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Protein Adducts of Iso[4]levuglandin E_2, a Product of the Isoprostane Pathway, in Oxidized Low Density Lipoprotein*

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Levuglandin (LG) E_2, a cytotoxic seco prostanoic acid co-generated with prostaglandins by nonenzymatic rearrangements of the cyclooxygenase-derived endoperoxide, prostaglandin H_2, avidly binds to proteins. That LGE_2-protein adducts can also be generated nonenzymatically is demonstrated by their production during free radical-induced oxidation of low density lipoprotein (LDL). Like oxidized LDL, LGE_2-LDL, but not native LDL, undergoes receptor-mediated uptake and impaired processing by macrophage cells. Since radical-induced lipid oxidation produces isomers of prostaglandins, isoprostanes (isoPs), via endoperoxide intermediates, we postulated previously that a similar family of LG isomers, isoLGs, is cogenerated with isoPs. Now iso[4]LGE_2-protein epitopes produced by radical-induced oxidation of arachidonic acid in the presence of protein were detected with an enzyme-linked immunosorbent assay. Iso[4]LGE_2-protein epitopes are also generated during free radical-induced oxidation of LDL. All of the LGE_2 isomers generated upon oxidation of LDL are efficiently sequestered by covalent adduction with LDL-based amino groups. The potent electrophilic reactivity of iso-LGs can be anticipated to have biological consequences beyond their obvious potential as markers for specific arachidonate-derived protein modifications that may be of value for the quantitative assessment of oxidative injury.

Oxidative modification of low density lipoprotein (LDL)[1] is considered a key step in the etiology of atherosclerosis (1, 2). Free radical-induced oxidation of LDL consumes polyunsaturated fatty esters and concomitanty generates lipid-derived electrophiles which modify LDL by covalent adduction with protein-based nucleophiles (3–5). Receptor recognition of the resulting protein modifications leads to uptake of the oxidized (ox) LDL by macrophages (6–8). Because uptake is unregulated and processing of oxLDL is inefficient, the macrophages become lipid-laden foam cells, progenitors of atherosclerotic plaques (9). To acquire a fundamental molecular level understanding of atherogenesis and other biological sequelae of oxidative injury, we are identifying the chemical structures of lipid oxidation products that bind with proteins.

Previously, we discovered derivatives of levulinaldehyde with prostaglandin side chains appended at the carbons α and β to the aldehyde group. Named levuglandins (10), *e.g.* LGE_2 (Fig. 1), these seco prostanoic acids are cogenerated with prostaglandins (PGs) (11–13) by rearrangements of the endoperoxide PGH_2 which occur readily ($t_{1/2}$ = 5 min at 37 °C) under the conditions of its cyclooxygenase (COX)-promoted biosynthesis from arachidonic acid (AA). LGE_2 binds avidly with proteins (14) forming a protein-bound pyrrole, LGE_2-pyrrole (15), as well as protein-protein (16, 17) and DNA-protein (18) cross-links. We recently reported mass spectral characterization of several lysine-based modifications that are generated by covalent adduction of LGE_2 with proteins (19).[2] Levels of LGE_2-protein adducts are markedly elevated in the blood of atherosclerosis and end stage renal disease patients *versus* healthy controls (21). Furthermore, LGE_2-modified LDL is recognized by macrophages, taken up, and inefficiently processed in close analogy and competition with oxLDL (8). In effect, LGE_2-modified LDL may function as a Trojan horse, fostering uptake but then compromising the ability of macrophage proteases to hydrolyze oxidatively damaged LDL protein.

Because COX only converts free AA into PGH_2, this pathway is regulated by enzymatic release of AA from AA-PC (22, 23). In contrast, a free radical pathway oxidizes AA-PC directly to produce phospholipid endoperoxides (24–26). We previously showed that LGE_2-protein adducts are also produced during free radical-induced oxidation of LDL (27). While the enzymatic pathway generates a single stereoisomer with *trans* disposed side chains, peroxy radical cyclization generates an isomeric mixture in which stereoisomers with *cis* disposed side chains predominate (28) as, for example, in the 2-lysophosphatidylcholine (PC) ester 8-*epi*-PGH_2-PC (Fig. 1). Rearrangement of 8-*epi*-PGH_2-PC would deliver 8-*epi*-LGE_2-PC. However, because the stereocenters at positions 8 and 9 are lost during Paal-Knorr condensation (29) of this LG-phospholipid with ly-

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[1] The abbreviations used are: LDL, low density lipoprotein; apo, apolipoprotein B; AA, arachidonic acid; BCA, bicinchoninic acid; BSA, bovine serum albumin; CEO, chicken egg ovalbumin; COX, cyclooxygenase; DMAB, *p*-(*N*,*N*-dimethylamino)benzaldehyde); ELISA, enzyme-linked immunosorbent assay; EPA, eicosapentaenoic acid; ETA, eicosatrienoic acid; γ-LA, γ-linolenic acid; HNE, (*E*)-4-hydroxy-2-nonenal; HODA, 9-hydroxy-12-oxo-10-dodecenoic acid; HOHA, 5-hydroxy-8-oxo-6-octenoic acid; HSA, human serum albumin; iso-LGs, isolevuglandins; isoPs, isoprostanes; KLH, keyhole limpet hemocyanin; LG, levuglandin; LA, linoleic acid; MDA, malondialdehyde; ON, 4-oxononanal; oxLDL, oxidized LDL; PBS, phosphate-buffered saline; PC, 2-lysophosphatidylcholine; PUFA, polyunsaturated fatty acid; PG, prostaglandins; HPLC, high performance liquid chromatography; LC/MS, liquid chromatography mass spectrometry.

[2] O. Boutaud, C. J. Brame, R. G. Salomon, L. J. Roberts, II, and J. A. Oates, submitted for publication.

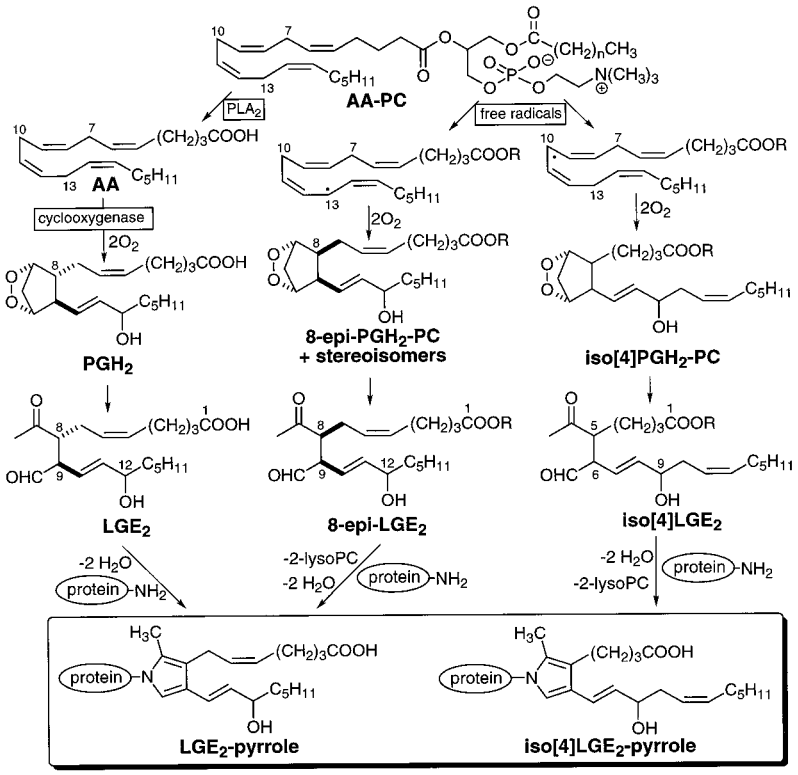

FIG. 1. **Cyclooxygenase (enzymatic) pathway and free radical-induced (nonenzymatic) route to LGs and iso-LGs via rearrangements of endoperoxide intermediates.**

syl amino groups of LDL protein, formation of a pyrrole adduct in conjunction with enzyme-catalyzed hydrolytic release of lysophosphatidylcholine (30–32) generates the same LGE_2-pyrrole as that formed by the cyclooxygenase pathway (Fig. 1).

