Protein Adducts of Iso[4]levuglandin E₂, a Product of the
Isoprostane Pathway, in Oxidized Low Density Lipoprotein*

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Levuglandin (LG) E₂, a cytotoxic seco prostanoid acid
co-generated with prostaglandins by nonenzymatic re-
arrangements of the cyclooxygenase-derived endoperox-
ide, prostaglandin H₂, avidly binds to proteins. That
LGₕ-protein adducts can also be generated nonenzy-
matically is demonstrated by their production during
free radical-induced oxidation of low density lipopro-
tein (LDL). Like oxidized LDL, LGₕ-LDL, but not native
LDL, undergoes receptor-mediated uptake and im-
paired processing by macrophage cells. Since radical-
induced lipid oxidation produces isomers of prosta-
glandins, isoprostanes (isoPs), via endoperoxide
intermediates, we postulated previously that a similar
family of LG isomers, isoLGS, is cogenerated with isoPs.
Now iso[4]LGₕ-protein epitopes produced by radical-
induced oxidation of arachidonic acid in the presence of
protein were detected with an enzyme-linked immu-
noassay. Iso[4]LGₕ-protein epitopes are also
generated during free radical-induced oxidation of LDL.
All of the LG₂ 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 biologi-
cal consequences beyond their obvious potential as
markers for specific arachidonate-derived protein mod-
fications that may be of value for the quantitative as-
essment 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 polysaturated
fatty esters and concomitantly 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 unregu-
lated and processing of oxLDL is inefficient, the macrophages
become lipid-laden foam cells, progenitors of atherosclerotic plaques (9). To acquire a fundamental molecular level understand-
ing of atherogenesis and other biological sequelae of oxida-
tive 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. LG₂
(Fig. 1), these seco prostanoids are cogenerated with pros-
taglandins (PGs) (11–13) by rearrangements of the endoperox-
disposed side chains of peroxo radical cyclization generates an isomeric
LGE₂-PC. However, be-

Omits Salomon, R. G. Som, M. J. Roberts, II, and J. A. Oates, submitted for publication.

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, cyclooxyge-

nase; DMAB, p-(N,N-dimethylamino)benzaldehyde); ELISA, enzyme-
linked immunosorbent assay; EPA, eicosapentaenoic acid; ETA, eico-
satrienioic acid; γ-LA, γ-linolenic acid; HNE, (E)-4-hydroxy-2-nonenal;
HODA, 9-hydroxy-12-oxo-10-dodecenoc acid; HOHA, 5-hydroxy-8-oxo-
6-ocenolic acid; HSA, human serum albumin; iso-LGs, isolevuglan-
dins; isoPs, isoprostanes; KLH, keyhole limpet hemocyanin; LG,
levuglandin; LA, linoleic acid; MDA, malondialdehyde; ON, 4-ox-
onoanal; oxLDL, oxidized LDL; PBS, phosphate-buffered saline; PC,
2-lysophosphatidylcholine; PUFA, polyunsaturated fatty acid; PG, pros-
taglandins; HPLC, high performance liquid chromatography; LC/MS,
liquid chromatography mass spectrometry.

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HODA, 9-hydroxy-12-oxo-10-dodecenoc acid; HOHA, 5-hydroxy-8-oxo-
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onoanal; oxLDL, oxidized LDL; PBS, phosphate-buffered saline; PC,
2-lysophosphatidylcholine; PUFA, polyunsaturated fatty acid; PG, pros-
taglandins; HPLC, high performance liquid chromatography; LC/MS,
liquid chromatography mass spectrometry.
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 LGE2-pyrrole as that formed by the cyclooxygenase pathway (Fig. 1).

