methods

A cell-free assay for detecting HDL that is dysfunctional in preventing the formation of or inactivating oxidized phospholipids

Mohamad Navab,[1] Susan Y. Hama, Greg P. Hough, Ganesamoorthy Subbanagounder, Srinivasa T. Reddy, and Alan M. Fogelman

Atherosclerosis Research Unit, Division of Cardiology, Department of Medicine, University of California, Los Angeles, Los Angeles, CA 90095

Abstract **We have developed a novel and rapid cell-free assay of the ability of HDL to prevent the formation of or inactivate oxidized phospholipids. HDL was tested for its ability to inhibit the oxidation of LDL, or inhibit the oxidation of L-α-1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC) by hydroperoxyoctadecadienoic acid (HPODE), or inactivate oxidized PAPC (Ox-PAPC). In each case the fluorescent signal generated in the presence of the test substances and the test HDL was determined. As little as 2.5 μg of normal human HDL cholesterol significantly inhibited the fluorescent signal generated by Ox-PAPC; results did not differ regardless of whether the HDL was prepared by gel electrophoresis, fast protein liquid chromatography, or dextran sulfate precipitation. HDL from each of 27 patients with coronary atherosclerosis failed to inhibit the fluorescent signal generated by a control LDL, whereas HDL from each of 31 matched normal subjects with the same levels of HDL cholesterol significantly inhibited the signal. Results from an established cell-based assay (Navab, M., S. Hama, 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. *J. Lipid Res.* 41: 1481–1494) were identical. HDL from the patients also failed to inhibit the fluorescent signal generated from PAPC plus HPODE (10 of 10 patients) whereas HDL from matched controls (8 of 8 patients) significantly inhibited the fluorescent signal. We conclude that this new assay has the potential to allow widespread testing of the hypothesis that HDL that is dysfunctional in preventing the formation or inactivating oxidized phospholipids may play an important role in the development of atherosclerosis.**—Navab, M., S. Y. Hama, G. P. Hough, G. Subbanagounder, S. T. Reddy, and A. M. Fogelman. **A cell-free assay for detecting HDL that is dysfunctional in preventing the formation of or inactivating oxidized phospholipids. *J. Lipid Res.* 2001.** 42: **1308–1317.**

Supplementary key words LDL • mildly oxidized LDL • HPODE • HPETE • atherosclerosis

We have reported evidence to support the hypothesis that the formation of mildly oxidized LDL by artery wall cells occurs in three steps, each of which can be inhibited by normal HDL (1, 2). The first and second steps required seeding molecules derived from the 12-lipoxygenase pathway, including hydroperoxyoctadecadienoic acid (HPODE) and hydroperoxyeicosatetraenoic acid (HPETE). The third step was postulated to occur when a critical threshold of the seeding molecules was reached in LDL and caused the oxidation of L-α-1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (PAPC) in LDL producing oxidized PAPC (Ox-PAPC).

Epidemiological studies indicate that HDL cholesterol levels are among the most predictive of risk factors for cardiovascular events (3–5). We have previously presented evidence that HDL is dysfunctional (i.e., fails to prevent the formation of biologically active LDL-derived oxidized phospholipids and fails to inactivate the biologically active LDL-derived oxidized phospholipids) and is in many instances proinflammatory (i.e., enhances the formation of the biologically active oxidized phospholipids) in mice susceptible to diet-induced atherosclerosis and fed an atherogenic diet (6), in apolipoprotein E (apoE) knockout mice (6), in mice overexpressing murine apoA-II (7), in mice transgenic for secretory phospholipase A_2 ($sPLA_2$) (8), in paraoxonase knockout mice (9), and in humans and rabbits with an acute-phase response (10). These studies were performed with a cell-based assay that requires endothelial cells, smooth muscle cells, and monocytes (11). We report here a novel and rapid test of HDL function that does not require cells but that gives results highly comparable to those of the previously described cell-based assay.

Abbreviations: DCFH, dichlorofluorescein; HPETE, hydroperoxyeicosatetraenoic acid; HPODE, hydroperoxyoctadecadienoic acid; PAPC, L-α-1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine; POVPC, 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-3-phosphorylcholine.

[1] To whom correspondence should be addressed.
e-mail: mnavab@mednet.ucla.edu

MATERIALS AND METHODS

Materials

Tissue culture materials and other reagents including PAPC and HPODE were obtained from sources previously reported (1, 2). Ox-PAPC and 1-palmitoyl-2-oxovaleryl-*sn*-glycero-3-phosphorylcholine (POVPC) were prepared and isolated as previously described (2). Dextran sulfate was obtained from Sigma (St. Louis, MO) and dichlorofluorescein diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR).

Human cells

Human aortic endothelial cells, and human aortic smooth muscle cells were isolated as previously described (11) and cocultures of these cells were prepared and maintained as previously reported (11). Monocytes were isolated by techniques previously described (12) 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).

Monocyte chemotaxis assay

Monocyte chemotaxis assays were performed as previously described (1). In general, the cocultures were treated with LDL or phospholipids in the absence or presence of HDL for 4 to 8 h. Solutions of LDL or HDL in phosphate-buffered saline were diluted with culture medium 199 (M199) containing 10% lipoprotein-deficient serum (LPDS) and added to coculture wells. HPODE (in ethanol) and PAPC and Ox-PAPC (in chloroform) were evaporated under a stream of argon, 37°C culture medium containing 10% LPDS was added, and tubes were vortexed intermittently for 1 min and the resulting medium containing PAPC plus HPODE or Ox-PAPC was added to culture wells and incubated at 37°C. The supernatants were collected and used for determination of lipid hydroperoxide levels. The cocultures were subsequently washed and fresh M199 without any additions was added and incubated for 18 h. This allowed for the collection of supernatants that were tested for monocyte chemotactic activity. At the end of incubation, the supernatants were collected from the cocultures, diluted 40-fold, and assayed for monocyte chemotactic activity as described previously (1). Briefly, the supernatants were added to a standard Neuroprobe chamber (NeuroProbe, Cabin John, MD), with isolated human peripheral blood 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.

Lipoproteins

All plasma samples were cryopreserved in sucrose prior to use as described (1). LDL and HDL were isolated by ultracentrifugation or by fast performance liquid chromatography (FPLC) as previously described (13). HDL was also prepared by standard agarose gel electrophoresis (14) or by precipitation with dextran sulfate (Sigma). For precipitation with dextran sulfate, Sigma HDL cholesterol reagent containing dextran sulfate and magnesium ions was dissolved in distilled water. Fifty microliters of dextran sulfate (1.0 mg/ml) was mixed with 500 μl of the test plasma and incubated at room temperature for 5 min and subsequently centrifuged at 3,000 *g* for 10 min. The supernatant containing HDL was used in the experiments. In some experiments dysfunctional patient HDL was enriched with vitamin E or purified paraoxonase, by incubation with a 5 to 20 μM concentration range of α-tocopherol acetate (Sigma), or with purified human paraoxonase 1 (a generous gift of B. N. La Du, University of Michigan Medical School, Ann Arbor, MI) at 1×10^{-2} units in a volume of 1.0 ml. The incubation was carried out in 10% LPDS, at 37°C and with gentle mixing for 2 h. The mixture was then filtered through 100-kD molecular mass cutoff membranes and HDL was reisolated in the >100-kDa fraction and used in the assays. Mildly oxidized LDL was generated by incubation with human artery wall cells as previously described (1), or by incubation in saline/air with gentle mixing at room temperature for 2 h.

