ASBMB
JOURNAL OF LIPID RESEARCH

Studies on oxidized low density lipoproteins. Controlled oxidation and a prostaglandin artifact

Hanfang Zhang,* W. Bruce Davis, Xunsheng Chen,* Ronald L. Whisler,** and David G. Cornwell[1,]***

Departments of Physiological Chemistry* and Internal Medicine,** The Ohio State University, Columbus, OH 43210

Abstract Low density lipoproteins (LDL), isolated by ultracentrifugal flotation, were oxidized (LDL_{OXID}) slowly during dialysis against 0.15 M NaCl and subsequent incubation in 96% air-4% CO_2 at 37°C. Butylated hydroxytoluene prevented LDL oxidation. LDL preparations from different sera were oxidized at different rates and the degree of lipid peroxidation was controlled by varying the incubation time. Mild oxidation did not alter the electrophoretic mobility of the LDL_{OXID} preparation. LDL_{OXID} contained lipid peroxides in neutral lipids, had increased amounts of lysophosphatidylcholine, and contained a number of complex oxidation products that were generated from the oxidation of free fatty acids. These oxidation products included large amounts of soluble material that cross-reacted with antibodies to PGE_2 but not 6-keto-$PGF_{1\alpha}$. The amount of cross-reacting material was proportional to the degree of lipid peroxidation. Cross-reacting material in LDL_{OXID} preparations was evidently formed from the oxidation of free fatty acids released from LDL, since cross-reacting material was also formed when a synthetic fat emulsion was oxidized in the presence of free arachidonic acid.—**Zhang, H., W. B. Davis, X. Chen, R. L. Whisler, and D. G. Cornwell.** Studies on oxidized low density lipoproteins. Controlled oxidation and a prostaglandin artifact. *J. Lipid Res.* 1989. **30:** 141-148.

Supplementary key words lipid peroxides • PGE_2 • 6-keto-$PGF_{1\alpha}$ • arachidonic acid • Intralipid® • radioimmunoassay

The potential role of lipid peroxidation in the pathogenesis of atherosclerosis continues to be an area of considerable interest and controversy (1-4). The susceptibility of isolated plasma low density lipoproteins (LDL) to lipid peroxidation was recognized over 30 years ago (5, 6) and recent studies (7-10) have identified lipid peroxides as malondialdehyde (MDA) in serum (3 to 7 nmol MDA/ml), and LDL (3 to 15 nmol MDA/mg LDL cholesterol). A number of studies describe specific effects of oxidized low density lipoproteins (LDL_{OXID}) that may be related to atherosclerosis. LDL_{OXID} are taken up by the scavenger receptor of the monocyte/macrophage and degraded by these cells more rapidly than native LDL (11-21).

We have recently begun a series of studies (22) with LDL_{OXID} that were prepared by controlled mild oxidation. The LDL_{OXID} preparations were characterized by differences in lipid peroxide content that were measured as thiobarbituric acid reactants (TBAR). LDL_{OXID} apparently contained lipid oxidation products with cross-reactivity in the radioimmunoassay (RIA) of prostanoids. Studies on the formation and properties of LDL_{OXID} and an oxidized synthetic fat emulsion Intralipid® (Il) are presented in this report.

MATERIALS AND METHODS

Materials

Arachidonic acid (AA) was purchased from Nu-Chek-Prep (Elysian, MN), purified by elution from a Unisil® column with hexane-ether 9:1 (v/v), and used only when thin-layer chromatography (TLC) showed that lipid peroxides were absent (23). Other reagents were obtained from the following sources: [5,6,8,11,12,14,15-^{3}H(N)]PGE_2 (200.0 Ci/mmol), 6-[5,8,9,11,12,13,15-^{3}H(N)]-keto-$PGF_{1\alpha}$ (163.5 Ci/mmol), and [U-^{14}C]AA (1.0 Ci/mmol) (New England Nuclear, Boston, MA); phosphatidylinositol (PI) and phosphatidylserine (PS) (Avanti Polar-Lipids, Inc., Birmingham, AL); phosphatidic acid (PA), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) (Sigma Chemical Co., St. Louis, MO); sphingomyelin (Sph) (Serdary, London,

Abbreviations: AA, arachidonic acid; BHT, butylated hydroxytoluene; C, cholesterol; CE, cholesteryl ester; FFA, free fatty acid; HPLC, high performance liquid chromatography; Il, Intralipid®; Il_{OXID}, oxidized Il; LDL, low density lipoproteins; LDL_{BHT}, unoxidized LDL; LDL_{OXID}, oxidized LDL; LPC, lysophosphatidylcholine; MDA, malondialdehyde; NL, neutral lipid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; RIA, radioimmunoassay; Sph, sphingomyelin; TBAR, thiobarbituric acid reactants; TG, triglycerides; TLC, thin-layer chromatography; TNS, 6-*p*-toluidino-2-naphthalenesulfonic acid; IM, indomethacin.

[1]To whom correspondence should be addressed.

Downloaded from www.jlr.org by guest, on June 19, 2012

Ontario, Canada); trilinolenin (Nu-Chek-Prep); butylated hydroxytoluene (BHT), and 1,1,3,3-tetramethoxypropane (Aldrich, Milwaukee, WI); Il (KabiVitrum, Alameda, CA). Tissue culture media was previously described (22–24).