Mouse peritoneal macrophages internalize and degrade LGE2-LDL if the molar ratio of LGE2 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 LGE2-LDL, supporting the conclusion that both LGE2-LDL and oxLDL are recognized by the same receptor. However, the ratio of LGE2 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 non-regioselectively 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-peroxyeicosatetraenoil radical could lead to iso[4]PGH2-PC and then iso[4]LGE2-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 PGH2 by free radical-induced oxidation was postulated previously to account for the formation of isoprostanes (24, 25). Thus, iso[4]PGH2-PC (12-H2-IsoP) is also the putative precursor of isoprostanes that have been designated 12-F2-IsoP, 12-E2-IsoP, and 12-D2-IsoP (33). In analogy with the chemistry of LGE2, we expected that iso[4]LGE2-PC would form iso[4]LGE2-pyrrole by covalent adduction to proteins and concomitant phospholipolysis (Fig. 1). We now report confirmation of this hypothesis. Thus, the generation of iso[4]LGE2-protein epitopes during \textit{in vitro} nonenzymatic free radical-induced oxidation of LDL was detected with an immunoassay using antibodies raised against an iso[4]LGE2-protein adduct. Since iso[4]LGE2 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<sub>r</sub> cutoff 14,000 number 2) for dialysis was obtained from Fisher Scientific Co. The following commercially available materials were used as received: AA, docosaheaxenoeic acid (DHA), eicosapentaenoic acid (EPA), eicosaatetraenoic 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 0.2M NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, 3.0 M NaCl, and 0.02% NaN<sub>3</sub> (w/w). This solution was diluted 20 times as needed. LGE2 (34), iso[4]LGE<sub>2</sub> (35), and 4-oxopentanal-BSA (36) were prepared as described previously. LDL was isolated (37) from human plasma and oxidized \textit{in vitro} to give oxLDL as described previously (27). HNE-HSA, NaCNBH<sub>3</sub>-reduced HNE-HSA, and MDA-HSA were prepared as described previously (38). ON-KLH antibodies (36) and LGE<sub>2</sub>-KLH antibodies (27) were prepared as described previously.

**Iso[4]LGE<sub>2</sub>-KLH Antigen**

A PBS solution containing 3.1 nm iso[4]LGE<sub>2</sub> (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...

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**Fig. 1.** Cyclooxygenase (enzymatic) pathway and free radical-induced (nonenzymatic) route to LGs and iso-LGs via rearrangements of endoperoxide intermediates.
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 bicinecinic acid (BCA) protein assay (39) using solutions of BSA as standards.

**Ehrlich Pyrrole Assay of Iso[4]LGE2-Protein Adducts**

An Ehrlich pyrrole assay (40, 41) was performed to determine the concentration of protein-bound iso[4]LGE2-derived pyroles as described previously for determining the concentration of LGE2-derived pyroles (15, 27). Tritium-labeled LGE2-HSA and LGE2-BSA were used as standards for the assay. The amount of LGE2 bound to HSA or BSA was determined by quantitative radiochemical analysis. The data for the standards (Fig. 2) fits the equation: 

\[
[\text{pyrrole (μmol)}] = 2.22 \times 10^{-3} + 10^{-2} \times \text{absorbance at 586 nm}
\]

The pyrrole concentration, 0.52 mM BSA-bound iso[4]LGE2, was determined using the Pierce BCA protein assay as described above. The data for the standards (Fig. 2) fits the equation:

\[
[\text{pyrrole (μmol)}] = 2.22 \times 10^{-3} + 10^{-2} \times \text{absorbance at 586 nm}
\]

A PBS solution containing 3.54 mM iso[4]LGE2 (1.6 mg, 4.54 μmol) and 0.2 mM BSA (15.2 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]LGE2, was determined by an Ehrlich assay using LGE2-BSA and LGE2-HSA as standards.

**Iso[4]LGE2-HSA Standard**

A PBS solution containing 3.55 mM iso[4]LGE2 (2.5 mg, 7.10 μmol) and 0.16 mM HSA (20.0 mg, 18.30 μmol of lysyl residues) was incubated at 37 °C for 12 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]LGE2, was determined by an Ehrlich assay using LGE2-BSA and LGE2-HSA as standards.