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. After signing a consent form approved by the Human Research Subject Protection Committee of the University of California, Los Angeles, the patient donated a fasting blood sample collected in a heparinized tube. HDL and LDL were isolated from blood samples from patients who had angiographically documented coronary atherosclerosis despite 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 receiving hypolipidemic medications and who were not diabetic, and who were not active smokers. All the patients had at least a 50% narrowing of at least one coronary artery. Normal volunteers were recruited to age and sex match the patients under a protocol approved by the Human Research Subject Protection Committee of the University of California, Los Angeles. The ability of the HDL from each patient/subject to protect LDL against oxidation by human artery wall cell cocultures was determined by techniques previously described (2). 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 (1).

Mice

C57BL/6J and C3H/HeJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were 4 to 6 months of age at the time of the experiments. The mice were maintained on a chow diet, containing 4% fat obtained from Ralston-Purina (St. Louis MO), or on an atherogenic diet, TD90221 containing 15.75% milk fat, 1.25% cholesterol, and 0.5% sodium cholate from Harlan Teklad (Madison, WI). In the first set of experiments the mice were placed on the atherogenic diet for 3 days before removal of blood to determine the ability of HDL to prevent LDL oxidation. In the second series of experiments the mice were exposed to second-hand cigarette smoke, using a Griffith device previously described (15). This unit consists of a programmable vacuum pump device that aspirates smoke from the mouthpiece of a lit cigarette at regular intervals. A rotary puffer intermittently exposes the lit cigarette to a vacuum pump, which then delivers the smoke to a distributing manifold as mainstream smoke. A recycle circuit allows the delivered amount of smoke from each puff to be regulated and to remain constant. Sidestream smoke generated at the burning cone of the cigarette in between puffs is collected and transferred to the distribution manifold. Snout smoke exposure is attained by restraining the mice in wire mesh compartments. These restraints are then connected to the smoke distribution manifold so as to expose the snout of the mouse to the cigarette smoke. The unit was set such that 1 puff was drawn from each cigarette every minute and 10 puffs per cigarette were delivered. The system can be adjusted to deliver air, fresh mainstream smoke, or sidestream smoke (second-hand smoke). For the present experiment, the

unit was adjusted to deliver sidestream smoke (1 part sidestream smoke:1 part air). Before the experiment, the mice were acclimated to second-hand smoke inhalation by gradually increasing the number of cigarettes delivered by the device from one to eight cigarettes over 3 days. On the day of the experiment, the mice received second-hand smoke at 1 puff/min by the unit, with a 20-min rest between cigarettes, for a total of eight cigarettes. The mice were then removed from the unit and blood samples were collected. HDL was isolated from blood obtained from the retro-orbital sinus, using heparin as an anticoagulant, at 2.5 U/ml blood, and under mild isoflurane anesthesia, adhering to the regulations set forth by the University of California Animal Research Committee.

Dichlorofluorescein (DCF) assay

DCFH-DA was dissolved in fresh methanol at 2.0 mg/ml and was incubated at room temperature and protected from light for 30 min. This results in the release of DCFH. On interaction with lipid oxidation products DCFH forms DCF, which produces intense fluorescence (16). Reagents were added to and incubated in 1 × 2.5 cm screw-cap polypropylene tubes. Fluorescence intensity was determined with a Farrand (Valhalla, NY) system 3 scanning spectrofluorometer set at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. A sensitivity level of 0.1 and slit widths of 2.5 and 10 nm were used for excitation and emission, respectively. For determining the capacity of HDL to inactivate previously oxidized PAPC, or the bioactive phospholipid POVPC, 10 μl aliquots of DCFH in methanol were placed in the polypropylene tubes and the methanol was evaporated under argon. Ten microliters of Ox-PAPC at 2.5 mg/ml or POVPC at 2.0 mg/ml in chloroform was added to each tube, mixed by vortexing, and evaporated under argon. Unless otherwise specified, 25 μl of saline, or HDL at 350 μg/ml, was added to the tubes, the volume was adjusted to 1.0 ml by addition of saline, and the mixture was vortexed for 1 min and incubated at room temperature in the dark. Fluorescence readings were obtained at the indicated time points. For experiments investigating the effect of HDL in preventing the oxidation of PAPC plus HPODE, 10 μl aliquots of DCFH in methanol were placed in the polypropylene tubes and the methanol was evaporated under argon. Ten microliters of PAPC at 2.5 mg/ml in chloroform was placed in each tube, mixed, and evaporated under argon. Subsequently 10 μl of HPODE at 0.1 mg/ml in ethanol was added and evaporated under argon. Unless otherwise specified, 25 μl of saline or HDL at 350 μg/ml was added to each tube, the volume was adjusted to 1.0 ml with saline, and the mixture was vortexed for 1 min and incubated at room temperature in the dark. Fluorescence readings were obtained at the indicated time points. For experiments investigating the protective effect of HDL on oxidation of LDL, 25 μl of LDL at 200 μg/ml was added to the tubes containing DCFH, followed by the addition of 25 μl of saline or HDL. The volume was adjusted to 1.0 ml, using normal saline, and the tubes were gently vortexed and incubated at room temperature in the dark. Fluorescence readings were obtained at the indicated time points. The assay was also adapted for analyzing a large number of samples with a plate reader (Spectra Max, Gemini YS; Molecular Devices, Sunnyvale, CA). Round-bottom polypropylene microtiter plates (Fisher Scientific, Pittsburgh, PA) were utilized in place of polypropylene tubes. Combinations of PAPC plus HPODE, or Ox-PAPC plus saline or HDL, were first prepared in polypropylene tubes and subsequently aliquoted into microtiter plates at 100 μl/well. The final concentrations of PAPC, HPODE, Ox-PAPC, and HDL were as described above unless otherwise specified. Thus, unless otherwise specified each well contained DCFH (2.0 μg), PAPC (25 μg) plus HPODE (1.0 μg), or Ox-PAPC (25 μg) and HDL at the indicated concentrations. A correlation coefficient of 0.901 was obtained between the values generated with the spectrofluorometer and those obtained with the plate reader. Values for intra- and interassay variability were 4.2 ± 1.3% and 6.3 ± 2.1%, respectively.

Other methods

The protein content of lipoproteins was determined by a modification (17) of the Lowry assay (18). Lipid hydroperoxide levels were measured by the assay reported by Auerbach, Kiely, and Cornicelli (19). Cholesterol levels were determined with a kit (Infinity reagent) obtained from Sigma. F_2-isoprostane (isoprostane 8-epi-$PGF_{2\alpha}$) levels were determined with an ELISA kit (EA84; Oxford Biomedical, Oxford, MI). Linoleic acid was oxidized by incubation with soybean lipoxygenase (SLO). Linoleic acid (100 μM) was incubated with 10 units of pure SLO bound to Sepharose beads for 4 h at 37°C with gentle mixing, followed by separation of the beads by centrifugation as previously described (1). LDL (250 μg) was combined with the SLO-treated linoleic acid and was incubated at 37°C for various lengths of time. The mixture was then added to tubes containing DCFH and incubated at room temperature for 2 h, followed by determination of fluorescence intensity. FPLC was performed as described previously (1). Statistical significance was determined with the Excel program and group means were compared for significant differences, using the Student's *t*-test for unpaired variates with $P < 0.05$ considered to be statistically significant. The significance of correlation coefficients was assessed by comparison of means performed by a planned approach. We did not use any corrections for multiple unplanned comparisons. All comparisons, whether significant or not, are reported here.

RESULTS

Dose response

To determine whether the signal generated by the interaction of Ox-PAPC with DCFH was dose dependent, Ox-PAPC was incubated with DCFH at the indicated concentrations and fluorescence intensity was determined after 2 h of incubation at room temperature. **Figure 1A** shows that the fluorescent signal generated by Ox-PAPC was dose dependent. There was a statistically significant ($P < 0.01$) difference between the signal generated by DCFH alone and DCFH plus Ox-PAPC for each concentration of Ox-PAPC used between 1 and 50 μg/ml. As seen in Fig. 1B, the correlation observed between the quantity of Ox-PAPC added and the increase in DCF fluorescence detected was highly significant ($P = 0.008$).

