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Discoveries

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Isolevuglandin Adducts in Disease

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Abstract

Significance: A diverse family of lipid-derived levulinaldehydes, isolevuglandins (isoLGs), is produced by rearrangement of endoperoxide intermediates generated through both cyclooxygenase (COX) and free radical-induced cyclooxygenation of polyunsaturated fatty acids and their phospholipid esters. The formation and reactions of isoLGs with other biomolecules has been linked to alcoholic liver disease, Alzheimer's disease, age-related macular degeneration, atherosclerosis, cardiac arythmias, cancer, end-stage renal disease, glaucoma, inflammation of allergies and infection, mitochondrial dysfunction, multiple sclerosis, and thrombosis. This review chronicles progress in understanding the chemistry of isoLGs, detecting their production *in vivo* and understanding their biological consequences. ***Critical Issues:*** IsoLGs have never been isolated from biological sources, because they form adducts with primary amino groups of other biomolecules within seconds. Chemical synthesis enabled investigation of isoLG chemistry and detection of isoLG adducts present *in vivo*. ***Recent Advances:*** The first peptide mapping and sequencing of an isoLG-modified protein present in human retina identified the modification of a specific lysyl residue of the sterol C27-hydroxylase Cyp27A1. This residue is preferentially modified by iso[4]LGE_2 *in vitro*, causing loss of function. Adduction of less than one equivalent of isoLG can induce COX-associated oligomerization of the amyloid peptide $A\beta_{1\text{-}42}$. Adduction of $isoLGE_2$ to phosphatidylethanolamines causes gain of function, converting them into proinflammatory $isoLGE_2$-PE agonists that foster monocyte adhesion to endothelial cells. ***Future Directions:*** Among the remaining questions on the biochemistry of isoLGs are the dependence of biological activity on isoLG isomer structure, the structures and mechanism of isoLG-derived protein–protein and DNA–protein cross-link formation, and its biological consequences. *Antioxid. Redox Signal.* 22, 1703–1718.

Background

Discovery in vitro

NATURAL PRODUCTS ARE generally discovered by isolation from biological sources, often guided by biological activity-based assays. *Isolevuglandins (isoLGs) are unusual natural products in that they have never been isolated from biological sources* (72). In 1977, we observed that decomposition of the bicyclic peroxide nucleus (2,3-dioxabicyclo[2.2.1]heptane) of the prostaglandin endoperoxide PGH_2 produces levulinaldehyde, and this reaction is especially rapid ($t_{1/2} = 5$ min at 37°C) in neutral aqueous solution (69, 78). Although the rapid nonenzymatic decomposition of PGH_2 in the aqueous environment of its biosynthesis was well known (54, 55, 64), levulinaldehyde derivatives with prostanoid side chains (levuglandins) had never been identified as products. The model study provided the inspiration to explore the biological ramifications of this chemistry, and in 1984 we showed that spontaneous rearrangement of PGH_2 produces not only two prostaglandins PGD_2 and PGE_2 but also two levuglandins, LGD_2 and LGE_2 (Fig. 1) (70, 77). Aided by a chemical synthesis (76) that facilitated studies of the chemistry of LGs, it soon emerged that *LGs rapidly (in seconds) form adducts with proteins* (74), and produce protein–protein (43) and DNA–protein cross-links (51). The formation of covalent adducts with proteins was first demonstrated with radiolabeled LGs that were prepared by chemical synthesis (74). Owing to their extraordinary proclivity for adduction to primary amino groups of biomolecules, unadducted LGs have never been found *in vivo*. Nevertheless, considerable progress has been made in understanding the chemistry of LGs, including determining the molecular structures of their covalent adducts (*vide infra*), confirming the production of LGs *in vivo*, and understanding its biological consequences.

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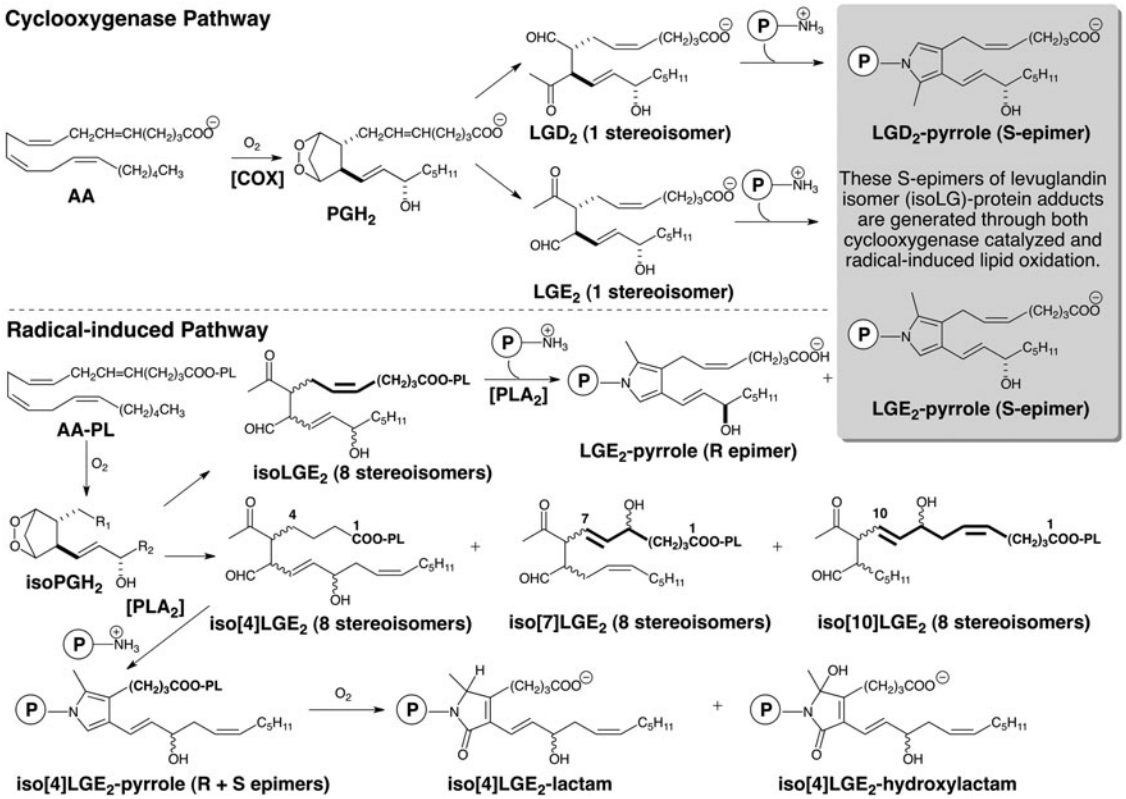

FIG. 1. The cyclooxygenase (COX) and radical-induced cyclooxygenation of arachidonate leads to the formation of isoLGs. The COX pathway generates LGE_2 as a single stereoisomer whereas the radical-induced pathway also generates LGE_2 as well as seven other stereoisomers (referred to collectively as $isoLGE_2$). Protein adduction generates a pyrrole adduct as a single allylic hydroxyl S-epimer from LGE_2 but both the R- and S-epimers of the allylic hydroxyl are produced from the eight stereoisomers of LGE_2. Only the radical-induced pathway generates *structural isomers* of LGE_2, for example, iso[4]LGE_2. Thus, protein and aminophospholipid adducts of iso[n]LGs are biomarkers of radical-induced lipid oxidation.

Figure 2 provides a historical perspective of key developments in this field.

In 1997, 20 years after discovering the endoperoxide to levulinaldehyde rearrangement, we secured the first evidence that LGs are generated *in vivo*. Using anti-LGE_2 antibodies raised against proteins modified with pure LGE_2 obtained by chemical synthesis, we detected disease-related elevations in levels of LGE_2 adducts in blood from patients with atherosclerosis (AS) or end-stage renal disease (RD) (79). That LG-protein adducts can be formed *via* a PGH synthase-dependent pathway (7) has now been demonstrated by activating human platelets with exogenous arachidonic acid or thrombin (8). Formation of these adducts is inhibited by indomethacin, a PGH synthase inhibitor, and is enhanced by an inhibitor of thromboxane synthase, an enzyme that metabolizes PGH_2. Thus, enzymatic cyclooxygenation of arachidonate leads to the production LGs in whole cells through a spontaneous rearrangement of PGH_2 that competes with its enzymatic conversion to thromboxane A_2.