**Immunization**

The immunogen, iso[4]LGE2-KLH (5.0 mg) containing 0.75 μmol of iso[4]LGE2 per mg of KLH, was diluted to 5 ml with PBS 7.4 PBS. An aliquot (500 μl) was emulsified in Freund’s complete adjuvant (500 μl). Each of two Pasteurella 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]LGE2-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. "Cross-reactivity of LGE2-Lysine Lactam and Hydroxylactam—A mixture of LGE2-lysine lactam and hydroxylactam adducts was obtained by incubating LGE2 with [3H]lysine (27,000 cpm/μg) under argon overnight at 37 °C. The mixture was applied to a C18 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/ethanol acetate (1:1, v/v, 10 ml) before elution with methanol/ethanol 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 C18 column

**Antibody Purification**

The iso[4]LGE2-KLH immune serum from the 73 day bleeding of rabbit 1, containing 34.4 mg/ml protein, as determined by absorbance at 280 nm (A280 = 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 Titer**—For determination of antibody levels in rabbit blood serum, iso[4]LGE2-BSA containing 10 mol of pyrrole/mol of protein, was used as coating agent. The iso[4]LGE2-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]LGE2-BSA adduct was used as coating agent and iso[4]LGE2-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]LGE2-HSA standard were run. The standard was prepared by diluting a 1.04 mM HSA-bound iso[4]LGE2 solution in PBS to 104 μl/m with pH 7.4 PBS. A serial dilution of factor 0.2 was used. Each well of the plate was coated with iso[4]LGE2-BSA solution (100 μl), prepared by diluting a solution containing 1.08 mM BSA-bound iso[4]LGE2 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]LGE2-HSA standard, the undiluted sample solution (150 μl) and aliquots (150 μl) of up to nine 1:10 serial dilutions with 5 μl/m 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 μg/14 μl/ml of 2% CEO). The remaining ELISA procedure and similar antibody binding inhibition studies with LGE2-KLH antibody, LGE2-BSA adduct as coating agent, and LGE2-HSA as standard were performed as described previously (21).
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. 4A) and containing the protonated molecular ion as well as ions resulting from sodium adduction and dehydration that occurs in the mass spectrometer. 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 mass spectrum of a lactam adduct is shown in C; it also displays ions corresponding to the molecular ion of the LGE2-lysine lactam as well as ions resulting from sodium adduction and dehydration.

Synthesis of Iso[4]LGE2-protein Adducts—Iso[4]LGE2 is a chemically sensitive vinylogous θ-hydroxy aldehyde that was freshly prepared for reaction with proteins (BSA, HSA, and KLH) to afford iso[4]LGE2-protein adducts. We previously showed that for high LGE2/protein ratios, Paal-Knorr condensation of LGE2 with ε-amino groups of lysyl residues of proteins gives mainly LGE2-derived protein-bound pyrrole (21). Earlier studies also demonstrated that quantitative analysis of LGE2-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 LGE2-pyrrole with DMB (15).

For the present study, iso[4]LGE2-protein adducts, rich in iso[4]LGE2-pyrrole, were prepared by exposing various proteins to an excess of iso[4]LGE2. The levels of protein-bound iso[4]LGE2-derived pyrrole in these adducts were determined by ELISA using LGE2-KLH (27), iso[4]LGE2-KLH, or ON-KLH (36) antibodies, respectively, in pH 7.4 PBS containing 0.001% Tween™ 20 and 0.2% CEO.

LGE2 and iso[4]LGE2-Protein Immunoreactivity in OxLDL—ELISA of oxLDL was performed the same as the inhibition assays, except in a dilution factor of 0.3 was employed. The starting concentration was the undiluted samples. The time dependence of appearance of protein-bound LGE2- and iso[4]LGE2-derived epitopes during oxidation of LDL was determined as described in our previous study of LGE2-pyrrole generation during oxidation of LDL (27).

