Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: steps 2 and 3

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Abstract **Treatment of human artery wall cells with apolipoprotein A-I (apoA-I), but not apoA-II, with an apoA-I peptide mimetic, or with high density lipoprotein (HDL), or paraoxonase, rendered the cells unable to oxidize low density lipoprotein (LDL). Human aortic wall cells were found to contain 12-lipoxygenase (12-LO) protein. Transfection of the cells with antisense to 12-LO (but not sense) eliminated the 12-LO protein and prevented LDL-induced monocyte chemotactic activity. Addition of 13(*S*)-hydroperoxyoctadecadienoic acid [13(*S*)-HPODE] and 15(*S*)-hydroperoxyeicosatetraenoic acid [15(*S*)-HPETE] dramatically enhanced the nonenzymatic oxidation of both 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC) and cholesteryl linoleate. On a molar basis 13(*S*)-HPODE and 15(*S*)-HPETE were approximately two orders of magnitude greater in potency than hydrogen peroxide in causing the formation of biologically active oxidized phospholipids (m/z 594, 610, and 828) from PAPC. Purified paraoxonase inhibited the biologic activity of these oxidized phospholipids. HDL from 10 of 10 normolipidemic patients with coronary artery disease, who were neither diabetic nor receiving hypolipidemic medications, failed to inhibit LDL oxidation by artery wall cells and failed to inhibit the biologic activity of oxidized PAPC, whereas HDL from 10 of 10 age- and sex-matched control subjects did. We conclude that *a*) mildly oxidized LDL is formed in three steps, one of which involves 12-LO and each of which can be inhibited by normal HDL, and *b*) HDL from at least some coronary artery disease patients with normal blood lipid levels is defective both in its ability to prevent LDL oxidation by artery wall cells and in its ability to inhibit the biologic activity of oxidized PAPC.**—Navab, M., S. Y. Hama, G. M. Anantharamaiah, K. Hassan, G. P. Hough, A. D. Watson, S. T. Reddy, A. Sevanian, G. C. Fonarow, and A. M. Fogelman. **Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: steps 2 and 3. *J. Lipid Res.* 2000.** 41: **1495–1508.**

Supplementary key words LDL • HDL • mildly oxidized LDL • MM-LDL • oxidized phospholipids • HPODE • HPETE • cholesteryl linoleate hydroperoxide • paraoxonase • atherosclerosis • antioxidant

We previous reported (1) that high density lipoprotein (HDL) but not apolipoprotein A-I (apoA-I), when added to human artery wall cell cocultures together with low density lipoprotein (LDL), prevented the oxidation of the LDL by the artery wall cells. In those experiments, the apoA-I was kept in the culture together with the artery wall cells and the LDL (1). Subsequently, in pursuing the mechanisms for the ability of HDL to protect LDL against oxidation by human artery wall cells, we observed that if the apoA-I was incubated with the cells and then removed prior to the addition of the LDL, the artery wall cells were then unable to oxidize the added LDL. This suggested to us that apoA-I might be able to remove from cells not only cholesterol and phospholipids but perhaps oxidized lipids as well. These preliminary findings, which have been reported in abstract form (2, 3), prompted us to perform the studies detailed in this article.

The experiments detailed in this and the accompanying article (3a) have led us to propose that the biologically active lipids in mildly oxidized LDL are formed in a series of three steps. The first step is the seeding of LDL with lipid oxidation products including those of the metabolism of linoleic and arachidonic acid. The evidence of the first step is presented in the accompanying article. In this article we present evidence regarding the second step in LDL

Abbreviations: ChC18:2, cholesteryl linoleate; ChC18:2-OOH, cholesteryl linoleate hydroperoxide; DMPC, 1,2-ditetradecanoyl-*rac*-glycerol-3-phosphocholine; 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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oxidation by artery wall cells. We demonstrate that 12-lipoxygenase (12-LO) protein is present in human artery wall cells and is required for the production of mildly oxidized LDL by the artery wall cells.

Stocker and colleagues (4, 5) have presented indirect evidence that lipoxygenases mediate the peroxidation of cholesteryl linoleate largely by a nonenzymatic process. We demonstrate in this article that the nonenzymatic oxidation of cholesteryl linoleate is greatly enhanced by the presence of 13-hydroperoxyoctadecadienoic acid [13(*S*)-HPODE]. We also propose in this article that the third step in the formation of mildly oxidized LDL is the nonenzymatic oxidation of LDL phospholipids that occurs when a critical threshold of "seeding molecules" (e.g., 13(*S*)-HPODE and 15-hydroperoxyeicosatetraenoic acid [15(*S*)-HPETE]) is reached in the LDL. We present evidence in this article to indicate that when these seeding molecules reach a critical level, they cause the nonenzymatic oxidation of a major LDL phospholipid, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC). This results in the formation of the three biologically active oxidized phospholipids: 1-palmitoyl-2-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) (6, 7). The experiments described in this article also indicate that in contrast to the case for normal HDL, HDL taken from patients with coronary artery disease, who show normal blood lipid levels, and were neither diabetic nor taking hypolipidemic medications, did not protect LDL against oxidation by human artery wall cells and failed to inhibit the biological activity of oxidized PAPC.

MATERIALS AND METHODS

Materials

The arachidonic acid analog 5,8,11,14-eicosatetraynoic acid (ETYA) was obtained from Biomol (Plymouth Meeting, PA). Polyclonal antiserum against recombinant human platelet-type 12-lipoxygenase was obtained from Cayman Chemical (Ann Arbor, MI). Monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Chemicon (Temecula, CA). Phosphorothioate oligonucleotides directed against human platelet-type 12-lipoxygenase were purchased from GIBCO-BRL (Gaithersburg, MD). The antisense oligonucleotide sequence used was 5′-CTCAGGAGGGTGTAAACA-3′, the corresponding sense oligonucleotide sequence was 5′-TGTTTACAC CCTCCTGAG-3′, and the scrambled oligonucleotide sequence used was 5′-AAGATTGCGCGACGATGA-3′. SuperFect reagent was purchased from Qiagen (Chatsworth, CA). All other materials were from sources described in the accompanying article.

Lipoproteins, cocultures, monocyte isolation, monocyte chemotaxis assays, and monocyte adhesion assays

Lipoproteins, cocultures, monocyte isolation, monocyte chemotaxis assays, and monocyte adhesion assays were prepared and/or performed as described in the accompanying article.

Patients and normal subjects

Blood samples were collected from patients referred to the cardiac catheterization laboratory at the Center for Health Sciences at the University of California, Los Angeles (Los Angeles, CA). After signing a consent form approved by the Human Research Subject Protection Committee of the University of California, Los Angeles, each patient donated a fasting blood sample collected in a heparinized tube. LDL and/or HDL were isolated by fast protein liquid chromatography (FPLC) from the blood samples collected from patients who had angiographically documented coronary atherosclerosis but who had normal total cholesterol (<200 mg/dl), LDL-cholesterol (<130 mg/dl), HDL-cholesterol (males >45 mg/dl, females >50 mg/dl), and triglycerides (<150 mg/dl), who were not taking hypolipidemic medications, and who were not diabetic. Data from some patients and some controls previously reported by us (8) have been included with additional new data. The inclusion of previously reported patients is explicitly indicated in the appropriate figure legend. HDL was isolated from each individual and paraoxonase activity was determined as previously described (8). The ability of the HDL from each subject to protect LDL against oxidation by human artery wall cell cocultures was then determined by techniques previously described (1, 8). The LDL used for testing the ability of HDL to protect LDL against oxidation by human artery wall cells was prepared from a normal donor and was aliquoted and cryopreserved in sucrose as previously described (9). To determine the capacity of HDL to inactivate oxidized phospholipids, in some cases 100 μg of oxidized PAPC (Ox-PAPC) per milliliter (9) was incubated in test tubes with HDL at 250 μg/ml in 10% lipoprotein-deficient serum (LPDS) in medium 199 (M199) at 37°C with gentle mixing. The HDL-Ox-PAPC mixture was then added to endothelial monolayers and monocyte binding was determined.

Effect of overexpression of 15-lipoxygenase in fibroblasts on the removal of 13(*S*)-HPODE by apoA-I

Fibroblasts that were transfected with vector alone or cells that overexpressed 15-LO were a generous gift of J. Witztum and P. Reaven. In the present experiments, the fibroblasts were incubated with or without apoA-I (100 μg/ml). After 3 h of incubation at 37°C with gentle mixing, the culture supernatants were removed, apoA-I was separated by FPLC, and the level of hydroperoxides was determined in lipid extracts of the culture supernatants and in lipid extracts of apoA-I.

Effect of lipoxygenase and cyclooxygenase inhibitors

Human artery wall cell cocultures were preincubated for 30 min with ETYA at a concentration of 10^{-8} mol/liter or with cinnamyl-3,4-dihydroxy-α-cyanocynamate (CDC; from Biomol) at a concentration of 10^{-8} mol/liter in M199 containing 10% LPDS. The cocultures were then washed and LDL was added at 250 μg/ml and incubated for 8 h. The supernatants were removed and assayed for Auerbach lipid hydroperoxide equivalents and monocyte chemotactic activity was determined as described in the accompanying article.

Western analysis for the detection of 12-LO

Human aortic endothelial cells (HAEC) and artery wall cell cocultures were prepared as described previously (1). Cells were harvested in a buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM sodium orthovanadate, 1% Triton X-100, aprotinin (1 μg/ml) and leupeptin (1 μg/ml). Samples were boiled for 10 min and protein concentrations were determined by the Bradford assay. Seventy-five micrograms of total protein from each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 5% stacking and 8% resolving gel). The proteins were blotted onto nitrocellulose membranes with a semidry transfer apparatus (Bio-Rad, Hercules, CA). The filters were incubated for 1 h in phosphate-buffered saline (PBS) containing 0.2% Tween 20 and 10% nonfat dried milk,

washed in PBS containing 0.2% Tween 20 and 1% nonfat dried milk, and incubated with a polyclonal anti-12-LO antibody (1:1,000 dilution) for 1 h, and Western analysis was performed with 12-LO antibody at a 1:1,000 dilution. After three additional washes in PBS containing 0.2% Tween 20 and 1% nonfat dried milk, the filters were incubated with a secondary antibody (anti-rabbit IgG conjugated to horseradish peroxidase; Sigma, St. Louis, MO) at a dilution of 1:4,000. Immunodetection was performed with ECL reagents (Pharmacia, Piscataway, NJ). The filters were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY).