LDL preparations

Individual units of freshly drawn human plasma were obtained from the Red Cross and converted to serum by the addition of $CaCl_2$. LDL (d 1.019–1.063 g/ml) were isolated by ultracentrifugation (6). A portion of the LDL preparation was dialyzed at 4°C for 16 hr against 400 vol of 0.15 M NaCl and then sterilized by passage through a 0.45-μm Millipore filter. Total cholesterol was measured by an established procedure (25); LDL concentrations are reported as μg cholesterol throughout this study. LDL_{OXID} were prepared by incubating sterilized LDL (ca. 4,000 μg cholesterol/ml) at 37°C in 96% air–4% CO_2 for various time intervals. The oxidation reaction was monitored by the disappearance of the yellow carotenoid color (6) and the appearance of TBAR. In some experiments, 10 μM indomethacin (IM) was added before oxidation. LDL_{BHT} were prepared by the immediate addition of 100 μM BHT to a second portion of the fresh LDL preparation before dialysis. This mixture was dialyzed for 16 hr against 400 vol of 0.15 M NaCl saturated with BHT and then sterilized by filtration. LDL_{BHT} were incubated at 37°C for the same number of hours as LDL_{OXID}. LDL_{OXID} and LDL_{BHT} were further characterized by ultracentrifugal flotation (6) and electrophoresis in agarose gel (26).

Oxidized Intralipid (Il_{OXID})

Intralipid diluted in saline, unlike LDL, was not oxidized during incubation at 37°C in 96% air–4% CO_2. However, large amounts of lipid peroxides (TBAR) were formed when 10 μM Fe^{3+} was added to the incubation system. Il_{OXID} containing different amounts of lipid peroxides were obtained by varying the incubation times.

Separation of lipid classes

Lipids were extracted from LDL_{BHT} and LDL_{OXID} with chloroform–methanol 2:1 (v/v) or LDL_{BHT} and LDL_{OXID} were applied directly to a TLC plate. Phospholipid classes were separated by TLC on Whatman LK5D plates. The developing solvent consisted of chloroform–methanol–40% methylamine 60:20:5 (v/v) (27). Phospholipid standards PI, LPC, Sph, PC, and PE were well separated. PS was not separated from PA. Neutral lipids moved near the solvent front and free AA separated as a distinct spot (R_f 0.75) in this system.

Neutral lipid (NL) classes were separated by TLC on Whatman LK6D plates. The developing solvent consisted of hexane–ether–acetic acid 80:20:1 (v/v). Cholesteryl esters (CE), triglycerides (TG), cholesterol (C), and free AA were well separated. Phospholipids remained at the origin in this system.

Lipid classes were visualized with iodine vapor or visualized under UV light after spraying with 1 mM 6-*p*-toluidino-2-naphthalenesulfonic acid (TNS) in buffer solution (28). Lipid peroxides were visualized by a starch–iodide spray (23).

High performance liquid chromatography (HPLC) was used as previously described (24) to separate labeled AA derivatives formed by oxidation. We used a Beckman model 334 chromatograph which consisted of dual Beckman Model 110A pumps (A and B), Altex 210A injector with a 20-μl loop, Beckman controller, Beckman 164 variable wavelength detector, and an Altex Model C-RIA integrator. HPLC was performed at room temperature on a 250 × 4.6 mm Ultrasphere-ODS (5 μm) reversed-phase column preceded by 35 × 4.6 mm guard column packed with ODS.

The elution solvents for HPLC consisted of various mixtures of acetonitrile–isopropanol–aqueous phosphoric acid (pH 2). Solvent compositions were: solvent A, HPLC grade water adjusted to pH 2 by phosphoric acid; solvent B_1, acetonitrile; solvent B_2, 3% isopropanol in acetonitrile. The flow rate of elution solvents was 1.0 ml/min. The elution steps were as follows: 0 to 14 min, 36.7% solvent B_1; 14 to 34 min, 60% solvent B_1; 34 to 41 min, 60% solvent B_2; 41 min to end, 90% solvent B_2. Fractions were collected at 0.5- or 1-ml intervals and the radioactivity in each fraction was counted.

Lipid peroxidation

Lipid peroxides were estimated as TBAR (24, 29). Preparations were diluted to a volume of 0.4 ml. Two ml of 0.25 N HCl containing 0.375% TBA and 15% trichloroacetic acid were added together with 10 μl of 5 mM Fe^{3+} as a catalyst to decompose lipid peroxides (29). The mixture was incubated at 97°C for 20 min, cooled, and centrifuged. The absorbance of the clear supernatant was measured at 532 nm. In some experiments, absorbance was converted to nmol (MDA) from a standard curve generated with 1,1,3,3-tetramethoxy-propane.

Prostanoids

PGE_2 and 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, were identified by HPLC and estimated by a standard RIA procedure (23, 24). Antibodies were kindly supplied by Dr. Lawrence Levine (Brandeis University, Waltham, MA). The cross-reactivity of the PGE antibody was: 6-keto-$PGF_{1\alpha}$, 0.4%; $PGF_{1\alpha}$, 0.76%; PGF_2, 0.31%; PGD_2, 0.051%; AA, 0.00045%. The cross-reactivity of the 6-keto-$PGF_{1\alpha}$ antibody was: PGE_2, 0.15%; PGD_2, 0.02%; $PGF_{2\alpha}$, 0.10%; AA, 0.005%.

Downloaded from www.jlr.org by guest, on June 19, 2012

RESULTS

Preparation and lipid peroxide content of LDL

Preliminary studies from our laboratory (22) showed that LDL were oxidized slowly at 4°C and that the rate of oxidation increased when preparations were incubated at 37°C in 96% air-4% CO_2. Oxidation was accompanied by the decrease and ultimate disappearance of the yellow carotenoid color that is characteristic of freshly prepared LDL (6). IM did not have any effect on LDL oxidation. Relative lipid peroxide levels were estimated by a sensitive TBA assay that used a Fe^{3+} catalyst to decompose lipid peroxides (29) and lipid peroxide levels were found to increase with the length of the incubation time.