Trapping ELISA Detection of Free LGE2—To detect any free LGE2 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 CuSO4. 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 LGE2 (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-LGE2 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 MgCl2 (1 mM) were added and the mixture was incubated for about 20 min at room temperature. The reaction was terminated by adding 3 μl 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 LGE2 (Fig. 5) showed a linear increase in absorbance with LGE2 concentration in the standard solutions. No absorbance was observed for any of the wells treated with ultrafiltrate from oxLDL.

**RESULTS**

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For the present study, iso[4]LGE2-protein adducts, rich in iso[4]LGE2-pyrrole, were prepared by exposing various proteins to an excess of iso[4]LGE2. The levels of protein-bound iso[4]LGE2-derived pyrrole in these adducts were determined by ELISA using LGE2-KLH (27), iso[4]LGE2-KLH, or ON-KLH (36) antibodies, respectively, in pH 7.4 PBS containing 0.001% Tween™ 20 and 0.2% CEO.
by Ehrlich assays (40, 41) using LGE₂-protein adducts as standards since the availability of radiolabeled LGE₂ allowed an accurate independent assessment of LGE₂ content in these standard samples. As expected, the chromophore generated by the condensation of iso[LGE²]-pyrrole with DMAB is very similar to that from LGE₂-pyrrole. Thus, the absorption maxima (λ_{max}) observed for the LGE₂-HSA-DMAB and iso[LGE²]-BSA-DMAB chromophores are 586 and 584 nm, respectively. It is reasonable to presume that the structurally similar Ehrlich chromophore derived from an iso[LGE²]-pyrrole has the same extinction coefficient as that derived from an LGE₂-pyrrole.

A linear correlation was obtained for a plot of pyrrole concentration versus absorbance at 586 nm for the DMAB chromophore of LGE₂-derived protein-bound pyrroles in LGE₂-BSA and LGE₂-HSA (see Fig. 2). The concentration of LGE₂-derived protein-bound pyrrole in LGE₂-HSA was taken to be equal to the total amount of protein-bound LGE₂ (0–500 nmol/sample) as determined by quantitative radiochemical analysis. This assumes a quantitative yield for pyrrole formation. Therefore, the use of LGE₂-protein-derived pyrrole as a standard for the Ehrlich assay provides an upper limit for the concentration of iso[LGE²] protein-derived pyrrole.

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**Lactam, and Hydroxylactam Epitopes in LGE₂-Protein Adducts**—Studies detailed elsewhere (19), employing mass spectral detection of lipid-modified lysine to characterize epitopes generated by covalent addition of LGE₂ with proteins, uncovered oxidative modifications that append one or two atoms of oxygen to protein-bound LGE₂-derived pyrroles. Thus, while LGE₂-lysine adduct containing the expected lysine-LGE₂-pyrrole could be prepared if oxygen is rigorously excluded, exposure to air or enzymatic proteolysis of LGE₂-protein adduct produced only mono- and dioxygenated lysine-LGE₂-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 LGE₂-pyrrole cross-react strongly with LGE₂-KLH antibodies. Thus, a sample containing a mixture of lysine-LGE₂-lactam and lysine-LGE₂-hydroxy lactam was isolated by HPLC from a Paal-Knorr condensation of LGE₂ with radiolabeled lysine and subsequent oxidation by adventitious oxygen. Quantitative radiochemical analysis in conjunction with an ELISA comparison of binding with LGE₂-KLH antibodies, showed 256% cross-reactivity for the hydroxylactam-lactam mixture relative to the LGE₂-HSA standard.