Mouse peritoneal macrophages internalize and degrade LGE_2-LDL if the molar ratio of LGE_2 to LDL protein (apoB) exceeds a threshold somewhere between 10:1 and 38:1 by a receptor mediated uptake that is completely inhibited by oxLDL (8). Furthermore, uptake of oxLDL is inhibited by LGE_2-LDL, supporting the conclusion that both LGE_2-LDL and oxLDL are recognized by the same receptor. However, the ratio of LGE_2 to apoB in oxLDL does not exceed 2:1. Nevertheless, the total modification of apoB by all of the isomeric levulinaldehyde derivatives produced by oxidation of AA might suffice to account for receptor recognition, uptake, and inefficient processing of oxLDL. Thus, because hydrogen atom abstraction readily occurs nonregioselectively at any doubly allylic methylene, we postulated that the free radical pathway not only can produce a stereoisomeric mixture of levulinaldehyde derivatives with PG side chains, *i.e.* iso-LGs, but also structurally isomeric levulinaldehyde derivatives with nonprostanoid side chains, *i.e.* iso[*n*]LGs. For example, hydrogen atom abstraction from the 10-position of AA-PC followed by cyclization of an intermediate 8-peroxyeicosatetraenoyl radical could lead to iso[4]PGH_2-PC and then iso[4]LGE_2-PC (Fig. 1), where the number in brackets signifies the length of the carboxylic side chain appended to a common 2,3-dioxabicyclo[2.2.1]heptane or levulinaldehyde core. The generation of phospholipid endoperoxides that are structural isomers of PGH_2 by free radical-induced oxidation was postulated previously to account for the formation of isoprostanes (24, 25). Thus, iso[4]PGH_2-PC (12-H_2-IsoP) is also the putative precursor of isoprostanes that have been designated 12-F_2-IsoP, 12-E_2-IsoP, and 12-D_2-IsoP (33). In analogy with the chemistry of LGE_2, we expected that iso[4]LGE_2-PC would form iso[4]LGE_2-pyrrole by covalent adduction to proteins and concomitant phospholipolysis (Fig. 1). We now report confirmation of this hypothesis. Thus, the generation of iso[4]LGE_2-protein epitopes during *in vitro* nonenzymatic free radical-induced oxidation of LDL was detected with an immunoassay using antibodies raised against an iso[4]LGE_2-protein adduct. Since iso[4]LGE_2 is formed by the isoprostane pathway but not by the COX pathway, the new antibody allows unambiguous assessment of the formation of iso-LGs from the isoprostane pathway. In a companion paper (19), we report mass spectral characterization of the covalent iso-LG-derived protein modifications that are generated during free-radical induced oxidation of LDL.

EXPERIMENTAL PROCEDURES

General Methods

Centrifugation was done on a Sorvall centrifuge at 5 °C and 2000 rpm. Absorbance values of enzyme-linked immunosorbent assays (ELISAs) were measured on a Bio-Rad Microplate Reader using dual wavelength (405 nm to read the plate and 650 nm as a reference).

Materials

Spectrapor membrane tubing (M_r cutoff 14,000 number 2) for dialysis was obtained from Fisher Scientific Co. The following commercially available materials were used as received: AA, docosahexaenioc acid (DHA), eicosapentaenoic acid (EPA), eicosatrienoic acid (ETA), γ-linolenic acid (γ-LA), linoleic acid (LA), chicken egg ovalbumin (CEO, grade V, 99%), bovine serum albumin (BSA, fraction V, 96–99%), human serum albumin (HSA, fraction V), and disodium *p*-nitrophenyl phosphate, were from Sigma; keyhole limpet hemocyanin (KLH, ICN Biochemicals); goat anti-rabbit IgG-alkaline phosphatase (Roche Molecular Biochemicals): *p*-(*N,N*-dimethylamino)benzaldehyde (DMAB, Aldrich, WI). Phosphate-buffered saline (PBS) was prepared from a pH 7.4 stock solution containing 0.2 M NaH_2PO_4/Na_2HPO_4, 3.0 M NaCl, and 0.02% NaN_3 (w/w). This solution was diluted 20 times as needed. LGE_2 (34), iso[4]LGE_2 (35), and 4-oxopentanal-BSA (36) were prepared as described previously. LDL was isolated (37) from human plasma and oxidized *in vitro* to give oxLDL as described previously (27). HNE-HSA, $NaCNBH_3$-reduced HNE-HSA, and MDA-HSA were prepared as described previously (38). ON-KLH antibodies (36) and LGE_2-KLH antibodies (27) were prepared as described previously.

Iso[4]LGE_2-KLH Antigen

A PBS solution containing 3.1 mM iso[4]LGE_2 (1.3 mg, 3.69 μmol) and 1.5 μM KLH (9.84 mg, 7.96 mg/ml, 4.92 μmol of lysyl residues) was incubated at room temperature for 1 h. The solution was then dialyzed

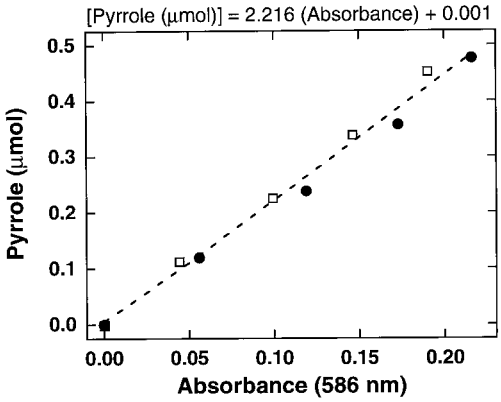

FIG. 2. **The correlation between LGE_2-bound to absorbance at 586 nm for the Ehrlich pyrrole assay, using DMAB and HCl, of LGE_2-protein adducts: LGE_2-BSA (●) and LGE_2-HSA (□).**

against PBS (3 × 1 liters over 60 h) at room temperature. After dialysis, the final volume of the solution was adjusted to 5 ml. The final protein concentration, 1.25 mg/ml, was determined by the Pierce bicinchoninic acid (BCA) protein assay (39) using solutions of BSA as standards.

Ehrlich Pyrrole Assay of Iso[4]LGE_2-Protein Adducts

An Ehrlich pyrrole assay (40, 41) was performed to determine the concentration of protein-bound iso[4]LGE_2-derived pyrroles as described previously for determining the concentration of LGE_2-derived pyrroles (15, 27). Tritium-labeled LGE_2-HSA and LGE_2-BSA were used as a standards for the assay. The amount of LGE_2 bound to HSA or BSA was determined by quantitative radiochemical analysis. The data for the standards (Fig. 2) fits the equation: [pyrrole (μmol)] = 2.22 (absorbance at 586 nm). The absorption maximum (λ_{max}) for LGE_2-HSA-derived and iso[4]LGE_2-BSA-derived Ehrlich pyrrole assay chromophores were 586 and 584 nm, respectively. The concentration of pyrrole was presumed to be equal to the concentration of LGE_2 that is bound to BSA or HSA. This assumes a quantitative yield of protein-bound LGE_2-derived pyrrole. Thus, the concentration of pyrrole estimated by Ehrlich assay is an upper limit for protein-bound iso[4]LGE_2-derived pyrrole. The pyrrole concentration in the iso[4]LGE_2-KLH solution is 0.52 mM KLH-bound iso[4]LGE_2.

Iso[4]LGE_2-BSA Coating Agent

A PBS solution containing 4.54 mM iso[4]LGE_2 (1.6 mg, 4.54 μmol) and 0.2 mM BSA (13.2 mg, 11.7 μmol of lysyl residues) was incubated at 37 °C for 12 h. The solution was then dialyzed against PBS (4 × 500 ml) over 48 h at room temperature. After dialysis, the final volume of the solution was adjusted to 6 ml. The final protein concentration, determined using the Pierce BCA protein assay as described above, was 1.85 mg/ml. The pyrrole concentration, 0.59 mM BSA-bound iso[4]LGE_2, was determined by an Ehrlich assay using LGE_2-BSA and LGE_2-HSA as standards.