Radical-induced cyclooxygenation produces isoLGE$_2$

There is an alternative mechanism for the formation of LGE_2. We had anticipated that a free radical pathway would also produce LGE_2 adducts. Nevertheless, we were surprised to find that free radical-induced oxidation of low-density lipoprotein (LDL) *in vitro* generated LGE_2-adduct immunoreactivity (80). Although free polyunsaturated fatty acids, for example, arachidonate released from arachidonyl phospholipids (PLs), are the substrates for cyclooxygenase (COX), we had expected that $isoLGE_2$ formed on oxidation of LDL would be esterified in PLs. Our LGE_2-protein antibodies did not detect protein adducts of the LGE_2 ester of 2-*lyso*-phosphatidylcholine (PC) prepared by chemical synthesis. Therefore, phospholipase activity associated with the LDL particle must hydrolyze the protein adducts of $isoLGE_2$-PC exposing the unesterified epitopes. Hydrolytic release of lyso-PC is known to accompany oxidation of LDL (88). Seven years later, in an animal model of oxidative injury, treatment of rats with CCl_4 was shown to cause the formation of protein adducts of isoLGs esterified in PLs that eventually undergo phospholipolysis *in vivo* (12). This is consistent with our earlier finding that protein adducts of an LGE_2-PL ester are substrates for snake venom PLA_2 (80).

Structural isomers of LGE$_2$: unique products of radical-induced cyclooxygenation

Detection of LGE_2 adducts *in vivo*, for example, the LGE_2-pyrrole (Fig. 1), with anti-LGE_2 antibodies cannot distinguish between their production through enzymatic cyclooxygenation and radical-induced cyclooxygenation because the exactly same LGE_2 isomer can be produced by either pathway. However, while the COX-promoted cyclooxygenation of arachidonate only generates LGE_2 and LGD_2 with prostanoid

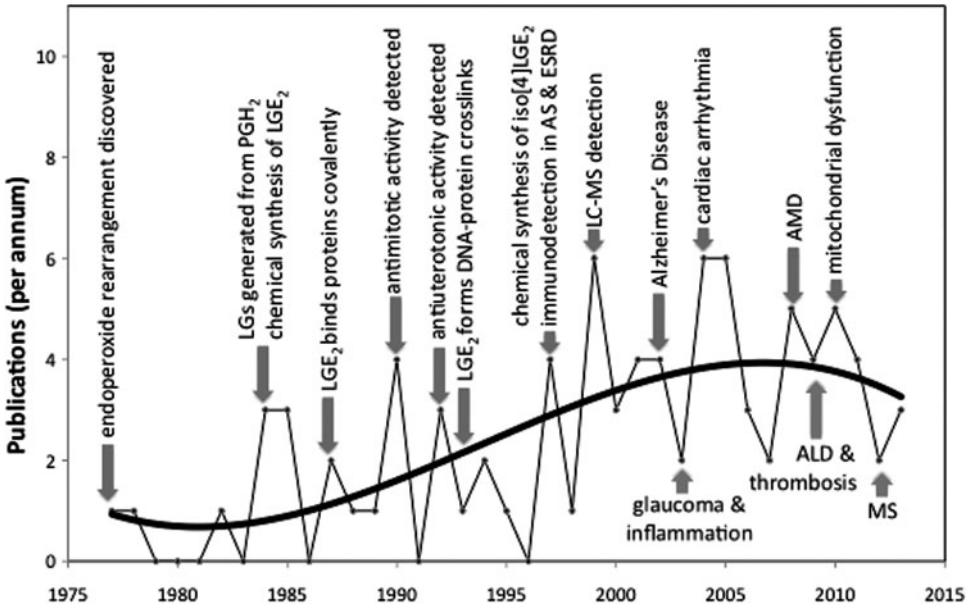

FIG. 2. A model study in 1977 led to our discovery that the prostaglandin endoperoxide PGH_2 rearranges nonenzymatically to levulinaldehyde derivatives with prostanoid side chains (levuglandins, LGs). Detecting the formation of LGs *in vivo* was complicated by their proclivity to rapidly form covalent adducts with primary amines. Finally, after two decades of research, the first evidence for the formation of LG-adducts *in vivo* emerged. We detected elevated levels of LGE_2-protein adduct immunoreactivity in blood from patients with atherosclerosis (AS) or end-stage renal disease (RD). That same year, we showed that LGs and a vast array of stereo and structural isomers, referred to collectively as isolevuglandins (isoLGs), are also generated *in vivo* through free radical-induced cyclooxygenation. Since then, our studies and those of others continue to expand the list of diseases associated with isoLGs, as well as understanding the mechanisms by which isoLGs contribute to pathology.

side chains, we postulated that 8 LGE_2 *stereoisomers* (referred to collectively as isoLGE$_2$) are only one of the eight structurally isomeric families of isoLGs (each also consisting of eight stereoisomers) that can be generated from AA-PC by nonenzymatic cyclooxygenation (80). The *structural isomers* of LGE_2 and LGD_2 are named with a bracketed numeral [n], for example, iso[4]LGE_2 (Fig. 1), to indicate the number of carbon atoms in their carboxylic side chains. Most importantly, these structural isomers with non prostanoid side chains and their protein adducts can only arise through free radical-induced cyclooxygenation. Using anti-iso[4]-LGE_2 antibodies, we secured the first unambiguous evidence that LG isomers are also produced *in vivo* through free radical-induced cyclooxygenation. Mean levels of both isoLGE$_2$- and iso[4]LGE$_2$-protein adducts in plasma from patients with AS ($n=16$) or RD ($n=8$) are about twice those in healthy individuals ($n=25$) (73, 79). These elevated levels are not related to variations in age, total cholesterol, or apoB. A linear correlation ($r=0.79$) between plasma isoLGE$_2$- and iso[4]LGE$_2$-protein adduct levels in all 49 individuals is consistent with a common free radical-induced mechanism for the production of both oxidized lipids *in vivo*. The correlation is even stronger ($r=0.86$) for patients with AS or RD. Furthermore, isoLG-protein adduct levels are more strongly correlated with disease than are total cholesterol or apoB, suggesting an independent defect that results in an abnormally high level of oxidative injury associated with AS and RD (63). Since then, the association of isoLGs with a growing list of diseases has emerged (Fig. 2).

It is important to understand that immunoreactivity detected with anti-LGE_2 or a single-chain (scFv) antibody that binds with isoLGE$_2$ adducts (22) does not provide a tool for unambiguous distinction between the COX and free radical-induced generation of isoLGs (71), because iso-LGE_2 and the LGE_2-pyrrole (S-epimer) are products of both pathways (Fig. 2). The conclusion that immunoreactivity detected with an isoLGE$_2$-specific ''D11 ScFv'' is indicative of free radical-induced oxidant stress is incorrect, because COX-mediated cyclooxygenation cannot be ruled out (22, 93). We recently generated a mouse monoclonal antibody (mAb) against iso[4]LGE$_2$-protein adducts that is highly selective for adducts of iso[4]LGE_2 versus those of isoLGE$_2$. Since iso[4]LGE$_2$ is only produced through free radical-induced cyclooxygenation, this anti-iso[4]LGE$_2$ mAb provides an excellent tool for unambiguously detecting the operation of that pathway, because it does not respond to products of the COX pathway. This antibody may also prove useful for enrichment of isoLG-modified proteins in biological extracts by standard immunoaffinity techniques, and thus facilitate proteomic identification of protein targets of isoLGs *in vivo*.

Throughout this review, I have eschewed the ''isoketal'' terminology introduced by others, because it is a misleading alternate nomenclature that refers to the products of the free radical-induced cyclooxygenation as ''isoketals'' to ''distinguish them from the products of the cyclooxygenase pathway'' (3). However this is a false dichotomy since the products of the COX pathway are also generated through free radical-induced cyclooxygenation. The claim that ''isoketals are…analogous to cyclooxygenase-derived levuglandins'' (2) is incorrect, because the levuglandins generated through COX and free radical-induced cyclooxygenation are the exact same molecules and not analogs. Levuglandins are a subclass of isoLGs (Fig. 1).