Peroxide levels (nmol MDA/200 μg cholesterol) were 0.7 ± 0.1 for LDL dialyzed against 0.15 M NaCl and 1.9 ± 0.1 when the dialyzed LDL were incubated until the yellow carotenoid color disappeared. Higher peroxide levels were attained by continuing the incubation for different time intervals after the disappearance of carotenoid color. LDL with peroxide levels equal to or greater than 0.7 are designated LDL_{OXID} in the present investigation.

With LDL_{BHT}, incubation did not destroy the carotenoid color and peroxide levels only reached 0.2 ± 0.03/200 μg cholesterol after incubation times exceeding 24 hr. We found previously that BHT prevented both lipid peroxidation and some decomposition of lipid peroxides to TBAR (24, 29). However, TBAR in LDL_{OXID} were only reduced 30% when 100 μM BHT was added at the end of the incubation period (data not shown). Thus, very low TBAR values in LDL_{BHT} showed that BHT prevented lipid peroxidation when it was added early in the isolation of LDL.

When LDL_{OXID} (2.2 nmol MDA/200 μg cholesterol) solutions were adjusted to a density of 1.10 g/ml and recentrifuged, all of the cholesterol was recovered in the top (LDL) fraction. Electrophoretic mobilities did not differ for LDL_{BHT} and LDL_{OXID} with 0.7 nmol MDA/200 μg cholesterol. The relative electrophoretic mobility of LDL_{OXID}, measured against LDL_{BHT}, increased to 1.1 when the peroxide level reached 1.9 nmol MDA/200 μg cholesterol.

LDL were always isolated from individual sera and these LDL preparations underwent oxidation at different rates. Data for the oxidation of three different sera are reported in **Fig. 1.** The carotenoid color disappeared more rapidly from LDL-C than either LDL-A or LDL-B during the incubation process. Although LDL were oxidized at different rates, it was possible to obtain LDL preparations with the same lipid peroxide content by incubating until the disappearance of carotenoid color and then varying the subsequent incubation time.

Lipid classes in LDL_{BHT} and LDL_{OXID}

Lipids were extracted from LDL_{BHT} (0.05 nmol MDA/200 μg cholesterol) and LDL_{OXID} (1.5 nmol MDA/200 μg cholesterol) and neutral lipid classes were separated by TLC. Both LDL_{BHT} and LDL_{OXID} contained CE (R_f 0.90), TG (R_f 0.56) and C (R_f 0.19) (**Fig. 2**). LDL_{OXID} appeared to contain less TG than LDL_{BHT}. FFA (R_f 0.20) were not found in either preparation. Lipid extracts from LDL_{BHT} did not stain for lipid peroxides.

Lipid extracts from LDL_{OXID} contained four new spots (R_f 0.42, 0.40, 0.37, and 0.26) (Fig. 2) and these spots stained for lipid peroxides. Autoxidized cholesteryl arachidonate contained lipid peroxides (R_f 0.43, 0.37, and 0.32) and autoxidized trilinolenin contained a major lipid peroxide (R_f 0.26) (data not shown). These lipid peroxides with R_f values similar to the lipid peroxides identified in LDL_{OXID} suggested that LDL_{OXID} contained oxidized CE and TG.

Phospholipid classes were also separated by TLC. Both LDL_{BHT} and LDL_{OXID} contained PC (R_f 0.60), Sph (R_f 0.45), and LPC (R_f 0.32) but the relative size of the LPC spot from LDL_{OXID} was much larger than the LPC spot from LDL_{BHT} (Fig. 2). A FFA standard (R_f 0.75) was distinguishable from other neutral lipids including autoxidized cholesteryl arachidonate and autoxidized trilinolenin (R_f 0.96) by the phospholipid solvent system. A NL spot was present but a FFA spot was absent from the TLC plates obtained with lipids from LDL_{BHT} and LDL_{OXID}. Phospholipid classes did not stain for lipid peroxides, but

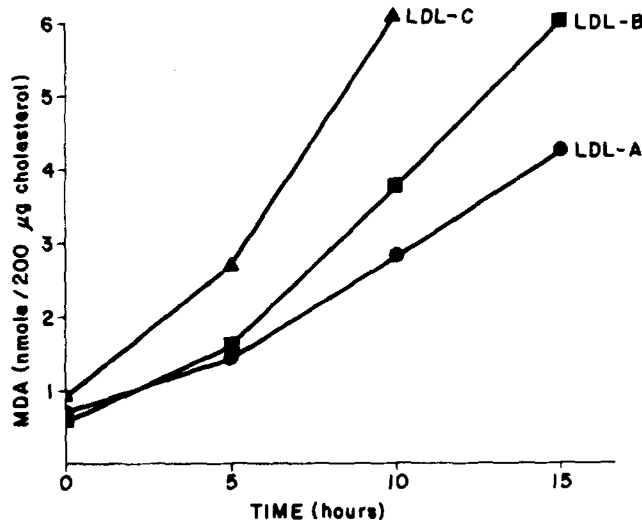

Fig. 1. LDL from individual sera are oxidized at different rates. Three LDL preparations (A, B and C) were isolated at the same time from three different sera, dialyzed against 0.15 M NaCl, sterilized, and then incubated at 37°C in 0.15 M NaCl. TBAR were measured at incubation times of 5, 10, and 15 hr and are reported as nmol MDA/200 μg cholesterol.

