Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: step 1

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Abstract **Apolipoprotein A-I (apoA-I) and an apoA-I peptide mimetic removed seeding molecules from human low density lipoprotein (LDL) and rendered the LDL resistant to oxidation by human artery wall cells. The apoA-I-associated seeding molecules included hydroperoxyoctadecadienoic acid (HPODE) and hydroperoxyeicosatetraenoic acid (HPETE). LDL from mice genetically susceptible to fatty streak lesion formation was highly susceptible to oxidation by artery wall cells and was rendered resistant to oxidation after incubation with apoA-I in vitro. Injection of apoA-I (but not apoA-II or murine serum albumin) into mice rendered their LDL resistant to oxidation within 3 h. Infusion of apoA-I into humans rendered their LDL resistant to oxidation within 6 h. We conclude that *1*) oxidation of LDL by artery wall cells requires seeding molecules that include HPODE and HPETE; *2*) LDL from mice genetically susceptible to atherogenesis is more readily oxidized by artery wall cells; and *3*) normal HDL and its components can remove or inhibit the activity of lipids in freshly isolated LDL that are required for oxidation by human artery wall cells.**—Navab, M., S. Y. Hama, C. J. Cooke, G. M. Anantharamaiah, M. Chaddha, L. Jin, G. Subbanagounder, K. F. Faull, S. T. Reddy, N. E. Miller, and A. M. Fogelman. **Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: step 1.** ***J. Lipid Res.*** **2000.** 41: **1481–1494.**

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HDL and its major apolipoprotein, apoA-I, are known to remove cholesterol and phospholipids from cells (1–4). Stocker and colleagues (5) have reported that cholesteryl ester hydroperoxides can be transferred from LDL to HDL, in part, mediated by cholesteryl ester transfer protein. Sattler and Stocker (6) demonstrated that there was a selective uptake of oxidized cholesteryl esters from HDL by rat liver parenchymal cells. Stocker and colleagues (7) reported that both apoA-I and apoA-II can reduce cholesteryl ester hydroperoxides via a mechanism that involves oxidation of specific methionine residues (8). However, a direct role for apoA-I in removing oxidized lipids from lipoproteins and cells has not previously been reported.

Sevanian and colleagues noted that a subpopulation of freshly isolated low density lipoprotein (LDL) that they have described as LDL^- contains lipid hydroperoxides (9). Parthasarathy (10, 11), Witztum and Steinberg (12, 13), Chisolm (14), Thomas and Jackson (15), Frei and colleagues (16, 17), and Thomas, Kalyanaraman, and Girotti (18) have studied LDL oxidation in vitro by metal ions and have hypothesized that LDL must be "seeded" with reactive oxygen species before it can be oxidized. Thomas and Jackson (15) and Parthasarathy (11) suggested a role for lipoxygenases (LO) in the seeding of LDL. We previously reported that defatted albumin was capable of removing biologically active lipids from mildly oxidized LDL (19). On the basis of the known lipid-binding properties of apolipoprotein A-I (apoA-I) (1–4), we reasoned that apoA-I was likely to be more effective than defatted albumin in binding and removing lipids. We, therefore, used apoA-I and apoA-I mimetic peptides to treat LDL. We hypothesized that if apoA-I could bind oxidized lipids and if the seeding molecules were oxidized lipids, then incubating apoA-I and LDL, followed by separation of the two, might result in the transfer of the seeding molecules from LDL to apoA-I, from which they could be extracted and identified. We found that both the neutral lipid and

Abbreviations: BL/6, C57BL/6J; C3H, C3H/HeJ; PAPC, L-α-1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PEIPC, 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phsophocholine; PGPC, 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine; PON, paraoxonase; POVPC, 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-3-phosphocholine; SHPF, standardized high power field.

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fatty acid fractions of the lipids extracted from apoA-I after incubation with LDL contained seeding molecules. The neutral lipid fraction is the fraction where cholesteryl ester hydroperoxides would be found. Because there is evidence that the lipoxygenase pathway can act to form cholesteryl ester hydroperoxides largely as a result of a nonenzymatic process mediated by the products of fatty acid oxidation and α-tocopherol (20–22), we concentrated our efforts on the fatty acid fraction of the lipids extracted from apoA-I after incubation with freshly isolated LDL. We present evidence that the seeding molecules present in freshly isolated LDL are derived, in part, from the cellular metabolism of linoleic acid [hydroperoxyoctadecadienoic acid (HPODE)] and arachidonic acid [hydroperoxyeicosatetraenoic acid (HPETE)] as originally predicted by Parthasarathy (10, 11) and in accord with the findings of Cyrus et al. (23) and Steinberg (24) that disruption of the 12/15-lipoxygenase gene diminished atherosclerosis in apoE-deficient mice. The experiments presented in this article also indicate that the seeding molecules in freshly isolated LDL can be removed and/or inactivated by normal high density lipoprotein (HDL) and its components (i.e., apoA-I, and paraoxonase). The experiments detailed in this and the following companion article (24a) led us to propose that the biologically active lipids (25, 26) in mildly oxidized LDL are formed in a series of three steps. The first step is the seeding of LDL with products of the metabolism of linoleic and arachidonic acid as well as with cholesteryl hydroperoxides. The second step is likely trapping of LDL in the subendothelial space and the accumulation of additional reactive oxygen species derived from nearby artery wall cells. We propose that the third step is the nonenzymatic oxidation of LDL phospholipids that occurs when a critical threshold of reactive oxygen species is reached, resulting in the formation of specific oxidized lipids that induce monocyte binding, chemotaxis, and differentiation into macrophages. The experiments described in this article focus on the first of these three steps, and the accompanying article presents data on the second and third steps.

MATERIALS AND METHODS

Materials

Tissue culture materials and other reagents were obtained from sources previously described (27–29). Acetonitrile, chloroform, methanol, ethyl acetate, acetic anyhydride, triethylamine, *tert*-butanol, polypropylene glycol, ammonium formate, formic acid, and water (all Optima grade) were obtained from Fisher Scientific (Pittsburgh, PA). Authentic L-α-1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC), arachidonic acid, linoleic acid, and other fatty acids were obtained from Avanti Polar Lipids (Alabaster, AL). The oxidized phospholipids derived from PAPC, including Ox-PAPC, and the oxidized phospholipids 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-3-phosphocholine (POVPC, m/z 594), 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC, m/z 610), and 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphocholine (PEIPC, m/z 828) were prepared and isolated as previously described (25, 26). 13(*S*)-HPODE, 15(*S*)-HPETE, and other eicosanoids were obtained from Biomol (Plymouth Meeting, PA). Human apoA-I and apoA-II, human and murine serum albumin, and soybean lipoxygenase were obtained from Sigma (St. Louis, MO) and were used for in vitro studies and for injection into mice. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic (SDS-PAGE) analyses demonstrated an approximately 90% purity for apoA-I and apoA-II preparations. ApoA-I peptide mimetics were synthesized as previously described (30–32). Human apoA-I/phosphatidylcholine discs for infusion into humans were prepared as previously described by ZLB Central Laboratory (Bern, Switzerland) (33–35). Purified paraoxonase was a generous gift from B. N. La Du of the University of Michigan (Ann Arbor, MI). In addition, two mutant recombinant paraoxonase preparations, which were unable to hydrolyze paraoxon, were kindly provided by R. Sorenson and B. N. La Du (36). Enzyme-linked immunosorbent assay (ELISA) kits for determination of fatty acid oxidation products and eicosanoids were purchased from Assay Designs (Ann Arbor, MI) and used according to the manufacturer instructions.

Lipoproteins

Low density lipoprotein (LDL, d = 1.019 to 1.063 g/ml) and high density lipoprotein (HDL, d = 1.063 to 1.21g/ml) were isolated on the basis of the protocol described by Havel, Eder, and Bragdon (37) from the blood of fasting normal volunteers after obtaining written consent under a protocol approved by the Human Research Subject Protection Committee of the University of California, Los Angeles (Los Angeles, CA). Lipoprotein-deficient serum (LPDS) was prepared by removing the pellet after HDL isolation, dialysis, and readjustment of the protein concentration to 7.5 g/100 ml. In some experiments butylated hydroxytoluene (BHT), 20 mM in ethanol, was added to freshly isolated plasma to a concentration of 20 μM and the lipoproteins were separated by fast protein liquid chromatography (FPLC), using methods previously described (29). The LDL, HDL, and LPDS had endotoxin levels below 20 pg/ml, which is well below the threshold needed for induction of monocyte adhesion or chemotactic activity. The concentrations of lipoproteins reported in this study are based on protein content.