Identification of specific proteins modified by isoLGs

Identification of the proteins that are modified *in vivo* by isoLGs was first attempted by Western blot analysis of two-dimensional SDS-PAGE using antibodies against adducts and subsequent proteomic analysis (39). Although the proteins identified co-migrate with immunoreactivity against specific adducts, proteomic evidence establishing the *in vivo* presence of isoLG-derived modifications of specific aminoacyl residues of proteins in biological samples had never been reported.

An arachidonic acid analogue with a terminal alkyne (alAA) was used to modify proteins in cultured cells by lipid oxidation products generated through exposure to iron ions and ascorbate or t-butylhydroperoxide (53). Extraction of the resulting covalently modified proteins was then achieved through cycloaddition of the alkyne with a biotin-linked azide. A target protein, that is, the cardiac sodium channel Na_v 1.5, in the extract was then identified by Western blot analysis using antibodies specific for the protein. The conclusion that covalent modification of the protein involved the generation and adduction an isoLG was supported by inhibition of protein modification by a scavenger that is particularly reactive toward isoLGs (53).

Recently, using targeted LC-MS/MS analysis of tryptic peptides from protein extracts of human retina, we demonstrated that peptide mapping and sequencing of an *in vivo* isoLG-modified protein can be used to unambiguously establish that a specific protein, cytochrome P450 (CYP)27A1 modified with an isoLG on a particular lysyl residue is present in the human retina (*vide infra*) (16).

Isolevuglandin structural diversity and the common core

One factor that complicates characterizing the biological occurrence of isoLGs and their pathological involvements in disease is the generation of a vast array of structural isomers through cyclooxygenation of polyunsaturated fatty acyls with 3, 4, 5, or 6 C=C bonds alternating with CH_2 groups, that is, doubly allylic methylenes (Fig. 3). We reported the chemical synthesis of 17-isoLGE_4 (84). Subsequently, evidence was obtained confirming the presence of protein adducts of isoLGE_4 or its stereo and structural isomers *in vivo* (3), and elevated levels of these protein adducts were detected in cerebrospinal fluid from multiple sclerosis patients (29).

Although hundreds of different isoLGs may be produced *in vivo*, the reactions that produce all isoLGs and their biological chemistry is expected to be very similar owing to the localization of their reactive functionality in a ''common core'' structure (highlighted in Fig. 4) as well as identical carboxyl and alkyl termini in their side chains. The isoLGs derived from various polyunsaturated fatty acyl PLs differ only by having various hydrocarbon chains inserted into their upper and lower side chains. These differences are not expected to influence the biological chemistry of the γ-ketoaldehyde core or the derived pyrroles. These pyrroles are electron rich and prone to oxidation. Our discovery of the free-radical induced formation of isoLG-derived protein adducts was subsequently confirmed by collaborative LC-MS/MS studies that we initiated with Roberts at Vanderbilt University. Those studies demonstrated the formation of LG isomers on free radical-induced *in vitro* oxidation of arachidonic acid (AA), and revealed that oxidation of the initial isoLG-pyrrole adducts generates lactams and hydroxylactams (13). These structures were identified by exhaustive proteolysis of isoLG-modified protein that delivered the corresponding lysyl lactams and hydroxylactams (Fig. 4). Additional complexity in the products arising from isoLGs is found for some isoLGs that have 1,4-diene moieties in their sidechains. Further oxidation can convert the 1,4-diene moieties to 1-hydroxy-2,4-dienes (5).

Besides differences in the isomeric composition of isoLGs produced through the COX and free radical-induced pathways, the cyclooxygenation substrates for these two pathways are also distinct. Free polyunsaturated fatty acids, for example, arachidonate released from arachidonyl PLs are the substrates for COX. That PL esters of polyunsaturated fatty acids are the primary targets *in vivo* for radical-induced cyclooxygenation was demonstrated in a rat model of oxidant injury to the liver that results in free radical-induced oxidation of polyunsaturated PLs (12). After exposure of rats to CCl_4, both lactam adducts and Schiff base adducts were detected in the liver. The lactam adducts were present as free acids, but >97% of the Schiff base adducts were apparently esterified to PLs because treatment of the proteins with base caused a 42-fold increase in the level of the Schiff base free acid detected (12). Thus, endoperoxide intermediates and the isoLGs generated through their rearrangement remain esterified in PLs that bind rapidly to proteins to generate Schiff base adducts. These metastable adducts undergo a slow subsequent dehydration to deliver pyrrole intermediates that are ultimately oxidized to produce hydroxylactam and lactam end products that were detected by exhaustive proteolysis that delivers lysyl hydroxylactams and lysyl lactams (Fig. 4).

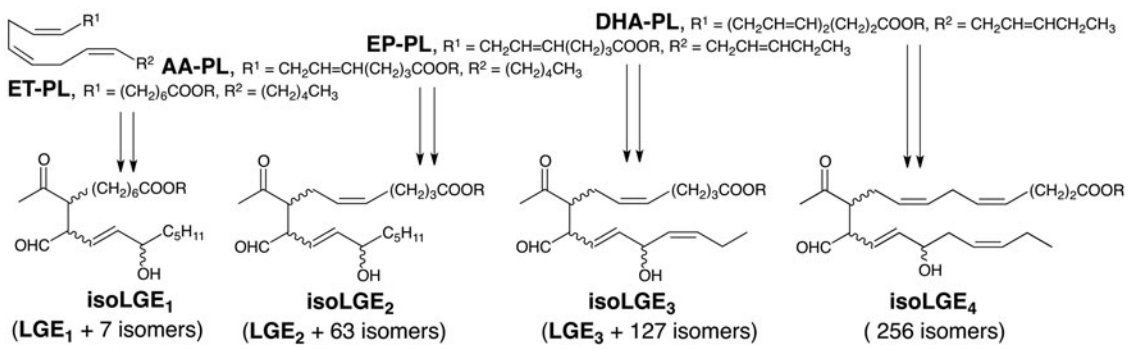

FIG. 3. Cyclooxygenation of polyunsaturated fatty acyls with 3, 4, 5, or 6 C=C bonds alternating with CH_2 groups (doubly allylic methylenes) leads to the formation of a vast array of structurally diverse isoLGs.

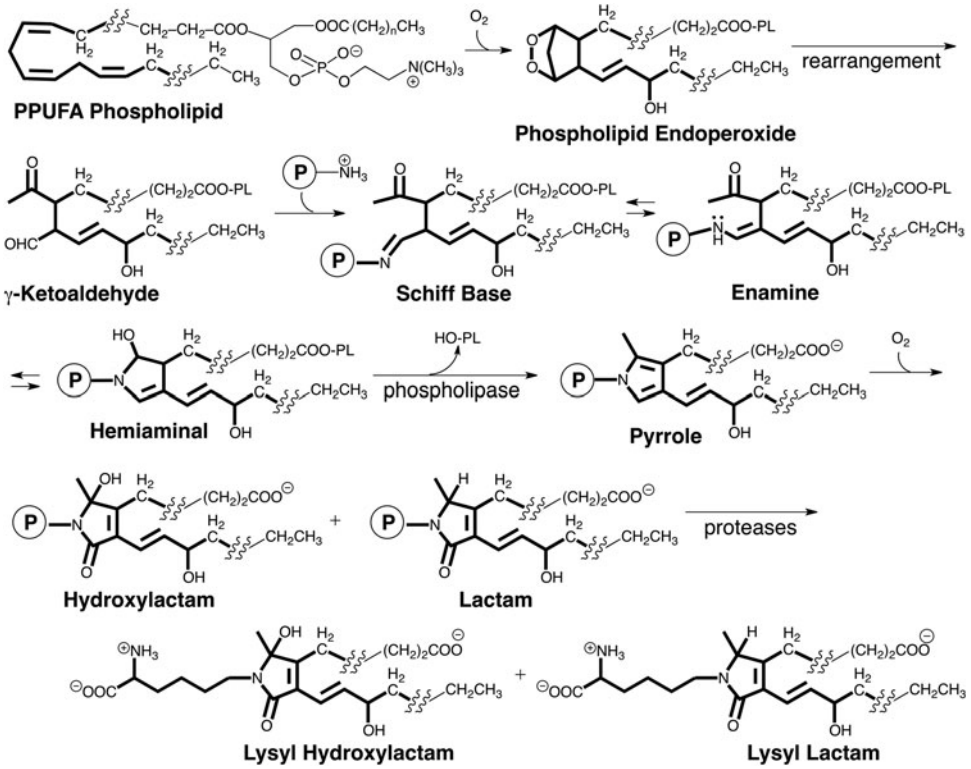

FIG. 4. The reactive functionality of all isoLGs is located in a "common core" structure (*highlighted*). This ensures that the chemistry of all isoLGs will be virtually identical.