Comparison of cell-free assay with artery wall cell coculture assay

We previously reported that when LDL was added to human artery wall cell cocultures there was an excellent correlation between the generation of lipid hydroperoxides in the culture supernatant as measured by the Auerbach assay and the secretion by the artery wall cells of monocyte chemotactic activity (monocyte chemoattractant protein 1) (1, 2). To determine the correlation between data obtained in the cell-free assay and the artery wall cell coculture assay, PAPC or Ox-PAPC at 6.25, 12.5, 25.0, 50.0, and 100 μg/ml was added to human aortic wall

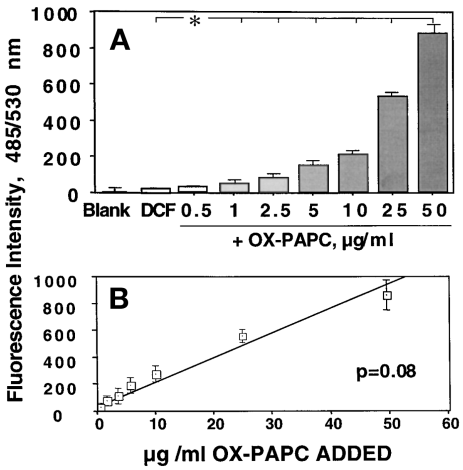

Fig. 1. A: Dose-dependent increase in DCF fluorescence by incubation with Ox-PAPC. Ox-PAPC was added as described in Materials and Methods. The values represent means ± SD of triplicate samples. The asterisk denotes a significant difference at a level of $P < 0.01$. B: The correlation between fluorescence intensity and the added concentration of Ox-PAPC is shown.

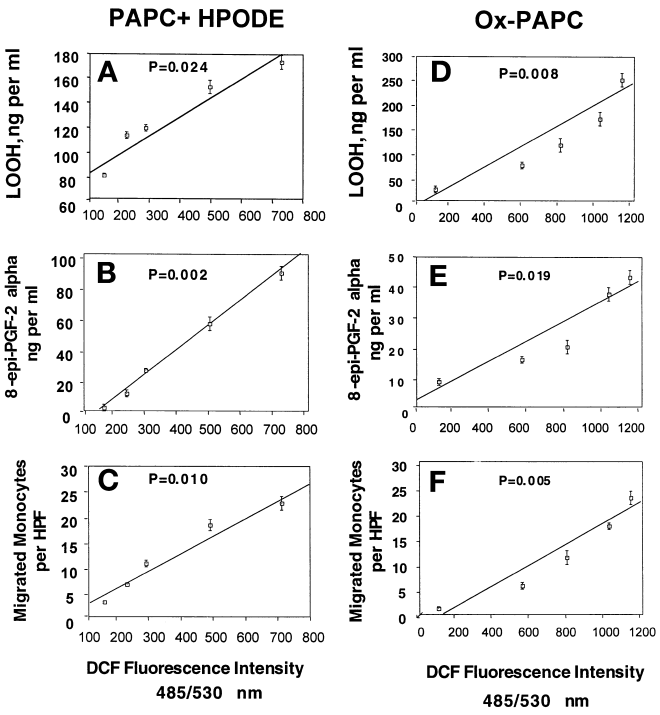

Fig. 2. Correlation of results obtained from different assays. PAPC or Ox-PAPC at 6.25, 12.5, 25.0, 50.0, or 100 µg/ml was added to human aortic wall cell cocultures or to test tubes without cells containing DCFH. The cocultures or test tubes that received PAPC also received HPODE (1 µg). The supernatants from the cocultures were removed and assayed for lipid hydroperoxides by the Auerbach assay (A and D). F_2-isoprostanes, another measure of lipid oxidation, were determined in the coculture supernatants by ELISA (B and E). Monocyte chemotactic activity was also determined in the supernatants from the cocultures (C and F). The values for each assay of the coculture supernatants were plotted on the y axis for each concentration of PAPC plus HPODE (A–C) or Ox-PAPC (D–F). The fluorescence intensity generated by the same concentration of PAPC plus HPODE or Ox-PAPC added to test tubes containing DCFH without cells was plotted on the x axis. Correlation coefficients greater than 0.9 were obtained for each comparison ($P < 0.024$ to $P < 0.019$). LOOH, Lipid hydroperoxide.

cell cocultures or to test tubes without cells containing DCFH. The cocultures or test tubes that received PAPC also received HPODE (1 µg). The supernatants from the cocultures were removed and assayed for lipid hydroperoxides by the Auerbach assay (**Fig. 2A** and **D**). F_2-isoprostanes, another measure of lipid oxidation, were determined in the coculture supernatants by ELISA (Fig. 2B and E). Monocyte chemotactic activity was also determined in the supernatants from the cocultures as described in Materials and Methods (Fig. 2C and F). The values for each assay of the coculture supernatants were plotted on the y axis for each concentration of PAPC plus HPODE (Fig. 2A–C) or Ox-PAPC (Fig. 2D–F). The fluorescence intensity generated by the same concentration of PAPC plus HPODE or Ox-PAPC added to test tubes containing DCFH without cells was plotted on the x axis. As shown in Fig. 2, there was a remarkable correlation between each measurement made of the coculture supernatant for each concentration of PAPC plus HPODE or Ox-PAPC added to the artery wall cells and the fluorescence intensity generated by the same concentration of PAPC plus HPODE or Ox-PAPC added to tubes without cells but containing DCFH ($P < 0.024$ to $P < 0.019$). Thus, the relative value of each assay on the supernatant from the artery wall cell cocultures in response to PAPC plus HPODE or Ox-PAPC was accurately predicted by the cell-free assay.

Effect of HDL and time

To investigate the inhibitory effect of HDL on the increase in DCF fluorescence due to Ox-PAPC or one of its major components, POVPC, HDL from healthy individuals was incubated with DCFH and Ox-PAPC (**Fig. 3A**) or DCFH and POVPC (Fig. 3B and C) and the fluorescence signal determined. The signal generated by Ox-PAPC in the presence of DCFH increased each hour from 1 to 4 h ($P < 0.01$) and the increase at each time point was completely inhibited by the presence of normal HDL ($P < 0.01$) (Fig. 3A).

Figure 3B shows a dose-dependent increase in fluorescence induced by POVPC. Figure 3C demonstrates a significant increase in fluorescence by 10 µg of POVPC over the value obtained by the auto-oxidation (air oxidation) of DCFH ($P < 0.01$) and normal HDL at concentrations of 6.25 to 25 µg/ml inhibited the POVPC-induced fluorescence to values below the signal generated by the auto-oxidation (air oxidation) of DCFH ($P < 0.01$).

Sensitivity of cell-free assay to HDL concentrations

Figure 4A demonstrates that as little as 2.5 µg/ml of normal HDL was able to significantly inhibit the signal generated by Ox-PAPC ($P < 0.01$). This was true, as also shown in Fig. 4A, whether HDL was isolated by agarose gel electrophoresis (open columns) or was separated from the apoB-containing lipoproteins by FPLC (hatched columns) or dextran sulfate precipitation (solid columns).