Downloaded from www.jlr.org by guest, on June 19, 2012
ASBMB
JOURNAL OF LIPID RESEARCH

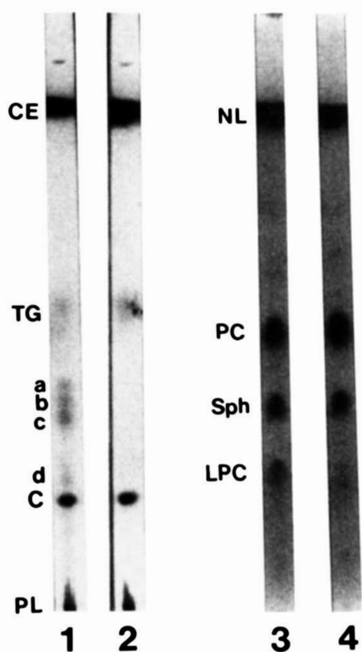

Fig. 2. Neutral lipids from LDL_{OXID} (lane 1) and LDL_{BHT} (lane 2) contain CE, TG, and C. LDL_{OXID} contains four additional spots (a, b, c, d) that stained for lipid peroxides (data not shown). Phospholipids from LDL_{OXID} (lane 3) and LDL_{BHT} (lane 4) contained NL, PC, and Sph. LDL_{OXID} also contained LPC and relatively less PC than LDL_{BHT}. NL from LDL_{OXID} stained for lipid peroxides (data not shown). Lipids were extracted by chloroform-methanol, separated by TLC, and visualized with iodine vapor. Spots were identified from the R_f values for authentic samples. See text for R_f values.

the neutral lipid spot (R_f 0.96) from LDL_{OXID} stained for lipid peroxides. These data showed that oxidation released fatty acids from PC resulting in the formation of increased amounts of LPC. The fatty acids were evidently converted to derivatives that did not separate as FFA on TLC.

The absence of a FFA spot on TLC suggested that FFA derivatives were formed from the FFA released during LDL oxidation. Labeled AA (0.4 μM) was added to a fresh LDL solution containing 7400 μg cholesterol/ml and this mixture was oxidized, extracted with acidified ethyl acetate, and the labeled products were separated by HPLC. The extract contained free AA and small amounts of a number of labeled compounds which eluted as broad peaks (**Fig. 3**). Thus HPLC showed that free AA was converted to a number of derivatives that could explain the absence of FFA in LDL_{OXID} when small amounts of FFA are released.

Immunoreactive products of LDL oxidation

Since prostanoids are synthesized through cyclic endoperoxides and lipid autoxidation forms a number of cyclic and acyclic peroxides (30, 31), the cross-reactivities of LDL_{OXID} containing FFA oxidation products were measured with antibodies to PGE_2 and 6-keto-$PGF_{1\alpha}$. LDL_{BHT} did not cross-react with antibodies to either prostanoid (data not shown). Cross-reactivity to PGE_2 antibodies varied directly with the initial degree of lipid peroxidation in the LDL_{OXID} preparations that were subsequently incubated for 24 hr in tissue culture media prior to measurement of PGE_2 levels by RIA (**Fig. 4**). Furthermore, immunoreactive materials were not the result of enzymatic prostanoid synthesis since a high concentration of IM had no effect on cross-reactivity (22). Lipid oxidation products showed unexpected specificity in that even highly oxidized LDL did not cross-react with antibodies to 6-keto-$PGF_{1\alpha}$ (Fig. 4).

An add-back experiment was performed to show that LDL_{OXID} did not destroy PGE_2 antibodies or interfere with their binding. PGE_2 was measured in media containing LDL_{OXID} (2.7 nmol MDA/200 μg cholesterol) and media containing PGE_2 generated by incubating cell cultures with AA (22–24). Media containing LDL_{OXID} (91 pg apparent PGE_2) were combined with AA media (88 pg de novo PGE_2) and PGE_2 levels were determined for these

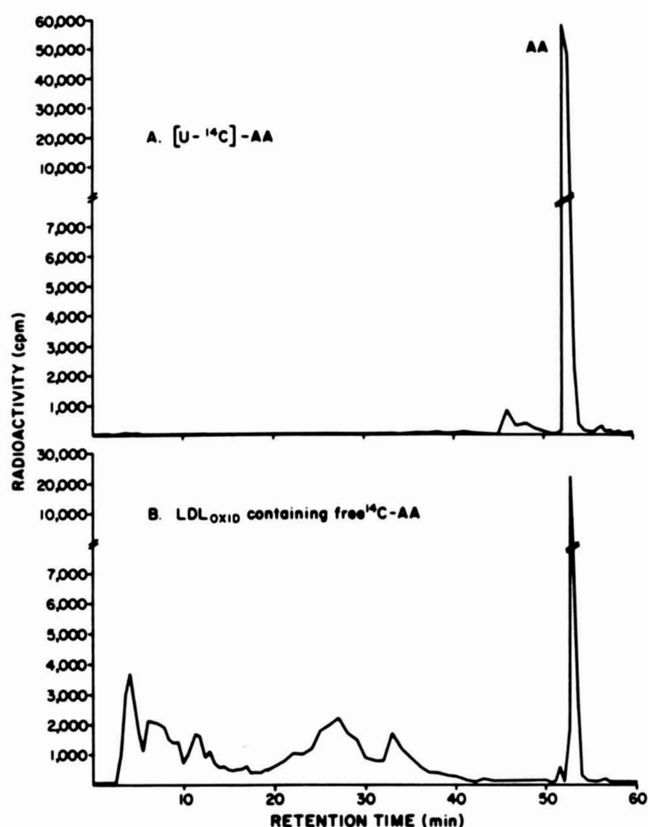

Fig. 3. [U-^{14}C]AA is converted to a number of derivatives during co-oxidation with LDL. Labeled AA (0.4 μM) and LDL (7400 μg cholesterol/ml) were co-oxidized to 3.7 nmol MDA/200 μg cholesterol. Labeled derivatives were extracted in acidified ethyl acetate and separated by HPLC using mixtures of acetonitrile-isopropanol-aqueous phosphoric acid.