Using LC–MS/MS, we showed that isoLG adducts that incorporate the amino group of phosphatidylethanolamines (PEs) into isoLG-hydroxylactams (Fig. 5) are generated *in vivo* (47). Quantitative analysis of these hydroxylactams is achieved with samples that are an order of magnitude smaller, and sample processing is much simpler and less time consuming than required for measuring protein-derived isoLG-lysyl lactams. *In vivo*, PEs incorporate a variety of acyl groups esterified to the 1 and 2-positions. Treatment of heterogeneous mixtures of isoLG-modified PEs with phospholipase A_2 generates mainly 1-palmitoyl-2-lysoPE-isoLG adducts (47). Even greater simplification is achieved by treating such mixtures of isoLG-PEs with methyl–amine (33) or sodium hydroxide (34) that converts them into isoLG adducts of glycerophosphate ethanolamine (GPEA, Fig. 5). Alternatively, treatment with PLD_2 converts the isoLG-PEs into isoLG adducts of ethanolamine (isoLG-EAs, Fig. 5) (90).

Interestingly, LGs also form covalent adducts with the guanidine group of protein arginyl residues, but the adducts are unstable, readily undergoing hydrolysis to produce ornithine residues (98). Stable adducts of LGs with the deoxycytidyl primary amino group in DNA have also been characterized (14).

Inflammation fosters the production of isoLGs

Using a mouse Candida sepsis model of inflammation, we observed 3.5- and 2.7-fold increases in iso[4]LGE_2 and iso-LGE_2 adducts of plasma proteins after pathogen exposure in wild-type mice (62). Myeloperoxidase (MPO) uses H_2O_2 together with NO_2^- to generate nitrogen dioxide, a free radical capable of initiating lipid peroxidation. That MPO contributes to the formation of isoLG protein adducts *in vivo* under inflammatory conditions is suggested by a 34% reduction in protein adduct content in plasma proteins from MPO knockout mice ($p = 0.003$) even though an increase might be anticipated because the inflammatory state is more severe (persistent) in the knockout mice.

In a murine model of allergic airway inflammation produced by sensitization to ovalbumin with subsequent daily aerosol challenge, increased isoLGE_2-adduct immunoreactivity was found in airway epithelial cells at 24 h after the first aerosol challenge and at 5 days in alveolar macrophages (93). Collagen surrounding airways and blood vessels, and airway and vascular smooth muscle, also exhibited increased immunoreactivity after ovalbumin challenge. High levels of iso-LGE_2-adducts appeared in epithelial cells within an hour, declining after 24 h and dropping to baseline levels by day 5 in spite of continual daily challenge. In contrast, baseline levels of isoLGE_2-adducts were present in alveolar macrophages after an hour, rising modestly by 24 h and reaching a maximum at day 5. These contrasting time courses suggest that isoLGs in macrophages arise from macrophage uptake and accumulation of isoLG adducts shed from epithelial cells or generated in the extracellular matrix. This conclusion is supported the observation that LGE_2-modified LDL binds with and is degraded on receptor-mediated endocytosis by macrophage cells (40). Both the COX and free radical-induced pathways could be involved in the production of the isoLGE_2 detected with the ScFv antibody used in this study. Stimulation of A549 cultured lung epithelial cells with the cytokine IL-1β followed by treatment with arachidonic acid results in upregulation of COX-2 and elevation of isoLGE_2-histone levels in the nucleus, and the formation of these isoLGE_2 (presumably the pure LGE_2 stereoisomer) adducts is inhibited by pretreatment of the cells with the nonsteroidal anti inflammatory drug indomethacin (15).

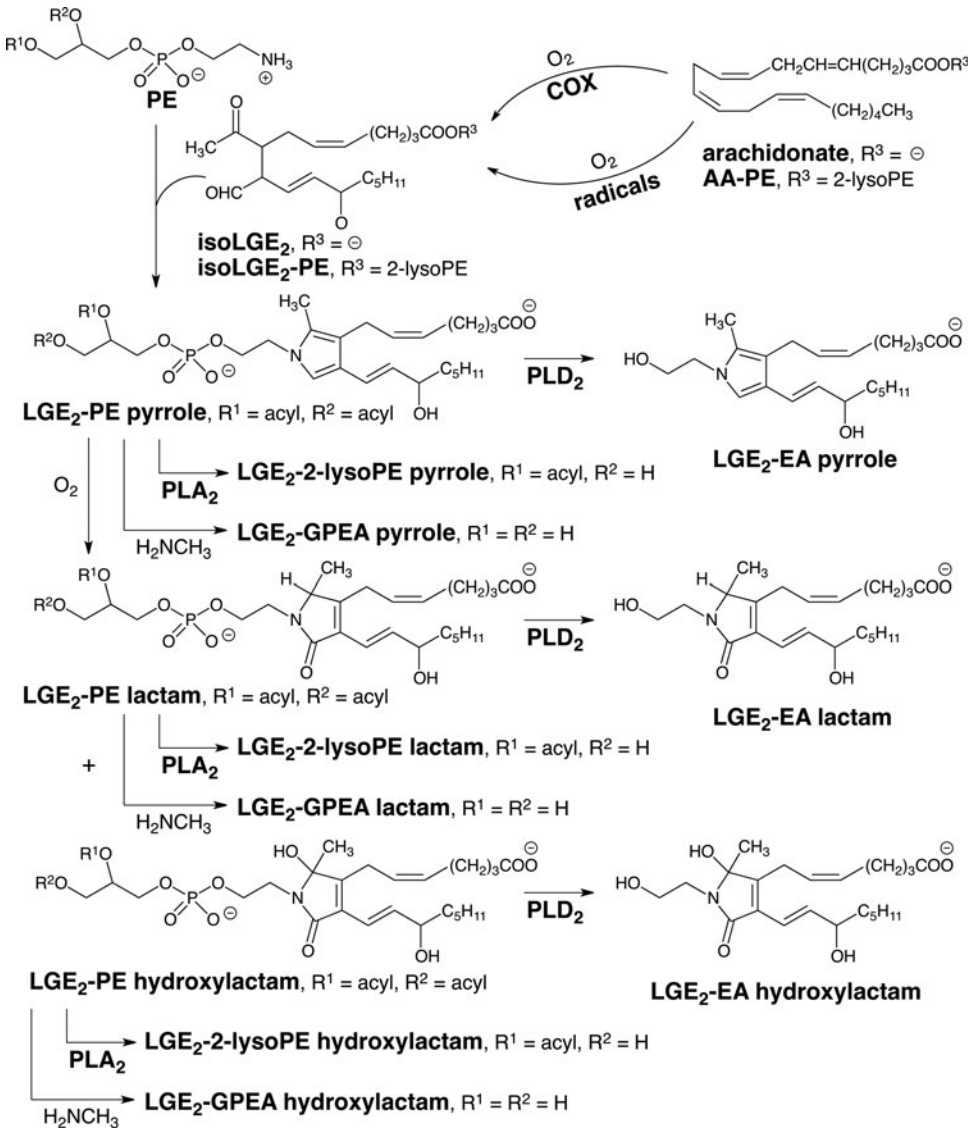

FIG. 5. IsoLG adducts that incorporate the amino group of phosphatidylethanolamines (PEs) complex into isoLG-hydroxylactams are generated *in vivo*. Analysis of the adducts of phospholipids with a mixture of acyl groups is simplified by phospholipase catalyzed conversion into 2-isoLG adducts of lyso phospholipids or ethanolamine. Treatment with methylamine or sodium hydroxide converts them into isoLG adducts of glycerophosphate ethanolamine.

