumen volume of vessel under the photometric window, (3) maximal initial fluorescence intensity of each TGRP perfusion trial, and (4) the concentration of lipid in the perfusion buffer.\(^{30}\)

A total of 16 carotid arteries from male SD rats were used to evaluate the effects of apoE (10 μg/mL), LpL (10 μg/mL), or apoE+LpL on lipid accumulation. Arteries were perfused with (1) DiI-TGRPs (control), (2) TGRPs+LpL or TGRPs+apoE, and (3) TGRPs+LpL+apoE. Each vessel served as its own control.

Histology

To determine whether DiI-lipid had accumulated on the artery endothelial surface or had penetrated into the arterial wall, we used fluorescence histology to image the vessels after the various treatments with apoE and/or LpL.\(^{30}\) Arteries were continuously perfused for 60 minutes with DiI-TGRPs alone, DiI-TGRPs+apoE, DiI-TGRPs+LpL, or DiI-TGRPs+apoE+LpL. This was followed by 30 seconds of perfusion with the buffer solution, which washes out lumen and weakly bound DiI-TGRPs on the endothelial cell surface. Remaining DiI-TGRPs within the arteries were detected by fluorescence microscopy on serial cross sections. The carotid artery segment was fixed with OCT (Tissue-Tec), placed on dry ice, and stored at –80°C. Frozen cryosections of the artery (4 μm thick) were examined with a Zeiss Axiophot MC80 equipped with a Dage DC 330 3CCD color camera interfaced with the Scion LG-3/PCI board and PowerPC 7200 MacIntosh. The same field was captured under fluorescent light with an excitation filter of 530 to 580 nm and an
emission filter of 650 nm (rhodamine filter) and under phase-contrast microscopy. By use of imaging software, the fluorescent image was layered over the phase-contrast image so that the location of the fluorescence could be determined.

Statistical Analysis
All statistical analyses used SigmaStat 2.0 software by Jandel Scientific. Differences between groups were analyzed by Student t test at a level of significance of \( P < 0.05 \). DiI-TGRP accumulation was calculated by using the average of 3 trials, with each trial consisting of 10 minutes of fluorescence perfusion followed by 10 minutes of fluorescence washout.

Results

Effects of ApoE on TGRP Accumulation
Arterial accumulations of control and apoE-containing TGRPs were compared. Initially, dose-response experiments were performed to establish the dose at which apoE induced accumulation of TGRPs. At 10 \( \mu \)g apoE/mL, using 100 \( \mu \)g NL/mL (TGs 50 \( \mu \)g/mL, cholesteryl ester 50 \( \mu \)g/mL), we found significant arterial accumulation of TGRPs. Additionally, in previous studies, we established that at 1 \( \mu \)g apoE/10 \( \mu \)g NL, there was maximal uptake of TGRPs in J774 macrophages.28 We calculated that at this ratio, there are 4 to 6 molecules of apoE per particle, an amount likely to cause saturation of apoE receptors (ie, LDL receptor, LDL-related receptor protein, etc) and allow for significant interactions of apoE particles and HSPGs, as occurs physiologically.28 An example of perfusion of an artery before (control) and after the addition of apoE (10 \( \mu \)g/mL) is illustrated in Figure 1. I, accumulation represents the amount of DiI-lipid that accumulated in the arterial wall during the 10-minute perfusion with the solution containing DiI-TGRP. Note that this accumulation is greater after the addition of apoE. Additionally, the minimal efflux of apoE particles from the arterial wall indicates increased TGRP binding in the presence of apoE with little change in arterial permeability, which would have been manifested as fluorescence intensity returning to baseline. On average, apoE caused a 220% increase in TGRP accumulation relative to control (2.7 ± 0.4 [apoE vs control] versus 8.8 ± 2.3 [apoE] ng NL · min\(^{-1}\) · cm\(^{-2}\) · cm\(^{-2}\); for LpL + apoE, 3.0 ± 0.5 (control) vs 19.1 ± 4.7 (LpL + apoE), \( P < 0.05 \).