Downloaded from www.jlr.org by guest, on June 19, 2012

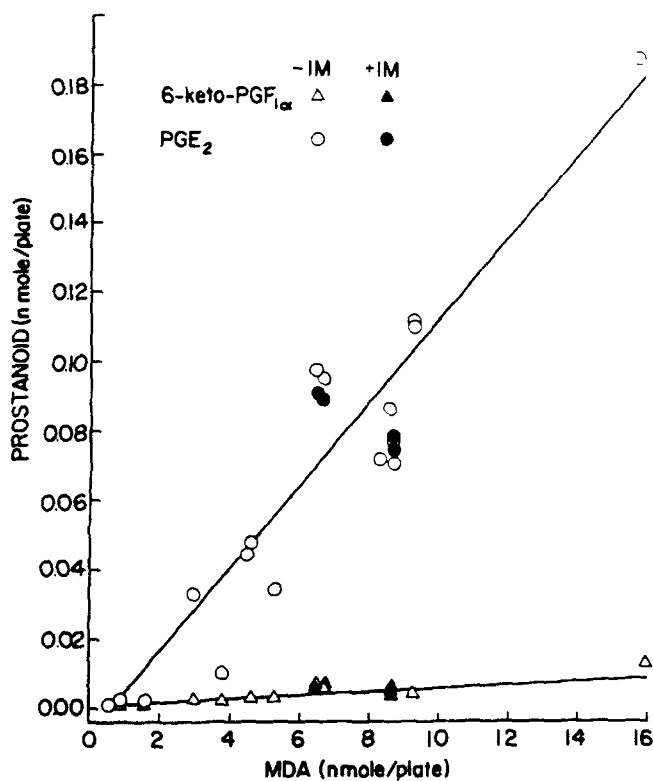

Fig. 4. Cross-reactivity of LDL_{OXID} to PGE_2 antibodies varies directly with lipid peroxide content and is not inhibited by IM. LDL_{OXID} does not cross-react significantly with 6-keto-$PGF_{1\alpha}$ antibodies. LDL from 17 individual sera were prepared with different levels of lipid peroxidation. LDL concentrations were adjusted to 800 μg cholesterol/ml and the LDL were incubated in tissue culture media alone for 24 hr at 37°C in the absence or presence of 10 μM IM. RIA was then used to estimate prostanoid levels. PGE_2 in the graph refers to immunoreactive PGE_2-like material.

mixtures. The PGE_2 level measured in the mixture was 105% of the PGE_2 level calculated from values obtained for the separate samples. These data showed that LDL_{OXID} did not interfere with the determination of PGE_2.

Cross-reactivity to PGE_2 antibodies was a function both of the initial degree of lipid peroxidation and of the incubation time. For example, 24-hr incubations of seven different LDL_{OXID} preparations adjusted to 800 μg/ml in tissue culture medium produced significant increases in immunoreactive PGE_2 (0.040 ± 0.002 nmol/plate at 0 hr compared to 0.088 ± 0.003 nmol/plate at 24 hr). We also extended the incubation time period to 72 hr. Data for two representative LDL_{OXID} preparations incubated for 72 hr in tissue culture media alone are compared in **Fig. 5.** Cross-reactivity to the PGE_2 antibody varied directly with the initial lipid peroxide levels in the LDL_{OXID}, and large increments in immunoreactive materials occurred during incubation.

Lipid peroxide levels were also measured during the experiments described in Fig. 5. In contrast to cross-reacting material, MDA levels (absorbance at 532 nm) did not increase with time when LDL_{OXID} were incubated in tissue culture media. These data showed that tissue culture media contained sufficient amounts of antioxidants to prevent further lipid peroxidation. Despite the absence of further lipid peroxidation, the increase in cross-reacting material over time occurred even in the presence of 100 μM BHT. In this context, after 24 hr incubation there was no significant difference in immunoreactive PGE_2 in incubations with or without BHT (0.084 ± 0.005 nmol/plate compared to 0.088 ± 0.003 nmol/plate). These experiments showed that preformed lipid peroxides in the LDL_{OXID} were converted during incubation in tissue culture media alone to products that expressed immunoreactivity toward PGE_2 antibodies.

Solubility of immunoreactive products from LDL_{OXID}

A LDL_{OXID} solution was adjusted to d 1.10 g/ml with KBr and LDL was re-isolated by ultracentrifugal flotation. The contents of the centrifuge tube were separated into top, middle, and bottom fractions and each fraction

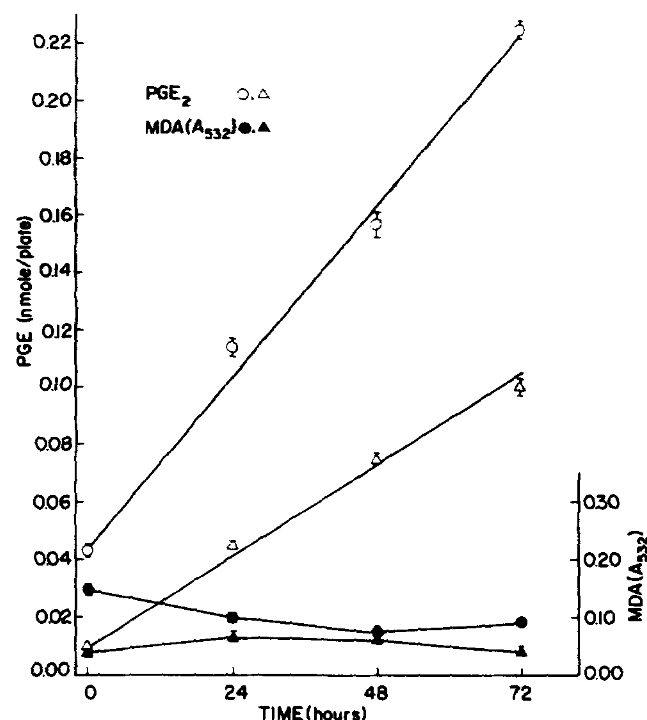

Fig. 5. Cross-reactivity of LDL_{OXID} to PGE_2 antibodies varies directly with both the initial lipid peroxide content and the incubation time. Lipid peroxide levels decrease or remain constant during the incubation period. Two LDL_{OXID} preparations were adjusted to 800 μg cholesterol/ml and incubated in tissue culture media alone for 0, 24, 48, and 72 hr at 37°C. Lipid peroxides were measured as TBAR (●, ▲) and immunoreactive PGE_2-like material was measured by RIA (○, △) after each time interval. The initial lipid peroxide content of one LDL_{OXID} preparation (●) was greater than the initial lipid peroxide content of the other LDL_{OXID} preparation (▲). Each data point in the graph was generated from two or three separate incubations of the LDL_{OXID} and is reported as mean ± SEM.