Cocultures

Human aortic endothelial cells (HAEC) and smooth muscle cells (HASMC) were isolated as previously described (27). The wells of microtiter plates (with reduced surface area, 0.15 cm^2) were treated with 0.1% gelatin at 37°C overnight. HASMC were added at a confluent density of 1×10^5 cells/cm^2. Cells were cultured for 2 days, at which time they had covered the entire surface of the well and had produced a substantial amount of extracellular matrix. HAEC were subsequently added at 2×10^5 cells/cm^2 and were allowed to grow, forming a complete monolayer of confluent HAEC in 2 days. In all experiments, HAEC and autologus HASMC (from the same donor) were used at passage levels of four to six.

Monocyte isolation

Monocytes were isolated by a modification of the Recalde method as previously described (38) from the blood of normal volunteers after obtaining written consent under a protocol approved by the Human Research Subject Protection Committee of the University of California, Los Angeles.

Monocyte chemotaxis assay

In general, the cocultures were treated with native LDL (250 μg/ml) in the absence or presence of HDL for 8 h. The supernatants were collected and used for determination of lipid hydroperoxide levels. The cocultures were subsequently washed and fresh culture medium 199 (M199) without any additions was added and incubated for an additional 8 h. This allowed the collection of monocyte chemotactic activity released by the cells as a result of stimulation by the oxidized LDL. At the end of incuba-

tion, the supernatants were collected from cocultures, diluted 40-fold, and assayed for monocyte chemotactic activity. Briefly, the supernatant was added to a standard Neuroprobe chamber (Neuroprobe, Cabin John, MD), with monocytes added to the top. The chamber was incubated for 60 min at 37°C. After the incubation, the chamber was disassembled and the nonmigrated monocytes were wiped off. The membrane was then air dried and fixed with 1% glutaraldehyde and stained with 0.1% Crystal Violet dye. The number of migrated monocytes was determined microscopically and expressed as the mean ± SD of 12 standardized high power fields counted in quadruple wells.

Monocyte adhesion assay

In brief, HAEC monolayers, in 48-well tissue culture plates, were incubated with the desired LDL or phospholipid for 4 h at 37°C as described (39). After washing, a suspension of human peripheral blood monocytes was added and incubated for 10 min. The loosely adherent monocytes were then washed away, the monolayers were fixed, and the number of adherent monocytes was counted in nine standardized high power microscopic fields.

Effect of 13(*S*)-HPODE on LDL oxidation

Freshly isolated LDL (250 μg) was incubated with pure 13(*S*)-HPODE (1.0 μg) in 10% LPDS in M199 for 4 h at 37°C with gentle mixing. LDL was reisolated by centrifugation and incubated with monolayers of human aortic endothelial cells. Supernatants were removed at time points ranging from 0 to 5 h and were assayed for lipid hydroperoxide content. The endothelial monolayer was washed after each time point and a monocyte suspension was added, incubated, washed, and the number of adherent monocytes determined.

Treatment of LDL with soybean lipoxygenase

Freshly isolated LDL (250 μg) was incubated with 10 units of pure soybean lipoxygenase bound to Sepharose beads for 4 h at 37°C with gentle mixing. LDL was reisolated by centrifugation and incubated with monolayers of HAEC. Supernatants were removed at time points ranging from 0 to 4 h and were assayed for lipid hydroperoxide content. The endothelial monolayer was washed after each time point and monocyte adhesion was determined.

Mice

C57BL/6J and C3H/HeJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were female (4–6 months of age at the time of the experiments). The mice were maintained on a chow diet (Purina Chow; Ralston-Purina, St. Louis, MO) containing 4% fat. LDL was isolated from groups of the lesion-susceptible C57BL/6 mice and the lesion-resistant C3H/HeJ mice, using blood obtained from the retroorbital sinus with heparin as an anticoagulant (2.5 U/ml blood) and under mild isoflurane anesthesia, adhering to the regulations set forth by the University of California Animal Research Committee.

Infusion of apoA-I into humans

After obtaining written informed consent and with IRB approval from St. Bartholomew's and the Royal London School of Medicine and Dentistry, apoA-I/phosphatidylcholine discs were infused at a dose of 40 mg of apoA-I/kg of body weight over 4 h, using the materials and protocol described by Nanjee et al. (33, 34) into six healthy male subjects. The lipid levels for these six volunteers (subjects 1, 2, 3, 4, 5, and 6) were, respectively: total cholesterol: 149, 160, 164, 209, 153, and 163 mg/dl; triglycerides: 176, 169, 95, 150, 121, and 153 mg/dl; LDL-cholesterol: 69, 73, 88, 117, 73, and 82 mg/dl; and HDL-cholesterol: 45, 54, 56, 62, 59, and 47 mg/dl. Plasma was prepared 2 h before and 6 h after the infusion, was cryopreserved as described (37), and LDL was isolated by FPLC in Los Angeles before the experiments. LDL isolated from plasma according to this protocol functions in a manner that is indistinguishable from freshly isolated LDL in vitro and in vivo (40).

Preparation of cholesteryl linoleate hydroperoxide

Peroxidation of cholesteryl linoleate was accomplished by combining cholesteryl linoleate dissolved in chloroform–methanol 2:1 with 70% *tert*-butyl hydroperoxide and incubating at room temperature with mixing for 24 h. The lipids were extracted with chloroform–methanol 2:1 with Folch wash and separated by silica gel thin-layer chromatography (TLC) with ethyl acetate–heptane 1:1 as the mobile solvent. In more recent experiments we have used cholesteryl linoleate hydroperoxide from Cayman Chemical, with highly similar results.

Solid-phase extraction chromatography

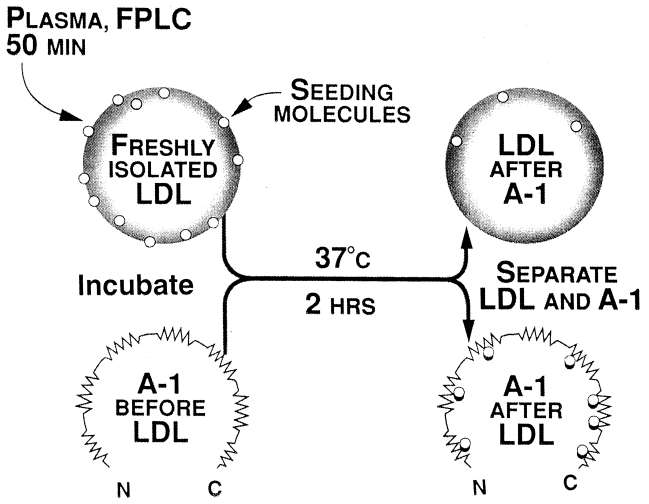
Solid-phase extraction chromatography was performed as previously described (41). In brief, the lipid extract from no more than 2.0 mg of LDL protein was resuspended in 250 μl of chloroform containing 0.01% BHT. Solid-phase extraction amino columns (Fisher) were conditioned by adding 3.0 ml of methanol followed by 6.0 ml of hexane, using a Vac-Elut manifold (Analytichem International, Harbor City, CA). The lipids were added to the column and neutral lipids (cholesterol, cholesteryl esters, cholesteryl ester hydroperoxides, triglycerides, diglycerides, and monoglycerides) were eluted with 3.0 ml of chloroform–isopropanol 2:1 (v/v). Free fatty acids were eluted with 3.0 ml of 3% acetic acid in diethyl ether, and phospholipids were eluted with 3 ml of methanol. The solvents were evaporated, the lipids were resuspended in chloroform–methanol 2:1 (v/v) with 0.01% BHT, covered with argon, and stored at −80°C. In these analyses the recovery of the C17:0 added as an internal standard was 92 ± 3%. Solid-phase extraction chromatography (SPEC) did not induce detectable levels of oxidation products of linoleic or arachidonic acids in duplicate experiments (data not shown). Pure linoleic acid and arachidonic acid were subjected to SPEC along with sham treatment of samples. The neutral lipids, phospholipids, and fatty acid subclasses were then analyzed by reversed-phase high performance liquid chromatography (HPLC). The levels of C18:2 and C20:4 (relative to internal standards included) after SPEC were similar to those after sham treatment (relative to internal standards included). In addition, there were no detectable levels of oxidation products of C18:2 or C20:4 (data not shown).

Thin-layer chromatography

Lipid thin-layer chromatography (TLC) was performed as described previously (19). Pure cholesterol oleate, cholesterol linoleate, unesterified cholesterol, arachidonic acid, linoleic acid, cardiolipin, phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine were used as standards.