Effect of antisense to 12-lipoxygenase on LDL-induced monocyte chemotaxis

Artery wall cocultures were set up in six-well plates as described previously (1). Antisense, sense, or scrambled oligonucleotides were used at a final concentration of 1 μM. For each transfection, an appropriate amount of the oligonucleotide was diluted in 200 μl of serum-free M199 medium in a 0.5-ml Eppendorf tube. Three microliters of SuperFect reagent was added to each tube, vortexed for 10 sec, and allowed to incubate at room temperature for 15 min to allow SuperFect reagent-DNA complex formation. During the incubation the artery wall cocultures were washed with PBS and supplemented with 0.8 ml of complete M199 medium. The transfection complexes were added to the wells and incubated for 2 h. The cocultures were washed in PBS and supplemented with complete M199 medium. Eighteen hours later the transfection protocol was repeated, and appropriate cocultures received LDL (250 μg/ml) to be oxidatively modified by the artery wall cells. Six hours later, supernatants were collected and were transferred to fresh untransfected cocultures that served as target for induction and release into supernatant of monocyte chemotactic activity under the influence of the transferred LDL. After incubation for 5 h, the coculture medium was changed to serum-free medium and incubated for an additional 6 h to accumulate monocyte chemotactic activity, which was then determined as described in Materials and Methods. In addition, the coculture lysates obtained from the first set that was used for modification of LDL were analyzed for 12-LO protein expression as described above.

Formation of oxidized phospholipids (POVPC, PGPC, and PEIPC) from PAPC by addition of 13(*S*)-HPODE or 15(*S*)-HPETE or hydrogen peroxide

13(*S*)-HPODE or 15(*S*)-HPETE or vehicle alone was added at various concentrations to PAPC, mixed and evaporated to form a thin film, and allowed to oxidize in air. In some experiments, PAPC was evaporated, forming a thin film, and allowed to oxidize in air with 100 μl containing hydrogen peroxide at various concentrations. The samples were extracted with chloroform–methanol 2:1 (v/v) (10) and in the case of the hydrogen peroxide experiments by addition of five parts chloroform–methanol 2:1 (v/v) to one part aqueous solution, mixing, and centrifugation. The chloroform phase was collected and analyzed by electrospray ionization mass spectrometry (ESI-MS) in the positive ion mode. The level of the remaining PAPC and the oxidized phospholipids that formed were determined and expressed in relation to the internal standard, 1,2-ditetradecanoyl-*rac*-glycerol-3-phosphocholine (DMPC, m/z 678.3).

Fast performance liquid chromatography and reversed-phase high performance liquid chromatography

Fast performance liquid chromatography and reversed-phase high performance liquid chromatography (RP-HPLC) were performed as described in the accompanying article. 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.

Electrospray ionization mass spectrometry

ESI-MS in the positive or negative ion mode was performed according to the protocol and conditions previously described (6, 7). Briefly, ESI-MS was performed with an API III triple-quadrupole biomolecular mass analyzer (Perkin-Elmer, Norwalk, CT) fitted with an articulated, pneumatically assisted nebulization probe and an atmospheric pressure ionization source (7). Positive ion flow injection analysis was done with acetonitrile–water–formic acid 50:50:0.1 (v/v/v) and negative ion flow injection analysis was done with methanol–water 50:50 (v/v) containing 10 mM ammonium acetate. For quantitative analysis, 1,2-ditetradecanoyl-*rac*-glycerol-3-phosphocholine (DMPC) or heptadecanoic acid were used as internal standards. Ions were scanned at a step size of 0.3 Da. Data were processed by software provided by PE SCIEX (PE Biosystems, Foster City, CA).

Inhibition of the bioactivity of oxidized phospholipids by paraoxonase

Oxidized PAPC, POVPC, PGPC, or PEIPC was incubated in M199 without or with purified human paraoxonase (1×10^{-2} U/ml) for 3 h with gentle mixing at 37°C. Paraoxonase was removed from the mixture by ultrafiltration with 30-kDa cutoff spin filters and the lipids were incubated with human aortic wall cocultures in M199 with 10% LPDS for 8 h at 37°C. The cocultures were washed and incubated with fresh medium for an additional 4 h at 37°C. The supernatants were analyzed for monocyte chemotactic activity.

Other methods

Protein content of lipoproteins was determined by a modification (11) of the Lowry assay (12). Lipid hydroperoxide levels were measured by the assay described by Auerbach, Kiely, and Cornicelli (13). In some experiments, where indicated, the lipid in culture supernatants containing LDL that was oxidized by the artery wall cell cocultures was extracted with chloroform–methanol and hydroperoxides determined by the Auerbach method. Peroxidation of cholesteryl linoleate was accomplished as described in the accompanying article. Paraoxonase activity was measured as previously described (14). 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 whether 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

The accompanying article demonstrated that LDL contains "seeding molecules" necessary for LDL oxidation by artery wall cells. We previously reported (1, 15) that freshly isolated LDL does not induce monocyte adherence to endothelial cells and does not induce monocyte chemotaxis whereas mildly oxidized LDL induces both (1, 15). The ability of mildly oxidized LDL to induce monocyte adherence and chemotaxis was based on the presence in the mildly oxidized LDL of three oxidized phospholipids with characteristic m/z ratios (m/z 594, 610,

and 828) (6, 7). Because freshly isolated LDL did not induce monocyte adherence or monocyte chemotactic activity, we concluded that the seeding molecules in freshly isolated LDL were by themselves insufficient to generate the three biologically active oxidized phospholipids, either because the level of these seeding molecules was less than some critical threshold or because additional and different seeding molecules were required to generate the biologically active oxidized phospholipids. Thus, we concluded that at least an additional step in the formation of mildly oxidized LDL was required beyond the initial seeding.

Step 2

ApoA-I, but not apoA-II, renders human artery wall cells unable to oxidize LDL. We previously reported that coincubation of human artery wall cells with apoA-I and LDL did not protect the LDL against oxidation by the artery wall cells (1). As shown in **Fig. 1**, these results were confirmed (compare coincubated A-I with cultures sham treated). However, when the human artery wall cocultures were first incubated with apoA-I and the apoA-I was then removed from the cocultures prior to the addition of LDL (cultures after A-I), the artery wall cells were not able to oxidize the LDL (Fig. 1A) and monocyte chemotaxis was prevented (Fig. 1B). In contrast to the case for apoA-I, when the cultures were first incubated with apoA-II and the apoA-II was then removed, the artery wall cocultures retained their ability to oxidize LDL (Fig. 1A) and induce monocyte chemotaxis (Fig. 1B) (cultures after A-II).

In other experiments, apoA-I was incubated with a first set of cocultures and then removed from the first set of cocultures and added to a second set of cocultures that had been identically treated (i.e., the second set of cocultures had been incubated with apoA-I, which was then removed). When LDL was added to this second set of cocultures, which contained apoA-I from the first set of cocultures, these reconstituted cocultures readily oxidized the LDL (Fig. 1A) and induced monocyte chemotaxis (Fig. 1B) (cultures after A-I + A-I after cultures).

Similar experiments were performed with apoA-II. ApoA-II was incubated with a first set of cocultures and then removed and added to a second set of cocultures

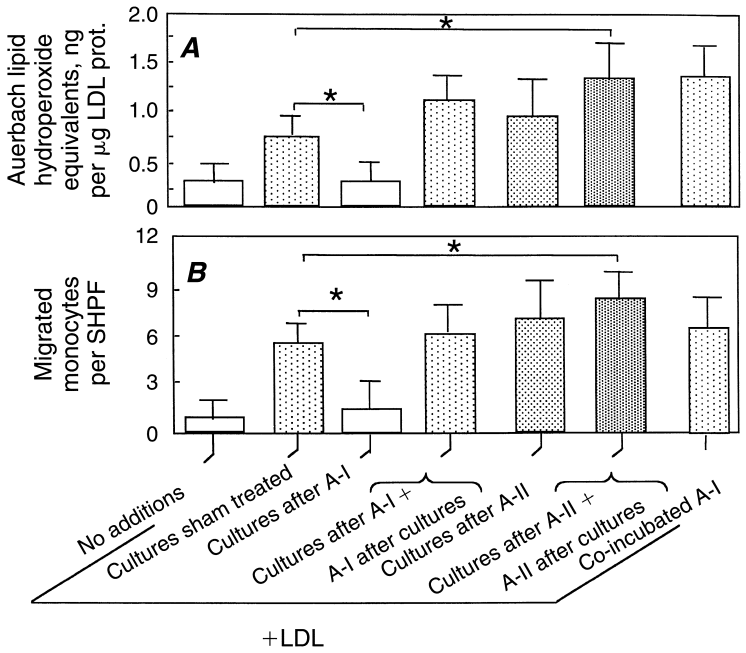

Fig. 1. ApoA-I removes substances from human artery wall cells and renders the cells unable to oxidize LDL. Cocultures were incubated with apoA-I or apoA-II (50 μg/ml) or were sham treated for 8 h. The conditioned media containing either apoA-I or apoA-II were removed and in some cases transferred to other cocultures that had been treated identically and served as target cocultures. LDL was added at 250 μg/ml to the target cocultures that had been sham treated (cultures sham treated), or to target cocultures that had been treated with apoA-I that had been removed (cultures after A-I), or treated with apoA-II that had been removed (cultures after A-II). LDL was also added at 250 μg/ml to target cocultures that had been treated with apoA-I or apoA-II and to which was added the conditioned medium containing either apoA-I or apoA-II from the first set of cocultures (cultures after A-I + A-I after cultures, cultures after A-II + A-II after cultures, respectively). The target cocultures were incubated for 8 h in M199 containing 10% LPDS and LDL with or without the additions (conditioned media) from the first set of cocultures. Some cocultures received LDL (250 μg/ml) plus apoA-I (50 μg/ml) at the start of the 8-h incubation and this was continued for a total of 16 h (co-incubated A-I). The supernatants were removed and assayed for Auerbach lipid hydroperoxide equivalents (A) and the cocultures were washed and fresh M199 without serum or LPDS was added and incubated for 8 h and assayed for monocyte chemotactic activity (B). Values represent means ± SD of three separate experiments utilizing LDL from different donors. Asterisks indicate significance at the level of $P < 0.01$

that had been identically treated (i.e., the second set of cocultures had been incubated with apoA-II, which was then removed). When LDL was added to this second set of cocultures, which contained apoA-II from the first set of cocultures, there was a significant increase in LDL oxidation by the artery wall cells (Fig. 1A) and a significant increase in LDL-induced monocyte chemotaxis (Fig. 1B) (cocultures after A-II + A-II after cultures).