Downloaded from www.jlr.org by guest, on June 19, 2012
ASBMB JOURNAL OF LIPID RESEARCH

was analyzed for cholesterol and immunoreactive products. All of the cholesterol was recovered in the top fraction. Apparent PGE_2 levels (ng/ml) were 33 before centrifugation and 32, 33, and 47 in the top, middle, and bottom fractions, respectively. These data showed that immunoreactive products were not bound to LDL_{OXID} since they were not concentrated in the LDL_{OXID} fraction by ultracentrifugation.

Immunoreactive products of Il oxidation

The cross-reactivity to PGE_2 antibodies of Il, a synthetic intravenous fat emulsion, was measured to ascertain whether cross-reactivity was a general result of lipid oxidation or the specific result of LDL oxidation. Il did not contain significant amounts of lipid peroxides and Il did not generate lipid peroxides during incubation in 96% air-4% CO_2 at 37°C. However, large amounts of lipid peroxides were formed when 10 μM Fe^{3+} was added to the incubation solution (**Table 1**). Il showed a low level of cross-reactivity to PGE_2 antibodies and cross-reactivity was not increased in Il_{OXID} (Table 1) even though MDA levels in Il_{OXID} were comparable to MDA levels in LDL_{OXID} (Fig. 4). The Fe^{3+} catalyst did not affect cross-reactivity since both MDA levels and cross-reactivities were enhanced when LDL was oxidized in media containing 10 μM Fe^{3+} (data not shown). The differences between Il and LDL showed that significant cross-reactivity was a property specific for the oxidation of LDL.

TABLE 1. Free AA and oxidation enhance the cross-reactivity of Il to PGE_2 antibodies

Incubation Time	MDA	Apparent PGE_2
hr	*nmol/plate*	*pmol/plate*
A. Il		
12	0.2	8.6 ± 0.15
24	0.2	12.3 ± 0.37
48	0.2	10.1 ± 1.00
Il + AA		
12	0.8	9.5 ± 0.37
24	0.2	12.2 ± 0.22
48	0.4	10.3 ± 0.81
B. Il_{OXID}		
12	7.7	14.9 ± 0.18
24	12.5	14.5 ± 0
48	10.1	13.6 ± 0
Il_{OXID} + AA		
12	7.1	16.0 ± 0.22
24	12.5	24.2 ± 1.41
48	10.1	28.0 ± 0.15

Il samples with and without 120 μM AA were incubated in saline alone (Il) or saline containing 10 μM Fe^{3+} (Il_{OXID}) for different time intervals and MDA was measured. Il or IL_{OXID} content was adjusted to 760 μg/ml with tissue culture media and incubation was continued for 24 hr. Two separate incubations were performed for each time point. Apparent PGE_2 was then measured (RIA) and reported as mean ± SEM. Data were subjected to a two-way analysis of variance (see text).

Since Il does not contain either free or bound AA, Il was mixed with free AA and cross-reactivity to PGE_2 antibodies was measured before and after lipid peroxidation (Table 1). Oxidation and AA effects were compared by a two-way analysis of variance and this analysis showed a strong interaction between free AA and oxidation in enhancing cross-reactivity (F 9.92, P 0.005). Free AA also increased cross-reactivity when it was added during LDL oxidation (data not shown). These results showed that the co-oxidation of free AA during both Il and LDL oxidation generated cross-reacting material.

DISCUSSION

Newly isolated LDL contain very little lipid peroxide but peroxides are readily formed during dialysis against 0.15 M NaCl and these preparations, which contain 0.7 ± 0.1 nmol MDA/200 μg cholesterol, must be considered as LDL_{OXID}. Oxidation is blocked by BHT and it is probable that LDL_{BHT} more nearly represents native LDL than LDL_{OXID}. Although the BHT experiments show that much of the native LDL that are present in plasma are not oxidized, these experiments do not eliminate the possibility that LDL_{OXID} are formed in plasma and other tissues and then taken up more rapidly than unoxidized LDL by cells (11-16).

Dialyzed LDL_{OXID} are capable of more extensive lipid peroxidation in which the TBAR content is increased to as much as 6 nmol MDA/200 μg cholesterol. Oxidation is controlled by incubating LDL_{OXID} under 96% air-4% CO_2 at 37°C for various time intervals and oxidation under these conditions is much slower than oxidation catalyzed by Cu^{2+}. The rate of mild oxidation is actually a sensitive measure of the susceptibility to autoxidation of LDL preparations from different human plasma samples. Rate studies on autoxidation show that it may be possible to generate circulating LDL with different peroxide levels and indeed plasma and isolated plasma lipoproteins from normal and diseased subjects contain different concentrations of lipid peroxides (4, 7-10).

LDL_{OXID} contain several lipid peroxides that are confined to esterified neutral lipids. Thus lipid extracts from LDL_{OXID} contained lipid peroxides that migrated as discrete spots with R_f values in the same region as spots from autoxidized cholesteryl arachidonate and trilinolenin. Lipid peroxides were not identified either in the region of the TLC plate occupied by simple fatty acid hydroperoxides or the phospholipid band that remained at the origin.