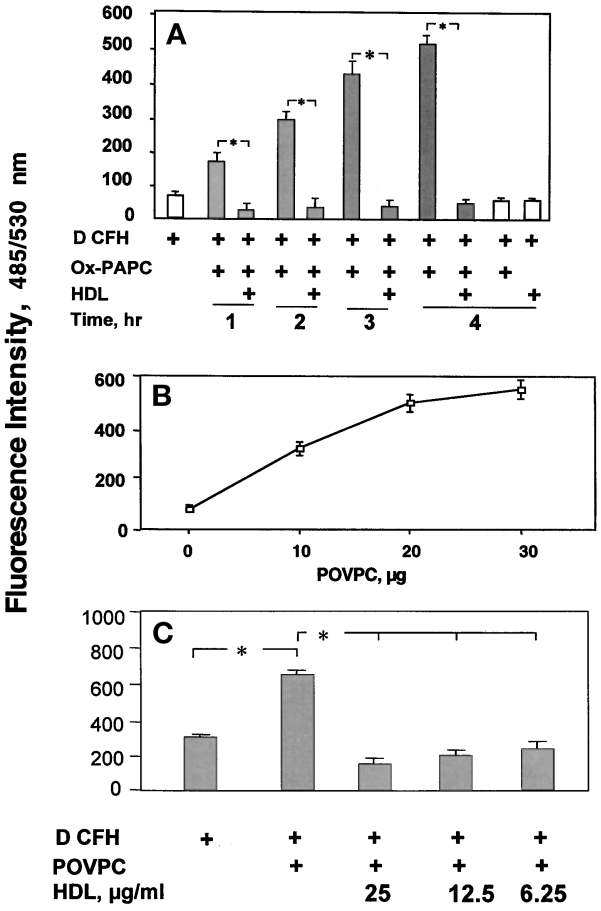

Fig. 3. Time-dependent increases in DCF fluorescence by Ox-PAPC, concentration-dependent increase in fluorescence by POVPC, and inhibition of Ox-PAPC- and POVPC-induced fluorescence by normal HDL. Ox-PAPC (25 μg) was added to tubes containing DCFH and dried under argon as described in Materials and Methods. HDL (prepared by FPLC), 25 μl (350 μg of cholesterol per ml) isolated from healthy individuals, was then added, and the volume was adjusted to 1.0 ml with saline and mixed by intermittent low speed vortexing for 1 min. Fluorescence intensity was determined at the indicated time points as described in Materials and Methods (A). POVPC was substituted for Ox-PAPC (in B and C) and was incubated with DCFH as described in Materials and Methods. In (C), saline or normal HDL (prepared by FPLC) was added together with POVPC at 10 μg per tube containing DCFH, incubated for 2 h, and fluorescence was determined as described in Materials and Methods. The values represent means ± SD of data from three separate experiments. Asterisks indicate a significant difference at the level of $P < 0.01$.

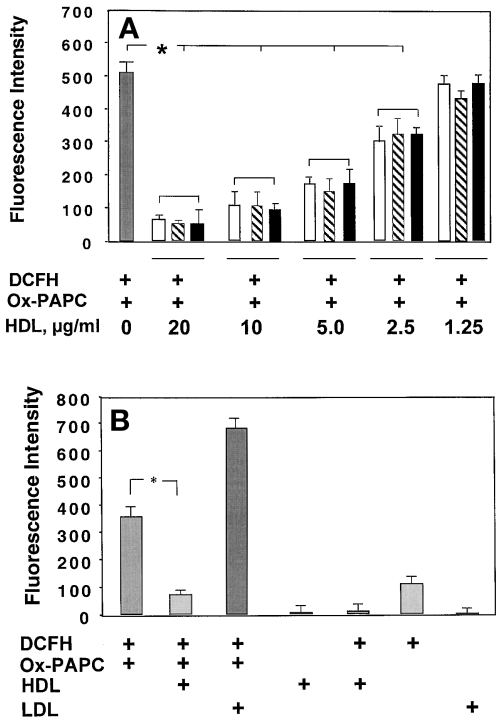

Fig. 4. Method of HDL preparation, HDL concentration, and effect of LDL. A: DCFH was placed in test tubes. Ox-PAPC (25 μg) was added and evaporated to form a thin film. Normal HDL isolated by electrophoresis (open columns), FPLC (hatched columns), or dextran sulfate precipitation (solid columns) was added at the indicated concentrations, and the volume was adjusted to 1.0 ml, mixed, and incubated at room temperature for 2 h. Fluorescence intensity was determined as described in Materials and Methods. The values represent means ± SD of data from thee separate experiments. The asterisk indicates a significant difference at a level of $P < 0.01$. B: Specificity of HDL. Aliquots of DCFH solution (20 μg each) were distributed in test tubes and evaporated as described in Materials and Methods. Ox-PAPC was added, mixed, and dried under a stream of argon. Normal human HDL (25 μg of cholesterol per ml) or LDL (25 μg of cholesterol per ml) was added, and the volume was adjusted to 1.0 ml with saline, mixed, and incubated at room temperature. Fluorescence intensity was determined 2 h later as described in Materials and Methods. The values represent means ± SD of data from three separate experiments. The asterisk indicates a significant difference at a level of $P < 0.01$.

The protective effect of HDL and HDL-containing fractions was virtually identical regardless of the method of HDL preparation (Fig. 4A).

Figure 4B demonstrates that the inhibition of the Ox-PAPC signal was specific to HDL and not observed with LDL. Indeed, as shown in Fig. 4B, LDL significantly increased the Ox-PAPC induced fluorescent signal ($P < 0.01$). Figure 4B also shows that neither HDL nor LDL by themselves generated a fluorescent signal. In other experiments the addition to LDL of linoleic acid treated with soy bean lipoxygenase dramatically increased the fluorescent signal generated by LDL in the presence of DCFH (data not shown).

Effects of antioxidants and apolipoproteins

We previously reported that vitamin E prevented coculture oxidation of LDL but failed to inhibit LDL that had already been oxidized by a first set of cocultures from inducing a second set of cocultures to produce monocyte chemotactic activity (11). Thus, antioxidants were shown to prevent the formation of the LDL-derived biologically active oxidized phospholipids, but once formed the antioxidants could not inactivate these oxidized phospholipids (11). The data in **Fig. 5** indicate that vitamin E and another potent antioxidant, lutein, both inhibited DCF fluorescence generated from PAPC plus HPODE ($P < 0.01$) (Fig. 5A). On the basis of our previous work (2) and the data presented in the present article, we conclude that PAPC plus HPODE generated less Ox-PAPC in the pres-

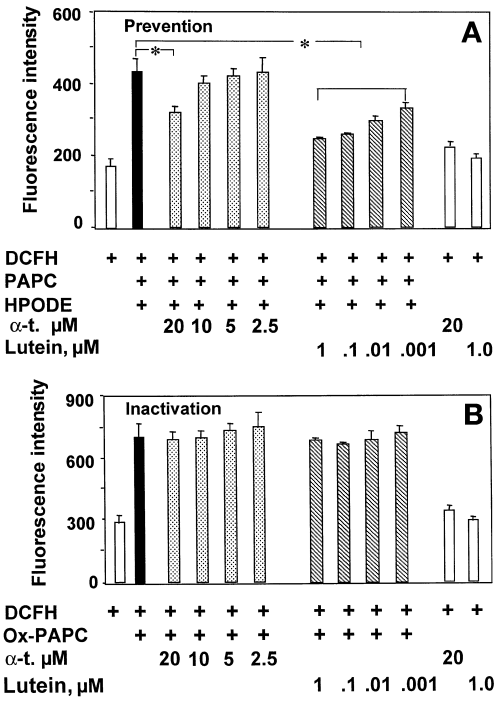

Fig. 5. Effect of antioxidants. A: Prevention of oxidation. DCFH (20 μg), HPODE (1.0 μg), and PAPC (25 μg) were added to tubes as described in Materials and Methods. α-Tocopherol (α-t.) or lutein was added at the indicated concentrations. The tubes were incubated at room temperature for 2 h and fluorescence intensity was determined as described in Materials and Methods. B: Inactivation of oxidized PAPC. Ox-PAPC (25 μg) was substituted for PAPC plus HPODE and treated as described for (A). The values represent means ± SD and the asterisks in (A) indicate $P < 0.01$.

