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Review

Oxidized phospholipids, isolevuglandins, and atherosclerosis

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Autoxidation of polyunsaturated phosphatidylcholines (PCs) generates isolevuglandins (isoLGs) through rearrangements of isoprostanoid endoperoxides. Within seconds, isoLGs are sequestered by covalent adduction with proteins. Murine plasma isoLG-protein levels increased at least 2.5-fold in response to inflammation. IsoLG-protein adducts accumulate *in vivo* providing a convenient dosimeter of oxidative stress. Elevated blood isoLG-protein levels present in atherosclerosis (AS) patients point to an independent defect that is not associated with total cholesterol levels, which results in an abnormally high level of oxidative injury in AS. Protein adduction and cross-linking caused by isoLGs can obstruct protein function. For example, it interferes with proteosomal degradation of proteins and, consequently, may result in apoptotic death of smooth muscle cells and destabilization of atherosclerotic plaques. Phospholipid autoxidation also generates biologically active oxidatively truncated PCs through fragmentation of dihydroperoxydienes that can be promoted by α-tocopherol. The oxidatively truncated PCs in oxidized low-density lipoprotein (oxLDL) contribute to the etiology of AS by inhibiting enzymatic activities required for normal processing of oxLDL by macrophages. They promote interactions of monocytes with endothelial cells that may foster migration of monocytes into the subendothelial space. They are also ligands for unregulated receptor-mediated uptake of oxLDL by monocyte macrophages leading to foam cell formation.

Keywords: Atherosclerosis / Enzyme inhibition / Isolevuglandins / Oxidatively truncated phospholipids / Protein cross-linking

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1 Introduction

It is an important challenge for biological entities to avoid damage caused by nonenzymatic processes, such as free radical-induced lipid oxidation, to which their component biomolecules are prone. Understanding those proclivities is the cornerstone of an approach that we are using to unravel the complexities of lipid oxidation *in vivo*. The present review focuses on a cascade of biologically important chemistry initiated by the autoxidation of polyunsaturated fatty acyl residues in phospholipids. Some of the biological consequences of this chemistry result from covalent adduction of lipid oxidation products to proteins that impede their function. Others are receptor-mediated responses triggered by oxidized phospholipids that serve as *oxidative messengers*. In some cases those responses are inappropriate and have pathological consequences.

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Abbreviations: AA, arachidonic acid; **AS**, atherosclerosis; **COX**, cyclooxygenase; **HNE**, 4-hydroxy-2(E)-nonenal; **HODA**, 9-hydroxy-12-oxo-10-dodecenoic acid; **HOOA**, 5-hydroxy-8-oxo-6-octenoic acid; **IsoLG**, isolevuglandin; **LA**, linoleic acid; **LDL**, low-density lipoprotein; **LG**, levuglandin; **OV**, oxovaleroyl; **ox**, oxidized; **PC**, phosphatidylcholine

2 Isolevuglandins (IsoLGs)

2.1 Prostanoid endoperoxides rearrange to give levulinaldehydes

IsoLGs are extremely reactive products of free radical-induced lipid oxidation. Because they are immediately sequestered by covalent adduction with proteins (*vide infra*), detecting their formation *in vivo* and characterizing their molecular structures was a redoubtable challenge. We gleaned insights from *in vitro* studies that guided our discovery of IsoLGs *in vivo* despite the fact that their formation is camouflaged by adduction to proteins.

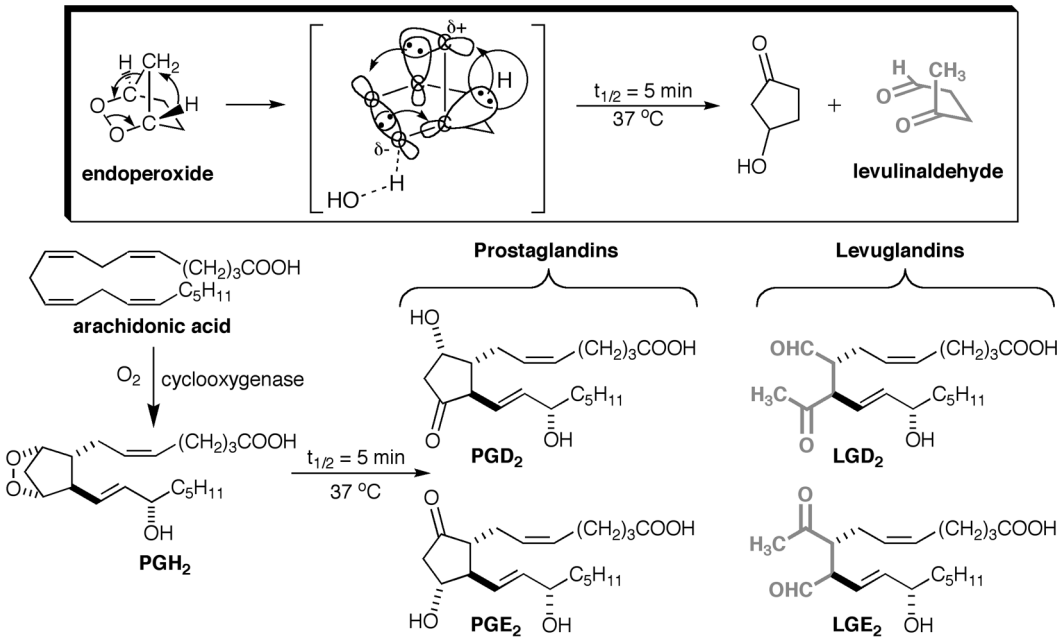

Figure 1. Rearrangement of the bicyclic endoperoxide nucleus of PGH_2 generates two levulinaldehyde derivatives, LGD_2 and LGE_2.

Spontaneous rearrangements of the prostaglandin endoperoxide PGH_2 were known to generate prostaglandins PGD_2 and PGE_2 [1, 2]. In a model study, we found that a fragmentation reaction of the bicyclic peroxide nucleus of the prostaglandin endoperoxide PGH_2 in neutral aqueous solution produces levulinaldehyde [3]. A polarized transition state (Fig. 1) is involved since hydrogen bonding by protic solvents accelerates the reaction by two orders of magnitude over the rate in aprotic solvents of similar polarity [4]. Isotopic labeling and kinetics experiments showed that water promotes intramolecular migration of a bridgehead hydride in concert with cleavage of C – C and O – O bonds [5]. These observations led us to predict that as yet unknown products should be readily formed by rearrangements of PGH_2. Inspired by the model studies, we then showed that rearrangement of PGH_2 in neutral aqueous solution not only generates PGD_2 and PGE_2 but also two levulinaldehyde derivatives with prostaglandin side chains, which we named levuglandin (LG) E_2 and LGD_2 (Fig. 1) [5]. We confirmed the structures of LGE_2 and LGD_2 by total syntheses [6, 7].