Iso[4]LGE_2-HSA Standard

A PBS solution containing 3.55 mM iso[4]LGE_2 (2.5 mg, 7.10 μmol) and 0.16 mM HSA (20.6 mg, 18.30 μmol of lysyl residues) was incubated at 37 °C for 16 h. The solution was then dialyzed against PBS (3 × 1 liters) for 48 h at room temperature. The final volume of the solution was adjusted to 10 ml. The final protein concentration, determined using Pierce BCA protein assay as described above, was 1.81 mg/ml. The pyrrole concentration, 0.52 mM HSA-bound iso[4]LGE_2, was determined by Ehrlich assay using LGE_2-BSA and LGE_2-HSA as standards.

Immunization

The immunogen, iso[4]LGE_2-KLH (5.0 mg) containing 0.75 μmol of iso[4]LGE_2 per mg of KLH, was diluted to 5 ml with pH 7.4 PBS. An aliquot (500 μl) was emulsified in Freund's complete adjuvant (500 μl). Each of two Pasturella free, New Zealand White rabbits (Hazelton) were inoculated intradermally into several sites on the back (200 μl) and rear leg (200 μl). Booster injections of iso[4]LGE_2-KLH with Freund's incomplete adjuvant were given every 21 days. Antibody titer was monitored 10 days after each inoculation by ELISA as described below.

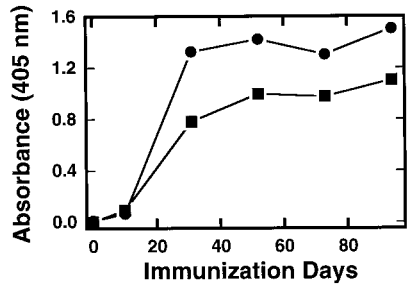

FIG. 3. **Antibody titer against iso[4]LGE_2-KLH (1:10,000 dilution).**

Antibody Purification

The iso[4]LGE_2-KLH immune serum from the 73 day bleeding of rabbit 1, containing 34.4 mg/ml protein, as determined by absorbance at 280 nm (A_{280} = 1.35 for 1.0 mg/ml), was purified using a protein G column as described previously (21). The resulting antibody solution (8.75 ml) contained 1.47 mg/ml purified IgGs. This corresponded to 13.3% of the total protein in the immune serum.

ELISA

For all ELISAs, unless otherwise noted, duplicates of each sample were run on the same plate.

Antibody Titers—For determination of antibody levels in rabbit blood serum, iso[4]LGE_2-BSA containing 10 mol of pyrrole/mol of protein, was used as coating agent. The iso[4]LGE_2-BSA conjugate (100 μl of a solution containing 4.4 mg/ml in pH 7.4 PBS) was added to each well of a sterilized Baxter ELISA plate. The plate was then incubated at 37 °C for 1 h in a moist chamber. After discarding the coating solution, each well was washed with PBS (3 × 300 μl), then filled with 1.0% CEO in PBS (300 μl), and incubated at 37 °C for 1 h to block remaining active sites on the plastic phase. Each well was washed with 0.1% CEO in PBS (300 μL) and then 100 μl of rabbit serum from each bleeding diluted 1:10,000 with 0.2% CEO in PBS, or 0.2% CEO in PBS without serum for a blank, was dispensed into the sample wells. Normal rabbit, *i.e.* prior to inoculation with antigen, serum diluted as above was employed as a negative response control. The ELISA was completed as described previously (21). The antibody titer rose abruptly after 3 weeks, reaching a plateau within about 30 days (Fig. 3).

Competitive Antibody Binding Inhibition Studies—For antibody binding inhibition studies to measure cross-reactivities, an iso[4]LGE_2-BSA adduct was used as coating agent and iso[4]LGE_2-HSA was used as a standard. On each ELISA plate, a blank, a positive control containing no inhibitor, and up to 10 serial dilutions of each inhibitor and the iso[4]LGE_2-HSA standard were run. The standard was prepared by diluting a 1.04 mM HSA-bound iso[4]LGE_2 solution in PBS to 104 μM with pH 7.4 PBS. A serial dilution of factor 0.2 was used. Each well of the plate was coated with iso[4]LGE_2-BSA solution (100 μl), prepared by diluting a solution containing 1.08 mM BSA-bound iso[4]LGE_2 in PBS to 432 nM with pH 7.4 PBS. The plate was covered with a plastic lid and placed in incubator at 37 °C for 1 h, and then allowed to come to room temperature. After discarding the supernatant, each well was washed with pH 7.4 PBS (3 × 300 μl) and then blocked by incubating 1 h at 37 °C with 300 μl of 1% CEO in pH 7.4 PBS. After coming to room temperature, the supernatant was discarded and the wells rinsed with 0.1% CEO in pH 7.4 PBS (300 μl). For each sample and the iso[4]LGE_2-HSA standard, the undiluted sample solution (150 μl) and aliquots (150 μl) of up to nine 1:10 serial dilutions with 5 mM pH 7.4 PBS were incubated in test tubes at 37 °C for 1 h with antibody solution (150 μl) that was prepared by adding the required amount of protein G column purified antibody (0.294 μg/ml) in pH 7.4 PBS to 0.2% CEO in pH 7.4 PBS (2.8 μl/14 ml of 2% CEO). The remaining ELISA procedure and similar antibody binding inhibition studies with LGE_2-KLH antibody, LGE_2-BSA adduct as coating agent, and LGE_2-HSA as standard were performed as described previously (21).

Cross-reactivity of LGE_2-Lysine Lactam and Hydroxylactam—A mixture of LGE_2-lysine lactam and hydroxylactam adducts was obtained by incubating LGE_2 with [^{3}H]lysine (27,00 cpm/μg) under argon overnight at 37 °C. The mixture was applied to a C_{18} SepPak cartridge (Waters) that had been preconditioned with methanol (5 ml) and water (10 ml). The SepPak was washed with heptane (10 ml) and heptane/ethyl acetate (1:1, v/v, 10 ml) before elution with methanol/ethyl acetate (2:3, v/v, 10 ml). The eluate was dried, resuspended in 10% aqueous methanol, and subjected to HPLC (4.6 × 250 mm Macrosphere 300 C_{18} column

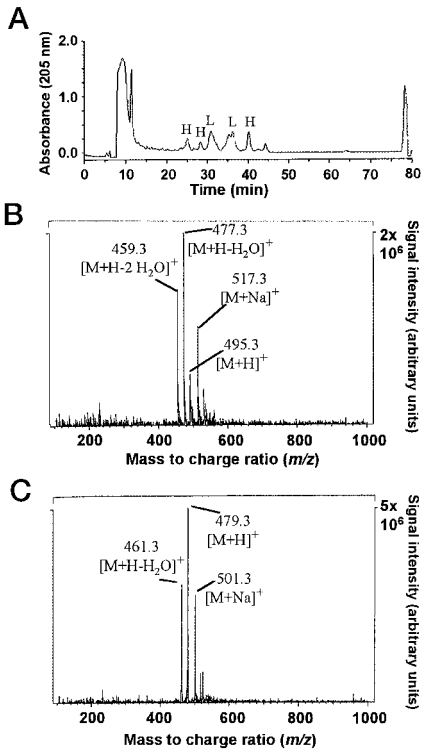

FIG. 4. **Analysis of LGE_2-lysine lactam and hydroxylactam adducts.** *A,* LGE_2-lysine adducts collected from solid phase extraction on a C_{18} Sep-Pak cartridge (Waters) were subjected to reverse phase HPLC. Compounds exhibiting absorbance at 205 nm were analyzed by LC/MS. Peaks corresponding to hydroxylactam adducts are labeled *H* and those corresponding to lactam adducts are labeled *L*. A typical mass spectrum of a hydroxylactam adduct is shown in *B* and contains the protonated molecular ion as well as ions resulting from sodium adduction and dehydration that occurs in the mass spectrometer. A typical spectrum of a lactam adduct is shown in *C*; it also displays ions corresponding to the molecular ion of the LGE_2-lysine lactam as well as ions resulting from sodium adduction and dehydration.