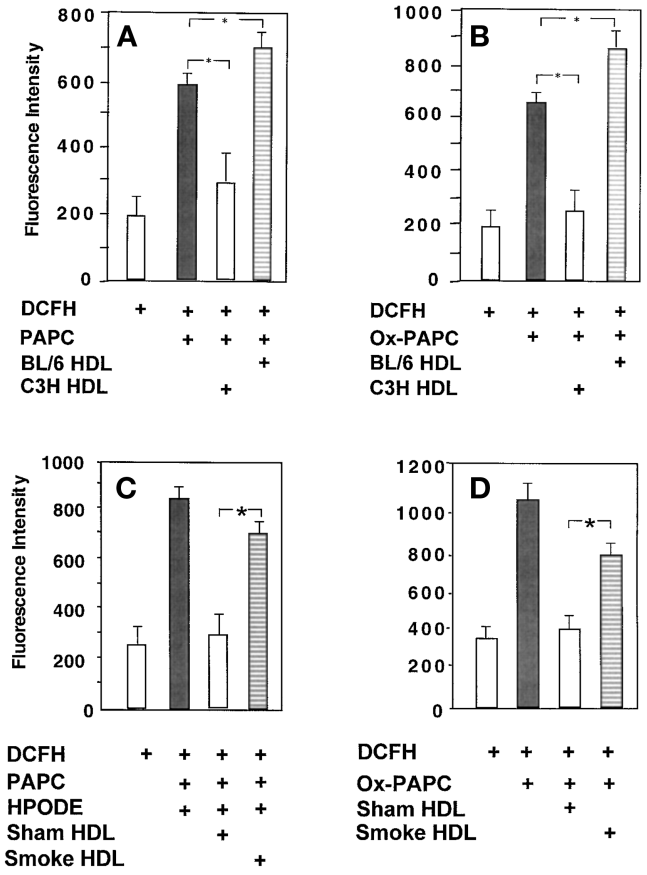

Fig. 6. Ability of the cell-free assay to detect subtle differences in HDL. A: HDL (prepared by FPLC) from C57BL/6J mice (BL/6 HDL) or C3H/HeJ mice (C3H HDL) fed an atherogenic diet for 3 days as described in Materials and Methods was added (25 μg of cholesterol per ml) to tubes containing DCFH and PAPC plus HPODE, mixed, and incubated for 2 h at room temperature as described in Materials and Methods. B: Ox-PAPC was substituted for PAPC plus HPODE and the ability of C3H HDL (prepared by FPLC) or BL/6 HDL (prepared by FPLC) to prevent the signal generated by Ox-PAPC was determined as described in Materials and Methods. C: PAPC at 25 μg/ml and (13*S*)-HPODE at 1.0 μg/ml were added to test tubes as described in Materials and Methods and incubated for 8 h at 37°C with gentle mixing. Some tubes received HDL (prepared by FPLC) (25 μg of cholesterol per ml) from the plasma of mice that were sham treated (Sham HDL) or mice that were exposed to second-hand cigarette smoke (Smoke HDL) as described in Materials and Methods. The tubes were incubated for an additional 4 h at 37°C. D: Ox-PAPC at 25 μg/ml together with HDL (prepared by FPLC) (25 μg of cholesterol per ml) from the sham- or smoke-treated animals was added to other tubes and incubated at 37°C for 4 h with gentle mixing. Aliquots of DCFH (20 μg) were placed in test tubes and dried as described in Materials and Methods. The mixtures of PAPC plus HPODE plus HDL (C) or Ox-PAPC plus HDL (D) were subsequently added to tubes containing DCFH. The tubes were incubated at room temperature for an additional 2 h and fluorescence intensity was determined as described in Materials and Methods. Means ± SD of triplicate samples are shown and the asterisks denote $P < 0.01$.

ence of vitamin E or lutein. However, analogous to the case for the coculture assay (11), both vitamin E and lutein failed to inhibit the signal generated by already formed Ox-PAPC (Fig. 5B).

We previously reported that coincubation of apoJ together with LDL and the artery wall cell cocultures prevented LDL oxidation (7) whereas coincubation of apoA-I did not (apoA-I was only effective in a preincubation) (2, 11). In the present studies we found that coincubation of apoJ with PAPC plus HPODE completely inhibited the oxidation of PAPC by HPODE and the subsequent increase in DCF fluorescence (data not shown). However, coincubation of apoA-I with PAPC plus HPODE did not prevent the oxidation of PAPC by HPODE and did not prevent the increase in DCF fluorescence (data not shown). Thus, the DCF assay accurately mirrored the coculture assay in regard to the effects of both antioxidants and apolipoproteins.

Ability of cell-free assay to detect subtle differences in HDL function

As shown in **Fig. 6**, similar to our previous observations with the artery wall cell cocultures (20), HDL from atherosclerosis-resistant mice (strain C3H/HeJ) being fed an atherogenic diet was capable of preventing the oxidation of PAPC by HPODE (Fig. 6A) as determined by the fluorescent signal. In contrast, HDL obtained from the atherosclerosis-susceptible strain (C57BL/6) fed the same atherogenic diet for only 3 days was not protective and even augmented the increase in DCF fluorescence intensity (Fig. 6A). Similarly, HDL from the C3H/HeJ mice, but not from the C57BL/6 mice (both having been fed

the atherogenic diet for 3 days), was capable of preventing the increase in DCF fluorescence by Ox-PAPC (Fig. 6B).

In other experiments we determined the effect of cigarette smoke on HDL function. As seen in Fig. 6C, HDL from sham-treated C57BL/6 mice fed a chow diet inhibited the signal generated by PAPC plus HPODE (Sham HDL; Fig. 6C) and also inhibited the signal generated by Ox-PAPC (Sham HDL; Fig. 6D). In contrast, HDL taken from the same mice after exposure to second-hand cigarette smoke was significantly less effective (Smoke HDL; Fig. 6C and D). [As noted above, the fluorescence observed for samples with DCFH alone resulted from DCFH auto-oxidation (air oxidation) during the course of the incubations].

Ability of cell-free assay to detect dysfunctional HDL isolated from humans with atherosclerosis

In the experiments shown in **Fig. 7A**, control LDL was presented to the human artery wall cell cocultures with HDL (at 2.18, 4.37, or 8.75 μg/ml) from 27 patients with documented coronary atherosclerosis with normal blood lipids, who were not diabetic or taking hypolipidemic medication, and who were not smokers (Patient HDL). The control LDL was also added to the cocultures together with HDL (at the same concentrations) from 31 age- and sex-matched normal controls (Normal HDL). In accordance with our previously published data (2, 6) the normal HDL markedly inhibited the generation of monocyte chemotactic activity at all concentrations tested ($P < 0.01$) whereas the patient HDL actually stimulated the production of monocyte chemotactic activity ($P < 0.01$) (Fig. 7A). In Fig. 7B, the control LDL was incubated in air-saline to generate mildly oxidized LDL either by itself (Control LDL) or together with HDL from the 27 patients described in Fig. 7A (Patient HDL) or with the HDL from their 31 age- and sex-matched normal controls (Normal HDL), and fluorescence was determined in the cell-free assay as described in Materials and Methods. At each HDL concentration, the normal HDL significantly decreased the fluorescent signal ($P < 0.01$) whereas the patient HDL significantly increased the fluorescent signal ($P < 0.01$). Thus, the results obtained with the cell-free assay were virtually identical to those obtained with the coculture assay (compare Fig. 7A and B).