2.2 LGs rapidly bind covalently with proteins

Detecting the production of LGs *in vivo* is complicated by rapid covalent adduction with proteins. We anticipated that LGs would react rapidly with primary amino groups in biological molecules because other γ-dicarbonyl compounds were known to undergo Paal – Knoor condensation with primary amines to form pyrroles, and to participate in irreversible binding to tissues [8 – 10]. Furthermore, irreversible covalent binding of unidentified metabolites from arachidonic acid (AA) to "tissue macromolecules" had been observed upon incubation with microsomes from a variety of cyclooxygenase (COX)-containing tissues. The known products from the PGH_2 intermediate of COX-induced bioconversion of AA could not account for this covalent binding [11, 12]. This led us to propose that "LGs are the unidentified metabolites of AA, generated by the COX pathway from PGH_2, which are responsible for covalent binding with microsomes from various tissues as well as with proteins" [13]. Using *in vitro* studies with radiolabeled LGE_2 we showed that LGs covalently bind avidly (within seconds) with proteins [13]. We quantitatively detected the production of protein-based LGE_2-derived pyrroles by the generation of a characteristic chromophore upon treatment of the LGE_2-protein adduct with the Ehrlich reagent [14]. The formation of an LG-derived pyrrole through the Paal – Knoor reaction, proceeds through a Schiff base and its enamine tautomer (Fig. 2). A subsequent study confirmed the rapid initial formation of lysine-LGE_2 Schiff base adducts by mass spectroscopic analysis [15]. Because they are electron rich, LG-derived pyrroles are highly susceptible to oxidation. Using mass spectroscopic analysis of protein digests, Brame *et al.* [16] discovered that these LGE_2-derived pyrroles are oxidized by air to relatively stable end products, lactams, and hydroxylactams (Fig. 2).

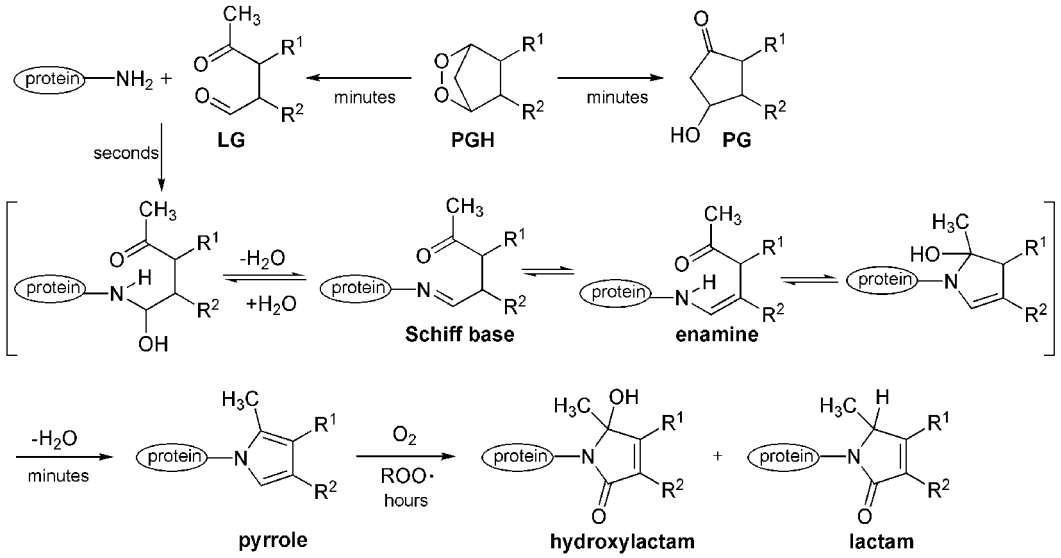

Figure 2. LGs form covalent adducts with proteins. Schiff base adducts are generated within seconds. Within minutes, cyclization and dehydration generates pyrroles and these are slowly oxidized to give lactams and hydroxylactams.

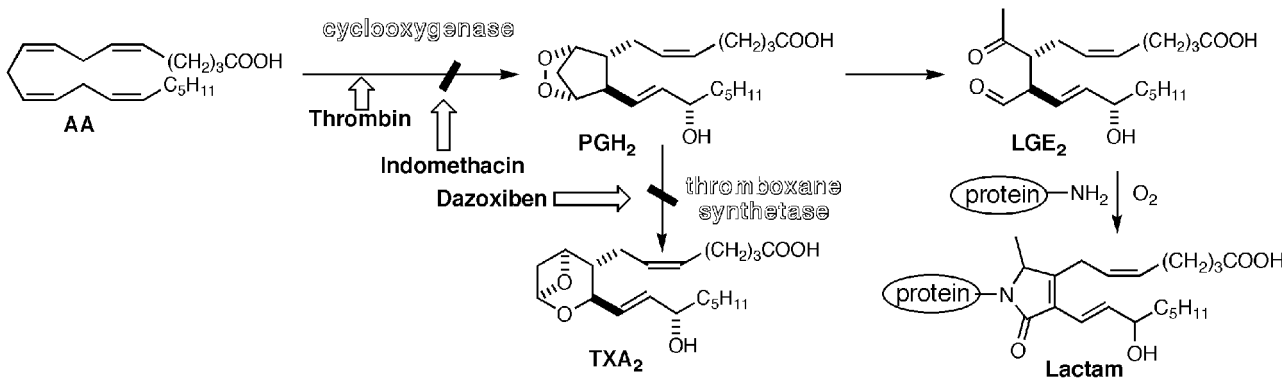

Figure 3. Activation of platelets with thrombin generates LGs through the COX pathway in competition with the production of thromboxanes.

2.3 Platelet activation generates LGs

The first evidence for the production of LGs in cells was provided by the observation that LGE_2-protein adducts are formed after platelet activation with thrombin [17]. The COX inhibitor indomethacin prevented the formation of these adducts while an inhibitor of thromboxane synthetase enhanced their production because the enzymatic conversion to thromboxanes no longer competed with the nonenzymatic rearrangement to LGE_2 (Fig. 3). Thus, LGE_2 is formed in platelets even in the presence of an enzyme that uses PGH_2 as a substrate. The ability of the nonenzymatic rearrangements to compete in a cellular environment with enzymatic conversion of PGH_2 to thromboxanes is noteworthy. LG production, and associated pathological sequelae, can be fostered by therapeutic interventions that inhibit enzymes, such as thromboxane synthetase, which consume PGH_2.

2.4 Free radical-induced lipid cyclooxygenation leads to the generation of a family of LG isomers: IsoLGs

Because LGs are rapidly sequestered by covalent adduction with proteins, we opted to detect their presence *in vivo* using antibodies that recognize their protein adducts [18]. We detected LGE_2-protein adducts in human cerebral vasculature and blood [19]. However, we cautioned that these studies could not distinguish between a COX-dependent origin and a possible alternative free radical-induced autoxidative biogenesis. Thus, we envisioned that the epitopes recognized by LGE_2-protein adduct antibodies could also be generated by free radical-induced cyclooxygenation of arachidonyl phospholipids [20]. This possibility was suggested by chemistry discovered in the late sixties that results in the generation of stereo and structural isomers of prostaglandins [21] – now referred to as isoprostanes