from Alltech; 10 min in 0.1% aqueous acetic acid, then 30% acetonitrile in 0.1% aqueous acetic acid; 1 ml/min). Fractions (1 ml) were collected and aliquots subjected to scintillation counting. Aliquots of fractions exhibiting UV absorbance at 205 nm (Fig. 4*A*) and containing radioactivity, which indicates the incorporation of lysine, were assessed by LC/MS (Fig. 4, *B* and *C*) by direct infusion using a Finnigan TSQ7000 spectrometer with the sheath gas held at 70 p.s.i., auxiliary gas at 10 p.s.i., and with 25 volts on the capillary, a capillary temperature of 220 °C, and the tube lens voltage at 90 V. Fractions deemed to contain only compounds with the molecular ion of the lactam (m/z 479.4) or hydroxylactam (m/z 495.4) adducts and were combined. None of these fractions contained detectable amounts of the LGE_2-derived pyrrole which would show $[MH]^+ = 463$, $[M + H - H_2O]^+ = 445$, or $[M + Na]^+ = 485$. The resulting mixture of lactam and hydroxylactam adducts was again analyzed by LC/MS, revealing no contamination by compounds with the molecular ion of the pyrrole adduct (m/z 463). The putative lactam and hydroxylactam adducts were also subjected to collision-induced dissociation, which resulted in fragmentation patterns consistent with the structures assigned to the compounds (19). A sample containing 50 ng each of LGE_2-lysine lactam and hydroxylactam was dissolved in PBS/EtOH (75 μl, 4:1, v/v) to give 140 pmol/well as final concentration. LGE_2-BSA was used as coating agent and LGE_2-HSA was used as standard to measure cross-reactivity. The ELISA was done as under "Competitive Antibody Binding Inhibition Studies."

Autoxidation of Polyunsaturated Fatty Acids (PUFAs) in the Presence of HSA—Fatty acid (2 mg) and HSA (30 mg, 0.45 μmol) were dissolved in 0.1 M PBS (10 ml). Autoxidation was started by addition of 20 mM sodium ascorbate (510 μl) and 0.8 mM $FeSO_4 \cdot 7H_2O$ (510 μl) (42). The solutions were incubated at 37 °C for 24 h under air. After incubation the reaction was quenched by adding 1 mM EDTA (200 μl) to each solution which then was dialyzed against pH 7.4 PBS (2 × 2 liters) for 40 h at room temperature. Samples of PUFAs that had been oxidized in the presence of HSA were analyzed for LGE_2-, iso[4]LGE_2-, and HNE-derived epitopes by ELISAs using LGE_2-KLH (27), iso[4]LGE_2-KLH, or ON-KLH (36) antibodies, respectively, in pH 7.4 PBS containing 0.001% Tween™ 20 and 0.2% CEO.

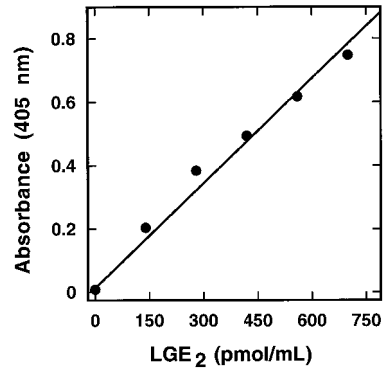

FIG. 5. **A standard curve for LGE_2 in an LGE_2-trapping ELISA.**

LGE_2- and Iso[4]LGE_2-Protein Immunoreactivity in OxLDL—ELISA of oxLDL was performed the same as the inhibition assays, except a dilution factor of 0.3 was employed. The starting concentration was the undiluted samples. The time dependence of appearance of protein-bound LGE_2- and iso[4]LGE_2-derived epitopes during oxidation of LDL was determined as described in our previous study of LGE_2-pyrrole generation during oxidation of LDL (27).

Trapping ELISA Detection of Free LGE_2—To detect any free LGE_2 that may be released upon oxidation of LDL, a trapping ELISA was done on the ultrafiltrate from oxLDL. Thus, LDL (0.5 mg/ml) was dialyzed at 5 °C for 5 h against pH 7.4 PBS (4 liters), and then for 12 h against fresh buffer (4 liters). The LDL was then incubated at 37 °C with 10 μM $CuSO_4$. The reaction product mixture was then filtered using an Ultrafree-CL filter unit (NMWL: 10,000) for 3 h in a Beckmann centrifuge at 5 °C and 4,000 rpm. Each well of a microtiter plate was coated with 100 μl of BSA (1 mg/ml) in pH 7.4 PBS and was incubated at 37 °C for 1 h. Following washing once with PBS, samples for a standard curve containing LGE_2 (0–35 pmol/well), or the filtrate from oxLDL were added to the wells (100 μl/well). After incubation for 3 h at 37 °C followed by washing once with PBS, each well was filled with 300 μl of 1% CEO for 1 h at 37 °C. After washing once with 0.1% CEO, 100 μl of KLH-LGE_2 antibody was added to each well and the plate was gently shaken for 1 h at room temperature. After three washes with 0.1% CEO, 100 μl/well of goat anti-rabbit IgG-alkaline phosphatase (1:1,000) was added and the mixture was incubated for 1 h at room temperature. After washing three times with 0.1% CEO, 100 μl of disodium *p*-nitrophenyl phosphate (10 mg) in water (11 ml, pH adjusted to 9.6 using NaOH) containing glycine (50 mM) and $MgCl_2$ (1 mM) were added and the resulting mixture was incubated for about 20 min at room temperature. The reaction was terminated by adding 3 M NaOH (50 μl) to each well, and the absorbance was read at 405 nm on a micro-ELISA plate reader. A standard curve, constructed from absorbance data for solutions containing 0–35 pmol/well of LGE_2 (Fig. 5) showed a linear increase in absorbance with LGE_2 concentration in the standard solutions. No absorbance was observed for any of the wells treated with ultrafiltrate from oxLDL.

RESULTS

Synthesis of Iso[4]LGE_2-protein Adducts—Iso[4]LGE_2 is a chemically sensitive vinylogous β-hydroxy aldehyde that was freshly prepared for reaction with proteins (BSA, HSA, and KLH) to afford iso[4]LGE_2-protein adducts. We previously showed that for high LGE_2/protein ratios, Paal-Knorr condensation of LGE_2 with ε-amino groups of lysyl residues of proteins gives mainly LGE_2-derived protein-bound pyrrole (21). Earlier studies also demonstrated that quantitative analysis of LGE_2-derived protein-bound pyrroles can be accomplished using the Ehrlich assay that measures the absorbance of a blue-green chromophore generated by the condensation of LGE_2-pyrrole with DMAB (15).

For the present study, iso[4]LGE_2-protein adducts, rich in iso[4]LGE_2-pyrrole, were prepared by exposing various proteins to an excess of iso[4]LGE_2. The levels of protein-bound iso[4]LGE_2-derived pyrrole in these adducts were determined

TABLE I
Ehrlich pyrrole assay of iso[4]LGE$_2$-protein adducts

Sample	Protein by BCA assay	Protein lysyl[a] groups	Iso[4]LGE$_2$ added	Pyrrole by Ehrlich assay	Pyrrole/protein	Pyrrole/protein
	mg	*μmol*	*μmol*	*μmol*	*μmol/mg*	*mol/mol*
Iso[4]LGE$_2$-BSA	11.1	9.86	3.69	3.54 ± 1.29	0.32	21 ± 8
Iso[4]LGE$_2$-KLH	6.25	3.13	4.54	2.62 ± 0.32	0.42	1257 ± 153
Iso[4]LGE$_2$-HSA	18.1	16.1	7.10	8.26 ± 0.19	0.46	30 ± 1

[a] The molecular weight (43) of KLH is taken to be 3×10^6 and number of lysyl residues in KLH, determined by TNBS assay, is 0.5 μmol/mg (44). For both BSA (FW = 66,430) and HSA (FW 66,437) the number of lysyl groups were calculated using the value of 59 mol of Lys/mol of protein.