Fast performance liquid chromatography

FPLC for the rapid and mild isolation of LDL and for reisolation of LDL and apoA-I after incubation as shown in **Scheme 1** was performed as previously reported (29). For the detection of cholesteryl linoleate hydroperoxide an Alltech Associates (Deerfield, IL) Alltima 250 × 4.6 mm, 5-μm RP-HPLC C_{18} column was used to separate and detect cholesteryl linoleate hydroperoxide at 234 nm and cholesteryl linoleate at 205 nm. The mobile solvent consisted of acetonitrile–2-propanol–water 44:54:2 (v/v/v) at 1.0 ml/min. Lipids were resuspended in the mobile solvent for injection.



Scheme 1. Removal of seeding molecules from LDL by apoA-I. Butylated hydroxytoluene (BHT) was added to freshly isolated plasma to a concentration of 20 μM and was fractionated by gel-filtration chromatography, using an FPLC system with two Superose 6 columns connected in series and eluting with normal saline. The fractions containing LDL were pooled. Purified apoA-I (100 μg/ml) was added to the LDL (0.3 to 1.0 mg/ml) and incubated for 2 h at 37°C with gentle mixing in normal saline. The LDL and apoA-I were then reisolated by FPLC or by centrifugation. Reisolated LDL is designated "LDL after A-1" and the reisolated apoA-I is designated "A-1 after LDL."

Reversed-phase high performance liquid chromatography

High performance reversed-phase liquid chromatography (RP-HPLC) was conducted according to the methods of Yamamoto and Ames (42), Kambayashi and colleagues (43), and A. Sevanian (personal communication). In brief, the analyses were performed by injecting isolated lipids resuspended in mobile solvent onto the column and eluting at a flow rate of 1.0 ml/min. Detection of fatty acid oxidation products was performed by UV absorbance with a diode array detector (Beckman Instruments, Palo Alto, CA) scanning from 200 to 350 nm or with an evaporative light scattering detector (ELSD) (SEDEX 55; Richard Scientific, Novato, CA). For quantitative analysis, pure authentic fatty acids, eicosanoids, and heptadecanoic acid were used as internal standards. Hypersil MOS-1 C8 (Alltech) or Supelcosil LC-18-DB (Supelco) columns were used for the separation of fatty acid oxidation products, and an Alltima C_{18} column (Alltech) was used for the separation of cholesteryl ester oxidation products. A solvent system composed of methanol–triethylamine 99.99:0.01 (v/v) was used to elute 13-HPODE, one consisting of a gradient of acetonitrile–water–acetic acid 60:40:0.1 (v/v/v) to acetonitrile–water–acetic acid 98:2:0.1 (v/v/v) was used to elute 12-HPETE, 15-HPETE, 13-HODE, 12-HETE, and 15-HETE, and one consisting of acetonitrile–2-propanol–water 44:54:2 (v/v/v) was used to elute cholesteryl linoleate hydroperoxide.

Electrospray ionization mass spectrometry

Electrospray ionization mass spectrometry (ESI–MS) in the positive or negative ion mode was performed according to the protocol and conditions previously described (25). The limit of detection for phospholipids in the positive ion mode was 0.2 pmol/μl and for fatty acids in negative ion mode was 1.0–2.0 pmol/μl.

Other methods

Protein content of lipoproteins was determined by a modification (44) of the Lowry assay (45). The levels of monocyte chemotactic protein 1 were determined by an ELISA as described previously (27). Lipid hydroperoxide levels were measured by the assay reported by Auerbach, Kiely, and Cornicelli (46). In some experiments, where indicated, the lipids in culture supernatant containing LDL that was oxidized by the artery wall cell cocultures was extracted with chloroform–methanol and hydroperoxides were determined by the Auerbach method. Paraoxonase (PON) activity was measured as previously described (47). Statistical significance was determined by model 1 analysis of variance (ANOVA). The analyses were carried out first by ANOVA in an Excel application to determine if differences existed among the group means, followed by a paired Student's *t*-test to identify the significantly different means, when appropriate. Significance is defined as $P < 0.05$.

RESULTS

ApoA-I and an apoA-I peptide mimetic remove seeding molecules from freshly isolated human LDL and render the LDL resistant to oxidation by human artery wall cells

Our human artery wall coculture system has been extensively characterized (27–29, 48–51). When LDL is added to this coculture it is trapped in the subendothelial space and is oxidized by the artery wall cells. As a result, three biologically active oxidized phospholipids are produced: POVPC, PGPC, and PEIPC with characteristic m/z ratios of 594, 610, and 828, respectively (25, 26). These three oxidized phospholipids account for the ability of mildly oxidized LDL to induce endothelial cells to bind monocytes, secrete the potent monocyte chemoattractant MCP-1 (monocyte chemotactic protein 1), and the differentiation factor macrophage colony-stimulating factor (M-CSF) (27, 52, 53). Conditioned medium from cocultures exposed to LDL was found to contain MCP-1 (27). When human monocytes were added to the LDL-treated cocultures, the monocytes bound to the endothelial cells and emigrated into the subendothelial space (27). Addition to the cocultures of neutralizing antibody to MCP-1 completely abolished LDL-induced monocyte chemotaxis (27). Thus, coculture monocyte chemotaxis is a highly sensitive bioassay for the formation of the biologically active oxidized phospholipids and the subsequent induction of MCP-1 (27–29, 49, 51).

ApoA-I is the major protein component of normal HDL. Because of its known ability to bind cholesterol and phospholipids (1–4) we hypothesized that apoA-I might also bind the seeding molecules in LDL. To test this hypothesis we utilized the protocol shown in Scheme 1. Butylated hydroxytoluene (BHT) was added to freshly drawn plasma and LDL was separated by FPLC and incubated for 2 h with apoA-I at 37°C. The LDL and apoA-I were then rapidly separated and studied. We refer to the LDL and apoA-I after separation as "LDL after A-I" and "A-I after LDL," respectively.

Figure 1 demonstrates that LDL after A-I could not be oxidized by a coculture of human artery wall cells. The data in Fig. 1 represent the means ± SD of those obtained

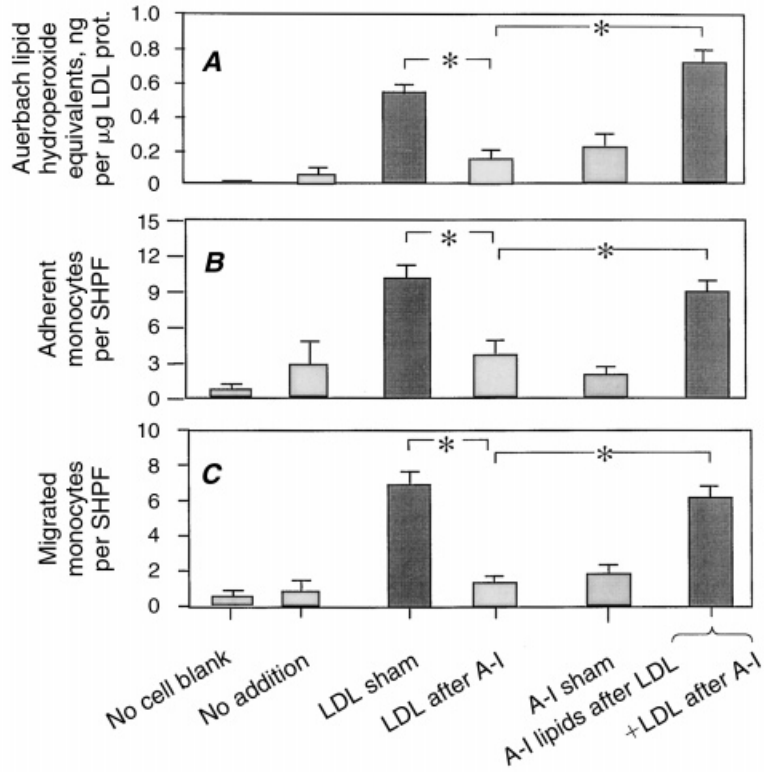


Fig. 1. Resistance of LDL to oxidation after incubation with apoA-I. LDL was rapidly isolated by FPLC from seven normal human donors and incubated at 0.3–1.0 mg/ml with apoA-I (100 μg/ml), followed by reisolation of the LDL and apoA-I as described in Scheme 1. Cocultures of artery wall cells were incubated with sham-treated LDL (LDL sham) or with LDL that was incubated with apoA-I and was reisolated (LDL after A-I), or with sham-treated apoA-I (A-I sham). To other coculture wells was added reconstituted LDL that was prepared by incubating "LDL after A-I" plus the lipids extracted from "A-I after LDL" (A-I lipids after LDL + LDL after A-I). The cocultures were incubated for 8 h at 37°C in the presence of 10% LPDS. The supernatants were collected and analyzed for Auerbach lipid hydroperoxide equivalents. Monocyte adhesion was determined on one set of the cocultures and the others were washed and incubated with culture medium without serum or LPDS for 16 h. This conditioned medium was collected and analyzed for monocyte chemotactic activity. (A) Lipid hydroperoxide levels of supernatants; (B) monocyte adherence; (C) values for monocyte chemotactic activity. The values are means ± SD of quadruplicate cocultures from seven separate experiments using LDL from seven different normal donors and cocultures and monocytes from different donors. The asterisks indicate $P < 0.0004$.