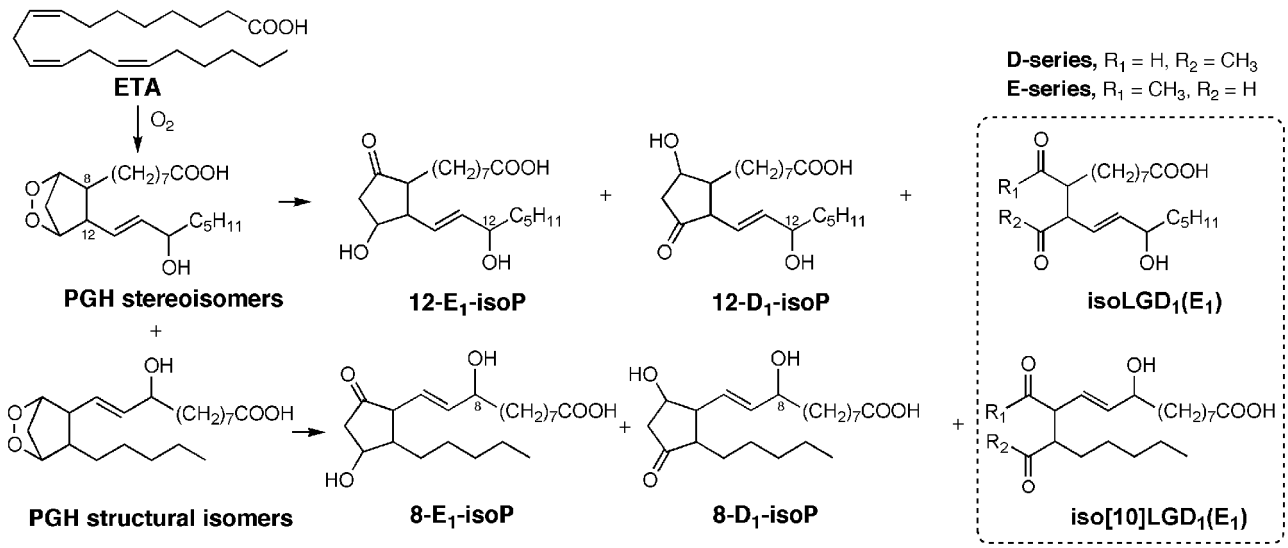

Figure 4. Free radical-induced oxidation of eicosatrienoic acid generates stereo and structural isomers of prostaglandins through putative endoperoxide intermediates that are also expected to rearrange to LG isomers.

(isoPs) – through free radical-induced cyclooxygenation of eicosatrienoic acid. Alternative rearrangements of the putative isoprostane endoperoxide intermediates involved could generate stereo and structural isomers of LGs (Fig. 4) that we now refer to collectively as isoLGs.

In vivo, AA is present mainly in the form of esters including those of PC undergo free radical-induced cyclooxygenation to generate isoPGH$_2$ phospholipid esters [22]. Mixtures of eight isoLGD$_2$-PC and eight isoLGE$_2$-PC *stereoisomers* are expected, two of which incorporate the exact same LGD$_2$ and isoLGE$_2$ molecules as are generated through the COX pathway (Fig. 5). Six isoLG-PC *structural isomers* that are each mixtures of eight stereoisomers are also expected. Importantly, these structural isomers are unique products of the nonenzymatic cyclooxygenation pathway that we designate collectively as iso[*n*]LGs where the number in brackets refers to the length of the nonprostanoid carboxylic side chain.

As mentioned above, we noted the possibility that an isoLG identical with LGE$_2$ may be formed *in vivo* by nonenzymatic phospholipid peroxidation through rearrangement of intermediate isoprostane endoperoxides, and therefore, protein adducts detected *in vivo* with our anti-LGE$_2$ protein antibodies might not be products of the COX pathway [20]. Confirmation of this hypothesis and our discovery of IsoLGs soon followed with the demonstration that LGE$_2$-protein adducts are generated upon free radical-induced oxidation of LDL [23]. We prepared LGE$_2$-PC by total synthesis and showed that our LGE$_2$-protein adduct antibodies do not cross react with protein adducts of LGE$_2$-PC, but do recognize the protein adducts after phospholipolysis through treatment with phospholipase A$_2$ [23]. Lipase activity associated with LDL particle apparently converts the initially formed protein adducts of the LGE$_2$-PC ester into adducts of the free acid LGE$_2$. Subsequent LC/MS studies confirmed the generation of isoLG-protein adducts upon autoxidation of LDL [16].

2.5 Elevated serum IsoLG-protein adduct levels are an independent risk factor for atherosclerosis (AS)

We used antibodies against iso[4]LGE$_2$-protein adducts [24, 25] and iso[7]LGD$_2$-protein adducts [26] to detect the formation of these unique products of the IsoLG pathway *in vivo*. Parenthetically, an alternative LC/MS method for analysis of isoLG-protein adducts [16] does not distinguish between those derived from different structurally isomeric isoLGs such as isoLGE$_2$ *versus* iso[4]LGE$_2$. The mean levels of iso[4]LG and iso[7]LG adducts, like those detected with antibodies raised against LGE$_2$-protein adducts [19], are elevated in plasma from individuals with AS compared to individuals with no cardiovascular disease (Fig. 6). In agreement with the proposition that all iso[*n*]LGs are produced through the isoprostane pathway through parallel competing free radical-induced cyclooxygenations of AA, plasma levels of iso[7]LGD$_2$-protein adducts for all individuals are strongly correlated with iso[4]LGE$_2$-protein adducts (r = 0.933). Although the COX pathway can contribute to the generation of LGE$_2$-protein adducts in cells (*vide supra*), the strong correlation (r = 0.877) observed with levels of iso[7]LGD$_2$-protein adducts supports the view that LGE$_2$-protein adduct immu-

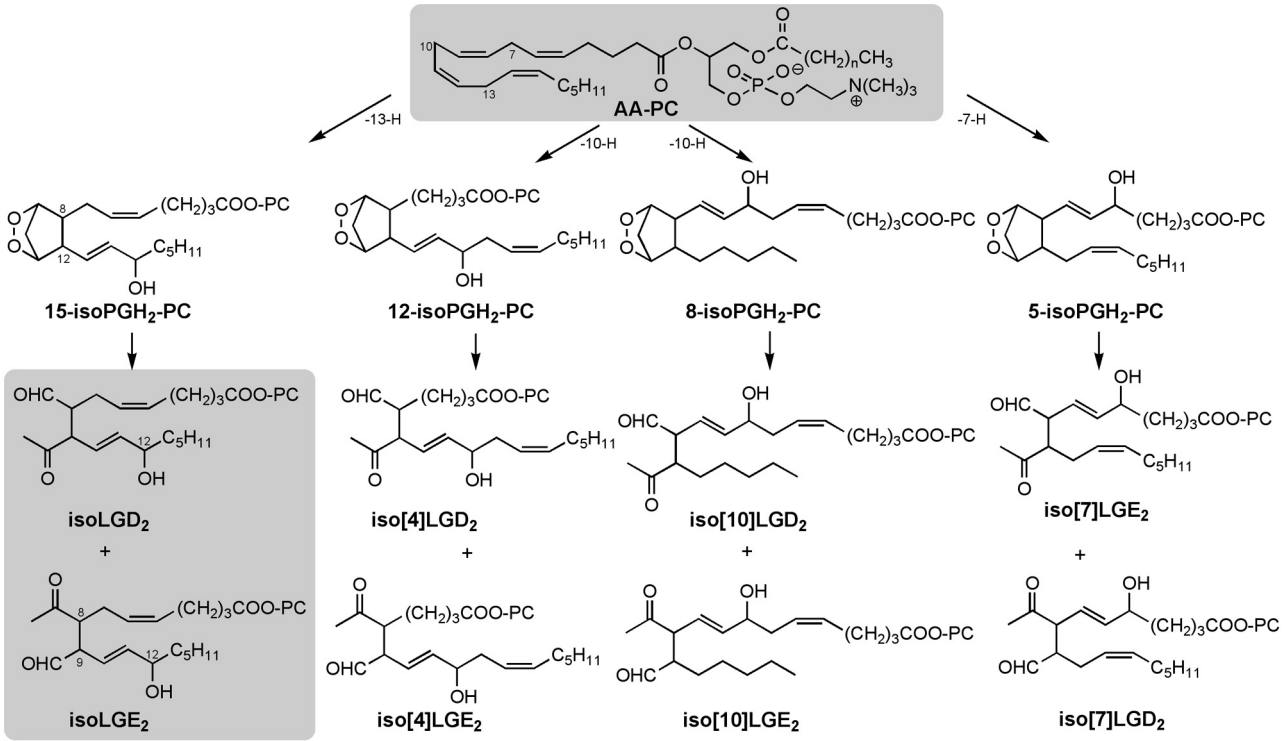

Figure 5. Free radical-induced oxidation of a PC ester AA generates phospholipids derivatives of LG stereoisomers (isoLGs) and structural isomers (iso[*n*]LGs).