Because the reduction in LDL oxidation and LDL-induced monocyte chemotaxis by apoA-I required that the apoA-I be removed from the cocultures after incubation with the cells and before the addition of LDL (compare cultures after A-I with co-incubated A-I), we conclude that apoA-I removed substances from the artery wall cell cocultures that were necessary for the LDL to be oxidized by the cocultures and induce monocyte chemotaxis. We also conclude that apoA-II was incapable of reducing LDL oxidation and LDL-induced monocyte chemotaxis, and, in fact, enhanced these (compare cultures after A-II with cultures after A-I).

Similar results were obtained when the cocultures were treated with an apoA-I peptide mimetic (**Fig. 2**). The cocultures were incubated with or without the apoA-I peptide mimetic 37pA, and the peptide was then removed by washing the cultures before the addition of LDL. Other cocultures were incubated with the control peptide 40P. Cocultures that had been incubated with the apoA-I peptide mimetic 37pA that was removed prior to the addition of LDL were unable to oxidize the added LDL (Fig. 2A) and did not induce monocyte chemotaxis (Fig. 2B). This was not the case when the cocultures were treated with the control peptide 40P. After treatment with the control peptide 40P, LDL was oxidized by the cocultures (Fig. 2A) and induced monocyte chemotaxis (Fig. 2B) to the same degree as sham-treated cocultures. We conclude that the apoA-I peptide mimetic 37pA removed substances from the artery wall cells that were necessary for LDL to be oxidized by the cocultures and induce monocyte chemotaxis.

HDL only or HDL-associated enzymes render human artery wall cells unable to oxidize LDL. We also tested whether whole HDL and its associated enzyme paraoxonase (PON) could alter the ability of artery wall cells to oxidize LDL. We incubated the artery wall cell cocultures with HDL, or purified PON and then removed these prior to the addition of LDL to the cocultures. Treatment of the artery wall cells with any of these two rendered the artery wall cells incapable of oxidizing LDL (**Fig. 3A**) and prevented LDL-induced monocyte chemotaxis (Fig. 3B). We conclude that in addition to apoA-I, HDL and PON can prevent hu-

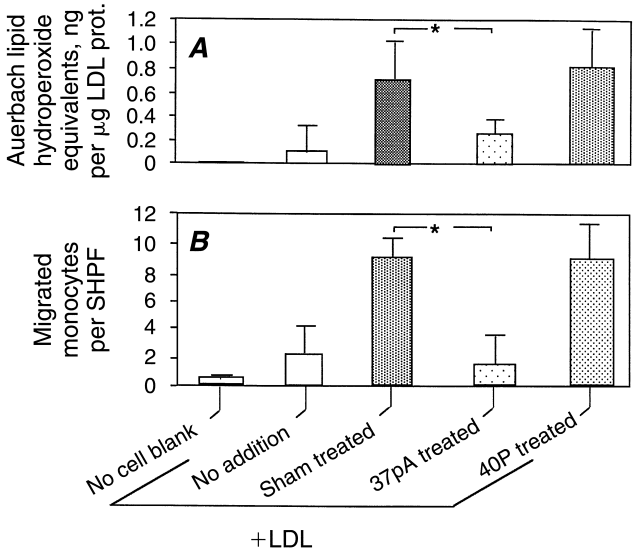

Fig. 2. An apoA-I peptide mimetic removes substances from human artery wall cells and renders the cells unable to oxidize LDL. Human aortic wall cocultures were incubated with medium alone (sham treated), with an apoA-I mimetic peptide at 100 μg/ml (37pA treated), or with control peptide at 100 μg/ml (40P treated) for 8 h. The cocultures were then washed and freshly isolated LDL was added and incubated in M199 containing 10% LPDS for an additional 8 h. The media were removed and assayed for Auerbach lipid hydroperoxide equivalents (A). The cocultures were then washed and incubated with culture medium without serum or LPDS for an additional 8 h and assayed for monocyte chemotactic activity (B). The data represent means ± SD of values obtained from quadruplicate cocultures in three separate experiments. Asterisks indicate significance at the level of $P = 0.01$.

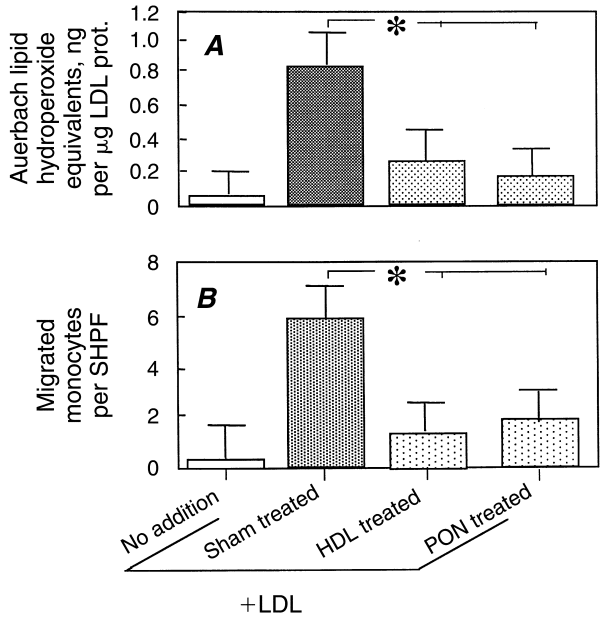

Fig. 3. HDL and its associated enzyme PON render human artery wall cells unable to oxidize LDL. Human aortic wall cocultures were incubated with medium alone (sham treated), with HDL at 350 μg/ml (HDL treated), or with purified paraoxonase at 1×10^{-2} U/ml (PON treated) for 8 h. The cocultures were then washed and freshly isolated LDL was added at 250 μg/ml and incubated in M199 containing 10% LPDS for an additional 8 h. The media 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 and the supernatant was collected and analyzed for monocyte chemotactic activity (B). The data represent means ± SD of values obtained from quadruplicate cocultures in three separate experiments. Asterisks indicate significance at the level of $P < 0.01$.

man artery wall cells from oxidizing LDL and inducing monocyte chemotaxis.

Linoleic acid, but not oleic acid, stimulates human artery wall cells to oxidize LDL. As noted above, we concluded that the seeding molecules in mildly oxidized LDL were by themselves insufficient to generate the three biologically active oxidized phospholipids that induce monocyte chemotaxis. We hypothesized that this might be because the level of these seeding molecules was less than some critical threshold or because additional and different seeding molecules were required to generate the biologically active oxidized phospholipids in LDL. We reasoned that if there was some threshold for the same seeding molecules to generate the oxidized phospholipids and hence monocyte chemotaxis and if these seeding molecules were in part derived from the metabolism of linoleic acid, then enriching the human artery wall cocultures with linoleic acid might be expected to enhance their ability to oxidize LDL and induce monocyte chemotaxis. Consequently, we incubated human artery wall cocultures with or without linoleic acid (C18:2), or oleic acid (C18:1), washed the cells, and allowed them to metabolize the fatty acids by incubating them for 3 h at 37°C in fresh medium that was not supplemented with the fatty acids. Subsequently, we tested the ability of these human artery wall cell cocultures to oxidize LDL and induce monocyte chemotaxis (**Fig. 4**). Incubating the artery wall cells with linoleic acid significantly enhanced the ability of the artery wall cells to oxidize LDL compared with oleic acid (Fig. 4A) and induced monocyte chemotaxis (Fig. 4B). In other experiments cocultures were incubated without LDL but with (+) or without (−) linoleic acid (C18:2) and the cells were washed and then incubated with or without apoA-I (Fig. 4C). The supernatants were removed and the apoA-I was separated by FPLC, and the lipid was extracted from the apoA-I. Lipid extracts of the culture supernatants from incubations without apoA-I were also obtained. Incubating the cocultures with linoleic acid dramatically increased the Auerbach lipid hydroperoxide equivalents in the lipid extract of the apoA-I (Fig. 4C) (compare apoA-I lipid extract of the cells incubated with C18:2 with apoA-I lipid extract of the cells incubated without C18:2). We conclude that incubating human artery wall cells with linoleic acid markedly enhances the cellular production of lipid hydroperoxides, which can be removed by apoA-I. We further conclude that incubation of human artery wall cells with linoleic acid but not oleic acid stimulates the oxidation of LDL by artery wall cells and stimulates LDL-induced monocyte chemotaxis. In other experiments, the studies described in Fig. 4 were performed with arachidonic acid. The results indicated that arachidonic acid was more potent (approximately an order of magnitude) than linoleic acid in stimulating the oxidation of LDL by artery wall cells (data not shown).