In a mouse model of alcoholic liver disease, free radical-induced oxidative damage owing to inflammation is indicated by an accumulation of iso[4]LG adducts in liver tissue. Here, there is no ambiguity as to COX versus free radical-induced generation of the isoLG detected, because iso[4]LGE_2 is only produced through free radical-induced cyclooxygenation. More than160% higher mean levels of iso[4]LGE_2-PE-hydroxylactam ($p < 0.001$) were detected by LC-MS/MS in liver homogenates from chronic ethanol-fed mice (32.4 ± 6.3 ng/g, $n = 6$) compared with controls (12.1 ± 1.5 ng/g, $n = 4$) (47). Immunohistochemical analysis of liver tissue from these mice found elevated levels of iso[4]LGE_2 and isoLGE$_2$ immunoreactivity in the ethanol-fed mice compared with controls (67). That isoLGE$_2$ was produced by free radical-induced lipid oxidation was confirmed by the failure of COX 1 or 2 deficiency to prevent the ethanol-induced generation of LGE_2 adducts. Pathological markers of ethanol-induced hepatic injury, including serum alanine aminotransferase, hepatic triglyceride, and cytochrome P450 (CYP)2E1, were elevated in response to ethanol feeding. Tumor necrosis factor (TNF)α can induce mitochondrial reactive oxygen species production in hepatocytes (23). Animals deficient in TNFα receptor 1 (TNFR1) (96) or treated with TNFα neutralizing antibody are protected from ethanol-induced lipid peroxidation and liver injury (41). IsoLG adduct formation was reduced in both TNFR1- and CYP2E1-deficient mice. In summary, ethanol feeding enhanced isoLG–protein adduct production *via* a TNFR1/CYP2E1-dependent, but COX-independent, mechanism in mouse liver.

Children born prematurely have significantly ($p = 0.008$) elevated mean levels of blood plasma iso[4]LGE_2-adduct immunoreactivity, 22.7 ± 5.9 nmol/ml ($n = 6$) compared with 15.3 ± 5.0 nmol/ml ($n = 16$) in children with no significant birth events (24). This suggests that chronic inflammation is a heretofore-unrecognized consequence of premature birth or postnatal treatment protocols that result in a failure to develop fully competent antioxidant defenses.

Pyridoxamine and analogues trap isoLGs inhibiting their adduction to proteins

By screening various primary amines as potential scavengers of γ-ketoaldehydes, a model γ-ketoaldehyde, 4-oxopentanal, was found to react extremely rapidly with pyridoxamine (PM), with a second-order rate constant at

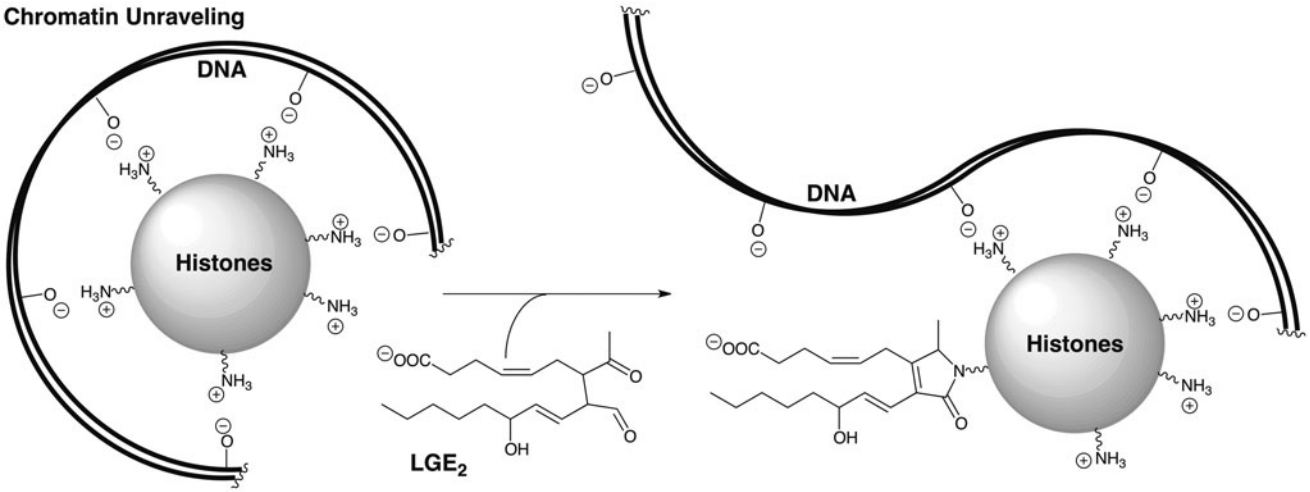

FIG. 6. Conversion of histone lysyl primary amino groups into pyrroles or lactams, neither of which is protonated at physiological pH, in conjunction with the addition of a negatively charged carboxyl group, could disrupt histone-DNA binding.

physiological pH being approximately 2300 times faster than that of Nα-acetyllysine (1). The extreme reactivity of PM was unique to γ-dicarbonyls owing to a mechanism involving facilitation by the phenolic proton of ring closure to deliver a pyrrole from an unstable initial Schiff base adduct. PM prevented adduction to ovalbumin and inactivation of RNase A and glutathione reductase by isoLGE$_2$, and inhibited the formation of isoLG-protein adducts in platelets activated with arachidonic acid (21). PM and lipophilic PM analogues, such as salicylamine, are noncytotoxic isoLG scavengers that block the formation of LG adducts of proteins in cells without inhibiting the catalytic activity of the COXs and thus without interfering with the formation of prostaglandins (97). They are useful for assessing the biological consequences of isoLG production *in vivo* (*vide infra*). The oral administration of salicylamine can be used to assess the contribution of isoLGs in animal models of disease (100). They also have been used in experimental animals *in vivo* to assess the pathophysiological contribution of levuglandins in diseases associated with COX upregulation, as have other primary amines that compete with protein lysyl ε-amino residues. Thus, glucosamine at doses that did not inhibit prostaglandin biosynthesis totally abolished formation of LG-protein adducts after induction in A549 lung cancer cells of COX-2 by IL-1β (11, 42, 89).

Pathological Activities of Isolevuglandins

Inhibition of tubulin polymerization and mitosis

LGE$_2$ inhibits the first synchronous cell division of fertilized sea urchin eggs with an ED$_{100}$ ~25 μ*M* (52). Tubulin is the major constituent protein of the microtubule, an important component of the eukaryotic cytoskeleton, and an integral part of the mitotic spindle. Presumably, LGE$_2$ can enter cells and bind to tubulin, thus preventing microtubule assembly and inhibiting mitosis. Indeed, LGE$_2$ inhibits GTP-induced assembly of bovine microtubule protein (~1.4 mg/ml, ~85% tubulin, and 15% microtubule-associated protein) with a slightly higher ED$_{100}$ ~80 μ*M* (52). The LG did not cause depolymerization of preassembled tubulin. Complete inhibition of assembly occurred on binding of only ~2 molecules of LG per tubulin dimer, one to each subunit. These observations suggest that a small number of key residues essential for microtubule assembly are modified by LGE$_2$ in the dimer state but are inaccessible to LGE$_2$ in the polymerized state.

Modification of histones and disruption of their nucleosomal complex with DNA

To stimulate COX-2 expression, cultured lung epithelial cells (A549) were treated with IL-1β or macrophages cells (RAW264.7) were treated with LPS and IFNγ. Subsequent addition of arachidonic acid resulted in the generation of LG-protein adducts of histones (15). Interestingly, a strong preference for modification of histone H4 and, to a lesser extent, of H3/H2B was observed. Changes in lysyl modifications of H4, in particular, are a common hallmark of human cancers. This discovery links inflammation and COX-2 expression that is associated with the development of many cancers, with histone modification by LGs. DNA is ''packaged'' into nucleosomes by winding the negatively charged phosphodiester-linked polynucleotide around histone octamers that are positively charged owing to protruding histone lysyl residues (Fig. 6). Conversion of the lysyl primary amino group into a pyrrole or lactam, neither of which is protonated at physiological pH, in conjunction with the addition of a negatively charged carboxyl group, could disrupt histone-DNA binding (Fig. 6). It is tempting to speculate that this may increase DNA transcriptional access to previously silent oncogenes and contribute to the development of cancer.

Modification of cytochrome c in mitochondria impairs respiration

Positively charged lysine residues on cytochrome c are responsible for its association with the negatively charged, cardiolipin-rich mitochondrial inner membrane as well as with cytochrome c oxidase and cytochrome c reductase (65). Modification of lysine residues on cytochrome c is expected

to weaken these interactions by converting positively charged lysyl amino groups into derivatives that lack a protonated amine, and introducing negatively charged carboxylates. Treatment of mitochondria with isoLGE$_2$ impairs maximal respiration that is restored by the addition of unmodified cytochrome c (87). Apparently, modification of cytochrome c by isoLGs reduces its affinity for the electron transport chain complex, thereby disrupting respiration.