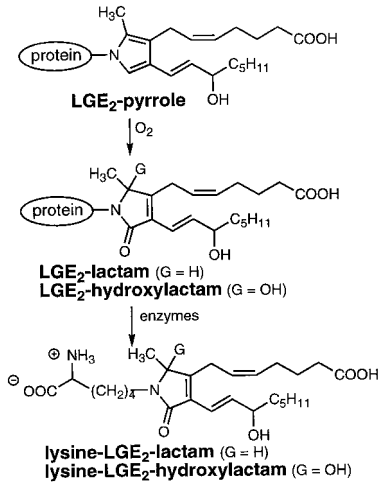

FIG. 6. **Oxidative conversion of LGE$_2$-pyrrole into lactam and hydroxylactam derivatives.**

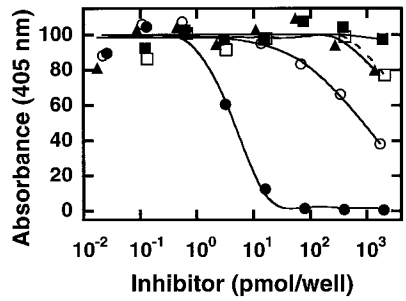

FIG. 7. **Inhibition curves showing cross-reactivity of iso[4]LGE$_2$-KLH antibody for iso[4]LGE$_2$-HSA (●), 4-oxopentanal-BSA (▲), LGE$_2$-HSA (○), LDL (□), and HSA (■) against iso[4]LGE$_2$-BSA as coating agent.**

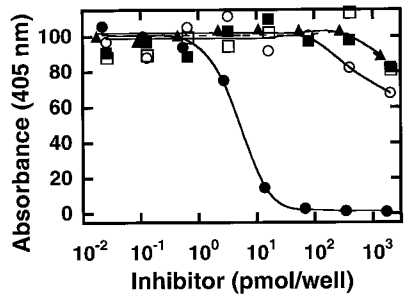

FIG. 8. **Inhibition curves showing cross-reactivity of LGE$_2$-KLH antibody for iso[4]LGE$_2$-HSA (○), 4-oxopentanal-BSA (▲), LGE$_2$-HSA (●), LDL (□), and HSA (■) against LGE$_2$-BSA as coating agent.**

by Ehrlich assays (40, 41) using LGE$_2$-protein adducts as standards since the availability of radiolabeled LGE$_2$ allowed an accurate independent assessment of LGE$_2$ content in these standard samples. As expected, the chromophore generated by the condensation of iso[4]LGE$_2$-pyrrole with DMAB is very similar to that from LGE$_2$-pyrrole. Thus, the absorption maxima (λ_{max}) observed for the LGE$_2$-HSA-DMAB and iso[4]LGE$_2$-BSA-DMAB chromophores are 586 and 584 nm, respectively. It is reasonable to presume that the structurally similar Ehrlich chromophore derived from an iso[4]LGE$_2$-pyrrole has the same extinction coefficient as that derived from an LGE$_2$-pyrrole.

A linear correlation was obtained for a plot of pyrrole concentration verses absorbance at 586 nm for the DMAB chromophore of LGE$_2$-derived protein-bound pyrroles in LGE$_2$-BSA and LGE$_2$-HSA (see Fig. 2). The concentration of LGE$_2$-derived protein-bound pyrrole in LGE$_2$-HSA was taken to be equal to the total amount of protein-bound LGE$_2$ (0–500 nmol/sample) as determined by quantitative radiochemical analysis. This assumes a quantitative yield for pyrrole formation. Therefore, the use of LGE$_2$-protein-derived pyrrole as a standard for the Ehrlich assay provides an upper limit for the concentration of iso[4]LGE$_2$-protein-derived pyrrole. The final protein concentrations in iso[4]LGE$_2$-protein adducts were determined by BCA protein assay (39) and the ratios of iso[4]LGE$_2$-pyrrole to protein were calculated (Table I).

Lactam, and Hydroxylactam Epitopes in LGE$_2$-Protein Adducts—Studies detailed elsewhere (19), employing mass spectral detection of lipid-modified lysine to characterize epitopes generated by covalent adduction of LGE$_2$ with proteins, uncovered oxidative modifications that append one or two atoms of oxygen to protein-bound LGE$_2$-derived pyrroles. Thus, while LGE$_2$-lysine adduct containing the expected lysine-LGE$_2$-pyrrole could be prepared if oxygen is rigorously excluded, exposure to air or enzymatic proteolysis of LGE$_2$-protein adduct produced only mono- and dioxygenated lysine-LGE$_2$-pyrrole. These oxidized pyrroles almost certainly are lactams and hydroxylactams (Fig. 6) generated by well known free radical-initiated reactions of molecular oxygen with electron-rich pyrroles (45, 46).

These oxidized derivatives of LGE$_2$-pyrrole cross-react strongly with LGE$_2$-KLH antibodies. Thus, a sample containing a mixture of lysine-LGE$_2$-lactam and lysine-LGE$_2$-hydroxylactam was isolated by HPLC from a Paal-Knorr condensation of LGE$_2$ with radiolabeled lysine and subsequent oxidation by adventitous oxygen. Quantitative radiochemical analysis in conjunction with an ELISA comparison of binding with LGE$_2$-KLH antibodies, showed 256% cross-reactivity for the hydroxylactam-lactam mixture relative to the LGE$_2$-HSA standard.

Specificity of LGE$_2$- and Iso[4]LGE$_2$-KLH Antibodies—Structural specificities were also examined for the LGE$_2$-KLH and iso[4]LGE$_2$-KLH antibodies to selectively recognize the LGE$_2$-HSA and iso[4]LGE$_2$-HSA standards, respectively. ELISA binding inhibition studies for cross-reactivity of iso[4]LGE$_2$-KLH antibody (Fig. 7) and LGE$_2$-KLH antibody (Fig. 8) with various haptens demonstrated excellent specificity for both antibodies. Thus, neither antibody recognizes a protein-bound

TABLE II
LGE_2-KLH and Iso[4]LGE_2-KLH antibody specificity

Antibody Coating	LGE_2-KLH LGE_2-BSA		Iso[4]LGE_2-KLH Iso[4]LGE_2-BSA	
Protein or epitope	IC_{50}	Cross-reactivity	IC_{50}	Cross-reactivity
	pmol/well	%	*pmol/well*	%
LGE_2-HSA	4.85	100	845	0.5
Iso[4]LGE_2-HSA	10,000	<0.1	4.2	100
Native LDL or HSA	ND[b]	ND	ND	ND
HNE-HSA[a]	ND	ND	8172	<0.1
HNE-HSA-reduced[a]	ND	ND	13087	<0.1
MDA-HSA[a]	ND	ND	ND	ND

[a] Prepared as described previously (38).
[b] ND, not detected.

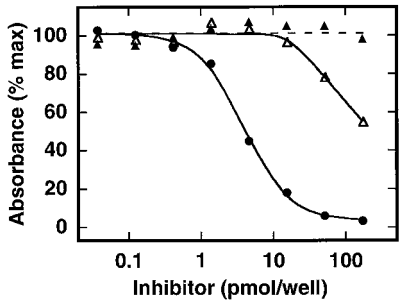

FIG. 9. **Inhibition curves for binding of anti-LGE_2-KLH to LGE_2-BSA by LGE_2-HSA standard (●), LGE_2-HSA generated during the oxidation of AA (△), but not LA (▲), in the presence of HSA.**

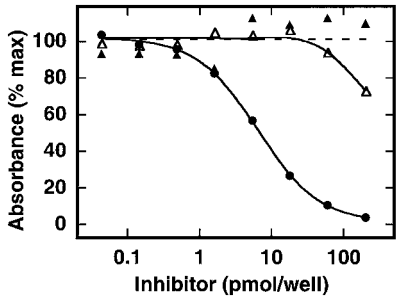

FIG. 10. **Inhibition curves for binding of anti-iso[4]LGE_2-KLH to iso[4]LGE_2-BSA by iso[4]LGE_2-HSA standard (●), iso[4]LGE_2-HSA generated during the oxidation of AA (Δ), but not LA (▲), in the presence of HSA.**

2-methylpyrrole, 4-oxopentanal-BSA (36), that lacks prostanoid or isoprostanoid side chains. The data presented in Table II establish that each of these antibodies shows outstandingly low cross-reactivity toward protein adducts of the structurally isomeric levulinaldehyde derivative. Thus, the LGE_2-KLH antibodies bind LGE_2-HSA 200 times more strongly than they bind iso[4]LGE_2-HSA, while the iso[4]LGE_2-KLH antibodies bind iso[4]LGE_2-HSA at least 2000 times more strongly than they bind LGE_2-HSA. Furthermore, cross-reactivity of either antibody toward HSA, native LDL, or HSA adducts of (*E*)-4-hydroxy-2-nonenal (HNE) or malondialdehyde (MDA) was not detected.