Figure 8A demonstrates that HDL from 10 of 10 of the 27 patients shown in Fig. 7 failed to prevent the signal generated from PAPC plus HPODE and in fact enhanced the signal (Patient HDL; Fig. 8) whereas HDL from 8 of 8 age- and sex-matched normal controls (Normal HDL) significantly inhibited the signal ($P < 0.01$). Figure 8B demonstrates that HDL from 8 of 8 of the 27 patients shown in Fig. 7 failed to prevent the signal generated by Ox-PAPC whereas HDL from 8 of 8 normal controls did ($P < 0.01$). Figure 8B also shows that supplementation of patient HDL with α-tocopherol did not prevent the signal generated by Ox-PAPC but supplementation of patient HDL with purified paraoxonase did ($P < 0.01$), in accordance with our previous findings that HDL supplemented with purified paraoxonase reverted to normal (7, 10).

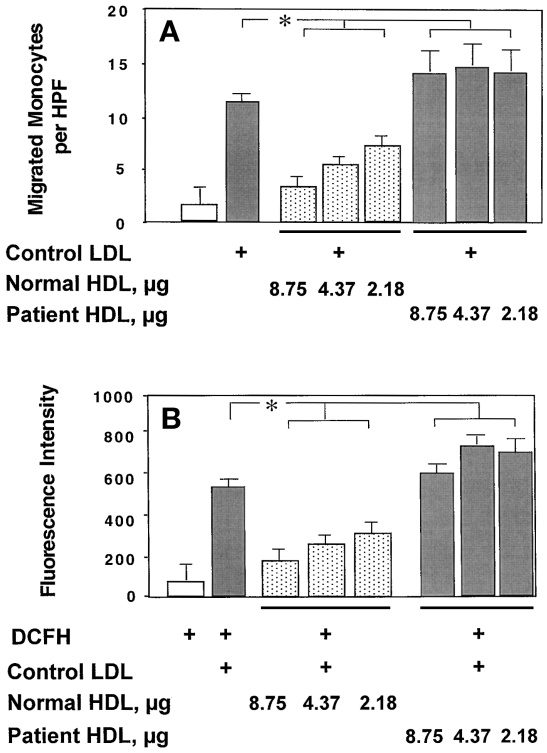

Fig. 7. Ability of the cell-free assay to detect dysfunctional HDL in humans, using LDL as the substrate. A: Tubes were prepared containing ultracentrifugally isolated control human LDL (25 μl, 250 μg of cholesterol per ml) alone, designated as Control LDL, or also containing HDL (at the indicated concentrations) obtained by FPLC from the plasma of 27 patients with documented coronary artery disease (Patient HDL) or HDL from 31 age- and sex-matched normals (Normal HDL). The mixtures were added to cultures of human artery wall cells and incubated for 8 h followed by washing of the cells and incubation in serumless medium for 18 h. Supernatants were then collected and assayed for monocyte chemotactic activity as described in Materials and Methods. HPF, High power field. B: The control LDL (25 μl, 250 μg/ml) was incubated in air/saline at room temperature for 2 h (to generate mildly oxidized LDL) either by itself (Control LDL) or together with HDL (at the concentration shown) obtained by dextran sulfate precipitation from the plasma of the 27 patients with documented coronary artery disease shown in (A) (Patient HDL) or HDL from the 31 age- and sex-matched normal subjects shown in (A) (Normal HDL). Fluorescence intensity was determined after 2 h of incubation at room temperature as described in Materials and Methods. The data represent means ± SD of values obtained from five separate experiments, each done in quadruplicate. Asterisks denote the presence of statistical significance at the level of $P < 0.01$.

DISCUSSION

HDL cholesterol levels are inversely related to risk for atherosclerotic events (3). This protective effect of HDL long has been assumed to be related to the ability of HDL to promote cholesterol efflux from cells (4). However, there have been reports that HDL contains components that can protect LDL against oxidation and it has been postulated that this ability of HDL to protect LDL against oxidation may be as important in its anti-inflammatory, antiatherogenic role as is reverse cholesterol efflux (1, 2, 6, 21, 22). One of the components of HDL that appears to

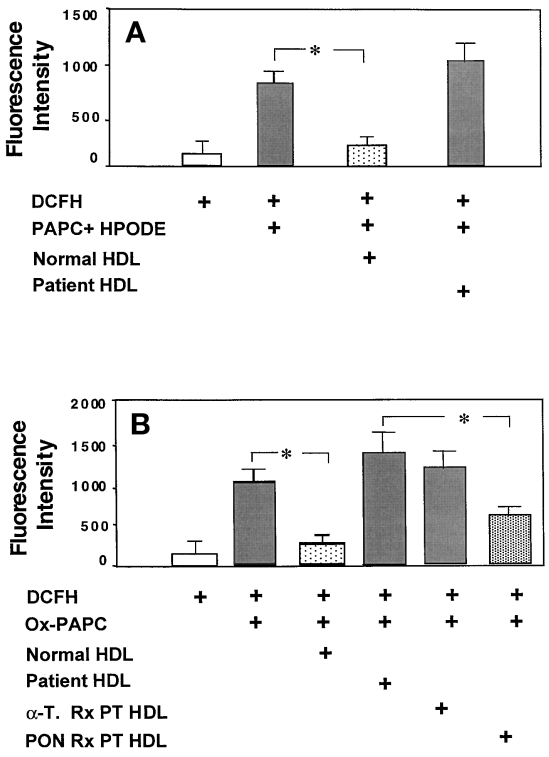

Fig. 8. Ability of the cell-free assay to detect dysfunctional HDL in humans, using PAPC plus HPODE or Ox-PAPC as the substrate. A: PAPC at 25 μg was incubated with 1.0 μg of (13*S*)-HPODE in polypropylene tubes with gentle mixing at 37°C (PAPC + HPODE). Some tubes received in addition the HDL-containing supernatant from a dextran sulfate precipitation (25 μl, 350 μg of cholesterol per ml) isolated from the plasma of 10 of the patients described in Fig. 7 (Patient HDL) or from the plasma of 8 age- and sex-matched controls (Normal HDL). The mixture was incubated at 37°C with gentle mixing for 2 h and subsequently added to tubes containing DCFH that was previously evaporated under argon as described in Materials and Methods. After brief vortexing the tubes were incubated at room temperature for 2 h and fluorescence intensity was determined as described in Materials and Methods. B: Ox-PAPC at 25 μg was incubated in polypropylene tubes alone (Ox-PAPC) or with the addition of the HDL-containing supernatant from a dextran sulfate precipitation (25 μl, 350 μg of cholesterol per ml) isolated from the plasma of eight of the patients described in Fig. 7 (Patient HDL) or from the plasma of eight age- and sex-matched controls (Normal HDL). The mixtures were incubated at 37°C with gentle mixing for 2 h and subsequently added to tubes containing DCFH that was previously evaporated under argon as described in Materials and Methods. After brief vortexing the tubes were incubated at room temperature for 2 h and fluorescence intensity was determined as described in Materials and Methods. In some experiments, the HDL-containing supernatant from a dextran sulfate precipitation of patient plasma (25 μl, 350 μg of cholesterol per ml) (referred to as Patient HDL) was incubated with 20 μM α-tocopherol (α-T. Rx PT HDL) or purified human paraoxonase 1 at 1×10^{-2} units (PON Rx PT HDL) for 2 h at 37°C with gentle mixing. The mixture was subsequently filtered through 100-kDa molecular mass cutoff membranes and the HDL-containing fraction was reisolated in the >100-kDa fraction and added to tubes containing Ox-PAPC (25 μg) and incubated at 37°C with gentle mixing for 2 h, and subsequently added to tubes containing DCFH that was previously evaporated under argon as described in Materials and Methods. After brief vortexing the tubes were incubated at room temperature for 2 h and fluorescence intensity was determined as described in Materials and Methods. The asterisks indicate significance at $P < 0.01$.