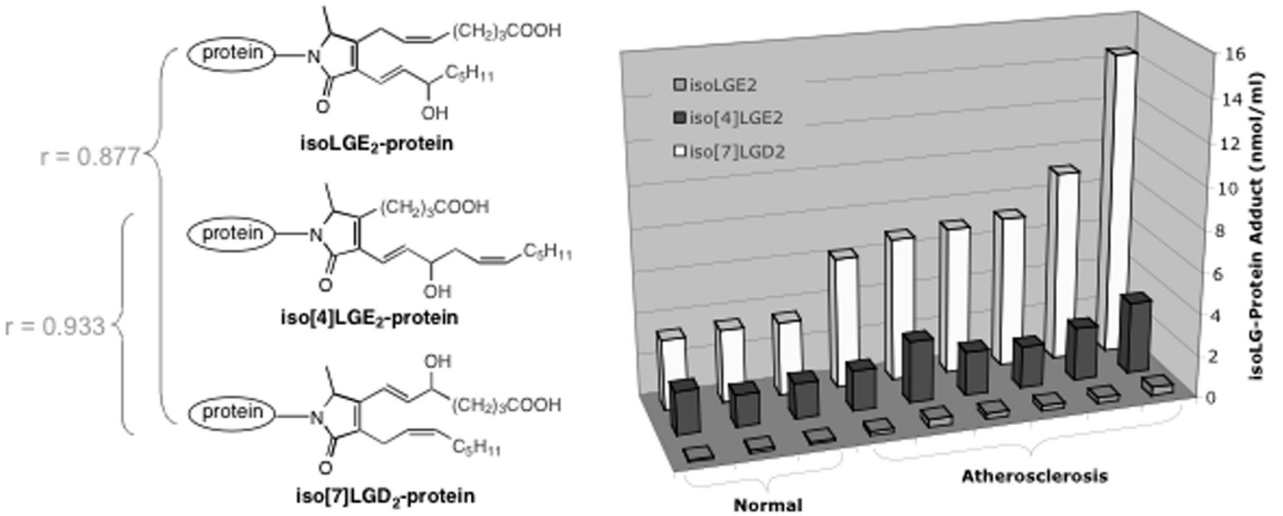

Figure 6. Levels of iso[4]LGE_2- and iso[7]LGD_2-protein adducts in blood from AS patients and healthy controls are strongly correlated with each other and with levels of isoLGE$_2$-protein adducts. Reproduced with permission from [26]. Copyright 2004 Am. Chem. Soc.

noreactivity arises in these individuals predominantly through the isoprostane pathway.

In agreement with the results shown in Fig. 6, another study [24] found higher levels of iso[4]LGE_2-protein adduct immunoreactivity in blood from AS patients who have undergone coronary artery bypass surgery than in blood from two populations of healthy individuals (*N*) of average age 33 ($p < 0.0001$) or 62 ($p < 0.002$) years (Table 1). For these individuals, we also determined plasma levels of two "risk factors" for cardiovascular disease, the protein apoB100 found in LDL and very LDL, and total choles-

Table 1. Levels (mean ± SD) of iso[4]LG-protein adduct, apoB, and total cholesterol

Cohort [average age]	*N* [33]	*N* [62]	AS [63]
Ages	33 ± 3	62 ± 7	63 ± 12
Population	15	10	16
Iso[4]LGE_2-protein (pmol/mL)	1441 ± 144	1691 ± 252	2224 ± 439
ApoB (mg/L)	724 ± 180	1046 ± 370	937 ± 206
Total cholesterol (mmol/L)	4.16 ± 0.87	5.78 ± 1.42	4.58 ± 0.59

terol. Since LDL cholesterol is a classical risk factor for AS, two aspects of the data in Table 1 are paradoxical: (1) levels of iso[4]LGE_2-protein adduct immunoreactivity are not correlated ($p < 0.1$) with those of apoB or total cholesterol, and (2) cholesterol is significantly elevated in older individuals *N*(62) who do not have AS compared with AS patients of similar ages. These data probably reflect the success of therapeutic interventions, including diet or drugs, which reduce total cholesterol levels for the AS patients but apparently do not reduce isoLG-protein adduct levels. If oxidative injury contributes to the progression of AS, lowering levels of total cholesterol may not be sufficient to prevent further oxidative injury. Iso[4]LGE_2-protein adduct levels appear to signal an independent defect which results in an abnormally high level of oxidative injury that is tightly associated with AS but not with total cholesterol levels. Therapeutic interventions that specifically lower levels of iso[4]LGE_2-protein adducts, *e.g.*, antioxidants, may be beneficial.

LGE_2-modified LDL, but not native LDL, is taken up and degraded by macrophage cells through a class of scavenger receptor which demonstrates ligand specificity for oxLDL [27]. The levels of LGE_2-protein adducts in human blood are lower than the threshold required for uptake. However, the cumulative effects of all eight structurally isomeric isoLG-protein adducts in oxLDL may make a significant contribution to recognition and endocytosis of oxLDL by macrophages resulting in their conversion into foam cells, progenitors of atherosclerotic plaques.

2.6 IsoLG-protein adducts are dosimeters of oxidative stress

Inflammation induced by *Candida* infection in mice causes a 3.5-fold increase in plasma levels of iso[4]LGE_2-protein adducts, but not in levels of F2-isoPs [28]. These disparate results indicate a fundamental difference between isoLGs and isoPs as markers of oxidative stress. Greatly increased levels of free isoPs in blood plasma, caused by oxidative stress associated with myocardial reperfusion, drop precipitously within minutes [29]. Thus, because they are rapidly cleared from the circulation, levels of isoPs represent a *snapshot* of oxidative stress at a particular time. In contrast, isoLGs are sequestered within seconds by reaction with proteins [13, 16]. The resulting isoLG-protein adducts accumulate over the lifetime of proteins. Since many proteins have half-lives of several weeks and even longer [30], it seems reasonable to presume that isoLG-protein adducts act like a dosimeter, providing a *cumulative index* for oxidative injury. Furthermore, because proteins that are modified by isoLGs resist proteasomal degradation (*vide infra*), the turnover of isoLG-protein adducts can be even slower than the unmodified proteins.

2.7 IsoLGs cross-link proteins

We observed that LGE_2 causes extensive protein oligomerization [31]. LGE_2 is orders of magnitude more effective in generating protein–protein cross-links than other AA-derived oxidized lipids known to cross-link proteins, *i.e.* malondialdehyde or 4-hydroxynon-2-enal. LGE_2 functions like molecular glue, sticking to a protein, and then bonding it through a covalent chemical link with another molecule of protein. Potentially, this can generate large heterogeneous aggregates of diverse proteins. It can also result in covalent linkages between proteins and nucleophilic functionality in small molecules. Thus, LGE_2-protein adducts covalently bind glycine [32]. *Nascent* LGE_2-protein adduct, which is formed within 5 min, binds nearly two equivalents of glycine. In this time frame the LGE_2-protein adduct is expected to exist primarily in the form of a Schiff base (see Fig. 2) that is slowly converted to lactam derivatives through an intermediate pyrrole [15]. A possible cross-linking mechanism identifies the LG-protein Schiff base adduct as the reactive electrophile that binds with a primary amino group in a second molecule of protein resulting in aminal formation (Fig. 7). The 2 : 1 LGE_2-protein-gly_2 adduct is probably a cyclic bis aminal. The ability of the LGE_2-protein adduct to bind glycine then slowly decreases, reflecting a competition, *interalia* with cross-linking and dehydration driven by the aromatization that accompanies pyrrole formation.