Further evidence of the role of lipoxygenase pathways. Thomas and Jackson (16) and Parthasarathy (17) suggested a role

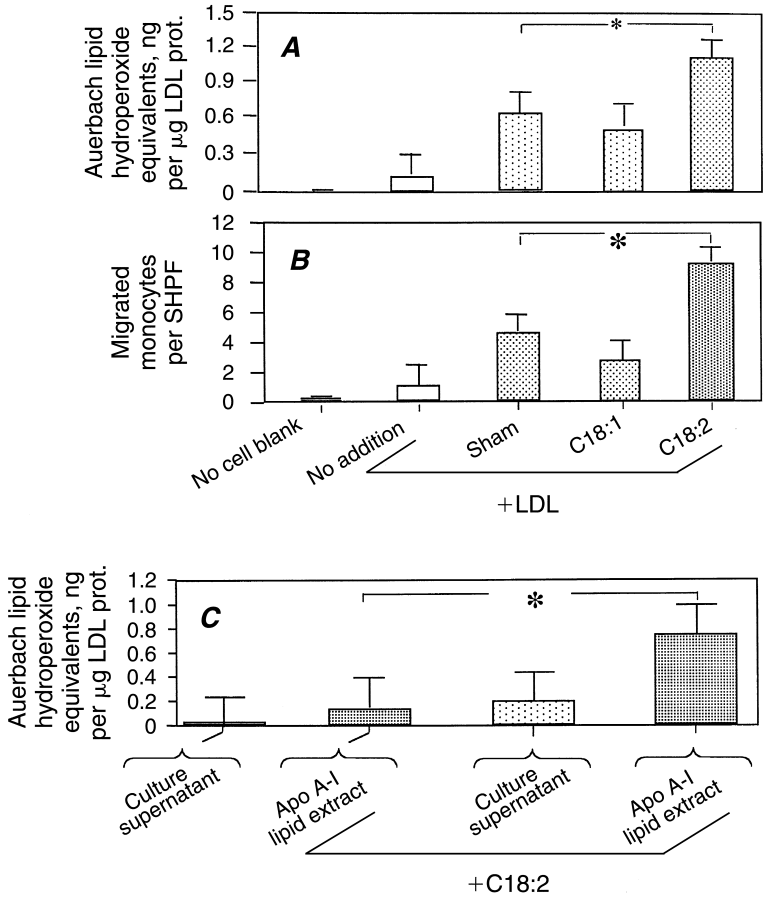

Fig. 4. Pretreatment of human artery wall cells with linoleic acid results in increased levels of Auerbach lipid hydroperoxide equivalents and monocyte chemotactic activity. Two sets of cocultures were incubated for 18 h at 37°C with 100 μM oleic acid (C18:1), or linoleic acid (C18:2) in M199 with 10% LPDS. The medium was removed and the cultures were washed three times. Fresh medium without fatty acids was added and the cultures were incubated at 37°C for an additional 3 h. LDL at 250 μg/ml was then added to one set of the cocultures in M199 containing 10% LPDS and incubated for 8 h. The medium was then removed and Auerbach lipid hydroperoxide equivalents (A) and monocyte chemotactic activity (B) were determined. To the second set of cocultures (C) apoA-I was added at 100 μg/ml and incubated for an additional 3 h with gentle mixing at 37°C. The supernatant was removed, apoA-I was separated by FPLC, and the Auerbach lipid hydroperoxide equivalents of the lipid extract of the supernatants that did not receive apoA-I (culture supernatant) and the lipid extract from apoA-I (apoA-I lipid extract) were determined as described in Materials and Methods and are expressed as nanograms per well. Values represent means ± SD of triplicate determinations in three separate experiments. The asterisks denote $P < 0.01$.

for lipoxygenase (LO) in the seeding of LDL and Sigari and colleagues (18) demonstrated that fibroblasts overexpressing 15-LO more readily oxidized LDL than fibroblasts transfected with vector alone. To further establish the ability of apoA-I to remove lipid hydroperoxide products of the LO pathway from cells, we incubated fibroblasts overexpressing LO and cells that were transfected with vector alone with apoA-I or without apoA-I as described in Materials and Methods. The supernatants were removed, the apoA-I was separated by FPLC, and the lipid was extracted from the apoA-I. Lipid extracts of the culture supernatants from incubations without apoA-I were also obtained. Without addition of apoA-I the lipid extracts of the supernatants from cells overexpressing LO contained only slightly more Auerbach lipid hydroperoxide equivalents compared with the control cells (data not shown). In contrast, the lipid extracts of apoA-I incubated with the cells overexpressing LO contained markedly more Auerbach lipoperoxide equivalents (5.1-fold more) than the lipid extracts of apoA-I incubated with the control cells (data not shown).

Preincubation of the cocultures with the lipoxygenase/cyclooxygenase inhibitor ETYA (1×10^{-8} mol/liter) prior to the addition of LDL as described in Materials and Methods resulted in an 80 ± 7% reduction in Auerbach lipid hydroperoxide equivalents and a 75 ± 10% decrease in LDL-induced monocyte chemotactic activity ($P < 0.008$, data not shown). Preincubation of human artery wall cocultures with the lipoxygenase inhibitor CDC (1×10^{-8} mol/liter) prior to the addition of LDL as described in Materials and Methods resulted in a 73 ± 6% reduction in Auerbach lipid hydroperoxide equivalents and a 74 ± 11% decrease in LDL-induced monocyte chemotactic activity ($P < 0.01$, data not shown). In additional experiments treatment of the cocultures with angiotensin II for 8 h resulted in a 76% increase ($P < 0.01$) in LDL-induced monocyte chemotactic activity (data not shown).

As shown in **Fig. 5**, HAEC and the cocultures of human aortic wall cells contained 12-LO protein (Fig. 5A). Transfection of the artery wall cells in culture with antisense to 12-LO eliminated the 12-LO protein (Fig. 5B). The sense oligonucleotides did not have any effect. To study the potential role of 12-LO in LDL-induced monocyte chemotactic activity LDL was first incubated with transfected artery wall cell cocultures that served for oxidative modification of LDL and supernatants were subsequently transferred to untransfected fresh cocultures that served as target cultures for induction of monocyte chemotactic activity. While incubation of the control artery wall cell cocultures with LDL resulted in a marked increase in monocyte chemotactic activity, transfection with the antisense to 12-LO resulted in complete inhibition of the LDL-induced increase in monocyte chemotactic activity in the target cocultures (Fig. 5C). Transfection with the sense oligonucleotides did not have an effect on LDL-induced monocyte chemotactic activity.

Taken together, these experiments suggest that artery wall cells produce reactive oxygen species, including those derived from the metabolism of linoleic and arachidonic

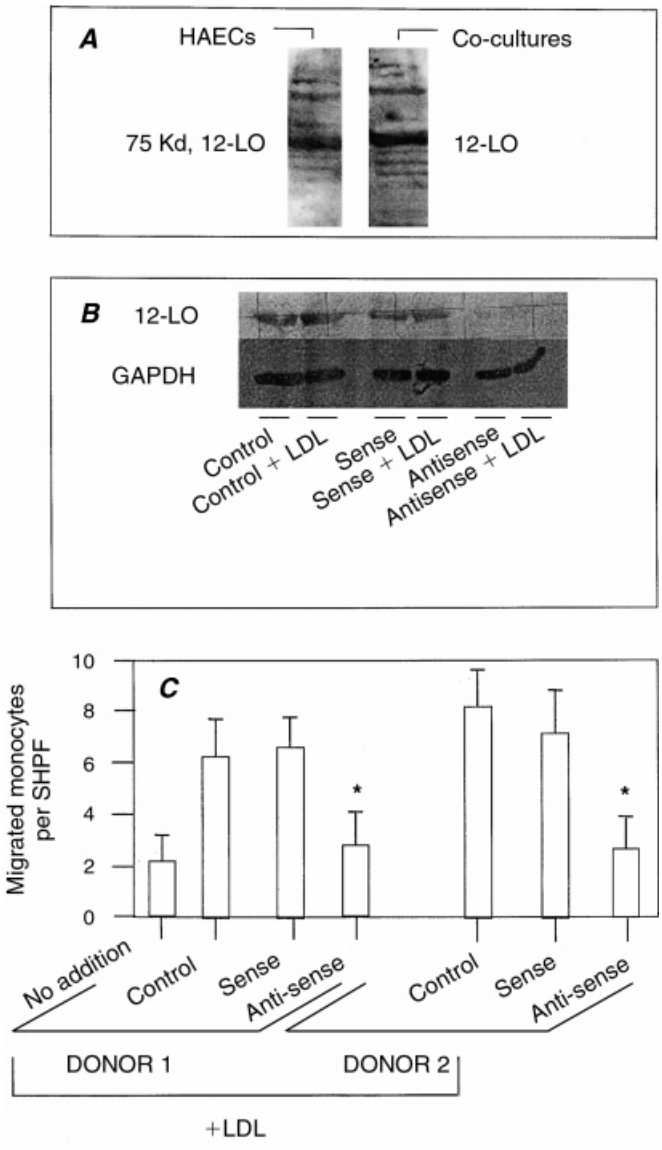

Fig. 5. 12-Lipoxygenase in human artery wall cells. (A) HAECs and artery wall cocultures express human platelet-type 12-lipoxygenase. From each sample 75 μg of total protein was subjected to SDS-PAGE and Western analysis with human platelet-type 12-LO antibody (Cayman) at 1:1,000 dilution. (B) Antisense oligonucleotides to 12-LO inhibit the accumulation of 12-LO protein in artery wall cocultures. Artery wall cocultures were transfected with either sense or antisense oligonucleotides to 12-LO as described in Materials and Methods. Total cell lysates were subjected to SDS-PAGE and to Western analysis using human platelet-type 12-LO antibody (Cayman) at 1:1,000 dilution. (C) Antisense oligonucleotides to 12-LO inhibit the formation of bioactive LDL and monocyte chemotactic activity. LDL was incubated with artery wall cell cocultures from two different heart donors. The cells were first transfected with vector (Control), antisense to 12-LO (antisense), or sense oligonucleotides (sense). After 6 h of incubation with the first set of cells for oxidative modification of LDL the supernatants were transferred to a second set of untransfected fresh cocultures that served as target cultures for induction of monocyte chemotactic activity and further incubated for 5 h. The cultures were then washed and incubated in medium M199 for an additional 5 h for the accumulation of monocyte chemotactic activity, which was assayed as described in Materials and Methods. The values represent means ± SD obtained in three separate experiments. Asterisks indicate significance at the level of $P < 0.01$.

acids, that are critical to the oxidation of seeded LDL. These experiments also suggest that HDL, apoA-I, and PON can remove or inhibit these substances and render the artery wall cells incapable of oxidizing the seeded LDL. Our hypothesis also proposes that when a critical level in LDL is reached by the further addition of reactive oxygen species by the artery wall cells to seeded LDL, the nonenzymatic oxidation of a major LDL phospholipid, PAPC, results in the formation of three biologically active oxidized phospholipids (POVPC, PGPC, and PEIPC) that induce monocyte binding and chemotaxis.