in seven of seven experiments using LDL taken from seven different normal individuals and using different cocultures and monocytes taken from different donors. Thus, these results are highly reproducible and demonstrate in Fig. 1A that the artery wall cells were unable to oxidize LDL after A-I. However, if the lipid extract from A-I after LDL was added back to LDL after A-I, it was readily oxidized (Fig. 1A). Also, as shown in Fig. 1, LDL after A-I did not stimulate monocyte adherence (Fig. 1B) or chemotaxis (Fig. 1C). However, when the lipid extract from A-I after LDL was added back to LDL after A-I the reconstituted LDL induced monocyte adherence (Fig. 1B) and chemotaxis (Fig. 1C) to the same degree as sham-treated LDL. Results that were highly similar to those shown in Fig. 1C were obtained when monocyte chemotactic protein 1 levels were measured by ELISA (data not shown).

The ability of apoA-I to bind lipids has been determined to be a function of its specific α-helical structure (54). Anantharamaiah and colleagues have synthesized apoA-I peptide mimetics that have been extensively characterized (30, 31). One of these peptide mimetics is known as 37pA with the amino acid sequence DWLKAFY DKVAEKLKEAFPDWLKAFYDKVAEKLKEAF. A peptide with the same amino acid sequence as 37pA but containing three extra amino acid residues [aspartic acid (D), glutamic acid (E), and proline (P)] at the N terminal that prevent the α-helix formation necessary for lipid binding has also been constructed by this group, using previously published methods (31). This control peptide, known as 40P, binds lipids poorly compared with 37pA. As shown in **Fig. 2**, after LDL had been incubated with and then separated from the apoA-I peptide mimetic 37pA, the LDL ("LDL after 37pA") was resistant to oxidation by the artery wall cells (Fig. 2A) and did not induce monocyte chemotactic activity (Fig. 2B). However, if the lipid extract from the peptide after incubation with the lipoprotein ("37pA after LDL") was added back to LDL after 37pA, it was readily oxidized (Fig. 2A). In contrast, "LDL after 40P" showed no reduction in LDL oxidation by the artery wall cells (Fig. 2A) and there was no reduction in LDL-induced monocyte chemotaxis (Fig. 2B). Thus, both apoA-I and its peptide mimetic 37pA were able to remove lipids from freshly isolated LDL that rendered the LDL resistant to oxidation by human artery wall cells and prevented LDL-induced monocyte chemotaxis.

Seeding molecules in freshly isolated LDL that are removed by apoA-I include 13-HPODE and 15-HPETE

To identify biologically active lipids associated with LDL that was rapidly isolated by FPLC in the presence of 20 μM BHT as indicated in Scheme 1, lipids were extracted from A-I after LDL. The lipids were analyzed by thin-layer chromatography. **Table 1** demonstrates the mean values for lipids transferred to apoA-I from LDL obtained from three normal individuals. There was detectable cholesteryl ester, unesterified cholesterol, and phosphatidylcholine that was transferred from LDL to apoA-I. Sphingomyelin was not detected on apoA-I after 2 h of incubation with LDL at 37°C with gentle mixing. In addition to TLC, the lipids that were extracted from apoA-I after incubation with and separation from LDL were fractionated by solid-phase extraction chromatography. The neutral lipid or fatty acid fractions were then added to cocultures together with either PAPC, a phospholipid present in LDL,

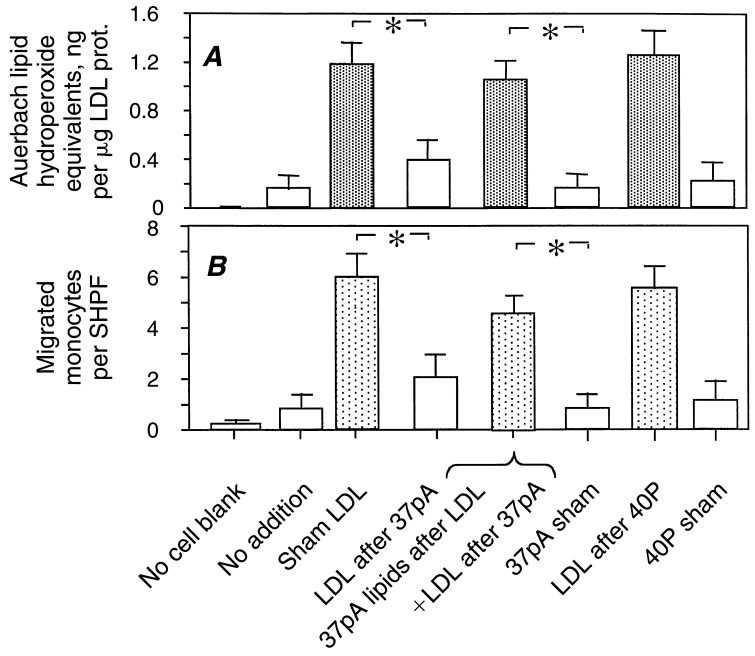


Fig. 2. Effect of pretreatment of LDL with apoA-I peptide mimetics on LDL oxidation and chemotactic activity. Freshly isolated LDL was incubated at 250–350 μg/ml with buffer (Sham LDL), with the apoA-I mimetic peptide 37pA at 100 μg/ml, or with the control peptide 40P at 100 μg/ml. The incubation was conducted in M199 for 2 h at 37°C with gentle mixing. LDL and the peptides were subsequently reisolated as described in Scheme 1. Cocultures of artery wall cells were incubated with sham-treated LDL (Sham LDL), or with LDL that was incubated with the apoA-I mimetic peptide (LDL after 37pA), or with the control peptide (LDL after 40P), sham-treated 37pA (37pA sham), or sham-treated 40P (40P sham). To other coculture wells was added reconstituted LDL that was prepared by incubating "LDL after 37pA" plus the lipids extracted from "37pA after LDL" (37pA lipids after LDL + LDL after 37pA). These additions were incubated with human artery wall cocultures for 8 h in the presence of 10% LPDS. The supernatants were collected and analyzed for Auerbach lipid hydroperoxide equivalents (A). The cocultures were then washed and incubated with culture medium without serum or LPDS for 8 h. The conditioned medium was then collected and analyzed for monocyte chemotactic activity (B). The data indicate means ± SD of values obtained from quadruplicate cocultures in three separate experiments. Asterisks indicate $P < 0.0014$.

or to LDL after A-I. Addition to the cocultures of PAPC or LDL after A-I did not stimulate lipid hydroperoxide formation or monocyte chemotactic activity (**Fig. 3**, open columns). However, addition to PAPC or to LDL after A-I of either the fatty acid fraction (Fig. 3A and B, solid columns) or the neutral lipid fraction (Fig. 3C and D, solid columns) extracted from A-I after LDL induced a dose-dependent increase in the formation of Auerbach lipid hydroperoxide equivalents and monocyte chemotaxis. In reversed-phase HPLC analyses, the neutral lipid fraction contained detectable levels of a peak coeluting with cholesterol linoleate hydroperoxide with a retention time of 14.15 min (data not shown). These experiments indicated that apoA-I removed lipids from freshly isolated LDL that was required for the artery wall cells to oxidize both PAPC and LDL. Addition of either the fatty acid or neutral lipid fractions recovered from A-I after LDL resulted in the oxidation of PAPC and LDL after A-I by the artery wall cells.