Previously we found that HSA binds ten equivalents of LGE_2 within 1 min [13]. We monitored the time course of intermolecular cross-linking of ovalbumin (OA) by incubating it with 15 equivalents of LGE_2, quenching the oligomerization after various time intervals by the addition of excess glycine, and fractionating the oligomers by SDS-PAGE. The formation of a "ladder" of various oligomers was readily apparent after 30 min (Fig. 8). Nevertheless, oligomerization of OA by LGE_2 can be completely prevented by adding a large excess of glycine to the reaction mixture 5 min after combining the protein and LGE_2. Such behavior is expected for a cross-linking mechanism involving rapid generation of a reactive electrophilic adduct between LGE_2 and monomeric protein, and a slower cross-linking reaction

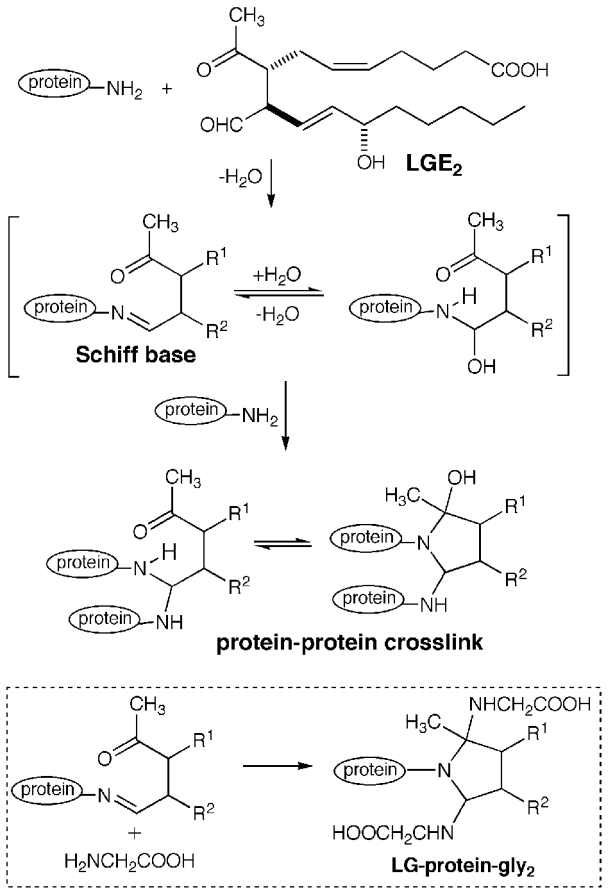

Figure 7. A probable mechanism for protein cross-linking by LGE_2 involves the formation of an aminal cross-link by addition of the ε-amino group of a lysyl residue in one protein with the Schiff base adduct of another protein.

of that "activated monomer" with a free primary amino group in another protein molecule.

The extraordinary ability of LGs and isoLGs to cross-link proteins is emerging as an important contributor to pathological sequelae of the aberrant overproduction of prostanoid endoperoxides through the COX pathway [33] as well as of the free radical-induced cyclooxygenation of lipids associated with oxidative stress [34]. For example, covalent modification of OA with as little as one equivalent of isoLGE$_2$ strongly curtailed its subsequent processing by the 20S proteasome [34]. Furthermore, isoLGE$_2$-OA competitively inhibited proteasomal chymotrypsin-like activity. In contrast, preincubation of the 20S proteasome with isoLGE$_2$ was much less effective at inhibiting the protease activity. Thus, the inhibitory effect is not primarily associated with modification of the protease, but rather with modification of its protein substrates. Apparently, isoLGE$_2$-modified proteins or peptides, containing lysyl lactam modifications or intermolecular cross-links, are poor substrates that competitively inhibit proteasomal degradation of other substrates (Fig. 9).

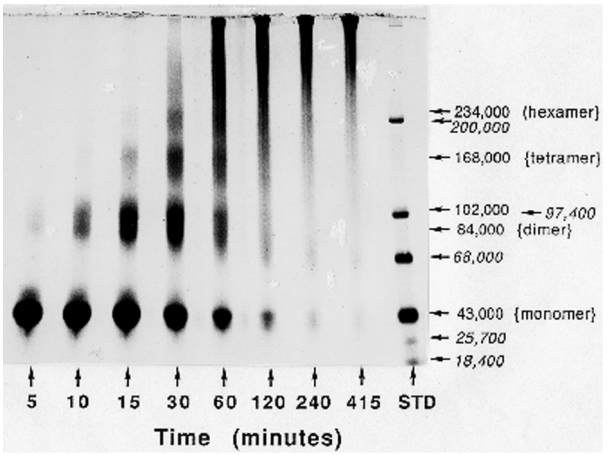

Figure 8. PAGE of ovalbumin after treatment with LGE_2 and then, after various time periods, with a large excess of glycine reveals the gradual oligomerization of the protein during several hours. Reproduced with permission from Ref. [31]. Copyright 1989 Elsevier.

IsoLGE$_2$ was especially effective at inhibiting chymotrypsin-like proteasomal activity when added to cell lysates, but in this case kinetic behavior was not consistent with competitive inhibition. Since direct adduction of LG isomers to the proteosome only caused weak inhibition, a novel mechanism of action may be operative. Thus, we postulate that nascent isoLG-protein adducts, generated *in situ* from cellular proteins, are potently electrophilic "activated monomers" capable of sticking to (cross-linking with) the proteasome resulting in irreversible binding of these toxic substrates. This scenario represents a novel mechanism for enzyme inactivation involving *in situ* nonenzymatic conversion of a protein into a suicide substrate that covalently binds to, and consequently irreversibly inhibits, its target enzyme (Fig. 9). Such poisoning of the proteasome by isoLGs may have important consequences in the etiology of AS. It might contribute to the accumulation of the proapoptotic protein Bax in atherosclerotic plaques, and thereby cause apoptotic death of smooth muscle cells [35]. In the plaque, these cells are the primary source of collagen needed to provide a stable fibrous cap. Consequent weakening and rupture of the fibrous cap, exposing its necrotic core, would promote thrombosis as would the apoptotic cells themselves.