Step 3

13(S)-HPODE and 15(S)-HPETE markedly enhance the oxidation of PAPC and cholesteryl linoleate. We previously reported that if PAPC was exposed to air for 48 h it would undergo auto-oxidation to produce the three biologically active phospholipids (POVPC, PGPC, and PEIPC) (6, 7). If products of the lipoxygenase pathway were involved in both the initial seeding of circulating LDL and the further seeding of LDL by artery wall cells necessary to reach a critical threshold that would cause the nonenzymatic oxidation of PAPC, then the addition of the products of the lipoxygenase pathway to PAPC should significantly increase the formation of the three biologically active oxidized phospholipids (POVPC, PGPC, and PEIPC). To test this hypothesis we measured the formation of the three biologically active oxidized phospholipids from PAPC as a function of time. As shown in **Fig. 6** the addition of 1.0 μg of 13(*S*)-HPODE to 10 μg of PAPC enhanced the formation of the three biologically active oxidized phospholipids at each time point sampled (POVPC, m/z 594, Fig. 6A; PGPC, m/z 610, Fig. 6B; PEIPC, m/z 828, Fig. 6C). The data in **Fig. 7A** demonstrate that addition of as little as 0.5 μg of 13(*S*)-HPODE to 10 μg of PAPC for 8 h significantly decreased the relative abundance of PAPC (m/z 782) and significantly increased the formation of the three biologically active oxidized phospholipids (m/z 594, 610, and 828). Figure 7B demonstrates that addition of as little as 0.5 μg of 15(*S*)-HPETE to 10 μg of PAPC for 8 h significantly decreased the relative abundance of PAPC (m/z 782) and significantly increased the formation of the three biologically active oxidized phospholipids (m/z 594, 610, and 828). Figure 7C shows that 8 mM hydrogen peroxide added to 10 μg of PAPC for 8 h dramatically decreased the relative abundance of PAPC and increased the formation of the biologically active phospholipids, while 2 and 4 mM hydrogen peroxide had no effect. Because the molecular weight of PAPC is 782 the molar ratio required for the enhanced oxidation of PAPC by hydrogen peroxide was approximately 62:1 (H_2O_2:PAPC) in the experiment described in Fig. 7C. Because the molecular weight of 13(*S*)-HPODE is 311 and the molecular weight of 15(*S*)-HPETE is 336.5, the molar ratio at which these products of the lipoxygenase pathway promoted the oxidation of PAPC is approximately 1:8. Thus, on a molar basis the ability of 13(*S*)-HPODE and 15(*S*)-HPETE to oxidize PAPC was more than two orders of magnitude greater than that of hydrogen peroxide under these conditions. Taken together these data indicate that 13(*S*)-HPODE and 15(*S*)-HPETE, products of linoleic and arachidonic acid metabolism, respectively, act as potent oxidizing agents and promote the noncatalytic oxidation of PAPC to yield the three biologically active oxidized phospholipids found in mildly oxidized LDL.

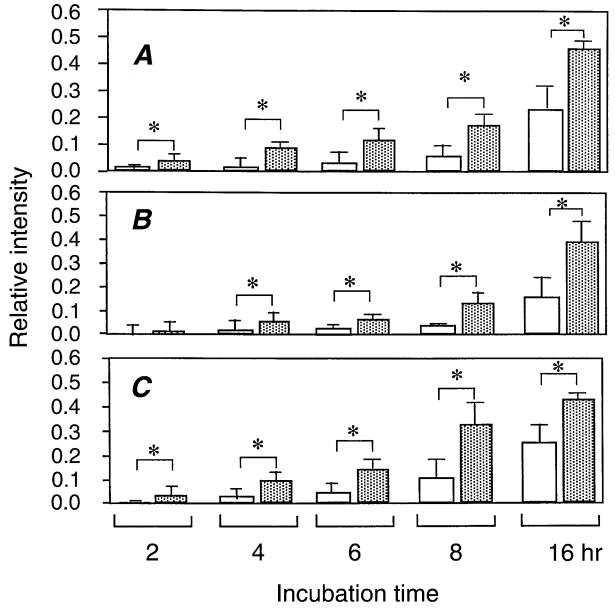

Fig. 6. 13(*S*)-HPODE accelerates the formation of bioactive oxidized phospholipids from PAPC. Ten micrograms of PAPC with 1.0 μg of 13(*S*)-HPODE (stippled columns) or with vehicle alone (open columns) were mixed and evaporated, forming a thin film, and allowed to oxidize in air for the times shown. After extraction with chloroform–methanol, the samples were analyzed by ESI-MS in the positive ion mode. The data represent the levels of 1-palmitoyl-2-oxovaleryl-*sn*-glycero-3-phosphocholine [POVPC, m/z 594; (A)], 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine [PGPC, m/z 610; (B)], and 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-*sn*-glycero-3-phosphocholine [PEIPC, m/z 828, (C)] relative to an internal standard (0.1 μg of DMPC) that was added with the PAPC. The values represent means ± SD of triplicate samples from four separate experiments. The asterisks indicate $P < 0.01$. 13(*S*)-HPODE alone did not give a signal for m/z 594, 610, or 828 (data not shown).

Stocker and colleagues (4, 5) presented indirect evidence to suggest that the lipoxygenase-mediated oxidation of cholesteryl linoleate is mediated primarily by a nonenzymatic process that involves products of the lipoxygenase pathway. The experiments in **Fig. 8** demonstrate that the presence of 13(*S*)-HPODE markedly stimulated the nonenzymatic formation of cholesteryl linoleate hydroperoxide (Ch18:2-OOH).

Paraoxonase inhibits the biologic activity of the three oxidized phospholipids, m/z 594, 610, and 828. We previously reported that antioxidants and HDL could prevent the formation of biologically active mildly oxidized LDL, but once formed HDL and antioxidants could not decrease the biologic activity of the mildly oxidized LDL (1). In these experiments, in contrast to those reported above where HDL was incubated with the cocultures before the LDL was added to the cocultures, we previously had

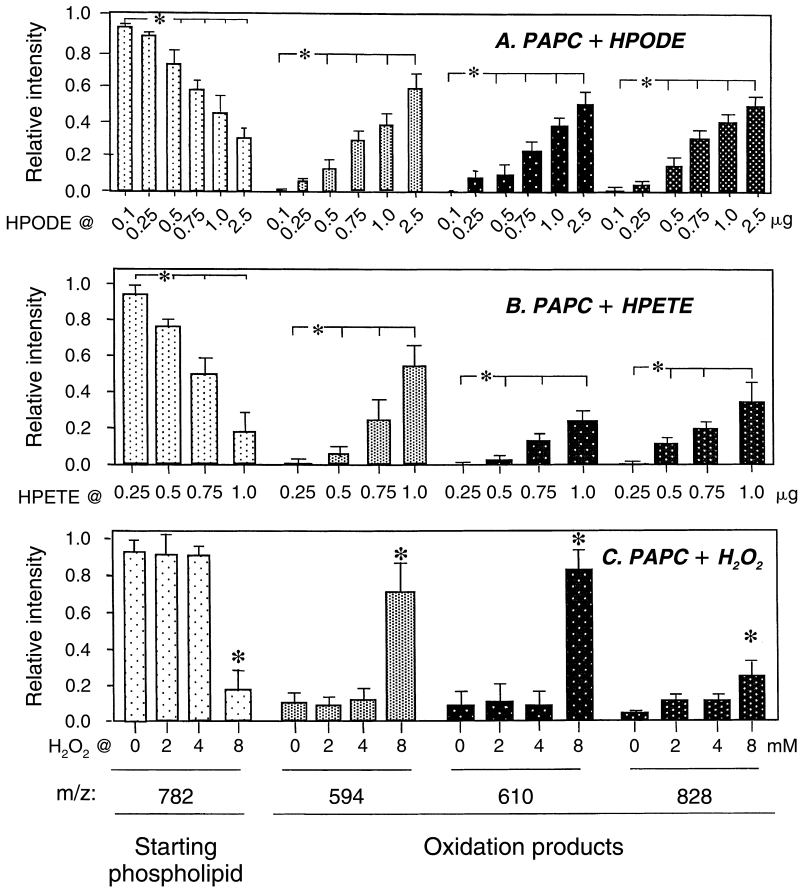

Fig. 7. 13(*S*)-HPODE, 15(*S*)-HPETE, or H_2O_2 accelerate in a dose-dependent manner the formation of oxidized phospholipids from PAPC. Ten micrograms of PAPC was mixed with the indicated micrograms of 13(*S*)-HPODE (A) or 15(*S*)-HPETE (B) and evaporated, forming a thin film, and allowed to oxidize in air for 8 h. In (C), 10 μg of PAPC was evaporated to form a thin film and H_2O_2 was added at the indicated concentrations and allowed to oxidize for 8 h. After extraction with chloroform–methanol, the samples were analyzed by ESI-MS in the positive ion mode. The data represent the levels of PAPC, m/z 782; POVPC, m/z 594; PGPC, m/z 610; and PEIPC, m/z 828 relative to an internal standard (0.1 μg of DMPC) that was added with the PAPC. The values represent means ± SD of triplicate samples in three separate experiments. 13(*S*)-HPODE alone, 15(*S*)-HPETE alone, or H_2O_2 did not give a signal for m/z 594, 610, or 828 (data not shown). Asterisks indicate significant differences at $P < 0.01$.