To further identify the fatty acids, freshly isolated LDL was incubated with or without apoA-I and then separated by ultrafiltration. After incubation with apoA-I, the lipids were extracted from the LDL (LDL after A-I) and from the apoA-I (A-I after LDL) in the presence of 0.01% BHT. Lipids were also extracted from apoA-I that was incubated without LDL (A-I sham) and from LDL that was not incubated with apoA-I (LDL sham). The extracted lipids were then fractionated by reversed-phase HPLC or by evaporative light scattering detection (ESLD) HPLC. ApoA-I that had not been incubated with LDL contained little if any 13-HPODE (**Fig. 4A**) and contained no 12-HPETE or 15-HPETE (Fig. 4E). In contrast, freshly isolated LDL that had been incubated without apoA-I (LDL sham) contained a major peak coeluting with pure authentic 13-HPODE and an unidentified nearby peak (Fig. 4B), and also contained peaks coeluting with 12- and 15-HPETE (Fig. 4F). LDL after A-I contained substantially less 13-

TABLE 1. Lipid transfer from freshly isolated LDL to apoA-I (mg/mg apoB)

	LDL		LDL after A-I	A-I after LDL	A-I sham	
Lipid Sample	t = 0	t = 2 h	(t = 2 h)	(t = 2 h)	t = 0	t = 2 h
Cholesteryl esters	1.8 ± 0.2	1.7 ± 0.1	1.6 ± 0.2	0.2 ± 0.1	ND	ND
Cholesterol	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.1 ± 0.1	ND	ND
Phosphatidylcholine	0.61 ± 0.1	0.51 ± 0.2	0.52 ± 0.3	0.1 ± 0.1	ND	ND
Sphingomyelin	0.2 ± 0.03	0.2 ± 0.1	ND	ND	ND	ND

Values are means ± SD of measurements from three separate experiments using LDL from three normal donors. Lipid extracts were analyzed by TLC as described in Materials and Methods and the band intensity was determined. All incubations were carried out in normal saline in the presence of 0.01% BHT at 37°C with gentle mixing. LDL, t = 0: freshly isolated LDL; LDL, t = 2 h: LDL incubated for 2 h; LDL after A-I, t = 2 h: LDL incubated with apoA-I; A-I after LDL, t = 2 h: apoA-I incubated with LDL followed by reisolation of apoA-I; A-I sham, t = 0: apoA-I incubated at time 0; A-I sham, t = 2 h: apoA-I incubated for 2 h. ND, Not detectable.

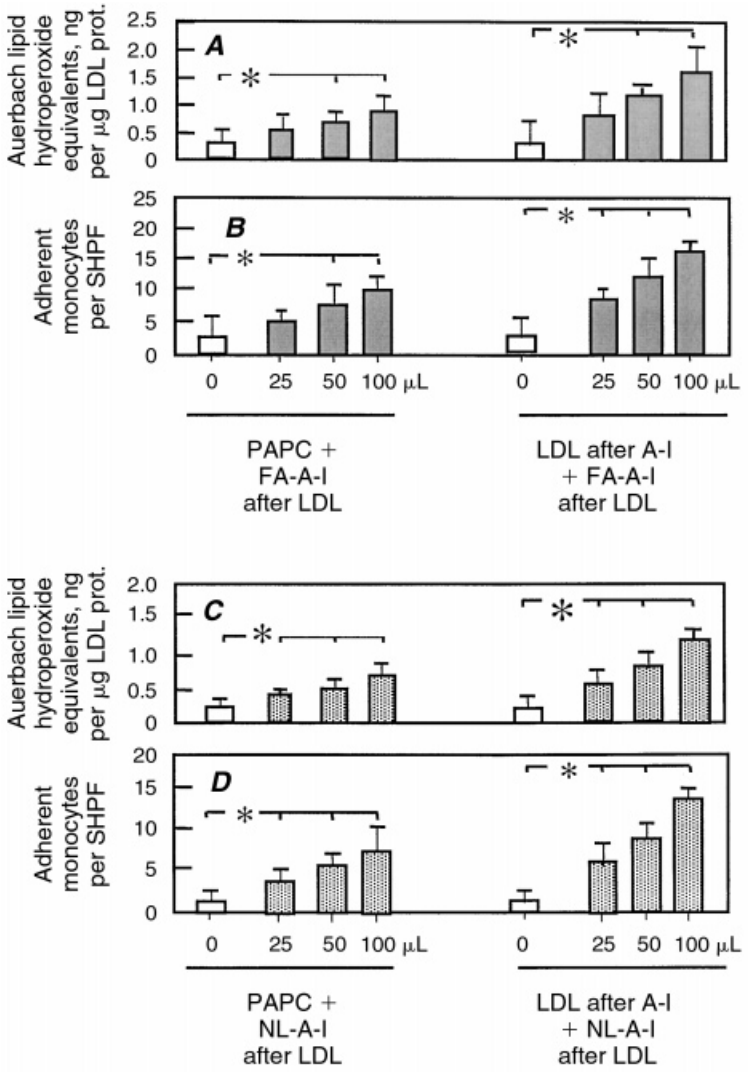


Fig. 3. Bioactivity of lipids extracted by apoA-I. Freshly isolated LDL (1 mg/ml) was incubated with apoA-I (100 μg/ml) and reisolated as indicated in Scheme 1. Lipids were extracted from "A-I after LDL" by chloroform–methanol extraction and separated by solid-phase extraction chromatography as described in Materials and Methods. The fatty acid (25 μg/10 μl) (FA) or neutral lipid (0.5 μg/μl) (NL) fractions were evaporated to dryness and were incubated with 200 μl of M199 containing 10% LPDS at 37°C for 5 min with intermittent gentle vortexing. Fatty acids [FA-A-I after LDL; (A and B)], or neutral lipids [NL-A-I after LDL; (C and D)], were then incubated at the indicated quantities with either 100 μg of PAPC or 250 μg of "LDL after A-I" in a total volume of 1 ml of M199 containing 10% LPDS at 37°C for 3 h. This treated PAPC or LDL in M199 containing 10% LPDS was then incubated with HAEC at 37°C for 4 h. The supernatants were removed and assayed for Auerbach lipid hydroperoxide equivalents (A and C) as described in Materials and Methods. The cells were washed and monocyte adhesion was determined (B and D) as described in Materials and Methods. The data are means ± SD from three separate experiments. The asterisks indicate significance at the level of $P < 0.01$.

HPODE (Fig. 4C) and less 12- and 15-HPETE compared with sham-treated LDL (Fig. 4G). Figure 4D and H demonstrates that 13-HPODE, 12-HPETE, and 15-HPETE, respectively, were transferred to apoA-I. Additional analyses using mass spectrometry confirmed the presence of HPODE, HPETE, and their oxidation and dehydration products in freshly isolated LDL, which were effectively removed by incubation with apoA-I (data not shown). The lipid extracts from LDL sham, LDL after A-I, and A-I after LDL were fractionated by reversed-phase HPLC and fractions containing materials coeluting with authentic 13-HPODE were analyzed by ESI-MS in the negative ion mode. This demonstrated the presence of ions at m/z 311.2 corresponding to HPODE, m/z 295 for HODE, and m/z 293.2 for a dehydration product of HPODE, that is, the loss of one molecule of water (data not shown). In addition, the lipid extracts from LDL sham, LDL after A-I, and A-I after LDL were fractionated by evaporative light scattering detector (ELSD) HPLC and fractions containing materials coeluting with authentic 12-HPETE, 15-HPETE, 12-HETE, and 15-HETE were analyzed by ESI-MS in the negative ion mode. Ions present in less abundance compared with those for the linoleic oxidation products and at m/z 335.2, corresponding to HPETE, m/z 319.0 for HETE, and m/z 317.5 for a dehydration product of HPETE were observed (data not shown). ELSD is more sensitive than RP-HPLC for detection of fatty acids and eicosanoids. The lipid extracts from A-I sham contained little if any detectable linoleic acid or arachidonic acid oxidation products. The lipid extracts from LDL sham, LDL after A-I, and A-I after LDL prior to HPLC fractionation also contained ions at m/z 255, m/z 279.1, and m/z 303.1 corresponding, respectively, to unoxidized palmitic, linoleic, and arachidonic acids (data not shown). There was no significant change in the level of lipid oxidation products in LDL + A-I at the end of the 2-h incubation as compared with freshly isolated LDL (data not shown).

As demonstrated in **Table 2**, there were detectable levels of 12-HPETE, 13-HPODE, 15-HPETE, 12-HETE, 13-HODE, and 15-HETE in the freshly isolated LDL before incubation. The level of these lipid oxidation products did not increase during the 2-h incubation, strongly suggesting that they were present in vivo and were not formed artifactually in vitro (Table 2).