Generation of LGE_2-HSA and Iso[4]LGE_2-HSA Immunoreactivity by Fe^{2+}-catalyzed Oxidation of AA but Not Linoleic Acid (LA) or Docosahexaenoic Acid (DHA)—In vitro free radical oxidations of a variety of PUFAs with iron and ascorbate were performed in the presence of HSA. Immunoreactive protein-bound epitopes were detected by ELISAs with LGE_2-KLH (Fig. 9) and iso[4]LGE_2-KLH (Fig. 10) antibodies in the reaction product mixture from AA but not in the reaction product mixture from LA. Similar experiments with γ-linolenic (γ-LA), DHA, ETA, and EPA acids revealed the generation of protein epitopes that cross-react with LGE_2-KLH and iso[4]LGE_2-KLH antibodies from γ-LA, ETA, and EPA, but not DHA (see below).

LGE_2-Protein and Iso[4]LGE_2-Protein Adduct Immunoreactivity in OxLDL—LDL was oxidized by dialyzing an aqueous solution of LDL in air against a buffer containing Cu^{2+}, an *in vitro* model (2) for physiological oxidation of LDL. Oxidation was halted after various time periods by sequestration of Cu^{2+} with Na_2EDTA added to an aliquot of the reaction mixture. After an induction period, during which the endogenous antioxidants presumably were consumed, immunoreactivity toward both LGE_2-KLH (Fig. 11) and iso[4]LGE_2-KLH (Fig. 12) antibodies increased rapidly, reaching a plateau after several hours. The immunoreactivity detected for LGE_2-protein and iso[4]LGE_2-protein epitopes in the oxLDL corresponded to a final ratio of 1:4, respectively.

Free LGE_2 Is Not Present in OxLDL—A trapping ELISA was used detect any free LGE_2 that might be present in the reaction product mixture generated by *in vitro* oxidation of LDL in the presence of Cu^{2+}. Free LGE_2 can be trapped by the protein coating agent (BSA) to give immunoreactive LGE_2-pyrrole epitope. Thus, a linear increase in absorbance was found for increasing concentrations of free LGE_2 (see Fig. 5). However, the wells treated with ultrafiltrate from oxLDL showed no absorbance, indicating that they contained no free LGE_2.

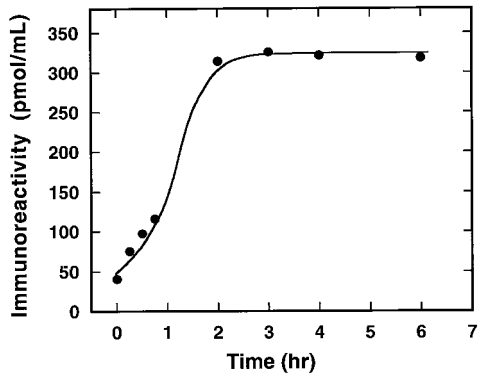

FIG. 11. **Generation of LGE_2-protein adducts by oxidation of LDL (0.5 mg/ml) with Cu^{2+} (10 μM).**

DISCUSSION

IsoLGs—The chemistry of LDL oxidation is quite complex. A plethora of lipid oxidation products is generated, and some of these covalently modify LDL protein, apolipoprotein (apo) B (3, 4). Two aldehydic fragmentation products, MDA and HNE, have been studied extensively because they form adducts with apoB, and because the MDA-LDL (47) and HNE-LDL (48) adducts could be atherogenic, in contrast with native LDL. Besides protein-bound HNE, free HNE is detectable in oxLDL. In a recent study, free HNE was quantitatively analyzed by an "HNE-trapping ELISA" based on the detection of epitopes generated when HNE is trapped by a protein that has been coated

onto an immunoplate (49). This study demonstrated that a considerable amount of free HNE is released from human plasma LDL upon Cu^{2+}-promoted oxidation. In contrast, employing an analogous LGE_2-trapping-ELISA, we now find *no evidence for the presence of free LGE_2* in LDL that has undergone Cu^{2+}-promoted oxidation. This is expected because, as we have noted elsewhere (8, 19), LGE_2 binds with proteins far more avidly than HNE. There is a physiological steady-state concentration of free HNE in human venous blood plasma (50, 51). In contrast, the generation of LGs and iso[*n*]LGs *in vivo* must be inferred from detection of protein-bound derivatives. Studies employing the new iso[4]LGE_2-KLH antibodies to detect iso[4]LGE_2-derived protein epitopes *in vivo* are in progress. Preliminary results show that these protein modifications are present in human blood plasma, confirming the hypothesis that a family of levulinaldehyde derivatives is generated *in vivo* by a free radical-induced oxidation of AA-PC (Fig. 13). Thus, nonregioselective hydrogen atom abstraction from the 7, 10, and 13 positions of an arachidonyl ester produces three regioisomeric pentadienyl radicals. These then react with molecular oxygen to afford four regioisomeric peroxyeicosatetraenoyl radicals that undergo peroxy radical cyclization (28, 52) to deliver four structurally isomeric endoperoxides. Besides the geometrically enforced *cis* relationship of the endoperoxide oxygens and a preference for peroxy radical cyclization to produce stereoisomers with *cis* disposed side chains (28), each structurally isomeric endoperoxide is expected to be generated as a mixture of 16 stereoisomers that are referred to collectively as isoPGH_2 or iso[*n*]PGH_2 where [*n*] specifies the number of carbon atoms in the carboxyl side chain of the non-prostanoid structural isomers. Each endoperoxide rearranges to form two structurally isomeric isoLGs or iso[*n*]LGs, designated as E series if the acetyl substituent is nearer than the formyl substituent to the carboxyl group or as D series if the formyl is nearer than the acetyl to the carboxyl.

Paal-Knorr condensation of the eight structurally isomeric

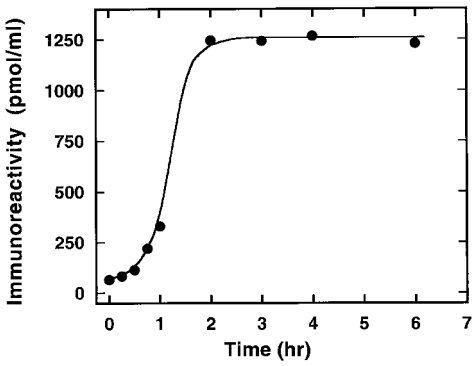

FIG. 12. **Generation of iso[4]LGE_2-protein adducts by oxidation of LDL (0.5 mg/ml) with Cu^{2+} (10 μM).**