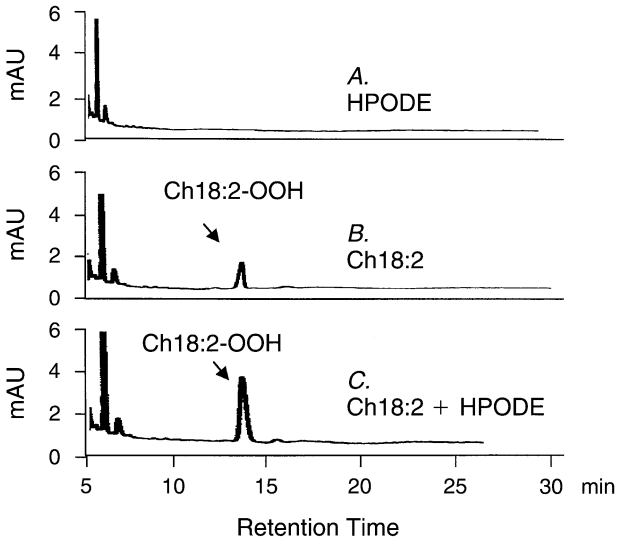

Fig. 8. 13(*S*)-HPODE stimulates the nonenzymatic formation of cholesteryl linoleate hydroperoxide (Ch18:2-OOH). 13(*S*)-HPODE at 0.5 μg/ml (A), cholesteryl linoleate at 10 μg/ml (B), or cholesteryl linoleate at 10 μg/ml together with 13(*S*)-HPODE at 0.5 μg/ml (C) in chloroform–methanol 2:1 (v/v) was briefly swirled to mix and evaporated to dryness under argon and allowed to undergo air oxidation in a laminar flow hood for 6 h. The lipids were solubilized in 50 μl of chloroform and analyzed for the presence of cholesteryl linoleate hydroperoxide (Ch18:2-OOH) by RP-HPLC as described in Materials and Methods.

added the HDL together with LDL to the cocultures. In other studies, we previously reported that platelet-activating factor acetylhydrolase (PAF-AH)(19) and PON (20) could destroy the biologic activity of mildly oxidized LDL if the enzymes were incubated with the LDL before addition to the cells. These studies were performed with mildly oxidized LDL, and not the specific oxidized phospholipids (i.e., oxidized PAPC or POVPC, PGPC, PEIPC). To directly test the ability of paraoxonase to destroy the biologic activity of each of the three oxidized phospholipids we incubated oxidized PAPC (Ox-PAPC), or POVPC, m/z 594; PGPC, m/z 610; or PEIPC, m/z 828 with or without purified paraoxonase as described in Materials and Methods. The enzyme was separated from the mixtures and the compounds were added to human artery wall cocultures. Incubation of Ox-PAPC, or POVPC; PGPC; or PEIPC with purified paraoxonase followed by separation of the paraoxonase from the compounds prior to presentation to the artery wall cell cocultures resulted in the inhibition of the biologic activity of each, that is, the loss of the ability to induce monocyte chemotactic activity (**Fig. 9**). Two mutant recombinant PON preparations, a generous gift of R. Sorenson and B. N. La Du (21) were unable to inactivate the biologically active phospholipids in this assay system (data not shown). PON that was inactivated by boiling at 100°C had no effect on the activity of the oxidized phospholipids (data not shown).

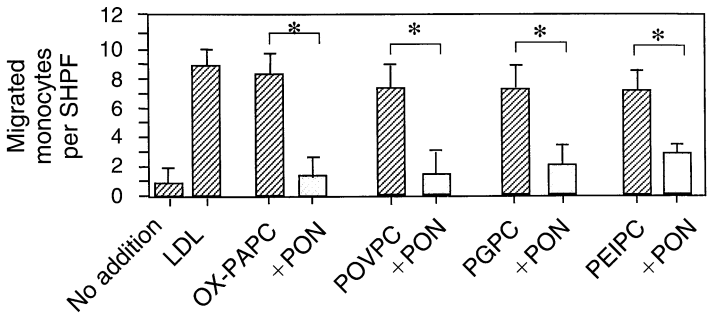

Fig. 9. Purified paraoxonase inhibits the bioactivity of the oxidized phospholipids. Oxidized PAPC (Ox-PAPC), POVPC (m/z 594), PGPC (m/z 610), and PEIPC (m/z 828) were incubated in test tubes in M199 without or with purified human paraoxonase at 1×10^{-2} U/ml (+PON) for 3 h with gentle mixing at 37°C. Paraoxonase was removed from the mixture and the lipids were incubated with human aortic wall cocultures in M199 with 10% LPDS for 8 h at 37°C. The cocultures were then washed and incubated with fresh medium without serum or LPDS for an additional 8 h at 37°C. The supernatants were removed and analyzed for monocyte chemotactic activity. Data represent means ± SD for quadruplicate cocultures in two separate experiments. Asterisks indicate significant differences at the level $P < 0.01$.

HDL from patients with coronary artery disease and with normal blood lipid levels, who were neither diabetic nor receiving hypolipidemic medications, failed to prevent LDL oxidation by artery wall cells and failed to destroy the biologic activity of oxidized PAPC. We previously reported (9) that after screening more than 250 patients with angiographically documented coronary artery disease, we identified 14 patients with angiographically documented coronary artery disease despite normal blood lipid levels and the absence of diabetes. These 14 had on average lower levels of paraoxonase activity despite their normal HDL-cholesterol levels compared with 19 age- and sex-matched controls (9). However, the differences between the patient's paraoxonase activity and normal controls did not reach statistical significance (9). We have now screened 520 additional patients and identified another 10 patients with normal lipid levels (total cholesterol, 171 ± 27 mg/dl; LDL-cholesterol, 109 ± 21 mg/dl; HDL-cholesterol, 58 ± 13 mg/dl for males and >65 ± 11 mg/dl for females; and triglycerides, 78 ± 23 mg/dl) who had angiographically documented coronary artery disease and who were neither diabetic nor receiving hypolipidemic medications. Combining the previously reported data with the new data we now see a statistically significant difference in paraoxonase activity between patients (n = 24) and controls (n = 29) (**Fig. 10A**).

Previously we were able to obtain sufficient sample from only 5 of the original 14 patients to test in our coculture system (9). We reported that HDL from these five did not protect against LDL-induced monocyte chemotactic activity in the human artery wall coculture system, while HDL from four control subjects did (see Fig. 12D in ref. 9). In our current studies we obtained HDL from an additional 10 normolipidemic patients with angiographically documented coronary artery disease, who were neither diabetic nor receiving hypolipidemic medications. The ability of HDL from these 10 patients and 10 age- and sex-matched normal subjects to modify the oxidation of a control LDL (i.e., LDL obtained from one normal subject, which was used in all the experiments) is shown in Fig. 10B. As shown in Fig. 10B, HDL taken from 10 of 10 of the patients did not protect the control LDL against oxidation by human artery wall cells. Indeed, on average the patient HDL actually increased control LDL oxidation, while HDL from 10 of 10 age- and sex-matched normal subjects markedly reduced control LDL oxidation by the artery wall cells.

Adding the data on monocyte chemotaxis from the 10 new patients and 10 normal subjects to that of the previously reported 5 patients and 4 age- and sex-matched normal subjects (Fig. 12D in ref. 9) yields a total of 15 patients and 14 normal subjects that have now been studied in the coculture system. In the experiments shown in Fig. 10C, HDL from 15 of 15 of these patients was unable to protect against LDL-induced monocyte chemotactic activity, while 14 of 14 of the controls had HDL that did.

Previously, we had not directly tested the ability of HDL from this subset of patients to inhibit the biologic activity of oxidized PAPC. Figure 10D demonstrates that the patients (none previously reported) had HDL that could not inhibit the biologic activity of oxidized PAPC (10 of 10 patients had HDL that did not inhibit the biologic activity of oxidized PAPC). Indeed, the HDL of the 10 patients on average increased the Ox-PAPC-induced monocyte adherence to HAEC (Fig. 10D). In contrast, HDL from 10 of 10 age- and sex-matched normal subjects markedly decreased the ability of Ox-PAPC to induce monocyte adherence to HAEC (Fig. 10D). Taken together, these data indicate that HDL from this subset of patients with coronary artery disease appears to be defective despite their normal plasma HDL-cholesterol levels.