LpL Addition to TGRP ± ApoE
In the absence of apoE, the addition of LpL (10 \( \mu \)g/mL) resulted in a 100% increase in TGRP accumulation relative to control (3.1 ± 0.1 [LpL control] versus 6.4 ± 0.9 [LpL] ng NL · min\(^{-1}\) · cm\(^{-2}\) · cm\(^{-2}\); for LpL, 276.9 ± 57.8 (LpL); for LpL + apoE, 16.0 ± 6.7 (control) vs 61.8 ± 28.3 (LpL), \( P < 0.05 \).


diagram
Heparin Modulation of Effects of LpL and ApoE

In separate studies, the role of HSPIs in the binding of lipid in the presence of LpL or apoE was studied by the addition of heparin (10 IU/mL) to the perfusate before the addition of LpL or perfusion of apoE-TGRP. On the addition of heparin, the effects of LpL on TGRP arterial accumulation were dramatically attenuated (3.4 ± 0.3 [LpL control], 58.7 ± 12.6 [LpL], 3.4 ± 0.3 [heparin control], and 5.7 ± 0.9 [LpL + heparin] ng NL · min⁻¹ · cm⁻²). Similarly, heparin was effective at significantly reducing the effect of apoE on TGRP accumulation (5.1 ± 0.7 [apoE control], 8.6 ± 0.1 [apoE], 2.8 ± 0.3 [heparin control], and 3.5 ± 0.2 [apoE + heparin] ng NL · min⁻¹ · cm⁻²). Hence, heparin significantly reduced apoE-induced TGRP accumulation and almost completely abolished LpL-induced TGRP accumulation.

Localization of TGRP in Vascular Wall

Figure 4 demonstrates the retention of TGRPs±apoE±LpL within the arterial wall. When Dil-TGRP was perfused alone, the fluorescent signal was found deep in the subendothelial space beyond the intimal layer (Figure 4A). The specific localization appears to be acellular and randomly distributed within the intimal-medial layer. The addition of apoE or LpL to the emulsion completely eliminated TGRP penetration deep into the arterial wall and increased the overall intensity of fluorescence accumulation on and in the endothelial cell layer (Figure 4B through 4D). Thus, these 2 proteins increased the affinity and amount of lipid binding to the endothelium and also prevented deep penetration of TGRPs into the arterial wall.

Discussion

The present study demonstrates the effects of apoE and LPL on TGRP accumulation in an artery. Although a number of studies have assessed the role of these 2 proteins on the interactions of lipoproteins with cells, this is the first study that illustrates their actions within an intact blood vessel. Specifically, our data demonstrate the following: (1) The addition of apoE to TGRP emulsion particles increases their adherence to the endothelial surface. (2) LpL also increases adherence, and the 2 proteins have more than an additive effect at increasing TGRP binding to the arterial wall. (3) Heparin will inhibit apoE- and LpL-mediated endothelial cell accumulation of TGRPs. (4) TGRP emulsion particles are deposited deeply within the subendothelial layer, and apoE or LpL inhibits this deep penetration.

TGRPs were generated from a lipid emulsion and complexed with apoE to form apoE-TGRP complexes. These particles are the size of IDL/LDL and have been used as model lipoproteins. Their unique advantage is that their composition is exactly known and can be altered, in this case, by the addition of apoE, LpL, or both. Thus, the use of these defined TGRP emulsions enabled us to perform perfusion studies that support the idea that apoE and/or LpL increases lipoprotein accumulation in vascular tissue. We observed a >200% increase in TGRP accumulation after apoE administration and a 100% increase after the addition of LpL. With apoE and LpL added together, there appeared to be more than an additive effect of apoE- and LpL-mediated TGRP accumulation with >500% increases in TGRP accumulation.

Our histological analysis confirms that apoE, LpL, or apoE + LpL increases TGRP accumulation in the endothelial layer of arteries. Additionally, we observed differential localization of TGRPs after the administration of apoE and/or LpL, resulting in a large reduction of TGRP accumulation deep in the arterial wall when either of these proteins were added to the lipid emulsion. Hence, although apoE and/or LpL increases TGRP accumulation in the endothelial layer, it effectively blocks deep penetration of TGRPs into the arterial wall. Additionally, arterial HSPIs appear to be important in...
mediating this effect of TGRP accumulation, inasmuch as the addition of heparin acts to attenuate LpL- and apoE-mediated TGRP accumulation. The greater attenuation of LpL-induced TGRP accumulation in the presence of heparin suggests that the effects of LpL on particle accumulation are largely due to HSPGs, whereas with apoE there appears to be other proteoglycans or receptors involved, such as the LDL receptor or LDL-related receptor protein. Further work is needed to exactly define what binding sites are involved in apoE binding to native endothelium. Coupled with our efflux data demonstrating that the increase in TGRP accumulation is the result of increased binding of TGRPs to the vascular wall and not the result of increased vascular permeability, our data further extend the concept that vascular wall HSPGs play a critical role in selectively binding lipoproteins in the presence of LpL and/or apoE.