be involved in this function of HDL is apoA-I. ApoA-I has been shown to be capable of removing seeding molecules from LDL that are required for its oxidation by artery wall cells in coculture (1). However, apoA-I must be removed from the LDL after interaction as the seeding molecules transferred from LDL to apoA-I can be utilized by LDL and artery wall cells if they continue to be coincubated with apoA-I (2). In contrast, a second component of HDL, apoJ, is protective even in a coincubation, suggesting that it sequesters the seeding molecules in a manner that does not allow oxidation to proceed (6). The other HDL components are enzymes that can act on oxidized phospholipids and include paraoxonase (21–26), platelet-activating factor acetylhydrolase (PAF-AH) (26, 27), and LCAT (28–32). A fourth HDL-associated enzyme that reduces organic hydroperoxides and is inhibited by physiologic concentrations of homocysteine is plasma GSH selenoperoxidase (33). Thus, HDL contains at least two apolipoproteins and four enzymes that may alter the oxidation of LDL. Previous cell-free studies of the function of HDL have demonstrated the ability of HDL to inhibit metal ion-dependent oxidation of LDL as measured by the generation of nonspecific oxidation markers (34). In a cell-based assay, Gowri and colleagues (35) found that HDL_2 from poorly controlled type II diabetic subjects was less effective in protecting LDL from oxidation by macrophages. Studies from this laboratory using human artery wall cell cocultures have demonstrated a consistent ability to show differences in the function of HDL. HDL from mice susceptible to diet-induced atherosclerosis and fed an atherogenic diet was shown to be dysfunctional and proinflammatory compared with HDL from the same mice fed a low-fat chow diet, or compared with HDL from mice genetically resistant to diet-induced atherosclerosis and fed either the chow or atherogenic diet (6). HDL from mice overexpressing murine apoA-II was shown to be dysfunctional, using the human artery wall cell coculture assay (7). Similarly, using the human artery wall coculture assay, HDL from mice transgenic for $sPLA_2$ was shown to be dysfunctional (8), as was HDL from paraoxonase knockout mice (9). HDL from humans was found to be proinflammatory 3 days after surgery, at the peak of the acute-phase response, and returned to normal 7 days after surgery as assessed with the coculture assay (10). Rabbits injected with croton oil to induce an acute-phase reaction also developed dysfunctional HDL as judged by their proinflammatory characteristics in the coculture assay (10). Unlike HDL from normal controls that was anti-inflammatory, HDL from patients with documented coronary atherosclerosis, who had normal levels of blood lipids and who were neither diabetic nor taking hypolipidemic medications, was dysfunctional in cell-based assays (2). To date, no cell-free assay has been reported to measure the ability of HDL to prevent the formation or inactivate already formed biologically active oxidized phospholipids without regard to a specific component of HDL (e.g., paraoxonase, PAF-AH, LCAT). Our previous data (2) strongly suggest that more than one component of HDL will be important in distinguishing normal from patient

HDL. Paraoxonase activity was on average significantly lower in the patient population (2) despite essentially identical HDL cholesterol levels, but there was considerable overlap as would be expected if more than one HDL component might be involved. Our previous studies (2) suggested that the cell-based assays that measured the function of whole HDL were much more discriminating than paraoxonase levels alone. However, the use of a cell-based assay is not practical for large-scale studies. The data presented here indicate that the cell-free assay described here gave results that were virtually identical to those obtained with the human artery wall cell-based assay.

As shown in Results, we have tested virtually every finding that has previously been reported with our cell-based artery wall coculture system in an effort to find discrepancies between the two assays. There was a striking concordance between the production of lipid hydroperoxides and monocyte chemotactic activity by human artery wall cells exposed to LDL or the biologically active LDL-derived oxidized phospholipids and the fluorescent signal generated by these oxidized phospholipids in the new cell-free assay (Fig. 2).

As noted above, we previously reported that normal HDL inhibited LDL-derived lipid hydroperoxide formation, LDL-induced monocyte adherence, and LDL-induced monocyte chemotactic activity by human artery wall cells (1, 2). We demonstrate here that normal HDL similarly inhibited the fluorescent signal generated by these oxidized phospholipids in the new cell-free assay whereas patient HDL did not (Figs. 3, 4, 7, and 8). The patients studied here were chosen because they had definite coronary atherosclerosis despite normal blood lipids (including normal HDL cholesterol levels) and because they were not diabetic, were not taking hypolipidemic medications, and were not smokers. Thus, they would not have been predicted to be at risk for atherosclerosis by conventional risk factor analysis. We believe that the results from these patients will be found to be applicable to most patients with atherosclerosis, but we cannot exclude the possibility that these patients represent a unique subset of patients.

Also as noted above, we previously reported (6) that when coincubated with artery wall cells, apoJ inhibited the production of LDL-derived oxidized phospholipids whereas apoA-I was not effective in a coincubation but was effective only in a preincubation. We report here that apoJ (but not apoA-I) inhibited the generation of a fluorescent signal when PAPC plus HPODE was coincubated with apoJ in the new cell-free assay.

Although antioxidants prevented the formation of biologically active LDL-derived oxidized phospholipids in human artery wall cell cocultures (11), once formed the biologic activity of the LDL-derived oxidized phospholipids could not be abolished by antioxidants (11). However, we also reported that purified paraoxonase could inactivate the LDL-derived oxidized phospholipids after they were formed and dysfunctional HDL supplemented with purified paraoxonase was restored to normal activity (7, 10). We report here that antioxidants prevented the fluorescent signal generated by PAPC plus HPODE in the new cell-free assay but did not prevent the fluorescent signal induced by Ox-PAPC (Fig. 5). We also report here that patient HDL supplemented with purified paraoxonase was restored to normal as judged by its ability to inhibit the fluorescent signal induced by Ox-PAPC (Fig. 8B).

Thus, we have thus far not succeeded in finding a discrepancy between the results obtained with our previously described cell-based assay and the new cell-free assay.

Indeed, the new cell-free assay was sensitive enough to detect changes in HDL from atherosclerosis-sensitive mice fed an atherogenic diet for only 3 days or from the same strain of mice exposed to second-hand cigarette smoke for a period of hours.

The data in Figs. 4, 7B, and 8 demonstrate that the HDL-containing supernatant from a dextran sulfate precipitation (currently performed in many clinical laboratories) was suitable for the cell-free assay described here. Therefore, we believe that the cell-free assay described in this article has the potential to allow widespread testing of the hypothesis that HDL that is dysfunctional in preventing the formation or inactivating oxidized phospholipids may play an important role in the development of atherosclerosis.

We thank Drs. Lawrence Yeatman, Jesse Currier, Jonathan Tobis, Kristin Kopelson, and Cathy McCloy for valuable assistance with patient studies, and Dr. Brian Van Lenten for valuable suggestions. We are grateful to Rachel Mottahedeh, Richard Jin, and Alan Wagner for excellent technical assistance and to Dr. Alan Garfinkel for advice on statistical analyses. This work was supported by USPHS grants HL 30568 and HL 34343, by a TRDRP grant from the State of California, and by the Laubisch, Castera, and M. K. Gray funds at UCLA.

Manuscript received 30 November 2000, in revised form 13 February 2001, and in re-revised form 6 April 2001.