DISCUSSION

We have demonstrated in this article that apoA-I and an apoA-I peptide mimetic were able to act directly on human artery wall cells and profoundly influence their ability to oxidize LDL (Figs. 1 and 2). In contrast, apoA-II was unable to prevent human artery wall cells from oxidizing

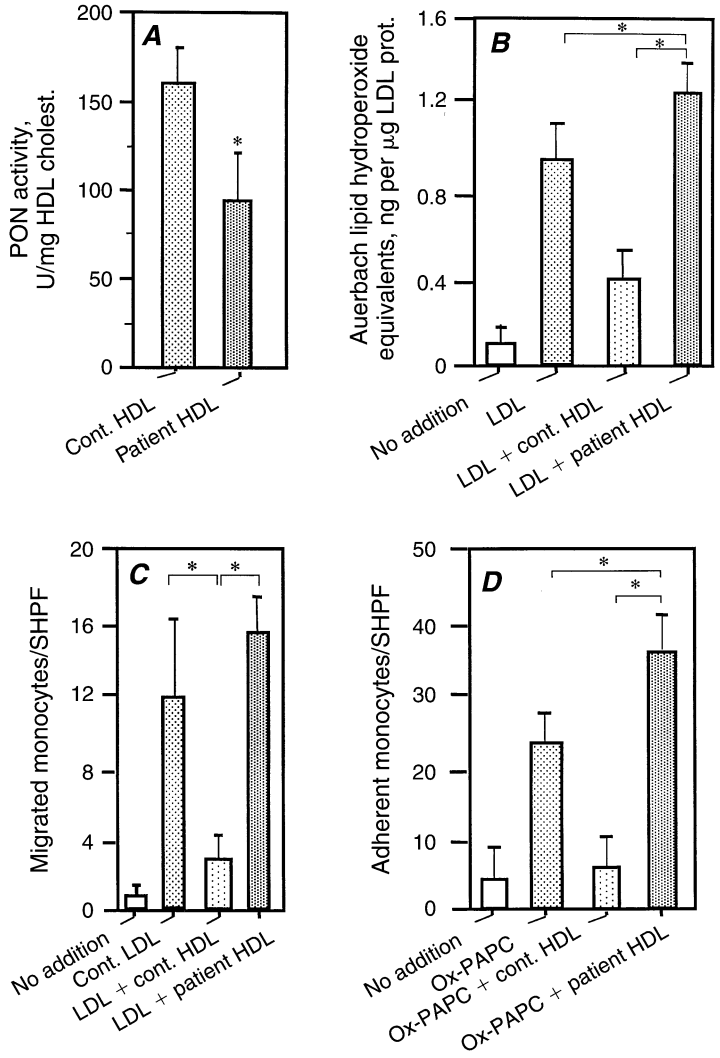

Fig. 10. HDL from patients with angiographically documented coronary atherosclerosis despite normal HDL-cholesterol levels is deficient in paraoxonase activity, does not protect LDL from oxidation by artery wall cells, and does not inhibit the biologic activity of oxidized phospholipids. These patients had angiographically documented coronary atherosclerosis, despite normal total cholesterol, triglycerides, LDL-cholesterol, and HDL-cholesterol levels. The patients were not diabetic or receiving hypolipidemic medications. Paraoxonase activity was determined as described in Materials and Methods for 24 patients and 29 age- and sex-matched normal subjects (A). Data from 14 previously reported patients (9) and from 19 previously reported normal subjects (9) are included in (A) together with data from an additional 10 patients and age- and sex-matched normal subjects. The ability of HDL from the additional 10 patients and controls to protect a control LDL against oxidation by artery wall cells is shown in (B) as determined by the formation of Auerbach lipid hydroperoxide equivalents as described in Materials and Methods and in (C) by monocyte chemotactic activity, which was determined as described in Materials and Methods. The data in (C) include data previously reported for 5 patients and 4 normal subjects (9) together with data from the additional 10 patients and their age- and sex-matched normal subjects. The data in (D) represent a new approach, namely the ability of patient and normal HDL (n = 10 for each group) to inhibit the biologic activity of oxidized PAPC (Ox-PAPC). In each instance Ox-PAPC (100 μg/ml) was incubated in test tubes with HDL (250 μg/ml) in 10% LPDS in M199 at 37°C with gentle mixing for 4 h. The HDL-Ox-PAPC mixture was then added to endothelial monolayers and monocyte binding was determined. Data represent means ± SD of quadruplicate cocultures from two to six separate experiments and the asterisks indicate a significant difference at the level of $P < 0.01$ for (A); $P < 0.02$ for LDL versus LDL + patient HDL, $P < 0.001$ for LDL + control (cont.) HDL versus LDL + patient HDL in (B); $P < 0.009$ for cont. LDL versus LDL + cont. HDL, $P < 0.008$ for LDL + cont. HDL versus LDL + patient HDL in (C); $P < 0.009$ for Ox-PAPC versus Ox-PAPC + patient HDL, $P < 0.01$ for Ox-PAPC + cont. HDL versus Ox-PAPC + patient HDL in (D).

LDL (Fig. 1). Similar to the case for LDL that was rendered resistant to oxidation after treatment with HDL or PON (see accompanying article), treating human artery wall cells with HDL or PON rendered the artery wall cells incapable of oxidizing LDL (Fig. 3). These experiments indicate that HDL and its associated enzymes can inhibit

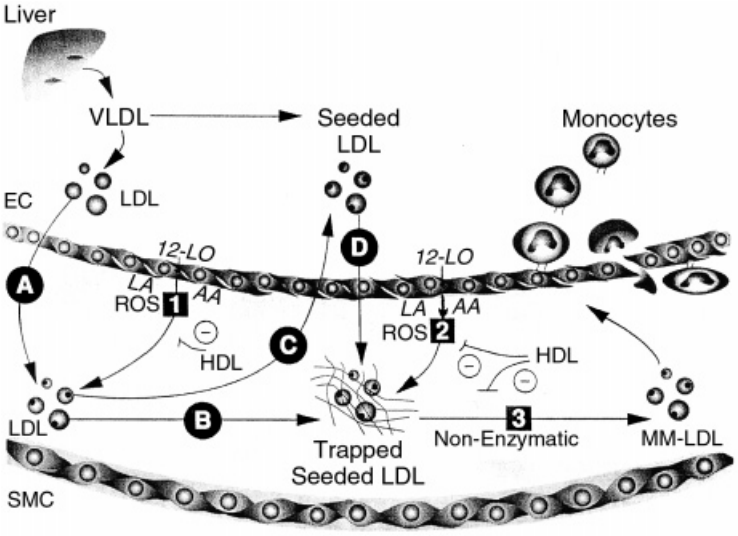

Fig. 11. A three-step model for LDL oxidation by artery wall cells: Step 1: LDL is seeded. Step 2: LDL is trapped in the artery wall and receives further seeding molecules. Step 3: When a critical level of seeding molecules relative to phospholipids is reached in the LDL, a nonenzymatic oxidation process generates POVPC, PGPC, and PEIPC. LDL that is formed from the hydrolysis of VLDL in the circulation may contain seeding molecules. Alternatively, LDL may enter the subendothelial space (A), where it is seeded with reactive oxygen species (ROS) delivered from the artery wall cells (likely by the action of 12-lipoxygenase (12-LO) on linoleic (LA) and arachidonic (AA) acids (step 1). While the diagram depicts this as occurring in the subendothelial space, step 1 might actually occur in the microcirculation. If the LDL is seeded in the subendothelial space it might remain there, becoming trapped in the extracellular matrix (B), or the seeded LDL could exit into the circulation (C) and reenter the subendothelial space at another site, where it would become trapped in the extracellular matrix (D). In step 2 the artery wall cells generate and transfer additional or different ROS to the trapped seeded LDL. This transfer could occur within the cell, at the cell surface, or in an adjacent protected microdomain. After this transfer of reactive oxygen species to the seeded and trapped LDL, a nonenzymatic propagation of lipid oxidation occurs (step 3). This results in the formation of specific oxidized phospholipids that induce NF-κB activation, monocyte binding, MCP-1 production, and M-CSF production and that are present in mildly oxidized LDL (minimally modified LDL; MM-LDL). As indicated, normal HDL is capable of blocking each and every step in the formation of MM-LDL.

human artery wall cells from contributing the additional reactive oxygen species necessary for circulating LDL to reach the critical threshold required to oxidize PAPC to the biologically active phospholipids.

The data in this article support a role for products of the lipoxygenase pathways in artery wall cells in the second step of the formation of mildly oxidized LDL and are consistent with the findings of Cyrus et al. (22) that disruption of the 12/15-lipoxygenase gene diminished atherosclerosis in apoE-deficient mice. They concluded that several mechanisms could explain their findings but favored one in which ". . . lipoxygenase-derived hydroperoxides or secondary reactive lipid species may be transferred across the cell membrane to 'seed' the extracellular LDL, which would then be more susceptible to a variety of mechanisms that could promote lipid peroxidation."

Our finding that platelet-type 12-LO protein is present in human artery wall cells and is required for human artery wall cells to modify LDL to a form that induces the artery wall cells to produce monocyte chemotactic activity provides support for the mechanism favored by Cyrus et al. (22) as described above.

The nonenzymatic oxidation of PAPC to form the three biologically active phospholipids (POVPC, PGPC, and PEIPC) was greatly enhanced by 13-HPODE and 15-HPETE (Figs. 5 and 6). Indeed, the ability of 13-HPODE and 15-HPETE to oxidize PAPC to these three biologically active phospholipids was more than two orders of magnitude more potent than that of hydrogen peroxide (Fig. 10). These results are consistent with the findings of Montgomery, Nathan, and Cohn (23), who found that the amount of hydrogen peroxide necessary to produce oxidation of LDL was two orders of magnitude greater than that produced by endothelial cells that oxidized LDL. The ability of 13-HPODE to stimulate the nonenzymatic formation of cholesteryl linoleate hydroperoxide (Ch18:2-OOH) (Fig. 8) is consistent with the results of Stocker and colleagues (4, 5) and suggests that products of the lipoxygenase pathway may be central in the formation of a variety of oxidized lipids as hypothesized by Cyrus et al. (22).

Stocker and colleagues (24, 25) also demonstrated that both apoA-I and apoA-II can reduce cholesteryl ester hydroperoxides via a mechanism that involves oxidation of specific methionine residues (25). In our experiments only apoA-I and not apoA-II was able to reduce the oxidation of LDL after injection into mice (see accompanying article). Moreover, only apoA-I and not apoA-II was able

to decrease the ability of human artery wall cells to oxidize LDL (Fig. 1).