Addition of authentic 13(*S*)-HPODE to freshly isolated

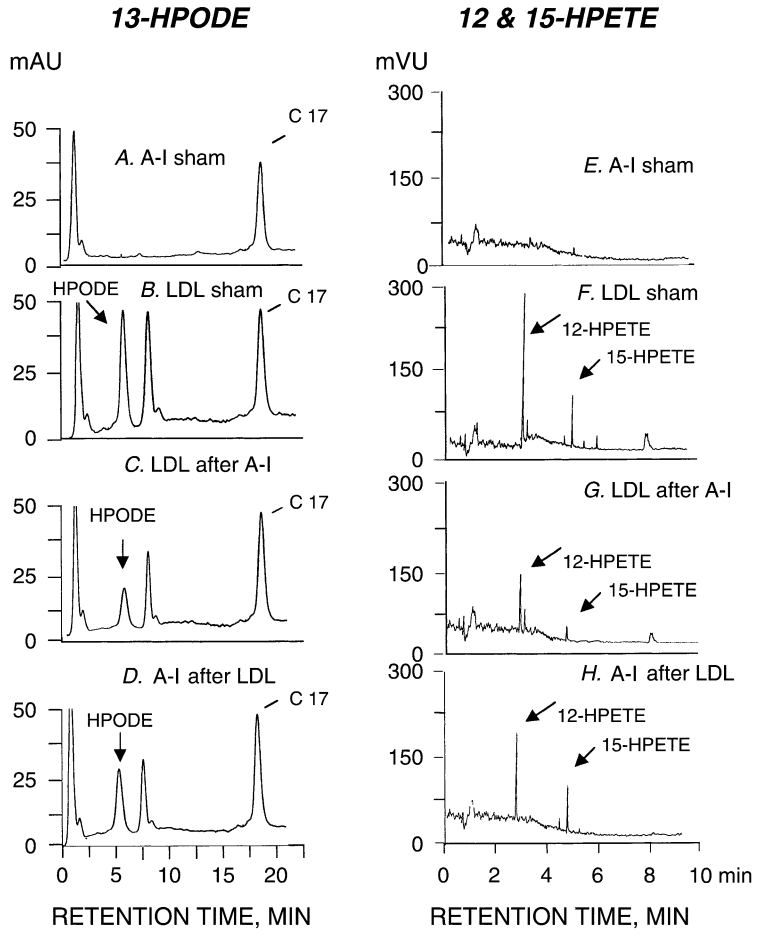


Fig. 4. Removal of 13-HPODE and 15-HPETE by apoA-I from LDL. Freshly isolated LDL (1 mg/ml) was incubated alone (LDL sham), or with apoA-I (100 μg/ml) in M199 for 2 h, with gentle mixing. For controls, apoA-I at 100 μg/ml was incubated alone in M199 for 2 h [A-I Sham; (A and E)] or freshly isolated LDL at 0.3 to 1 mg/ml was incubated alone in M199 for 2 h (LDL Sham; (B and F)] with gentle mixing at 37°C. The LDL and apoA-I were then reisolated by centrifugation, using Millipore molecular weight cutoff filters (100 kDa). Lipids were extracted from apoA-I and from LDL and were analyzed by reversed-phase HPLC for 13-HPODE and by evaporative light scatter detection (at 300 mVU) for 12- and 15-HPETE and other eicosanoids. Approximately 210 μg of LDL lipids was used for each analysis. (C) and (G) demonstrate the decrease in the 13-HPODE and 12- and 15-HPETE peaks in LDL after incubation with apoA-I (LDL after A-I) and (D) and (H) demonstrate the increase in 13-HPODE and 12- and 15-HPETE, respectively, in the lipid extract from apoA-I after incubation with and separation from LDL (A-I after LDL).

LDL as described in Materials and Methods increased the formation of Auerbach lipid hydroperoxide equivalents when the LDL was added to HAEC and also increased monocyte adherence to HAEC (data not shown).

In other experiments 10 μg of 13-HPODE was dried down and resuspended with LDL, 250 μg of protein/ml in saline. A second sample was sham treated. Mixtures were incubated for 60 min at 37°C with gentle mixing. ApoA-I (100 μg) was added to one aliquot of LDL (preincubated with 13-HPODE) and incubated for 60 min at 37°C with mixing. All other mixtures were sham treated. ApoA-I sham, 100 μg/ml, was included as a control. ApoA-I was separated from the LDL by ultrafiltration. ApoA-I after LDL, LDL sham, and apoA-I sham were extracted with ethyl acetate and reconstituted in ethanol, 100 μl. Ten-microliter aliquots were injected into an RP-HPLC (C_8 column), and the area under the curve at 234 nm was calculated from a standard curve consisting of 13(*S*)-HPODE. Seventy percent of the added 13-HPODE associated with the LDL and 28% subsequently transferred to apoA-I (data not shown). These experiments provide further evidence that HPODE readily associates with LDL and that substantial amounts are readily transferred to apoA-I within 1 h at 37°C. The results of experi-

TABLE 2. Level of octadecanoids and eicosanoids in freshly isolated LDL (pmol/100 μg LDL protein)

LDL Isolated from Subject	12-HPETE		13-HPODE		15-HPETE		12-HETE		13-HODE		15-HETE	
	t = 0	t = 2 h	t = 0	t = 2 h	t = 0	t = 2 h	t = 0	t = 2 h	t = 0	t = 2 h	t = 0	t = 2 h
1	170	181	320	301	1,200	1,109	ND	ND	200	210	ND	ND
2	60	35	170	115	171	156	21	ND	31	ND	ND	ND
3	280	198	534	870	3,800	2,980	80	95	2	ND	30	35
4	171	24	172	25	173	25	20	ND	40	ND	80	90
5	61	39	280	290	480	495	60	50	570	450	100	111
6	401	365	300	265	601	565	5	ND	20	ND	130	140
7	1,300	1,254	1,540	1,101	200	187	770	580	50	ND	30	40

Values are from LDL isolated from seven normal control subjects by FPLC and incubated in saline containing 0.01% BHT at 350 μg/mL for 2 h at 37°C with gentle mixing. Lipids were extracted and analyzed by evaporative light scatter detection HPLC. The area under the peaks coinciding with pure authentic icosanoids were determined and quantitated relative to the authentic pure lipids used. ND, Not detectable.

TABLE 3. Transfer of 13-HPODE from LDL to apoA-I (pmol)

Sample	Subject 1	2	3	4
A-I sham	ND	ND	ND	ND
LDL sham	210	982	654	762
LDL after A-I	80	353	214	302
A-I after LDL	90	547	324	363

Values are from four separate experiments using freshly isolated LDL from four normal donors. Incubations were carried out in normal saline in the presence of 0.01% BHT at 37°C with gentle mixing for 2 h. Lipids were extracted as described in Materials and Methods and reversed-phase HPLC was performed. The area under the curve for the peak corresponding to 13-HPODE was determined on the basis of the signal obtained for known amounts of pure authentic 13-HPODE used. A-I sham, apoA-I incubated alone; LDL sham, LDL incubated alone; LDL after A-I, LDL incubated with apoA-I followed by reisolation of LDL; A-I after LDL, apoA-I incubated with LDL followed by reisolation of apoA-I. ND, Not detectable.

ments showing the transfer of endogenous 13-HPODE from LDL to apoA-I are shown in **Table 3**.

13(*S*)-HPODE is the product of lipoxygenase activity on linoleic acid (55, 56). Because the major unsaturated fatty acid in LDL is linoleic acid, freshly isolated LDL was incubated with or without soybean lipoxygenase. After incubation with and then separation from the soybean lipoxygenase as described in Materials and Methods, the LDL was added to HAEC cultures. The LDL that was incubated with and then separated from soybean lipoxygenase significantly increased the formation of Auerbach lipid hydroperoxide equivalents in the culture supernatants and also increased monocyte adherence to HAEC as compared with LDL incubated without soybean lipoxygenase (data not shown).

Taken together these experiments indicate that the seeding molecules in freshly isolated LDL that are removed by apoA-I include HPODE and HPETE.