3 Oxidized phospholipids

3.1 γ-Hydroxyalkenal phospholipids bind covalently with proteins

The γ-hydroxyalkenal 4-hydroxy-2(E)-nonenal (HNE) is a well-known biologically active product [36] that is gener-

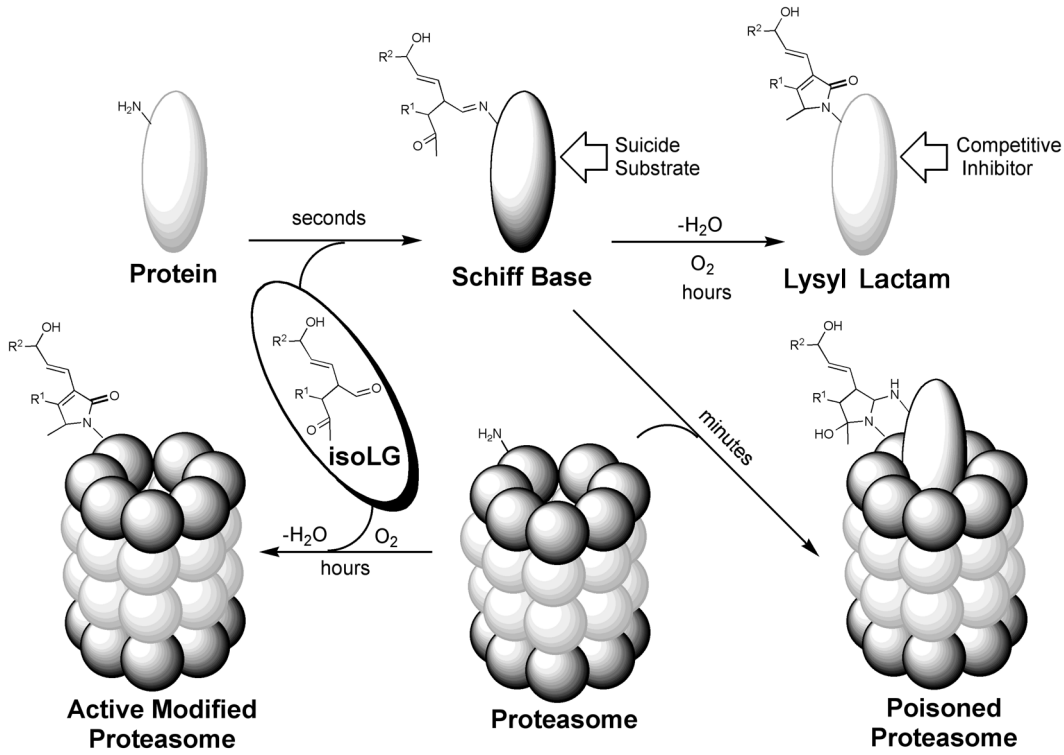

Figure 9. A mechanistic hypothesis for inhibition of proteasomal chymotrypsin-like activity by isoLGs: modification by preincubation with isoLGs generates lysyl lactam-modified (or intramolecularly cross-linked) proteins that competitively inhibit protease activity, whereas Schiff base modified proteins generated *in situ* in cells are suicide substrates that noncompetitively inhibit protease activity by cross-linking with the proteasome.

ated *in vivo* through oxidative fragmentation of either linoleates or arachidonates (Fig. 10). Sayre *et al.* [37] found that HNE forms 2-pentylpyrrole (PP) adducts that incorporate the ε-amino group of protein lysyl residues. Mean PP adduct levels in plasma from individuals with AS are double those found in healthy controls with no cardiovascular disease [38]. We postulated that analogous alternative oxidative fragmentations of linoleoyl and arachidonyl PCs generate γ-hydroxyalkenal phospholipids, and that these form pyrrole derivatives upon adduction to proteins. Therefore, we prepared and used polyclonal rabbit antibodies to detect such ω-carboxyalkylpyrroles in oxLDL and human plasma [39]. Mean levels of ω-carboxyheptylpyrrole derivatives of plasma proteins are significantly elevated in blood from individuals with AS.

3.2 Oxidized LDL contains oxidatively truncated phospholipids

We prepared 2-OV-PC and the glutaryl monoester G-PC (Fig. 11) by total syntheses to confirm the structures assigned by mass spectroscopic analysis of these oxidatively truncated phospholipids isolated from oxLDL [40]. A total synthesis of the 5-hydroxy-8-oxo-6-octenoic acid (HOOA) ester HOOA-PC [41], a putative intermediate in the generation of ω-carboxypropylpyrrole (CPP) protein epitopes (see Fig. 10), facilitated the subsequent identification of this phospholipid analog of HNE in oxLDL [42]. Both OV-PC and HOOA-PC induce binding of monocytes to endothelial cells [42]. Presumably this binding promotes infiltration of monocyte macrophages into the subendothelial space where they become foam cells through unregulated endocytosis of oxLDL. HOOA-PC also induces expression of interleukin (IL-8) and monocyte chemotactic protein (MCP)-1 in endothelial cells, chemokines that promote interaction with monocytes. Both HOOA-PC and OV-PC also inhibit E-selectin expression induced by lipopolysaccharide.

3.3 α-Tocopherol can promote fragmentation of dihydroperoxydienes

Dihydroperoxyoctadecadienoates (diHPODEs) are putative intermediates in the oxidative fragmentation of linoleic acid (LA) that generates γ-hydroxyalkenals. Two mechanistic alternatives for the fragmentation of diHPODEs are (1) a polar Hock rearrangement followed by hydrolysis and (2) homolysis of the doubly allylic hydroperoxy group to an alkoxy radical that undergoes a β-scission (Fig. 12).

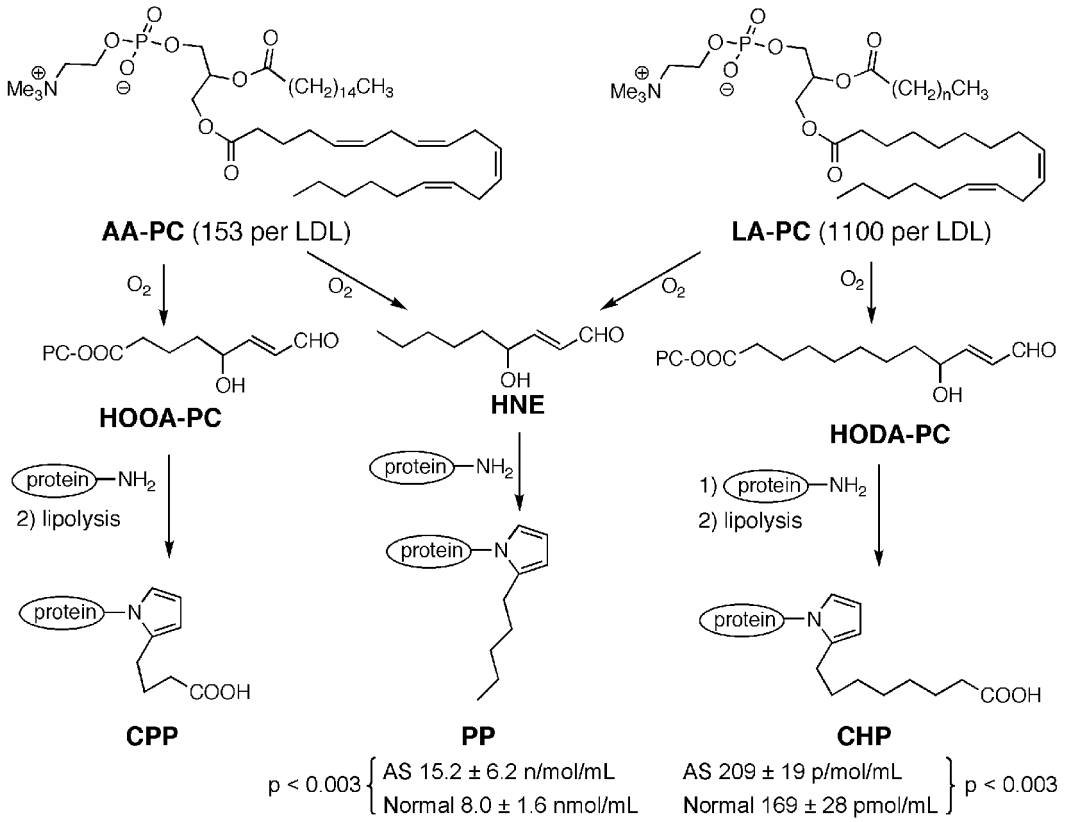

Figure 10. Oxidative fragmentation of polyunsaturated phospholipids generates γ-hydroxyalkenals that react with proteins to form various pyrrole derivatives. Levels of those derivatives are elevated in blood from AS patients compared to individuals with no cardiovascular disease.