Disruption of the blood–brain barrier

The potent cytotoxicity of isoLGs can damage the blood–brain barrier. Direct injection of 100 nmol of isoLGE$_2$ into the substance of the cerebral hemisphere of rats caused pallor and loss of cellular constituents typical of necrotic cells, and the marginal area became hypercellular because of the infiltration by macrophages (82). A dose-dependent extravasation of Evans Blue revealed loss of blood–brain barrier integrity.

DNA-protein cross-linking

LGE$_2$ causes repair-resistant DNA–protein cross-links and cell death (LD$_{50}$ = 230 n*M*) in V79 Chinese hamster lung fibroblasts (51). After removal of any unadducted free LGE$_2$ and the return of the cultures to normal growth medium, additional DNA-protein cross-links continued to form over the ensuing 6–24 h. The results suggest that LG adducts to DNA or protein are not repaired, but react further at sites on protein or DNA in close proximity to the initial adducts, forming cross-links in a slow phase of the process.

Protein-protein cross-linking

Treatment of proteins with isoLGs generates protein–protein cross-links (43). The consumption of monomeric ovalbumin by reaction with 15 equivalents of LGE$_2$ is almost complete within 2 h. Initially, polyacrylamide gel electrophoresis of the reaction product reveals that the amount of dimer rises rapidly, reaching 30% within 2 min, but then drops, and the formation of a ''ladder'' of various oligomers is readily apparent after 30 min. Only high-molecular-weight oligomers are present after 4 h. Malondialdehyde or 4-hydroxynon-2-enal caused no discernable oligomerization under the same conditions. Thus, isoLGE$_2$ is orders of magnitude more effective in generating protein–protein cross-links than these other electrophilic products from arachidonate oxidation that bind with and cross-link proteins.

Although many molecules of isoLGE$_2$ bind within 1 min to each molecule of protein, protein–protein crosslinking is completely prevented by adding a large excess of glycine to the reaction mixture at 5 min after combining the protein and isoLG, resulting in protein-glycine crosslinks (44). Thus, isoLGs serve as 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 such as glycine and spermine (6), a polyamine that is abundant in both the cytosol and nucleus. It is likely, although as yet unproven, that isoLGs also cross-link proteins with ethanolamine PLs resulting in anchoring proteins to cell membranes.

COX promoted oligomerization of amyloid $\beta_{1\text{-}42}$: Alzheimer's disease

Accumulation of amyloid β (Aβ) in the brain is associated with the pathogenesis of Alzheimer's disease (AD) (28, 83). Dimers and higher oligomers of Aβ are abundant in regions exhibiting neuronal pathology (50, 66), and soluble oligomers of A$\beta_{1\text{-}42}$ are cytotoxic to neurons (45, 81, 94). Incubation of monomeric A$\beta_{1\text{-}42}$ with PGH$_2$ or LGE$_2$ caused the formation of oligomers, resembling protofibrils in contrast with the slower spontaneous oligomerization of this peptide that produced long fibrils (10). The average amount of LG-protein adducts is 12-fold higher in AD brains than in control brains with the magnitude of the increase correlating with the pathological evidence of severity (99). That the LG-protein adducts isolated from AD brain do not result from free radical-induced lipid oxidation was indicated by LC-MS/MS chromatogram of the isoLG-lysine lactam obtained through exhaustive proteolysis of AD brain proteins that exhibited a single peak (99). In contrast, the isoLG-lysine lactam obtained from the mixture of isolevuglandin isomers generated by radical-catalyzed oxygenation of arachidonic acid exhibited multiple peaks. After 24 h incubation with isoLGE$_2$, A$\beta_{1\text{-}42}$ becomes toxic to primary cultures of mouse cerebral neurons; whereas A$\beta_{1\text{-}42}$ itself incubated for 24 h without the levuglandin exhibited little toxicity. The oligomers formed from a reaction of A$\beta_{1\text{-}42}$ with isoLGE$_2$ exhibit immunochemical similarity with neurotoxic amyloid-derived diffusible ligands.

Most remarkably, the formation of these neurotoxic oligomers occurred at a molar ratio as little as 0.1 LGE$_2$ per A$\beta_{1\text{-}42}$ demonstrating that intermolecular cross-linking cannot be the sole mechanism for oligomerization (9). It is tempting to speculate that *modification of A$\beta_{1\text{-}42}$ by LGE$_2$ serves as a seed to accelerate oligomerization*. These observations support the view that a 60%–80% reduction in risk of developing AD associated with the use of nonsteroidal antiinflammatory drugs for at least 2 years (42, 89) involves the inhibition of isoLG production through the COX pathway. These observations laid the foundation for a new biochemical paradigm for the participation of the COX enzymes in AD. A recent study on the effect of an isoLG scavenger on dementia in mice added important support for this view. Thus, oral administration of salicylamine to a mouse model of dementia, protected the mice against the development of age-related deficits in spatial working memory (20). This observation suggests that trapping of isoLGs may mitigate the development of cognitive dysfunction in patients with AD.

Inhibition of proteolysis: neurodegeneration and glaucoma

Covalent modification of proteins, for example, ovalbumin (OA) or the amyloid peptide A$\beta_{1\text{-}40}$, with isoLGE$_2$ conferred resistance to processing by the 20S proteasome (19). Furthermore, both isoLGE$_2$-OA and isoLGE$_2$-A$\beta_{1\text{-}40}$ competitively inhibit the chymotrypsin-like activity of the 20S proteasome for processing nonadducted proteins. Similarly, a ubiquinated iso[4]LGE$_2$-modified protein, that is, calpain-1, inhibited 26S proteasome activity, while unmodified calpain-1 had no effect (31). Presumably, the bulky isoLG adduct sterically hinders the processive passage of the protein through the peptide tunnel of the proteosome where its protease activity resides. This competitively inhibits access of nonadducted

proteins to the active site (19). Covalent modification of the 20S proteasome with isoLGE$_2$ only causes a minor decrease in its protease activity (19). Presumably, this is because the active site, which is buried within the central cavity of this multienzyme complex, is relatively inaccessible compared with other proteasomal lysine residues.

In vivo, modification of the proteasome by isoLGs occurs in the presence of other proteins. In a model of this environment, isoLGE$_2$ was added to cell lysates. Under these biomimetic conditions, proteosomal activity is strongly inhibited, but the kinetic behavior is not consistent with competitive inhibition (19). When newly generated, a nascent isoLGE$_2$-protein adduct can bind nearly two equivalents of glycine (44). It is possible that this strong inhibition involves cross-linking of protein substrates with the proteasome. The nascent isoLG-protein adduct, for example, a Schiff base, can serve as an ''activated monomer'' that is capable of sticking to (cross-linking with) the proteasome, resulting in irreversible binding of the isoLG-modified substrate (Fig. 7). Thus, adduction with isoLGE$_2$ might convert proteins into ''suicide substrates'' that covalently bind to, and consequently irreversibly inhibit, their target enzyme (102). However, direct evidence for this inhibition mechanism, for example, characterization of an enzyme cross-linked with its substrate by an isoLG, has not been reported.

Proteasomal degradation of proteins is an important regulatory mechanism. Inhibition of the proteasome can promote accumulation of ubiquinated proteins (57) and phosphorylated neurofilaments (49), and foster apoptosis (48, 92). For example, isoLG-protein adduction to the 26S proteasome might inhibit the ATP-dependent degradation of ubiquinated proteins and, thus, contribute to the accumulation of ubiquitinated proteins present in the neurofibrillary tangles that are a hallmark of AD. Addition of isoLGE$_2$ to neuroglial cells *in vitro* dose dependently inhibited proteasomal activity ($IC_{50} = 330\ nM$) and induced cell death at higher concentrations ($LC_{50} = 670\ nM$). Since this concentration was 100-fold lower than that previously reported for 4-hydroxynon-2-enal, isoLGs are ''among the most potent neurotoxic products of lipid oxidation heretofore identified'' (19). It is tempting to speculate that a similar process could convert a signaling peptide into an irreversible agonist whose binding with its receptor would result in dysregulation of a signaling pathway, because the receptor would remain in a permanently activated state.