Other investigators found that the PC fraction decreased and the LPC fraction increased when LDL were oxidized extensively by cells or Cu^{2+} (13, 16, 20, 21) and

Downloaded from www.jlr.org by guest, on June 19, 2012

an increase in LPC was also shown as a result of mild oxidation in the present study. The formation of increased LPC was shown in those studies to be the result of enhanced phospholipase A_2 activity. Since FFA and simple fatty acid hydroperoxides were not detected on TLC, FFA were evidently converted to a number of complex oxidation products such as the oxidation products that were separated by HPLC after the co-oxidation of LDL and labeled AA.

Autoxidation forms a number of acyclic and cyclic lipid peroxides (30, 31) which have different properties in LDL_{OXID}. Lipid peroxides in esterified neutral lipids such as cholesteryl esters and triglycerides are evidently the source of TBAR, since fatty acid hydroperoxides were not identified. The susceptibility of cholesteryl esters in LDL to lipid peroxidation is interesting in that early investigators identified fatty acid oxidation products in cholesteryl esters from aortas of subjects with severe atherosclerosis (32). The accumulation of these lipids in plaques may provide indirect evidence for circulating LDL_{OXID}.

Complex oxidation products that cross-react with antibodies to PGE_2 are not unique to LDL_{OXID}. Similar material is generated when the synthetic fat emulsion Il is co-oxidized with free AA. However, significant amounts of the immunoreactive material are not generated during the oxidation of Il alone. FFA released from PC during LDL oxidation are evidently converted to unbound oxidation products that cross-react specifically with antibodies to PGE_2.

A number of studies have indicated that LDL stimulate PGE_2 synthesis and either stimulate or inhibit PGI_2 synthesis (8, 9, 33-37). Prostanoids are usually measured by RIA and the formation of material cross-reacting with PGE_2 but not 6-keto-$PGF_{1\alpha}$ (the stable derivative of PGI_2) that we report here may affect the interpretation of data from other published studies.

This study was supported in part by a Research Challenge Grant from the Ohio State University. We appreciate statistical analyses by Dr. Larry Sachs.

Manuscript received 12 October 1987, in revised form 25 July 1988, and in re-revised form 3 October 1988.

REFERENCES

1. Glavind, J., S. Hartman, J. Clemmensen, K. E. Jessen, and H. Dam. 1952. Studies on the role of lipoperoxides in human pathology. II. The presence of peroxidized lipids in the atherosclerotic aorta. *Acta Pathol. Microbiol. Scand.* **30:** 1-6.
2. Woodford, F. P., C. J. F. Bottcher, K. Oette, and E. H. Ahrens, Jr. 1965. The artifactual nature of lipid peroxides detected in extracts of human aorta. *J. Atheroscler. Res.* **5:** 311-316.
3. Cornwell, D. G., J. C. Geer, and R. V. Panganamala. 1975. Development of atheroma and the lipid composition of the deposit. *In* Pharmacology of Lipid Transport and Atherosclerotic Processes. IEPT Section 24, Vol. 1. E. J. Masoro, editor. Pergamon Press, Oxford. 445-483.
4. Yagi, K. 1982. Assay for serum lipid peroxide level and its clinical significance. *In* Lipid Peroxidation in Biology and Medicine. K. Yagi, editor. Academic Press, New York. 223-242.
5. Ray, B. R., E. O. Davisson, and H. L. Crespi. 1954. Experiments on the degradation of lipoproteins from serum. *J. Phys. Chem.* **58:** 841-846.
6. Oncley, J. L., K. W. Walton, and D. G. Cornwell. 1957. A rapid method for the bulk isolation of β-lipoproteins from human plasma. *J. Am. Chem. Soc.* **79:** 4666-4671.
7. Lee, D. M. 1980. Malondialdehyde formation in stored plasma. *Biochem. Biophys. Res. Commun.* **95:** 1663-1672.
8. Szczeklik, A., and R. J. Gryglewski. 1980. Low density lipoproteins (LDL) are carriers for lipid peroxides and inhibit prostacyclin (PGI_2) biosynthesis in arteries. *Artery.* **7:** 488-495.
9. Szczeklik, A., R. J. Gryglewski, B. Domagala, A. Zmuda, J. Hartwich, E. Wozny, M. Grzywacz, J. Madej, and T. Gryglewska. 1981. Serum lipoproteins, lipid peroxides and prostacyclin biosynthesis in patients with coronary heart disease. *Prostaglandins.* **22:** 795-807.
10. Bittolo-Bon, G. B., G. Cazzolato, M. Saccardi, and P. Avogaro. 1987. Presence of a modified LDL in humans: effect of vitamin E. *In* Clinical and Nutritional Aspects of Vitamin E. O. Haysishi and M. Mino, editors. Elsevier Science Publishers B.V., Amsterdam. 109-120.
11. Fogelman, A. M., Schechter, J. Seager, M. Hokom, J. S. Child, and P. A. Edwards. 1980. Malondialydehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. *Proc. Natl. Acad. Sci. USA.* **77:** 2214-2218.
12. Haberland, M. E., A. M. Fogelman, and P. A. Edwards. 1982. Specificity of receptor-mediated recognition of malondialdehyde-modified low density lipoproteins. *Proc. Natl. Acad. Sci. USA.* **79:** 1712-1716.
13. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1983. Enhanced macrophage degradation of biologically modified low density lipoprotein. *Arteriosclerosis.* **3:** 149-159.
14. Baker, D. P., B. J. van Lenten, A. M. Fogelman, P. A. Edwards, C. Kean, and J. A. Berliner. 1984. LDL, scavenger, and β-VLDL receptors on aortic endothelial cells. *Arteriosclerosis.* **4:** 248-255.
15. Nagelkerke, J. F., L. Havekes, V. W. M. Van Hinsbergh, and T. J. C. Van Berkel. 1984. In vivo catabolism of biologically modified LDL. *Arteriosclerosis.* **4:** 256-264.
16. Steinbrecher, U. P., S. Parthasarathy, D. S. Leaks, J. L. Witztum, and D. Steinberg. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA.* **81:** 3883-3887.
17. Haberland, M. E., and A. M. Fogelman. 1985. Scavenger receptor-mediated recognition of maleyl bovine plasma albumin and the demaleylated protein in human monocyte macrophages. *Proc. Natl. Acad. Sci. USA.* **82:** 2693-2697.
18. Heinecke, J. W., L. Baker, H. Rosen, and A. Chait. 1986. Superoxide-mediated modification of low density lipoprotein by arterial smooth muscle cells. *J. Clin. Invest.* **77:** 757-761.
19. Ivanov, V. O., S. N. Preobrazhensky, V. P. Tsibulsky, V. R. Bakaev. V. S. Repin, and V. N. Smirnov. 1985. Liposome