**Specificity of LGE₂- and Iso[LGE²]-KLH Antibodies**—Structural specificities were also examined for the LGE₂-KLH and iso[LGE²]-KLH antibodies to selectively recognize the LGE₂-HSA and iso[LGE²]HSA standards, respectively. ELISA binding inhibition studies for cross-reactivity of iso[LGE²]-KLH antibody (Fig. 7) and LGE₂-KLH antibody (Fig. 8) with various haptons demonstrated excellent specificity for both antibodies. Thus, neither antibody recognizes a protein-bound...
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 LGE2-KLH antibodies bind LGE2-HSA 200 times more strongly than they bind iso[4]LGE2-HSA, while the iso[4]LGE2-KLH antibodies bind iso[4]LGE2-HSA at least 2000 times more strongly than they bind LGE2-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 LGE2-HSA and Iso[4]LGE2-HSA Immunoreactivity by Fe2+−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 LGE2-KLH (Fig. 9) and iso[4]LGE2-KLH (Fig. 10) antibodies in the reaction product mixture from AA but not in the reaction product mixture from LA. Similar experiments with ω-linolenic (ω-LA), DHA, ETA, and EPA acids revealed the generation of protein epitopes that cross-react with LGE2-KLH and iso[4]LGE2-KLH antibodies from ω-LA, ETA, and EPA, but not DHA (see below).

LGE2-Protein and Iso[4]LGE2-Protein Adduct Immunoreactivity in OxLDL—LDL was oxidized by dialyzing an aqueous solution of LDL in air against a buffer containing Cu2+, an in vitro model (2) for physiological oxidation of LDL. Oxidation was halted after various time periods by sequestration of Cu2+ with Na2EDTA added to an aliquot of the reaction mixture. After an induction period, during which the endogenous antioxidants presumably were consumed, immunoreactivity toward both LGE2-KLH (Fig. 11) and iso[4]LGE2-KLH (Fig. 12) antibodies increased rapidly, reaching a plateau after several hours. The immunoreactivity detected for LGE2-protein and iso[4]LGE2-protein epitopes in the oxLDL corresponded to a final ratio of 1:4, respectively.

Free LGE2 Is Not Present in OxLDL—A trapping ELISA was used to detect any free LGE2 that might be present in the reaction product mixture generated by in vitro oxidation of LDL in the presence of Cu2+. Free LGE2 can be trapped by the protein coating agent (BSA) to give immunoreactive LGE2-pyrrole epitope. Thus, a linear increase in absorbance was found for increasing concentrations of free LGE2 (see Fig. 15). However, the wells treated with ultrafiltrate from oxLDL showed no absorbance, indicating that they contained no free LGE2.

**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 Cu²⁺-promoted oxidation. In contrast, employing an analogous LGE₂-trapping-ELISA, we now find no evidence for the presence of free LGE₂ in LDL that has undergone Cu²⁺-promoted oxidation. This is expected because, as we have noted elsewhere (8, 19), LGE₂ 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]LGE₂-KLH antibodies to detect iso[4]LGE₂-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, non-regioselective 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 peroxycosatetraenoyl 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 isoPGH₂ or iso[n]PGH₂ 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]LGE₂-protein adducts by oxidation of LDL (0.5 mg/ml) with Cu²⁺ (10 μM).

FIG. 13. The iso[n]LG cascade of levulinaldehyde derivatives generated by free radical-induced oxidation of AA-PC.
isoLGs and iso[\(\eta\)]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 \(\varepsilon\)-amino group of protein lysyl residues. 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[\(\eta\)]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 polysaturated fatty acid in LDL. We now have a panel of five antibodies that specifically detect epitopes produced by the addition 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-oxo-10-decenenoic acid (HODA) 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-dodecenolic acid (HODA) are a selective marker for LA oxidation in the presence of protein.

HNE-pyrrole epitope (detected with ON-KLH antibody) was also detected in the reaction product mixture from in vitro oxidation of LA in the presence of HSA. 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 \(\gamma\)-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 \(\gamma\)-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 \(\gamma\)-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 LGE\(_2\)-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 isoster-derivative 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 prostanoic side chains appended to neighboring \(sp^2\) carbons on a five-membered ring, then 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 polycyonal 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 haptns, 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**

| Antibody                   | Immunoreactivity (pmol/ml) | Hapten, Protein adduct |
|---------------------------|---------------------------|------------------------|
| LGE\(_2\)-KLH             | ND (-)                    | LGE\(_2\)-pyrrole      |
| Iso[4]LGE\(_2\)-KLH       | ND (--)                   | IsoLGE\(_2\)-pyrrole   |
| DODA-KLH                  | 44 (+)                    | HODA-pyrrole           |
| DOOA-KLH                  | ND (-)                    | HOA-pyrrole            |
| ON-KLH                    | 276 (+)                   | HNE-pyrrole            |
|                           | 390 (+)                   |                        |

* ND, not detected.