Reactive oxygen species play a pathogenic role in primary open-angle glaucoma by fostering changes that reduce permeability of the trabecular meshwork (TM) tissue and, consequently, impede aqueous humor outflow, resulting in elevated intraocular pressure. We found elevated levels of calpain-1 in glaucomatous TM (31). However, calpain activity in glaucomatous TM is only about 50% of that in controls. This paradox is explicable by the facts that isoLG-protein adducts accumulate in human glaucomatous TM and that modification by isoLGs renders calpain-1 inactive. Thus, treatment of calpain-1 with iso[4]LGE$_2$ *in vitro* results in covalent modification, inactivation, the formation of high-molecular-weight aggregates, and resistance to proteasomal digestion. Iso[4]-LGE$_2$-modified calpain-1 undergoes ubiquitination (32), and its loading impairs the cellular proteasome activity (31). Apparently, interference with proteasomal activity, owing to protein modification by isoLGs, contributes to glaucoma pathophysiology by decreasing the ability of the TM to modulate outflow resistance. Thus, lipid-derived oxidative protein modifications interfere with the housekeeping and/or regulatory functions of proteases, that is, calpain-1 and the proteasome. Upregulation of calpain-1 expression in a futile attempt to ameliorate the problem, exacerbates the pathology owing to efficient inactivation of the newly generated enzyme by adduction of isoLGs. In contrast, higher calpain-1 activity, which appears to be under translational control, was observed in glaucomatous optic nerve compared with control (32). Therapeutic neuroprotection strategies using calpain-1 inhibitors will require consideration of such anatomic differences in its activity and biosynthesis.

Elevated levels of iso[4]LGE$_2$-adducts are present in astrocytes derived from the glaucomatous optic nerve head compared with those from controls. To model the environment

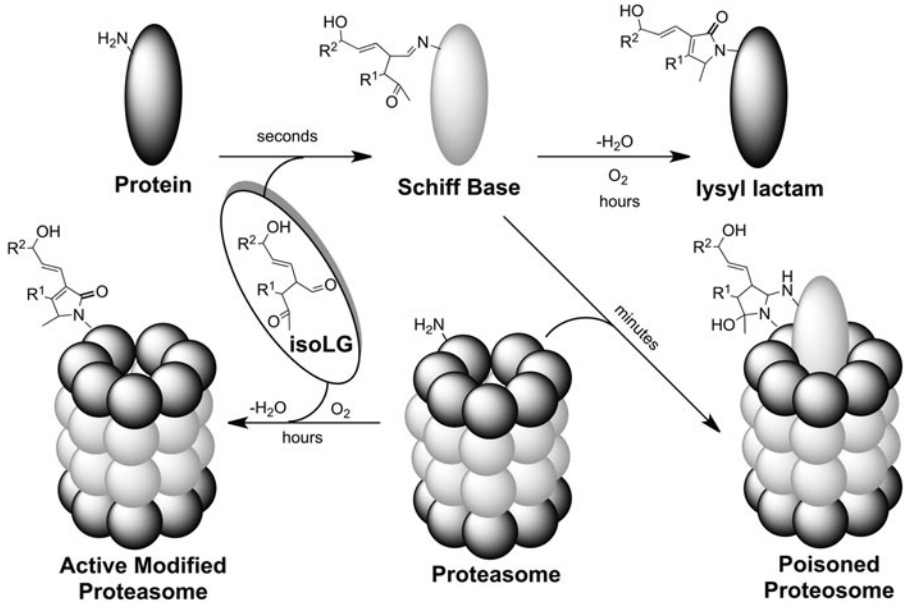

FIG. 7. Hypothetical endogenously generated suicide substrate mechanism for irreversible inhibition of the proteasome.

in primary open-angle glaucoma, astrocytes isolated from rat brain cortex and human optic nerve were subjected to 0–150 mmHg pressure for 3 h. Levels of iso[4]LGE_2-modification increased in direct proportion to a pressure of 50% above that found in control cells (30). Longer exposure resulted in a large increase in levels of iso[4]LGE_2-adduct immunoreactivity. These observations suggest that chronic and/or prolonged exposure to pressure can contribute to the elevated iso[4]LGE_2-protein adduct immunoreactivity observed in glaucomatous astrocytes. Apparently, reduced aqueous outflow caused by accumulation of isoLG-modified proteins causes increased intraocular pressure that promotes the generation of more isoLGs.

Inhibition of an oxidase: age-related macular degeneration

Patients with age-related macular degeneration (AMD) have mean blood levels of iso[4]LGE_2-PEs (5.2 ± 0.4 ng/ml, $n = 15$) that are elevated $\sim 53\%$ ($p < 0.0001$) compared with those of healthy volunteers (3.4 ± 0.1 ng/ml, $n = 15$) (37). The formation of cholesterol-rich deposits in the retina (drusen) are a hallmark of AMD (46, 68). Mitochondrial cytochrome P450 (CYP)27A1 is a sterol C27-hydroxylase that eliminates cholesterol and likely 7-ketocholesterol from the retina. Recently, we found that modification of this protein with iso[4]LGE_2 *in vitro* diminished enzyme activity (16). Only three Lys residues, Lys^{134}, Lys^{358}, and Lys^{476}, readily interacted with the isoLG *in vitro*. Analysis of a protein extract from a human retinal sample by proteolysis followed by multiple reaction monitoring demonstrated that the peptide $AVLK^{358}$ ($-C_{20}H_{26}O_3$)ETLR was present in the retinal protein extract. Such protein modification presumably impairs sterol elimination, contributing, for example, to cholesterol-rich lesions associated with AMD. Subsequently, the specificity of isoLG modification was confirmed, providing direct evidence that isoLG adduction impairs enzyme activity and supporting the hypothesis that isoLG modification in the retina is detrimental to CYP27A1 enzyme activity, potentially disrupting cholesterol homeostasis. Thus, the effect of Lys^{358} modification on enzyme activity was investigated by characterizing catalytic properties of Lys^{358} as well as Lys^{476} CYP27A1 mutants both before and after isoLG treatment (17). The $K^{358}R$ mutant was less susceptible to isoLG-induced loss of catalytic activity than the wild type (WT), whereas the $K^{476}R$ mutant was nearly as vulnerable as the WT.24 Thus, modification of Lys^{358}, a residue involved in redox partner interactions, is the major contributor to isoLG-associated loss of CYP27A1 activity.

To test whether scavenging of isoLGs could prevent modification of retinal proteins, mice were exposed to a bright light that caused retinal isoLG-adduct formation (18). In contrast, pretreatment of mice with PM, an efficient scavenger of γ-ketoaldehydes (vide supra), suppressed the accumulation of retinal isoLG adducts, and morphological changes in photoreceptor mitochondria were not as pronounced as in untreated animals (18). These experiments demonstrate that preventing the damage to biomolecules by lipid peroxidation products, a novel concept in vision research, is a viable strategy to combat oxidative stress in the retina. Whether this strategy can be of clinical utility for the prevention of the pathological sequelae of isoLG generation in human subjects deserves clinical investigation.

Modification of ion channels: cardiac arrythmia

AT-1 cells are an atrial tumor myocyte cell line that expresses a model integral membrane potassium ion (K^+) channel that is relevant to oxidative ischemia/reperfusion injury-induced cardiac dysrhythmias (85, 95). IsoLGE_2 caused a pronounced dose-dependent inhibition of the rapidly activating delayed rectifier K^+ current Ikr ($IC_{50} = 2.2\ \mu M$) (12). Activating and deactivating currents were suppressed equally, suggesting that these effects were neither voltage- nor gating dependent. Moreover, Ikr was not restored with washing, suggesting that the inhibition was not caused by a reversible allosteric effect but rather by an irreversible covalent modification of channel proteins. The inhibition was not manifested immediately, requiring 60 min to achieve full inhibition when tested at a concentration of 10 μM IsoLGE_2. Because isoLGEs adduct to proteins within seconds, the more prolonged time course for Ikr inhibition may be caused by time-dependent diffusion into the membrane and/or cross-linking of channel proteins.