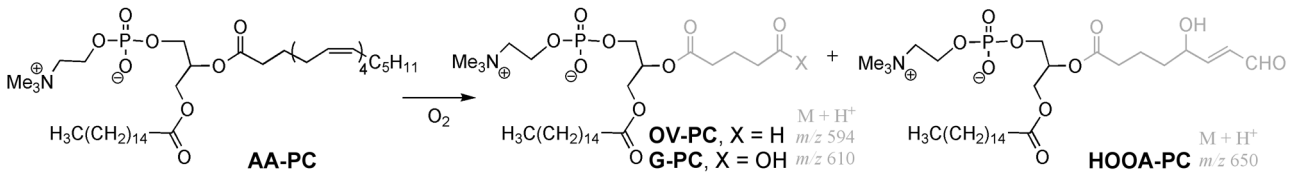

Figure 11. Various oxidatively truncated phospholipids are generated by free radical-induced oxidation of arachidonyl AA-PC.

Previous studies on the chemistry of PUFA hydroperoxides relied upon α-tocopherol to trap alkoxy radical intermediates and prevent their fragmentation through β-scission [43]. However, we discovered that α-tocopherol stoichiometrically induces the fragmentation of 9,12-diHPODE to generate 9-hydroxy-12-oxo-10-dodecenoic acid (HODA), KODA, and α-tocopherylquinone (Fig. 13). We postulate that this chemistry involves reductive cleavage of a hydroperoxide to generate an alkoxy radical and concomitant oxidation of the α-tocopherol. Oxidation of α-tocopherol through reactions with hydroperoxides is known [44 – 46]. Subsequent β-scission of the intermediate alkoxy radical delivers HODA. The reaction was suppressed by diethylenetriaminepentaacetic acid, a metal ion chelator. This indicates that the reaction is catalyzed by traces of redox active transition metal ions [44, 45]. This discovery underscores the complexity of the biologically important chemistry of vitamin E as a potential inhibitor/promoter of lipid oxidation and the reactions of lipid hydroperoxides that result in the generation of biologically active oxidized lipids. The ability of vitamin E to promote fragmentation of 9,12-diHPODE is reminiscent of a previous finding that vitamin C promotes the oxidative fragmentation of lipid hydroperoxides [47].

3.4 γ-Hydroxyalkenal phospholipids impair processing of oxLDL-containing endosomes in macrophages

Internalization of oxLDL in macrophages by receptor-mediated endocytosis reduces lysosomal cathepsin B activ-

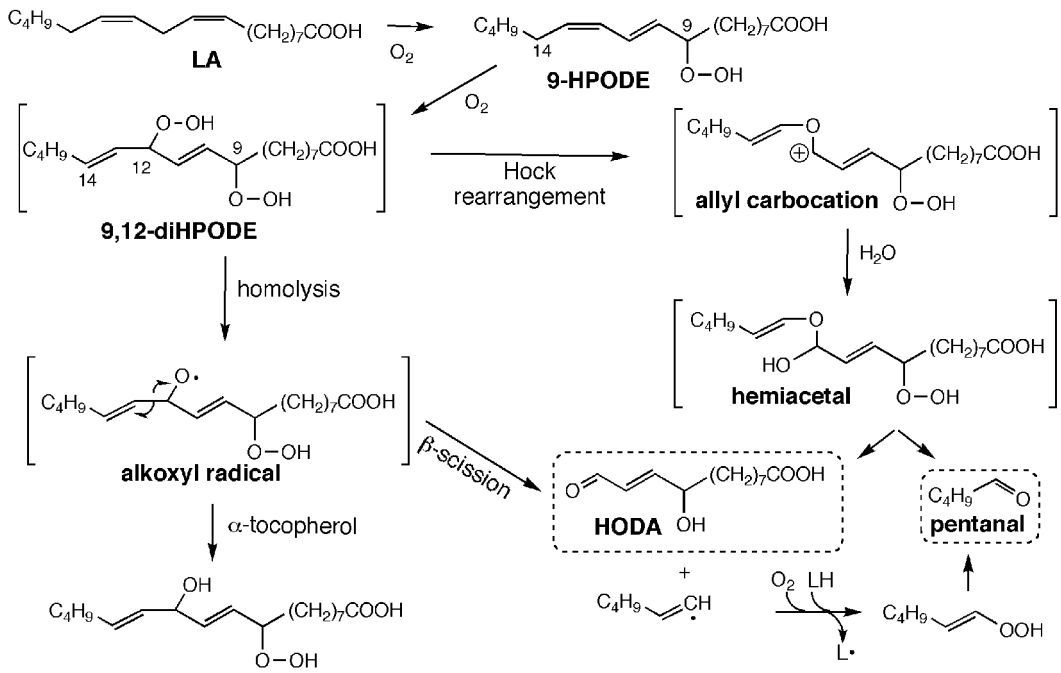

Figure 12. Putative polar and free radical mechanisms for the fragmentation of the dihydroperoxydiene 9,12-diHPODE that could explain the formation of HODA during free radical-induced oxidation of LA.

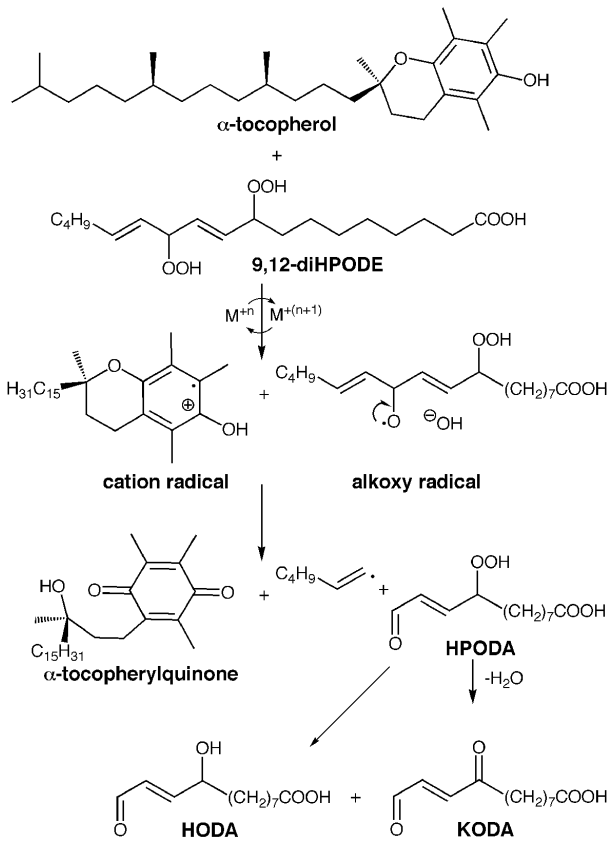

Figure 13. Vitamin E reduces 9,12-diHPODE with the generation of fragmentation products, HODA and KODA, as well as α-tocopherylquinone.