Freshly isolated LDL from mice that are genetically susceptible to atherosclerosis are highly susceptible to oxidation by human artery wall cells and are rendered resistant to oxidation by human apoA-I

When fed an atherogenic diet, C57BL/6J (BL/6) mice develop fatty streak lesions in their aorta while C3H/HeJ (C3H) mice do not, despite equivalent levels of apoB-containing lipoproteins (57, 58). We previously have presented evidence to suggest that the lesion-susceptible BL/6 mice are under oxidative stress (48, 51, 59). A logical consequence of this hypothesis might be increased susceptibility to oxidation of LDL from the BL/6 mice compared with LDL from the lesion-resistant C3H mice. On a low-fat chow diet the two strains of mice have similar low levels of plasma LDL and the lesion-susceptible BL/6 mice have higher levels of plasma HDL (57). To test our hypothesis we incubated freshly isolated LDL from the two strains, both of which were on the low-fat chow diet, with and without human apoA-I and then separated the LDL and apoA-I and incubated them with human artery wall cell cocultures. As shown in **Fig. 5**, LDL incubated without apoA-I (LDL sham) from the lesion-sensitive BL/6 mice was more readily oxidized (Auerbach assay) by the artery

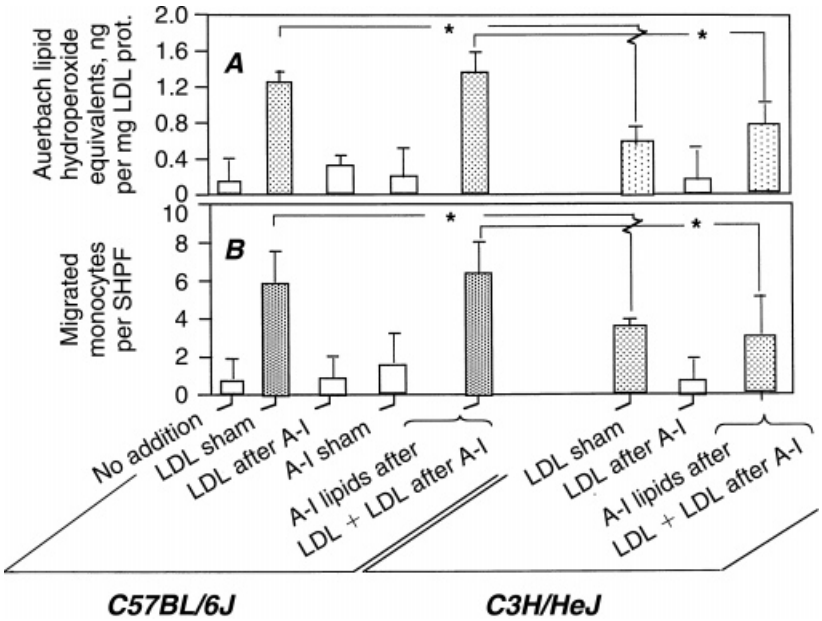


Fig. 5. Seeding molecules in LDL from C57BL/6 and C3H/HeJ mouse strains on a chow diet. LDL was isolated from plasma obtained from groups (n = 5 for each group) of the lesion-susceptible C57BL/6 (BL/6) mice and lesion-resistant C3H/HeJ (C3H) mice. The LDL was incubated (at 100 μg/ml) with human apoA-I (at 100 μg/ml) with gentle mixing at 37°C and then reisolated by FPLC as indicated in Scheme 1. Reconstitution of LDL with lipids removed by apoA-I was carried out as described in Fig. 1 and LDL was incubated with aortic wall cell cocultures. The abbreviations are the same as in Fig. 1. (A) Data on Auerbach lipid hydroperoxide equivalents formed; (B) monocyte chemotactic activity that was induced. The values shown are means ± SD of values from quadruplicate cocultures in each of two separate experiments. The asterisks indicate $P < 0.015$.

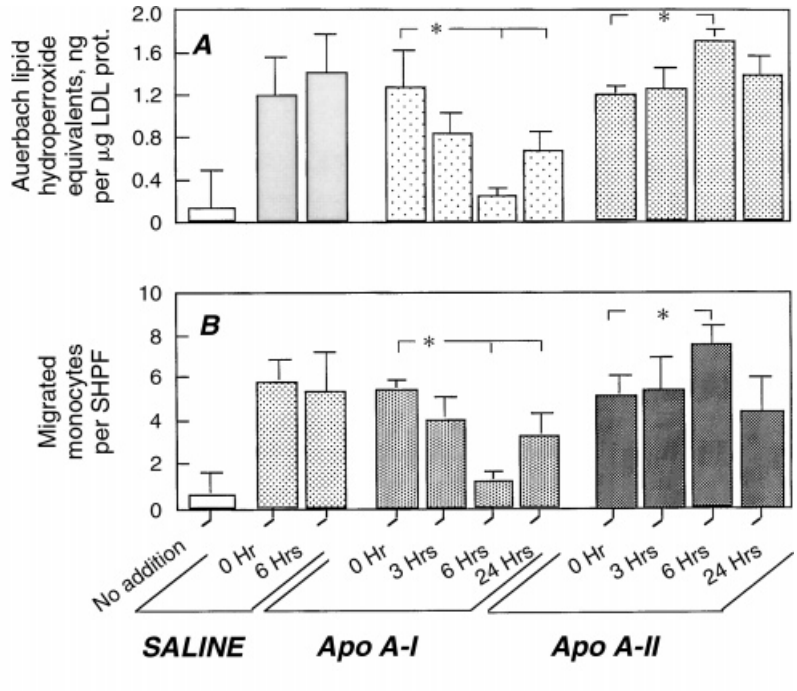


Fig. 6. Injection of apoA-I (but not apoA-II) into mice renders the mouse LDL resistant to oxidation by human artery wall cells. Groups of C57BL/6 mice (n = 5) were injected in the tail vein with 100 μg per animal of apo-AI or apoA-II, or with saline alone. Blood samples were removed at the indicated time points, and LDL was isolated and incubated with cocultures for 8 h. Culture supernatants were assayed for Auerbach lipid hydroperoxide equivalents (A) and for monocyte chemotactic activity (B) as described in Materials and Methods. The data represents means ± SD of quadruplicate samples from four separate experiment. The asterisks indicate $P < 0.01$ as compared with 0 time.

wall cells than was the case for the LDL from the lesion-resistant C3H mice (Fig. 5A). In contrast, LDL after A-I from both the lesion-sensitive BL/6 and the lesion-resistant C3H mice were resistant to oxidation (Auerbach assay) by the artery wall cells (Fig. 5A). On the other hand, if the lipids were extracted from A-I after LDL, and added back to LDL after A-I, the reconstituted LDL was oxidized (Auerbach assay) by the artery wall cells to the same degree as was the case for the sham-treated LDL (Fig. 5A). Similar results were obtained for LDL-induced monocyte chemotaxis (Fig. 5B). The data in Fig. 5 indicate that the difference in the ability of artery wall cells to oxidize LDL from the lesion-sensitive BL/6 mice compared with LDL from the C3H mice is due to lipids in their LDL that can be removed by apoA-I. These data also indicate that this difference is present while the animals are on the low-fat chow diet.

Injection of human apoA-I, but not human apoA-II, into mice renders the mouse LDL resistant to oxidation by human artery wall cells

To test the ability of apoA-I to alter the potential oxidative state of LDL in vivo, we injected 100 μg of apoA-I or apoA-II or saline alone into mice via their tail veins. Blood was removed immediately (0 h) or 3, 6, or 24 h after injection. LDL was isolated by FPLC and incubated with human artery wall cocultures and the formation of Auerbach lipid hydroperoxide equivalents and monocyte chemotactic activity was determined. **Figure 6** demonstrates that the freshly isolated LDL from BL/6 mice that had been injected with apoA-I 3 to 6 h earlier was resistant to oxidation by human artery wall cells and that this resistance persisted for up to 24 h (Fig. 6A). In contrast, the LDL obtained immediately after injection (0 h) or 6 h after injection of saline alone, or 6 h after injection of apoA-II, was not resistant to oxidation by the artery wall cells (Fig. 6A). Similar results were obtained for monocyte chemotactic activity (Fig. 6B). PON activity in plasma and HDL increased by approximately 20% 6 h after injection of apoA-I but did not change after injection of apoA-II (data not shown). Thus, as was the case for the in vitro studies described above, apoA-I injected in vivo (but not apoA-II) was able to dramatically decrease the oxidation of LDL.

Infusion of human apoA-I into humans renders their LDL resistant to oxidation by human artery wall cells

As indicated above in Fig. 6, injection of apoA-I into mice rendered their LDL resistant to oxidation by artery wall cells. **Figure 7** describes a parallel study in humans. Blood was taken from six healthy subjects (one with mildly increased levels of triglycerides, 176 mg/dl, as indicated in Materials and Methods) two h before and six h after infusion of apoA-I. LDL was isolated from the plasma at each time point and incubated with human artery wall cell cocultures. As shown in Fig. 7A, in six of six subjects, the LDL isolated 6 h after the infusion of apoA-I was much more resistant to oxidation (Auerbach assay) by artery wall cells as compared with the LDL 2 h before the infusion. Similar results were obtained for LDL-induced monocyte chemotactic activity (Fig. 7B). PON activity in plasma and HDL was increased by approximately 20% 6 h after the infusion as compared with 2 h before the infusion (data not shown). These data indicate that, as was the case for the mice, injection of apoA-I into humans rendered their LDL resistant to oxidation by human artery wall cells.