### Table IV

**Oxidation of PUFA (% of value for AA) generated by oxidation of PUFA in the presence of HSA**

| PUFA      | Iso[4]LGE\(_2\)-KLH | LGE\(_2\)-KLH | ON-KLH | LDL\(^a\) | Serum\(^b\) |
|-----------|----------------------|--------------|--------|-----------|-------------|
| LA        | ND\(^c\)             | 71           | 37     | 17 (19.6) | 22.9        |
| \(\gamma\)-LA | 23                  | ND           | 37     | 2.7 (4.8) | 2.2         |
| Docosahexaenoic | ND                 | ND           | ND     | 4.8 (7.1) | 11.0        |
| AA        | 100                  | 100          | 100    | 10 (1.4)  | 3.1         |
| ETA       | ND                   | 14           | ND     | 0.9 (5.6) | 0.7         |
| EPA       | 17                   | 16           | ND     |           |             |

\(^a\) 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.
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 LGE2 and Iso[4]LGE—Previously, we used quantitative radiochemical analysis to accurately determine the amount of LGE2 contained in protein adduct standards. Because radiolabeled iso[4]LGE2 is presently not available, we had to employ a less direct method to determine the amount of iso[4]LGE2-derivable pyrrole present in the iso[4]LGE2-iso[4]LGE2-HSA standard. While the Ehrlich assay is not sensitive enough to detect the low concentrations of iso[4]LGE2-HSA because this standard was used to calculate the concentration of iso[4]LGE2-pyrrole determined for the iso[4]LGE2-iso[4]LGE2-HSA standard. The Ehrlich assay is not sensitive enough to detect the low concentrations of iso[4]LGE2-derivable pyrrole present in human blood or generated upon oxidation of LDL, it was feasible to use this assay to compare the concentrations of LGE2-derived and iso[4]LGE2-derived pyroles in the protein adducts prepared as standards. The iso[4]LGE2 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 LGE2-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 LGE2, one molecule of BSA was found to bind a maximum of about 16 molecules of LGE2 (14). It seems reasonable to expect that a similar limit would apply to binding of iso[4]LGE2. Especially important is the concentration of iso[4]LGE2-pyrrole determined for iso[4]LGE2-HSA because this standard was used to calculate the amount of iso[4]LGE2-pyrrole in oxLDL samples. The 30:1 ratio determined indirectly by Ehrlich assay for iso[4]LGE2-HSA seems to overestimate the actual levels by factor of two. The concentrations of iso[4]LGE2-protein adduct indicated in the figures and tables must be interpreted in light of this caveat.

Possible Etiological Importance of LGs and Iso[4]LGs in Atherosclerosis—With mouse peritoneal macrophages, we previously showed that the covalent adduct of LGE2 with human LDL (LGE2-LDL) is internalized and degraded if the molar ratio of LGE2 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 LGE2-LDL. This result suggests that LGE2-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 LGE2-protein adduct are generated per mole of apoB (27). Nevertheless, it is reasonable to anticipate that macrophage recognition of iso[4]LGE2-LDLs will be similar to that of LGE2-LDL, and that total levels of LG and iso[4]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 LGE2-LDL exhibits an inefficiency similar to that found for oxLDL and, therefore, that incompletely processed LGE2-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 LGE2 binds avidly (within minutes) with proteins (14), and the reaction of LGE2 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[4]LG-protein adducts are expected to be “sticky,” readily forming protein-protein cross-links by binding to additional protein-based nucleophilic residues. It is tempting to speculate that such cross-links with proteolytic enzymes interfere with processing of oxLDL.

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