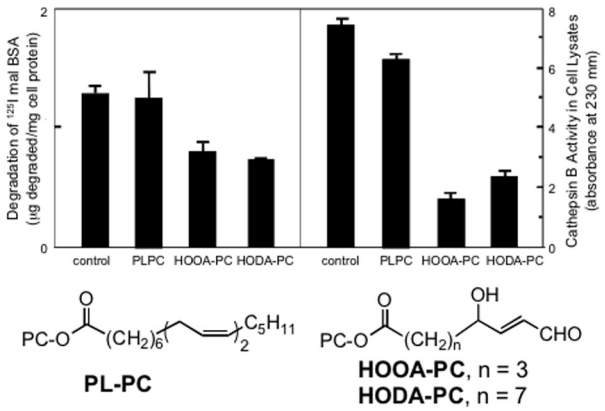

Figure 14. γ-Hydroxyalkenal phospholipids HOOA-PC and HODA-PC inhibit proteolysis of endocytosed protein in macrophage cells (left panel) and cathepsin B activity in cell lysates (right panel).

ity impairing intracellular protein degradation by this thioprotease [48]. Hoff *et al.* [49] detected the γ-hydroxyalkenal phospholipid HOOA-PC in atherosclerotic lesions and oxLDL. They found that HOOA- and HODA-PC reduce cellular cathepsin B activity and thereby impair the ability of macrophages to degrade protein that has been internalized by receptor-mediated endocytosis (Fig. 14). This enzyme inhibition presumably results from the ability of the electrophilic lipid oxidation products HOOA- and HODA-PC to block enzymatic activity through covalent modification of essential nucleophilic functionality, *i.e.* a

thiol in cathepsin B. Thus, oxLDL can act like a Trojan horse, delivering toxic electrophiles into macrophages where they attack an army of digestive enzymes and other proteins that are essential for processing of oxLDL.

Another mechanism by which toxic electrophiles from oxLDL might cause poor degradation of proteins internalized in macrophages is the perturbation of intracellular trafficking. Rab5a is a protein believed to be critical for fusion of phagosomes – early endosomes to form phagolysosomes – late endosomes. Hoff *et al.* found that HODA-PC inhibits PTM, *i.e.* isoprenylation and/or proteolytic cleavage, of immature Rab5a (a 25 kDa doublet) into the active 23 kDa doublet of mature Rab5a required for endosome fusion [49].

3.5 Oxidatively truncated PCs promote CD36-mediated endocytosis of oxLDL

To identify oxidized phospholipids in oxLDL that promote its binding and endocytosis by macrophage cells through the scavenger receptor CD36 [50], we fractionated oxPhCs that compete with oxLDL for binding CD36 receptors on embryonic kidney epithelial cells transfected with adenovirus. One of the three active fractions from the oxidation of AA-PC coeluted with an authentic sample of HOOA-PC that was available through our total synthesis (Fig. 12). The identity of this oxPC was confirmed by LC/MS comparison with an authentic synthetic sample and several derivatives [51]. We postulated that the less polar active fraction is the corresponding keto aldehydic PC ester of 5-keto-8-oxo-6-octenoic acid (KOOA-PC). This was confirmed by total synthesis and subsequent LC/MS comparisons. Similarly, total synthesis followed by LC/MS comparisons established that the active fraction that is more polar than HOOA-PC is a mixture of the dicarboxylic monoesters HOdiA-PC and KOdiA-PC (Fig. 15). Analogous oxPCs, *i.e.* HODA-PC, KODA-PC, HDdiA-PC, and KDdiA-PC, are derived from PCs that incorporate LA. Biological testing of the individual pure oxPhCs prepared by total syntheses showed that the dicarboxylic monoesters are the strongest ligands for CD36 and that other oxPCs that lack a γ-oxygenated-α,β-unsaturated carbonyl array possess little or no affinity for this scavenger receptor [51]. Only a few molecules of HDdiA-PC in cholesterol-containing liposomes prepared from unoxidized LDL lipids are sufficient to trigger macrophage binding, uptake, metabolism, cholesterol accumulation, and foam cell formation [52].

4 Conclusions and future prospects

A molecular level understanding of the pathological involvements of lipid oxidation in the etiology of AS is rapidly emerging. IsoLGs, γ-ketoaldehydes generated by a unique

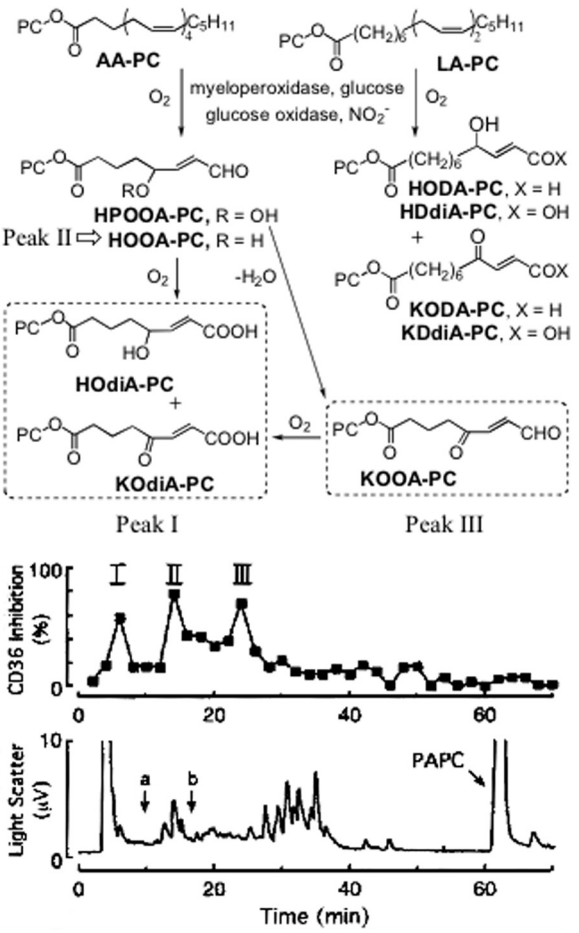

Figure 15. HPLC separation of oxidation products from free radical-induced oxidation of AA-PC gave three fractions containing oxidatively truncated phospholipids that inhibit binding of oxLDL with the scavenger receptor CD36. Molecular structures of the active phospholipids were established by LC/MS comparison with authentic samples and various derivatives that were prepared by unambiguous total syntheses.

rearrangement of lipid endoperoxides produced by free radical-induced cyclooxygenation of arachidonyl phospholipids, avidly bind covalently with and cross-link proteins. It seems likely, although largely unexplored, that these reactions interfere with many normal cellular functions. Plasma isoLG-protein adduct levels provide a cumulative index of oxidative injury, and elevated levels are associated with AS. IsoLG-protein adduct levels are independent of those of total cholesterol, a classical risk factor for AS.

Oxidatively truncated phospholipids present in oxLDL can trigger receptor-mediated cellular responses such as endocytosis of oxLDL and promotion of protein expression. Phospholipid analogs of HNE, incorporating a γ-hydroxyalkenal functional array, are reactive electrophiles that modify proteins, thereby interfering with their normal functions such as proteolysis or intracellular trafficking. Oxida-

tively truncated phospholipids can also contribute to atherogenesis by fostering infiltration of monocyte macrophages into the subendothelial space where they become foam cells through unregulated endocytosis. They can also contribute to foam cell formation by impairing the processing of oxLDL. Since vitamin E (α-tocopherol) reacts with dihydroperoxydienes causing fragmentation to give reactive γ-hydroxyalkenals, high serum concentrations of vitamin E (or of vitamin C that can regenerate vitamin E from its oxidized forms) may promote fragmentation of lipid hydroperoxides *in vivo*. There is some evidence for the presence in oxLDL of cholesteryl esters that incorporate oxidatively truncated lipids [53]. The presence of analogous cholesteryl esters of IsoLGs in the nonpolar core of oxLDL particles seems likely but remains to be confirmed. Such products of lipid oxidation could contribute to the pathological effects of oxidized lipoproteins that transport them into phagocytes as toxic cargo that is released when these cells disassemble the oxidatively damaged particles.

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