HDL or HDL-associated enzyme renders LDL resistant to oxidation by human artery wall cells

To test the ability of whole HDL and its components other than apoA-I, such as PON, to render LDL resistant

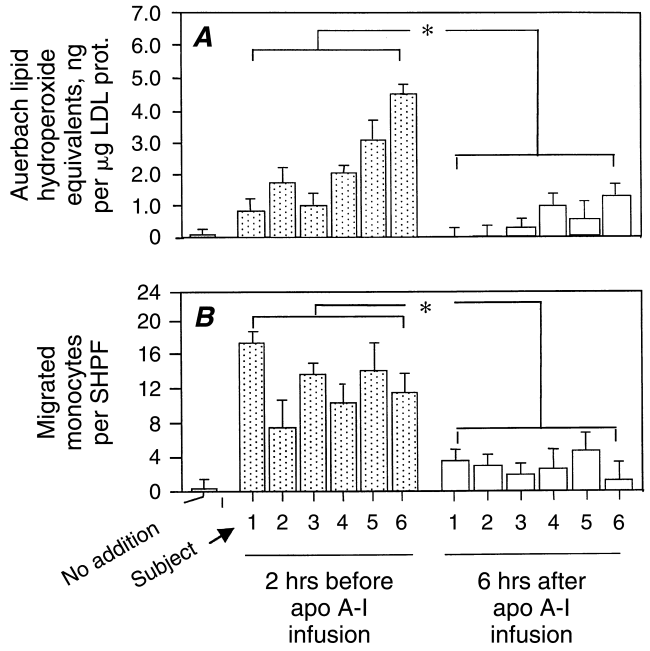


Fig. 7. Infusion of human apoA-I into humans renders their LDL resistant to oxidation by human artery wall cells. Six individuals (described in Materials and Methods) were infused with human apoA-I/phosphatidylcholine discs at 40 mg of apoA-I/kg body weight during a 4-h period. Plasma was prepared 2 h before and 6 h after the start of the infusion (i.e., 2 h after completion of the infusion). LDL was isolated by FPLC and incubated (at 100 μg/ml) with cocultures for 8 h. Culture supernatants were collected and subjected to lipid extraction and were assayed for Auerbach lipid hydroperoxide equivalents (A). The cocultures were washed and incubated in culture medium without serum or LPDS for 8 h and the conditioned medium was analyzed for monocyte chemotactic activity (B). Means ± SD of quadruplicate cocultures are presented and asterisks indicate $P < 0.0173$ for (A); $P < 0.0077$ for (B).

to oxidation by artery wall cells, LDL was incubated with or without HDL, or PON, as described in Materials and Methods and then separated from these and incubated with human artery wall cell cocultures. Incubation with HDL, or PON, rendered the LDL resistant to oxidation (Auerbach assay) by artery wall cells compared with sham-treated LDL (data not shown). Similar results were obtained for LDL-induced monocyte chemotactic activity (data not shown). Thus, HDL and its associated enzyme PON can render LDL resistant to oxidation by artery wall cells.

DISCUSSION

The data presented in this article demonstrate a role for HDL and its components, apoA-I and PON, in regulating the first step in a three-step process that leads to the formation of mildly oxidized LDL. Parthasarathy (10, 11), Witztum and Steinberg (12, 13), Chisolm (14), and Thomas and Jackson (15) hypothesized that LDL must be "seeded" with reactive oxygen species before it can be oxidized. Spector and colleagues (55, 56, 60) have demonstrated that the lipoxygenase pathway is active in artery wall cells, and Parthasarathy et al. (10, 11, 61) emphasized the possibility that hydrogen peroxide or its lipid hydroperoxide equivalent may play an important role in seeding LDL. The findings of Cyrus et al. (23) that disruption of the 12/15-lipoxygenase gene diminished atherosclerosis in apoE-deficient mice are consistent with this hypothesis and the data in this article.

We found that freshly isolated LDL from mice on a chow diet and that are genetically susceptible to the development of atherosclerosis was more readily oxidized by artery wall cells than was the case for LDL taken from mice that are genetically resistant to the development of atherosclerosis. The LDL from both strains of mice was rendered resistant to oxidation by artery wall cells after apoA-I treatment (Fig. 5), and the levels of oxidation of LDL after treatment with apoA-I were not significantly different for the two strains (Fig. 5). This may indicate that the genetic difference in susceptibility to develop atherosclerosis may be due, in part, to a difference in the level of seeding molecules in the LDL of these two mouse strains.

The in vitro ability of apoA-I (Figs. 1 and 6) and an apoA-I peptide mimetic (Fig. 2) to render LDL resistant to oxidation by artery wall cells was also demonstrated to apply in vivo in both mice (Fig. 6) and humans (Fig. 7). In mice, within 3 h of injection of apoA-I, LDL was rendered resistant to oxidation by artery wall cells and this state of protection persisted for up to 24 h (Fig. 6). In contrast to the case for apoA-I, injection of apoA-II did not protect LDL against oxidation by artery wall cells (Fig. 6). In humans, infusion of apoA-I into six of six men rendered their LDL resistant to oxidation by artery wall cells within 6 h of the infusion (Fig. 7).

Not only was apoA-I capable of favorably altering the susceptibility of LDL to oxidation by artery wall cells but so was HDL itself and the HDL-associated enzyme, PON. Aviram and colleagues (62, 63) demonstrated that PON has peroxidase activity, which in part may explain the role of PON in protecting against atherosclerosis in mouse models (48, 51) and in epidemiological studies (64–66). The article by Dansky and colleagues (67) suggested that there was benefit to overexpression of apoA-I in apoE-deficient mice without an increase in PON activity. However, as acknowledged by the authors of this study (67), they limited their experiments to the first 8 weeks of life. Aviram and colleagues reported that serum PON activity declined in apoE-deficient mice after 3 months of age, coincident with increases in aortic lesion area and serum lipid peroxidation (62). The mice studied by Plump, Scott, and Breslow (68) were killed at 4 or 6 months of age, when the data of Aviram et al. would suggest that PON activity would be reduced. Dansky and colleagues (67) also reported that lipid retention in the artery wall and monocyte adherence to the endothelium were not different at 8 weeks and concluded that the benefit of apoA-I was limited to a later time in lesion development. It should be noted that Dansky and colleagues (67) did not measure monocyte adherence but measured instead CD11a adherence, which is not specific for monocytes. In addition, Dansky and colleagues (67) used mice with a genetically mixed background for most of their experiments

and did not measure monocyte/macrophages in the subendothelial space. On the basis of our data, we would predict that apoA-I overexpression might reduce the susceptibility of LDL to oxidation independent of any change in PON activity. However, we saw an approximately 20% increase in PON activity 6 h after injection of apoA-I (but not apoA-II) in mice and a similar small increase in humans 6 h after infusion of apoA-I.

Sevanian and colleagues (9) reported increased levels of cholesterol oxides in LDL$^-$. Our finding (Fig. 3) that the neutral lipid extracted from "A-I after LDL" could restore the ability of artery wall cells to oxidize "LDL after A-I" are consistent with Sevanian's observations. Our results on the fatty acid fractions extracted from A-I after LDL (Figs. 4 and 5) indicate that metabolites of the linoleic and arachidonic acids can also act as LDL seeding molecules. Review of Fig. 4 reveals that LDL after A-I still contained a detectable level of 13-HPODE. However, this level was not sufficient to allow LDL after A-I to be oxidized by human artery wall cells (Figs. 2, 4, 6, and 7). Because the stepwise addition of either the neutral lipid or fatty acid fractions from A-I after LDL to LDL after A-I restored its ability to be oxidized by the artery wall cells (Fig. 3), we conclude that there is a critical threshold for the seeding molecules that is necessary for oxidation.

Stocker and colleagues (7, 8) demonstrated that both apoA-I and apoA-II can reduce cholesteryl ester hydroperoxides via a mechanism that involves oxidation of specific methionine residues (8). In our experiments only apoA-I, and not apoA-II, was able to reduce the oxidation of LDL after injection into mice (Fig. 6). These results suggest that the mechanism of protection of apoA-I in our studies was different from that investigated by Stocker and colleagues (7, 8). HDL has been demonstrated to be a strong inverse predictor of risk for atherosclerosis (69). It has been shown to reduce atherosclerosis in animal models when infused (70) and when associated with the overexpression of apoA-I (68). However, the overexpression of apoA-II has been demonstrated to enhance atherosclerosis (50, 71, 72). The studies reported here are consistent with these published reports and indicate that apoA-I but not apoA-II is capable of removing seeding molecules from freshly isolated LDL.

In the accompanying article we present evidence that normal HDL and its components can also inhibit the second and third steps in the formation of mildly oxidized LDL.

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