Downloaded from www.jlr.org by guest, on June 19, 2012

uptake by cultured macrophages mediated by modified low-density lipoproteins. *Biochim. Biophys. Acta.* **846:** 76–84.

20. Parthasarathy, S., U. P. Steinbrecher, J. Barnett, J. L. Witztum, and D. Steinberg. 1985. Essential role of phospholipase A_2 activity in endothelial cell-induced modification of low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **82:** 3000–3004.
21. Steinbrecher, U. P., J. L. Witztum, S. Parthasarathy, and D. Steinberg. 1987. Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL. *Arteriosclerosis.* **7:** 135–143.
22. Zhang, H., K. H. Jones, W. B. Davis, R. L. Whisler, R. V. Panganamala, and D. G. Cornwell. 1987. Oxidized low density lipoproteins in smooth muscle cell cultures: differential effects on prostanoid synthesis and viability. *In* Clinical and Nutritional Aspects of Vitamin E. O. Hayaish and M. Mino, editors. Elsevier Science Publishers B.V., Amsterdam. 89–100.
23. Huttner, J. J., E. T. Gwebu, R. V. Panganamala, G. E. Milo, D. G. Cornwell, H. M. Sharma, and J. C. Geer. 1977. Fatty acids and their prostaglandin derivatives: inhibitors of proliferation in aortic smooth muscle cells. *Science.* **197:** 289–291.
24. Morisaki, N., J. A. Lindsey, J. M. Stitts, H. Zhang, and D. G. Cornwell. 1984. Fatty acid metabolism and cell proliferation. V. Evaluation of pathways for the generation of lipid peroxides. *Lipids.* **19:** 391–394.
25. Witte, D. L., D. A. Barrett, and D. A. Wycoff. 1974. Evaluation of an enzymatic procedure for determination of serum cholesterol with the Abbott ABA-100. *Clin. Chem.* **20:** 1282–1286.
26. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9:** 693–700.
27. Hadjiagapiou, C., and A. A. Spector. 1987. Docosahexaenoic acid metabolism and effect on prostacyclin production in endothelial cells. *Arch. Biochem. Biophys.* **253:** 1–12.
28. Johns, M., R. W. Keenan, and P. Horowitz. 1982. Use of 6-*p*-toluidino-2-naphthalenesulfonic acid to quantitate lipids after thin-layer chromatography. *J. Chromatogr.* **237:** 522–524.
29. Gavino, V. C., J. S. Miller, S. O. Ikharebha, G. E. Milo, and D. G. Cornwell. 1981. Effect of polyunsaturated fatty acids and antioxidants on lipid peroxidation in tissue cultures. *J. Lipid Res.* **22:** 763–769.
30. Pryor, W. A., J. P. Stanley, and E. Blair. 1976. Autoxidation of polyunsaturated fatty acid. II. A suggested mechanism for the formation of TBA-reactive materials from prostaglandin-like endoperoxides. *Lipids.* **11:** 370–379.
31. Porter, N. A. 1986. Mechanisms for autoxidation of polyunsaturated lipids. *Acc. Chem. Res.* **19:** 262–268.
32. Brooks, C. J. W., G. Steel, J. D. Gilbert, and W. A. Harland. 1971. Lipids of human atheroma. Part 4. Characterisation of a new group of polar sterol esters from human atherosclerotic plaques. *Atherosclerosis.* **13:** 223–237.
33. Pomerantz, K. B., A. R. Tall, S. J. Feinmark, and P. J. Cannon. 1984. Stimulation of vascular smooth muscle cell prostacyclin and prostaglandin E_2 synthesis by plasma high and low density lipoproteins. *Circ. Res.* **54:** 554–565.
34. Beitz, J., M. Panse, S. Fischer, C. Hora, and W. Forster. 1983. Inhibition of prostaglandin I_2 (PGI_2) formation by LDL-cholesterol or LDL-peroxides? *Prostaglandins.* **26:** 885–892.
35. Giessler, C., J. Beitz, P. Mentz, and W. Forster. 1986. The influence of lipoproteins (LDL and HDL) on PGI_2 formation by isolated aortic preparations of rabbits. *Prostaglandins Leukotrienes Med.* **22:** 221–234.
36. Yokode, M., T. Kitz, Y. Kikawa, T. Ogorochi, S. Narumiya, and C. Kawai. 1988. Stimulated arachidonate metabolism during foam cell transformation of mouse peritoneal macrophages with oxidized low density lipoprotein. *J. Clin. Invest.* **81:** 720–729.
37. Triau, J. E., S. N. Meydani, M. Meydani, P. Libby, and E. J. Schaefer. 1986. Oxidized low density lipoproteins (OX-LDL) stimulate prostacyclin (PGI_2) production by adult human vascular endothelial cells. *Fed. Proc.* **45:** 347 (abstract).

Downloaded from www.jlr.org by guest, on June 19, 2012