The effect of apoE on plasma lipoprotein and hepatic metabolism has been extensively studied.32 Less clear is the role of apoE in extracellular tissue, such as the arterial wall. A number of studies have demonstrated that apoE acts to increase tissue lipoprotein accumulation,1,18,33 although it was not clear whether this is true in arterial tissue. Our group has recently published a study that looked at the effect of apoE on human HDL3 accumulation in mouse carotid arteries and in arterial smooth muscle cell extracellular matrix (ECM).18 Similar to the present study, apoE was found to increase HDL3 arterial tissue and ECM binding. This effect was eliminated in ECM in the presence of chondroitin ABC lyase, consistent with the idea that matrix proteoglycans mediate the binding of apoE particles via their glycosaminoglycan residues.

If apoE plays an antiatherogenic role in arterial tissue, the observation that apoE increases TGRP accumulation is seemingly paradoxical, given the accepted role of apoE in attenuating atherosclerosis. The present study indicates increased accumulation of TGRP accumulation in the endothelial layer; however, deep penetration of TGRPs into the arterial wall was completely prevented. Thus, these observations suggest a new antiatherogenic role for apoE not previously described. Such an effect may also facilitate lipid efflux and reverse cholesterol transport from the arterial wall.9 Hence, regardless of the location or source of apoE, one could infer an atheroprotective effect of apoE in the arterial wall.

LpL plays a supportive role in conjunction with apoE in the delivery and metabolism of TG-rich lipoproteins.1 Unlike apoE, which is a major component of plasma lipoproteins, LpL interacts with lipoproteins predominantly occur on the surface and within the walls of blood vessels. For larger arteries and especially atherosclerotic vessels, LpL expression is most robust by lesional macrophages.34,35 Its enzymatic actions, assisted by apoC-II, to hydrolyze TGs and phospholipids are essential for lipoprotein metabolism by the liver and vascular tissue.19 Additionally, its nonenzymatic role of establishing high-affinity interactions between lipoproteins and ECM, by forming a molecular bridge, is key for lipoprotein processing and removal.21,25,36 This latter role of LpL may have the effect of increasing cellular lipoprotein uptake in macrophages that generate LpL and may accelerate atheroma formation. Additionally, because macrophages also synthesize apoE within the arterial wall, LpL and apoE could both increase lipid retention deep within the vascular wall. Hence, atherogenic and antiatherogenic effects of LpL are possible and may be related to the localization and source of LpL.

Contrary to our previous studies investigating the binding and catalytic effect of LpL on human VLDL metabolism and trafficking within the arterial wall,32 in the present study, we were solely interested in the ability of LpL to bind and retain a synthetic TG-rich emulsion particle. Similar to our previous study, the addition of LpL resulted in large increases in arterial lipid accumulation. However, in the present study, we see that in the absence of lipolysis, the penetration of lipid deep into the arterial wall is abolished on the addition of LpL. The increase in LpL-induced accumulation of TGRPs was maximal when LpL was added directly to TGRPs versus when the arterial wall was preincubated and coated with LpL. These results suggest that more LpL is bound to the surface of TGRPs than is bound to the endothelial surface.

In the present study, apoE and LpL acted to increase TGRP accumulation in rat carotid arteries, an effect mediated chiefly by increased TGRP retention by extracellular HSPGs. Because the location of accumulation was the luminal surface of the arterial wall, we hypothesize this action of plasma-derived apoE or LpL to be antiatherogenic. When the epidemiological evidence of the atheroprotective effects of apoE and LpL is considered, a better understanding of the vascular wall effects of apoE and LpL could help in developing therapeutic strategies effective in thwarting premature atherosclerosis. Mechanistic studies elucidating the pathogenesis and pathophysiology of atherogenesis will be important in a disease that is responsible for enormous morbidity and mortality in western societies.

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