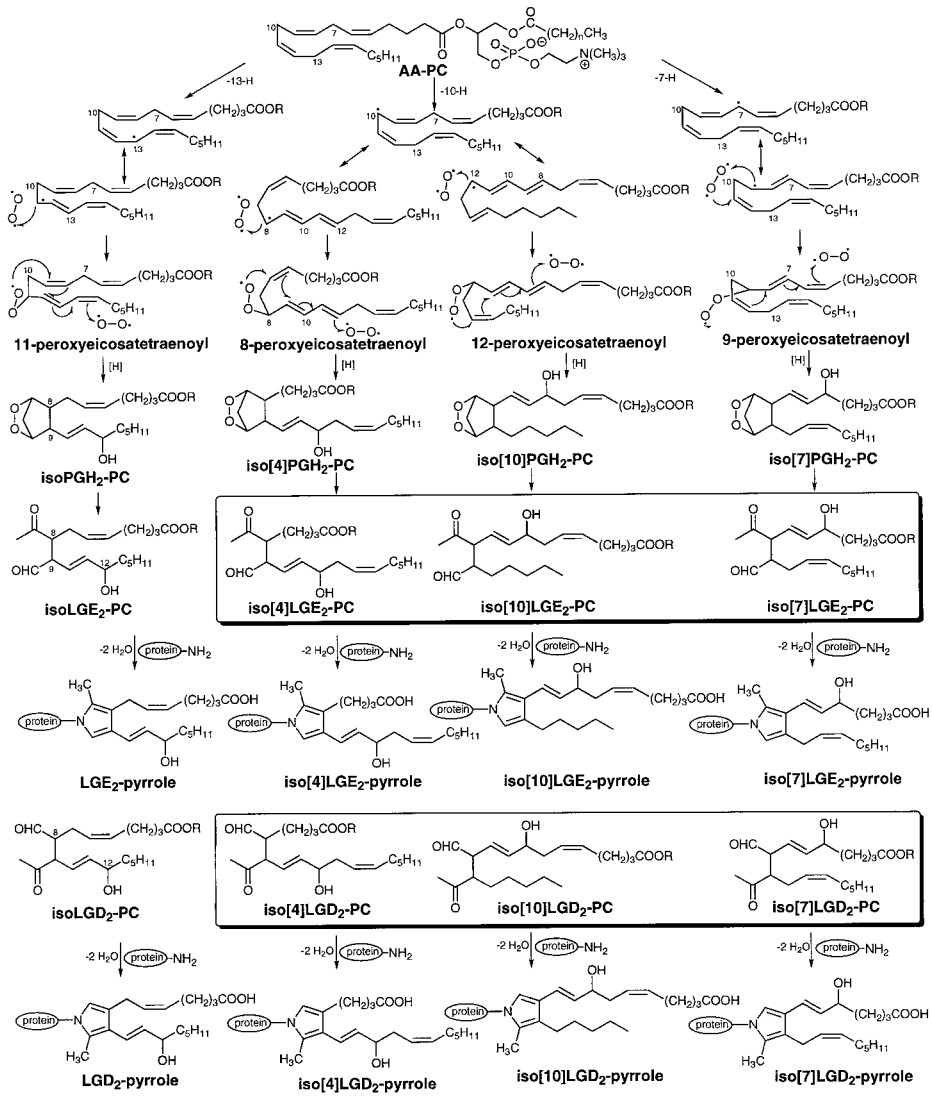

FIG. 13. **The iso[*n*]LG cascade of levulinaldehyde derivatives generated by free radical-induced oxidation of AA-PC.**

isoLGs and iso[*n*]LGs with protein primary amino groups produces eight different pyrrole epitopes. We previously reported chemical evidence for the initial formation of pyrroles that incorporate the ϵ-amino group of protein lysyl residues (15). Our recent studies employing mass spectral detection of lipid-modified lysine uncovered the facile oxidation of LGE_2-derived pyrroles leading to lactam and hydroxylactam derivatives, and confirmed that isoLG-derived lysyl group modifications are present in oxLDL (19). Those studies also demonstrated the formation of LGD_2 epimers in the free radical-induced oxidation of AA. Since LGD_2-protein and LGE_2-protein adducts can be produced by the enzymatic COX pathway, only detection of iso[*n*]LG-protein adducts, *i.e.* with nonprostanoid side chains, can provide unambiguous evidence for the operation *in vivo* of the free radical-promoted oxidative pathway summarized in Fig. 13. We now have two orthogonal polyclonal rabbit antibodies, *i.e.* that recognize and strongly discriminate between, LGE_2-protein and iso[4]LGE_2-protein adducts.

As expected, LGE_2-protein and iso[4]LGE_2-protein immunoreactivity are produced by free radical oxidation of AA but not LA, the most abundant polyunsaturated fatty acid in LDL. We now have a panel of five antibodies that specifically detect epitopes produced by the adduction of different lipid oxidation products with proteins (Table III). In the reaction product mixture from *in vitro* oxidation of AA in the presence of HSA, pyrrole epitopes derived from HNE and 5-hydroxy-8-oxo-6-octenoic acid (HOOA) were detected previously. HNE-pyrrole was also detected in the reaction product mixture from *in vitro* oxidation of LA in the presence of HSA. On the other hand, pyrrole epitopes derived from 9-hydroxy-12-oxo-10-dodecenoic acid (HODA) are a selective marker for LA oxidation in the presence of protein.

HNE-pyrrole epitope (detected with ON-KLH antibody) was generated in the oxidation of ω-6 but not ω-3 PUFAs in the presence of HSA. Thus, the ω-6 acids LA, γ-LA, AA, and ETA all afforded immunoreactivity detectable with ON-KLH antibodies while the ω-3 acids DHA and EPA did not (Table IV). Although LA and AA are the major PUFAs in normal human serum phospholipids, oxidative cleavage of ETA *in vivo* may produce significant amounts of HNE.

Oxidation of γ-LA, ETA, and EPA in the presence of HSA produces protein epitopes that cross-react with LGE_2-KLH and iso[4]LGE_2-KLH antibodies. The levels of ETA and EPA in human LDL vary greatly with diet (Table IV) and, therefore, the LDL from some individuals can contain levels of these PUFAs that may contribute significantly to the generation of LGE_2-KLH or iso[4]LGE_2-KLH immunoreactivity. The selective generation of iso[4]LGE_2-KLH immunoreactivity from γ-LA, LGE_2-KLH immunoreactivity from ETA, and both LGE_2-KLH and iso[4]LGE_2-KLH immunoreactivity from EPA (Table IV) is a reasonable consequence of the fact that only a close structural analogue of iso[4]LGE_2 is expected to be generated upon oxidation of γ-LA, a LGE_2 analogue upon oxidation of ETA, and analogues of both iso[4]LGE_2 and LGE_2 upon oxidation of EPA (Fig. 14).

LGE_2 and Iso[4]LGE_2 Epitope Families—Owing to concerns that LG-derived protein-bound pyrroles would be readily modified by oxidation, our earliest efforts to detect LGE_2-derived protein epitopes immunologically relied upon cross-reactivity of those epitopes with antibodies raised against a stable pyrazole isostere-derived antigen (53). Quite unexpectedly, the immunoreactivity generated by the reaction of LGE_2 with proteins showed no decrease over several weeks. While this could be the result of some stabilizing influence of the protein matrix on an otherwise readily oxidizable pyrrole hapten, we recognized the possibility of an alternative explanation. Thus, if the molecular fragment responsible for antibody recognition is preserved in secondary products derived from the initially formed pyrroles, *e.g.* the corresponding lactam or hydroxylactam (Fig. 6), in particular two prostanoid side chains appended to neighboring sp^2 carbons on a five-membered ring, then large changes in antibody binding need not accompany transformations of the LGE_2-pyrrole into these secondary products. Thus, in contrast with the excellent discrimination for variations in the side chains appended to the pyrrole ring at positions 3 and 4, both the LGE_2-KLH and iso[4]LGE_2-KLH antibodies could show a high tolerance for modifications at the 2 and 5 positions of the pyrrole ring. Furthermore, the LGE_2-pyrrole and iso[4]LGE_2-pyrrole antigens most probably were oxidized after administration to rabbits, and therefore, some or all of the LGE_2-KLH and iso[4]LGE_2-KLH antibodies in the polyclonal mixtures were raised against lactam or hydroxylactam epitopes. Since the side chains on the pyrrole, lactam, and hydroxylactam epitopes are appended to coplanar sp^2-hybridized carbons, they are restricted to the same coplanar geometry. This conformational rigidity is probably responsible for the excellent discrimination by LGE_2-KLH and iso[4]LGE_2-KLH antibodies for LGE_2- and iso[4]LGE_2-derived haptens, respectively. Thus, although the *functionality* in the side chains of LGE_2- and iso[4]LGE_2-protein adducts is the same, the different lengths of the side chains

TABLE III
Oxidation of LA or AA in the presence of HSA

LA (7.13 mmol) or AA (6.6 mmol) and HSA (0.45 mmol) in pH 7.4 PBS (0.1 M) were incubated at 37 °C in the presence of ascorbate (0.9 mM) and $FeSO_4$ (37 μM) for 24 h under air.