The inhibition of the biologic activity of Ox-PAPC and its components (POVPC, PGPC, and PEIPC) by PON (Fig. 9) and by normal HDL but not by HDL from patients with angiographically proven atherosclerosis despite normal plasma HDL-cholesterol levels (Fig. 10), suggests that an abnormality in HDL may be responsible, at least in part, for the atherosclerosis in this relatively rare subset of patients. A role for PON in the pathogenesis of atherosclerosis was first suggested by the work of Mackness et al. (26) and Ayub et al. (27) and has been supported by the work of a number of laboratories including ours (28–30). We report in this article that normolipidemic patients with coronary artery disease who were neither diabetic nor receiving hypolipidemic medications had significantly lower levels of PON activity compared with age- and sex-matched normal subjects (Fig. 10A). However, there was overlap in PON activities of the patients and normal subjects. In contrast, HDL from 10 of 10 patients failed to protect control LDL against oxidation by human artery wall cells (Fig. 10B) and failed to inhibit the biologic activity of oxidized PAPC (Fig. 10D) while HDL from 10 of 10 age- and sex-matched normal subjects did. These findings suggest to us that the difference in patient and control HDL cannot be completely explained by differences in PON activity.

The data presented in this and the accompanying article demonstrate a role for HDL and its components, apoA-I and PON, in regulating each and every step in a three-step process that leads to the formation of mildly oxidized LDL and that is diagrammed in **Fig. 11.** Understanding the mechanisms for the formation of mildly oxidized LDL and the role of HDL and its components in preventing the formation and inhibiting the biologic activity of mildly oxidized LDL may lead to new therapeutic strategies for the prevention and treatment of atherosclerosis and the clinical syndromes that result from this inflammatory process.

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REFERENCES

1. Navab, M., S. Imes, S. Hama, G. Hough, L. Ross, R. Bork, A. J. Valente, J. A. Berliner, D. C. Drinkwater, M. Laks, and A. M. Fogelman. 1991. Monocyte transmigration induced by modification of low density lipoprotein in cocultures of human aortic wall cells is due to induction of monocyte chemotactic protein-1 synthesis and is abolished by high density lipoprotein. *J. Clin. Invest.* **88:** 2039–2046.
2. Hama, S., L. Jin, M. Navab, and A. M. Fogelman. 1997. Apolipoprotein A-I can remove lipid molecules from native LDL rendering it resistant to oxidation by cultured artery wall cells. *Circulation.* **96:** I-485 (Abstract).
3. Hama, S., G. Hough, L. Jin, B. J. Van Lenten, G. M. Anantharamaiah, A. D. Watson, K. Faull, M. Navab, H. Laks, and A. M. Fogelman. 1998. Apolipoprotein A-I mimic peptides inhibit oxidation of low density lipoprotein by artery wall cells and the resulting monocyte interactions. *Circulation.* **98:** I-252 (Abstract).

3a. 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. 2000. Normal high densisty lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: step 1. *J. Lipid Res.* 41:1481–1494.

4. Neuzil, J., J. M. Upston, P. K. Witting, K. F. Scott, and R. Stocker. 1998. Secretory phospholipase A2 and lipoprotein lipase enhance 15-lipoxygenase-induced enzymic and nonenzymic lipid peroxidation in low-density lipoproteins. *Biochemistry.* **37:** 9203–9210.
5. Upston, J. M., J. Neuzil, P. K. Witting, R. Alleva, and R. Stocker. 1997. Oxidation of free fatty acids in low density lipoprotein by 15-lipoxygenase stimulates nonenzymic, alpha-tocopherol-mediated peroxidation of cholesteryl esters. *J. Biol. Chem.* **272:** 30067–30074.
6. Watson, A. D. 1999. Structural identification of a novel proinflammatory epoxyisoprostane phospholipid in mildly oxidized low density lipoprotein. *J. Biol. Chem.* **274:** 24787–24798.
7. Watson, A. D., N. Leitinger, M. Navab, K. F. Faull, S. Hörkkö, J. L. Witztum, W. Palinski, D. Schwenke, R. G. Salomon, W. Sha, G. Subbanagounder, A. M. Fogelman, and J. A. Berliner. 1997. Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induces monocyte/endothelial interactions and evidence for their presence in vivo. *J. Biol. Chem.* **272:** 13597–13607.
8. Navab, M., S. Hama-Levy, B. J. Van Lenten, G. C. Fonarow, C. J. Cardinez, L. W. Castellani, M-L. Brennan, A. J. Lusis, and A. M. Fogelman. 1997. Mildly oxidized LDL induces an increased apolipoprotein J/paraoxonase ratio. *J. Clin. Invest.* **99:** 2005–2019.
9. Rumsey, S. C., N. F. Galeano, Y. Arad, and R. J. Deckelbaum. 1992. Cryopreservation with sucrose maintains normal physical and biological properties of human plasma low density lipoproteins. *J. Lipid Res.* **33:** 1551–1561.
10. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
11. Lorenzen, A., and S. W. Kennedy. 1993. A fluorescent based protein assay for use with a microplate reader. *Anal. Biochem.* **214:** 346–348.
12. Lowry, O. H., M. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
13. Auerbach, B. J., J. S. Kiely, and J. A. Cornicelli. 1992. A spectrophotometric microtiter-based assay for detection of hydroperoxy derivatives of linoleic acid. *Anal. Biochem.* **201:** 375–380.
14. Gan, K. N., A. Smolen, H. W. Eckerson, and B. N. La Du. 1991. Purification of human serum paraoxonase/arylesterase. Evidence for one esterase catalyzing both activities. *Drug Metab. Dispos.* **19:** 100–106.
15. Berliner, J. A., M. C. Territo, A. Sevanian, S. Ramin, J. A. Kim, B. Bamshad, M. Esterson, and A. M. Fogelman. 1990. Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J. Clin. Invest.* **85:** 1260–1266.
16. Thomas, C. E., and R. L. Jackson. 1991. Lipid hydroperoxide involvement in copper-dependent and independent oxidation of low density lipoproteins. *J. Pharmacol. Exp. Ther.* **256:** 1182–1188.
17. Parthasarathy, S. 1994. Modified Lipoproteins in the Pathogenesis of Atherosclerosis. R. G. Landes, editor. Austin, TX. 91–119.
18. Sigari, F., C. Lee, J. L. Witztum, and P. D. Reaven. 1997. Fibroblasts that overexpress 15-lipoxygenase generate bioactive and minimally modified LDL. *Arterioscler. Thromb. Vasc. Biol.* **17:** 3639–3645.
19. Watson, A. D., M. Navab, S. Hama, A. Sevanian, S. Prescott, D. Stafforini, T. McIntyre, B. N. La Du, A. M. Fogelman, and J. A. Berliner. 1995. Effect of platelet activating factor acetylhydrolase on the formation and action of minimally oxidized low density lipoprotein. *J. Clin. Invest.* **95:** 774–782.
20. Watson, A. D., J. A. Berliner, S. Hama, B. N. La Du, K. Faull, A. M. Fogelman, and M. Navab. 1995. Protective effect of HDL associated paraoxonase-inhibition of the biological activity of minimally oxidized low density lipoprotein. *J. Clin. Invest.* **96:** 2882–2891.
21. Sorenson, R. C., S. L. Primo-Parmo, C. L. Kuo, S. Adkins, O. Lockridge, and B. N. La Du. 1995. Reconsideration of the catalytic cen-

ter and mechanism of mammalian paraoxonase/arylesterase. *Proc. Natl. Acad. Sci. USA.* **92:** 7187–7191.
22. Cyrus, T., J. L. Witztum, D. J. Rader, R. Tangirala, S. Fazio, M. F. Linton, and C. D. Funk. 1999. Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apoE-deficient mice. *J. Clin. Invest.* **103:** 1597–1604.
23. Montgomery, R. R., C. F. Nathan, and Z. A. Cohn. 1986. Effects of reagent and cell-generated hydrogen peroxide on the properties of low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **83:** 6631–6635.
24. Garner, B., P. K. Witting, A. R. Waldeck, J. K. Christison, M. Raftery, and R. Stocker. 1998. Oxidation of high density lipoproteins. I. Formation of methionine sulfoxide in apolipoproteins AI and AII is an early event that accompanies lipid peroxidation and can be enhanced by alpha-tocopherol. *J. Biol. Chem.* **273:** 6080–6087.
25. Garner, B., A. R. Waldeck, P. K. Witting, K. A. Rye, and R. Stocker. 1998. Oxidation of high density lipoproteins. II. Evidence for direct reduction of lipid hydroperoxides by methionine residues of apolipoproteins AI and AII. *J. Biol. Chem.* **273:** 6088–6095.
26. Mackness, B., M. I. Mackness, S. Arrol, W. Turkie, and P. N. Durrington. 1998. Effect of the human serum paraoxonase 55 and 192 genetic polymorphisms on the protection by high density lipoprotein against low density lipoprotein oxidative modification. *FEBS Lett.* **423:** 57–60.
27. Ayub, A., M. I. Mackness, S. Arrol, B. Mackness, J. Patel, and P. N. Durrington. 1999. Serum paraoxonase after myocardial infarction. *Arterioscler. Thromb. Vasc. Biol.* **19:** 330–335.
28. Shih, D. M., L. Gu, S. Hama, Y-R. Xia, M. Navab, A. M. Fogelman, and A. J. Lusis 1996. Genetic-dietary regulation of serum paraoxonase expression and its role in atherogenesis in a mouse model. *J. Clin. Invest.* **97:** 1630–1639.
29. Shih, D. M., L. Gu, Y-R. Xia, M. Navab, W-F. Li, S. Hama, L. W. Castellani, C. E. Furlong, L. G. Costa, A. M. Fogelman, and A. J. Lusis. 1998. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature.* **394:** 284–287.
30. Castellani, L. W., M. Navab, B. J. Van Lenten, C. C. Hedrick, S. Y. Hama, A. M. Goto, A. M. Fogelman, and A. J. Lusis. 1997. Overexpression of apolipoprotein AII in transgenic mice converts high density lipoproteins to proinflammatory particles. *J. Clin. Invest.* **100:** 464–474.