REFERENCES

1. Navab, M., S. Hama, 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 HDL inhibits three steps in the formation of mildly oxidized LDL—step 1. *J. Lipid Res.* **41:** 1481–1494.
2. 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. 2000. Normal HDL inhibits three steps in the formation of mildly oxidized LDL—steps 2 and 3. *J. Lipid Res.* **41:** 1495–14508.
3. Miller, G. J., and N. E. Miller. 1975. Plasma-high-density-lipoprotein concentration and development of ischaemic heart disease. *Lancet.* **1:** 16–19.
4. Oram, J. F., and S. Yokoyama. 1996. Apolipoprotein-mediated removal of cellular cholesterol and phospholipids. *J. Lipid Res.* **37:** 2473–2491.
5. Kwiterovich, P. O., Jr. 1998. The antiatherogenic role of high-density lipoprotein cholesterol. *Am. J. Cardiol.* **82:** 13Q–21Q.
6. Navab, M., S. Y. Hama-Levy, B. J. Van Lenten, G. C. Fonarow, C. J. Cardinez, G. P. Hough, L. W. Castellani, M-L. Brennan, B. N. La Du, A. J. Lusis, and A. M. Fogelman. 1997. Mildly oxidized LDL induces an increased apolipoprotein J/paraoxonase ratio. *J. Clin. Invest.* **99:** 2005–2019.
7. Castellani, L., M. Navab, S. Y. Hama, C. C. Hedrick, and A. J. Lusis. 1997. Overexpression of apolipoprotein AII in mice converts high density lipoprotein to a pro-inflammatory particle. *J. Clin. Invest.* **100:** 464–474.

8. Leitinger, N., A. D. Watson, S. Y. Hama, B. Ivandic, J. H. Qiao, J. Huber, K. F. Faull, D. S. Grass, M. Navab, A. M. Fogelman, F. C. de Beer, A. J. Lusis, and J. A. Berliner. 1999. Role of group II secretory phospholipase A2 in atherosclerosis. 2. Potential involvement of biologically active oxidized phospholipids. *Arterioscler. Thromb. Vasc. Biol.* **19:** 1291–1298.
9. Shih, D., X. P. Wang, Y. S. Hama, M. Navab, A. M. Fogelman, and A. J. Lusis. 1998. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature.* **394:** 284–287.
10. Van Lenten, B. J., S. Y. Hama, F. C. de Beer, D. M. Stafforini, T. M. McIntyre, S. M. Prescott, B. N. La Du, A. M. Fogelman, and M. Navab. 1995. Anti-inflammatory HDL becomes pro-inflammatory during the acute phase response. Loss of protective effect of HDL against LDL oxidation in aortic wall cell cocultures. *J. Clin. Invest.* **96:** 2758–2767.
11. Navab, M., S. Imes, S. Y. Hama, G. P. Hough, L. A. Ross, R. W. Bork, A. J. Valente, J. A. Berliner, D. C. Drinkwater, H. 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.
12. Fogelman, A. M., K. Sykes, B. J. Van Lenten, M. C. Territo, and J. A. Berliner. 1988. Modification of the Recalde method for the isolation of human monocytes. *J. Lipid Res.* **29:** 1243–1247.
13. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins of human serum. *J. Clin. Invest.* **43:** 1345–1343.
14. Nobel, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9:** 693–700.
15. Nene, S., H. Gelabert, W. Moore, W. Quinones-Baldrich, A. Santibanez-Gallerani, and L. Ignarro. 1997. Cigarette smoking increases endothelial-derived vasorelaxation in the rat carotid artery in a dose-dependent manner. *J. Surg. Res.* **71:** 101–106.
16. Rosenkranz, A. R., S. Schmaldienst, K. M. Stuhlmeier, W. Chen, W. Knapp, and J. Zlabinger. 1992. A microplate assay for the detection of oxidative products using 2′,7′-dichlorofluorescein-diacetate. *J. Immunol. Methods.* **156:** 39–45.
17. Lorenzen, A., and S. W. Kennedy. 1993. A fluorescent based protein assay for use with a microplate reader. *Anal. Biochem.* **214:** 346–348.
18. 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.
19. 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.
20. Shih, D. M., L. H. Gu, Y. S. Hama, M. 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.
21. Watson, A. D., M. Navab, S. Y. Hama, A. Sevanian, S. M. Prescott, D. M. Stafforini, T. M. 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.
22. Mackness, M. I., S. Arrol, and P. N. Durrington. 1991. Paraoxonase prevents accumulation of lipoperoxides in low density lipoprotein. *FEBS Lett.* **286:** 152–154.
23. Aviram, M., M. Rosenblat, C. L. Bisgaier, R. S. Newton, S. L. Primo-Parmo, and B. N. La Du. 1998. Paraoxonase inhibits high-density lipoprotein oxidation and preserves functions. A possible peroxidative role for paraoxonase. *J. Clin. Invest.* **101:** 1581–1590.
24. Aviram, M., E. Hardak, J. Vaya, S. Mahmood, S. Milo, A. Hoffman, S. Billicke, D. Draganov, and M. Rosenblat. 2000. Human serum paraoxonases (PON1) Q and R selectively decrease lipid peroxides in human coronary and carotid atheroslcerotic lesions. PON1 esterase and peroxidase-like activities. *Circulation.* **101:** 2510–2517.
25. Shi, D. M., Y-R. Xia, X-P. Wang, E. Miller, L. W. Castellani, G. Subbanagounder, H. Cheroutree, K. F. Faull, J. A. Berliner, J. L. Witztum, and A. J. Lusis. 2000. Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis. *J. Biol. Chem.* **275:** 17527–17535.
26. Stafforini, D. M., G. A. Zimmerman, T. M. McIntyre, and S. M. Prescott. 1993. The platelet activating factor acetylhydrolase from human plasma prevents oxidative modification of low density lipoprotein. *Trans. Assoc. Am. Physicians.* **105:** 44–63.
27. Watson, A. D., J. A. Berliner, S. Y. Hama, B. N. La Du, K. F. Faull, A. M. Fogelman, and M. Navab. 1995. Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. *J. Clin. Invest.* **96:** 2882–2891.
28. Goyal, J., K. Wang, M. Liu, and P. V. Subbaiah. 1997. Novel function of lecithin-cholesterol acyltransferase. Hydrolysis of oxidized polar phospholipids generated during lipoprotein oxidation. *J. Biol. Chem.* **272:** 16231–16239.
29. Liu, M., R. W. St Clair, and P. V. Subbaiah. 1998. Impaired function of lecithin:cholesterol acyltransferase in atherosclerosis-susceptible White Carneau pigeons: possible effects on metabolism of oxidized phospholipids. *J. Lipid Res.* **39:** 245–254.
30. Subramanian, V. S., J. Goyal, M. Miwa, J. Sugatami, M. Akiyama, M. Liu, and P. V. Subbaiah. 1999. Role of lecithin-cholesterol acyltransferase in the metabolism of oxidized phospholipids in plasma: studies with platelet-activating factor acetylhydrolase-deficient plasma. *Biochim. Biophys. Acta.* **1439:** 95–109.
31. Vohl, M. C., T. A. Neville, R. Kumarathasan, S. Braschi, and D. L. Sparks. 1999. A novel lecithin acyltransferase antioxidant activity prevents the formation of oxidized lipids during lipoprotein oxidation. *Biochemistry.* **38:** 5976–81.
32. Itabe, H., R. Hosoya, K. Karasawa, S. Jimi, K. Saku, S. Takebayashi, T. Imanaka, and T. Takano. 1999. Metabolism of oxidized phosphatidylcholines formed in oxidized low density lipoprotein by lecithin-cholesterol acyltransferase. *J. Biochem.* **126:** 153–161.
33. Chen, N., Y. Liu, C. D. Greiner, and J. L. Holtzman. 2000. Physiologic concentrations of homocysteine inhibit the human plasma GSH peroxidase that reduces organic hydroperoxides. *J. Lab. Clin. Med.* **136:** 58–65.
34. Parthasarathy, S., J. Barnett, and L. Fong. 1990. High-density lipoprotein inhibits the oxidative modification of low-density lipoprotein. *Biochim. Biophys. Acta.* **1044:** 275–283.
35. Gowri, M. S., D. R. Van der Westhuyzen, S. R. Bridges, and J. W. Anderson. 1999. Decreased protection by HDL from poorly controlled type 2 diabetic subjects against LDL oxidation may be due to the abnormal composition of HDL. *Arterioscler. Thromb. Vasc. Biol.* **19:** 2226–2233.