Antibody	Immunoreactivity (pmol/ml)		Hapten, Protein adduct
	LA oxidation	AA oxidation	
LGE_2-KLH	ND[a] (−)	313 (+)	LGE_2-pyrrole
Iso[4]LGE_2-KLH	ND (−)	475 (+)	IsoLGE_2-pyrrole
DODA-KLH	44 (+)	ND (−)	HODA-pyrrole
DOOA-KLH	ND (−)	2 (+)	HOOA-pyrrole
ON-KLH	276 (+)	390 (+)	HNE-pyrrole

[c] ND, not detected.

TABLE IV
Immunoreactivity (% of value for AA) generated by oxidation of PUFAs in the presence of HSA

PUFA (2.0 mg) and HSA (0.45 μmol) in pH 7.4 PBS (0.1 M, 10 ml) were incubated 24 h at 37 °C in the presence of $FeSO_4$ (37 μM) and ascorbate (0.9 mM) under air. Immunoreactivity is relative to HSA-iso[4]LGE_2, HSA-LGE_2, and HSA-ON standards.

PUFA	Antibody			Composition	
	Iso[4]LGE_2-KLH	LGE_2-KLH	ON-KLH	LDL[a]	Serum[b]
LA	ND[a]	ND	71	17 (19.6)	22.9
γ-LA	23	ND	37		0.1
Docosahexaenoic	ND	ND	ND	2.7 (4.8)	2.2
AA	100	100	100	4.8 (7.1)	11.0
ETA	ND	23	14	10 (1.4)	3.1
EPA	17	16	ND	0.9 (5.6)	0.7

[a] Weight percent in LDL phospholipids calculated from data for individuals on a corn oil (fish oil) diet (56).
[b] Weight percent in normal human serum phospholipids (20).
[c] ND, not detected.

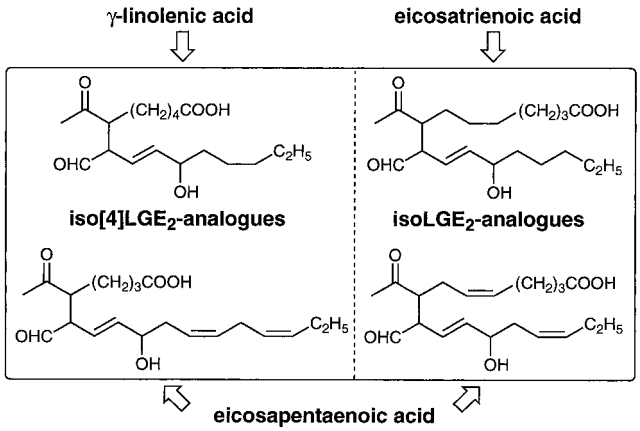

FIG. 14. **Structural analogues of iso[4]LGE_2 and LGE_2 derivable from γ-LA, ETA, and EPA.**

and restriction of conformational possibilities for their disposition results in strong but geometrically different interactions of the polar functional groups in each side chain with the respective antibodies.

Quantitative Analysis of LGE_2 and Iso[4]LGE_2—Previously, we used quantitative radiochemical analysis to accurately determine the amount of LGE_2 contained in protein adduct standards. Because radiolabeled iso[4]LGE_2 is not presently available, we had to employ a less direct method to determine the amount of iso[4]LGE_2-derived pyrrole present in the iso[4]LGE_2-KLH antigen, iso[4]LGE_2-BSA coating agent, and iso[4]LGE_2-HSA standard. While the Ehrlich assay is not sensitive enough to detect the low concentrations of iso[4]LGE_2-derived pyrroles present in human blood or generated upon oxidation of LDL, it was feasible to use this assay to compare the concentrations of LGE_2-derived and iso[4]LGE_2-derived pyrroles in the protein adducts prepared as standards. The iso[4]LGE_2 to protein ratios, *i.e.* 21, 30 and 1257 mol/mol, calculated for the BSA, HSA, and KLH adducts, are higher than found previously for analogous LGE_2-protein adducts, *i.e.* 10.5, 11.9, and 951 mol/mol of BSA, HSA, and KLH (21). Furthermore, in an earlier study, when BSA was exposed to a large excess (125 equivalents) of tritium-labeled LGE_2, one molecule of BSA was found to bind a maximum of about 16 molecules of LGE_2 (14). It seems reasonable to expect that a similar limit would apply to binding of iso[4]LGE_2, Especially important is the concentration of iso[4]LGE_2-pyrrole determined for iso[4]LGE_2-HSA because this standard was used to calculate the amount of iso[4]LGE_2-pyrrole in oxLDL samples. The 30:1 ratio determined indirectly by Ehrlich assay for iso[4]LGE_2-HSA seems to overestimate the actual levels by factor of two. The concentrations of iso[4]LGE_2-protein adduct indicated in the figures and tables must be interpreted in light of this caveat.

Possible Etiological Importance of LGs and Iso[n]LGs in Artheriosclerosis—With mouse peritoneal macrophages, we previously showed that the covalent adduct of LGE_2 with human LDL (LGE_2-LDL) is internalized and degraded if the molar ratio of LGE_2 to LDL protein, apoB, exceeds a threshold somewhere between 10:1 and 38:1 (8). OxLDL, but not acetyl-LDL that is recognized by the prototypical scavenger receptor, efficiently competed for receptor binding and uptake of LGE_2-LDL. This result suggests that LGE_2-LDL was recognized by a class of scavenger receptor that demonstrated ligand specificity for oxLDL but not for acetyl-LDL. However, our previous study of LDL oxidation found that only 1–2 mol of LGE_2-protein adduct are generated per mole of apoB (27). Nevertheless, it is reasonable to anticipate that macrophage recognition of iso[*n*]LG-LDLs will be similar to that of LGE_2-LDL, and that total levels of LG and iso[*n*]LG protein adducts in oxLDL are sufficient to account for the recognition and uptake of oxLDL by human monocyte-macrophages in the arterial wall, a key step in the etiology of atherosclerosis. Thus, substantial evidence now suggests that atherosclerotic plaques form when monocytes are recruited into the arterial intima to become macrophages where they grow into bloated, lipid-laden foam cells by accumulating large amounts of oxLDL (1, 9, 54).

Studies on the localization of immunoreactive LG-protein and iso[4]LG-protein epitopes in human atherosclerotic plaques are in progress in our laboratories. The details of these studies will be reported in due course. Since deficient processing of oxLDL in macrophages leads to foam cell formation, it is especially noteworthy that processing of LGE_2-LDL exhibits an inefficiency similar to that found for oxLDL and, therefore, that incompletely processed LGE_2-LDL accumulates in macrophages (8). The resistance to lysozomal degradation of oxLDL which accumulates in macrophages may be a consequence of continued oxidative modification or aggregation of the particles which occurs *following* uptake (55). In this regard, it is especially pertinent that LGE_2 binds avidly (within minutes) with proteins (14), and the reaction of LGE_2 with proteins generates reactive electrophilic intermediates that are responsible for a slower process, protein-protein cross-linking (14, 17). In other words, LG- and iso[*n*]LG-protein adducts are expected to be "sticky," readily forming protein-protein cross-links by binding to additional protein-based nucleophiles. It is tempting to speculate that such cross-links with proteolytic enzymes interfere with processing of oxLDL.

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Protein Adducts of Iso[4]levuglandin E_2, a Product of the Isoprostane Pathway, in Oxidized Low Density Lipoprotein

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