The cardiac sodium channel $Na_V1.5$ is a key determinant of electrical impulse conduction in cardiac tissue (53). Acute myocardial infarction leads to diminished sodium channel availability, both because of decreased channel expression and because of greater inactivation of channels already present. Myocardial infarction leads to significant increases in reactive oxygen species and lipoxidation products. The effects of reactive oxygen species on $Na_V1.5$ function in whole hearts can be modeled in cultured myocytes, where oxidants shift the availability curve of I_{Na} to hyperpolarized potentials, decreasing cardiac sodium current at the normal activation threshold. Both the general oxidant tert-butylhydroperoxide and IsoLGE_2 potentiate inactivation of cardiac Na^+ channels in human embryonic kidney (HEK)-293 cells and cultured atrial (HL-1) myocytes (26). Furthermore, IsoLGE_2were generated in the epicardial border zone of the canine healing infarct, an arrhythmogenic focus where Na^+ channels exhibit similar inactivation defects. These observations suggested that Na^+ channel dysfunction evoked by lipid peroxidation is a mechanism for ischemia-related conduction abnormalities and arrhythmias. Further studies confirmed that exposure to oxidants induces lipoxidative modification of $Na_V1.5$ by isoLGs and demonstrated that PM and analogues, which act as isoLG scavengers, block voltage-dependent changes in sodium current by the oxidant tert-butylhydroperoxide, both in cells heterologously expressing $Na_V1.5$ and in a mouse cardiac myocyte cell line (HL-1) (53).

IsoLG-modified ethanolaminephospholipids: AS

Elevated levels of LG-protein adduct immunoreactivity in blood from patients with atherosclerosis provided the first evidence for the formation and pathological significance of LG-adducts *in vivo* (75). The data in Table 1 paradoxically show that cholesterol levels are significantly elevated in older individuals (N[62]) who do *not* have AS compared with atherosclerosis patents (AS[63]) of a similar average age. This probably reflects the success of therapeutic interventions, including diet or drugs, that reduce total cholesterol levels, but apparently not isoLG levels, for the AS patients. The elevated isoLG-adduct levels, detected by immunoassay with anti-isoLG antibodies, indicate a defect that results in an abnormally high level of oxidative injury that is associated with AS but is independent of the classical risk factors, total

TABLE 1. LEVELS (MEAN ± SD) OF isoLGs, apoB, AND TOTAL CHOLESTEROL

Cohort [average age]	*N[33]*	*AS[63]*	*N[62]*
Ages	33 ± 3	63 ± 12	62 ± 7
Population	15	16	10
isoLGE$_2$-adduct (pmol/ml)	132 ± 47	287 ± 62	121 ± 42
iso[4]LGE$_2$-adduct (pmol/ml)	1441 ± 144	2224 ± 439	1691 ± 252
ApoB (mg/L)	724 ± 180	937 ± 206	1046 ± 370
Total cholesterol (m*M*)	4.16 ± 0.87	4.58 ± 0.59	5.78 ± 1.42

cholesterol, or LDL. That defect is almost certainly an elevated susceptibility toward oxidative injury. While LDL and total cholesterol levels may correspond with those of fatty acyl precursors of oxidized lipids, isoLG adduct levels represent a direct measure of oxidative injury that is intimately associated with the formation of foam cells. Because LG-protein and isoLG-protein adducts can accumulate for days or weeks over the lifetimes of proteins (86), which are extended by their resistance to proteasomal degradation (*vide supra*), they represent a convenient dosimeter that provides a *cumulative index of oxidative stress*. More recently, we showed that isoLG adducts that incorporate the amino group of PEs into isoLG-hydroxylactams (4) are also potential biomarkers for assessing risk for oxidative stress-stimulated diseases (47).

Radical-induced oxidation of LDL to an oxidized form (oxLDL) is accompanied by the formation of isoLG-adducts, and it fosters massive unregulated endocytosis by a class of macrophage receptors that recognize the lipid-based protein modifications, but not native LDL (25). This uptake, and impaired processing, leads to accumulation of large quantities of partially digested lipoprotein and globules of cholesterol esters in the macrophage cells, giving them the appearance of being filled with foam. The resulting ''foam cells'' are the progenitors of fatty streaks that evolve into atherosclerotic plaques. We found that LGE$_2$-modified LDL binds with and is degraded by receptor-mediated endocytosis by macrophage cells (40). Both binding and uptake, leading to degradation, are blocked by an excess of oxLDL, suggesting that LGE$_2$-derived modifications generated on oxidation of LDL contribute to binding and uptake of oxLDL by macrophages. It must be noted here that this biological activity is also elicited by other products of lipid oxidation. Thus, we subsequently identified a family of oxidatively truncated PLs whose binding with macrophage CD36 receptors also promotes endocytosis of oxLDL (60, 61).

Platelet hyper reactivity produces a prothrombotic state that plays an important role in the pathogenesis of AS. Incubation of platelets with 1 n*M* isoLGE$_2$ potentiates platelet aggregation induced by collagen, and is accompanied by the formation of thromboxane B2, and phosphorylation of both cytosolic phospholipase (cPL)A$_2$ and p38 MAPK. Since isoLGE$_2$ can be produced by both COX-mediated and free radical-induced cyclooxygenation, arachidonate oxidation through either or both of these pathways could produce such platelet activation. Some lipid-derived protein modifications, such as carboxyalkypyrrole derivatives of protein lysyl residues, induce a prothrombotic state by receptor-mediated responses, for example, by serving as agonists of the toll-like receptor 9 (59). Alternatively, the activation of cPLA$_2$ through phosphorylation may be a consequence of isoLG-induced modification of platelet plasma membrane aminophospholipids and proteins that could conceivably alter the membrane structure and affect membrane fluidity which could promote activation of PLA$_2$ (2). It is noteworthy that, since activation of platelets by exogenous agonists is accompanied by COX-dependent formation of isoLGE$_2$-protein adducts (8), isoLGE$_2$ can promote its own generation. In other words, isoLGE$_2$ could function as the mediator of an autocatalytic cycle that provides a mechanism for amplifying the effects of isoLGE$_2$ on thrombosis.

PE is a major target of endogenously generated isoLGs. MPO is an oxidative enzyme released by activated neutrophils that associates with circulating high-density lipoprotein (HDL) (38) and is an important generator of isoLG-modified plasma protein *in vivo* (62) Incubation of HDL with MPO generated at least 10-fold greater amounts of isoLG-PE than isoLG-protein adducts (34).

PE adducts of isoLGE$_2$ (isoLGE$_2$-PE) dose dependently induce cytotoxicity (LC$_{50}$ 2.2 μM) in human umbilical vein endothelial cells (HUVECs) (90). As noted earlier, ''inflammation fosters the generation of isoLGs.'' Recently, it was reported that the opposite is also true, that is, the generation of isoLGs fosters inflammation. Thus, isoLGE$_2$-PE also fosters the expression of inflammatory cytokines and endoplasmic reticulum (ER) stress signaling in HUVECs (35). It induced expression of adhesion molecules and increased MCP-1 and IL-8 mRNA in HUVECs. Chemical inhibitors of the ER stress response reduce the extent to which isoLGE$_2$-PE activates inflammatory responses (35). This is consistent with a developing paradigm linking oxidized lipids, ER stress, inflammation, and AS (36). ER stress response proteins are markedly increased in atherosclerotic lesions (91). In addition, fluorescently labeled isoLGE$_2$-PE rapidly internalized to the ER (35). IsoLGE$_2$-PE induced C/EBP homologous protein CHOP and BiP expression and p38 MAPK activity, and inhibitors of ER stress reduced isoLGE$_2$-PE - induced C/EBP homologous protein CHOP and BiP expression as well as EC activation, consistent with isoLGE$_2$-PE inducing ER stress responses that have been previously linked to inflammatory chemokine expression. Thus, isoLGE$_2$-PE is a potential mediator of the inflammation induced by lipid peroxidation. Furthermore, isoLGE$_2$-PE induces monocyte adhesion to endothelial cells (35). Endothelial cell activation and monocyte adhesion initiate AS by promoting migration of monocytes into the subendothelial space where they become foam cells, the precursors of atherosclerotic plaques.

Since isoLGE$_2$ is also produced through COX-mediated cyclooxygenation, these biological activities of isoLGE$_2$-PE also link COX activity to ER stress and inflammation. Blocking the contribution of the COX pathway to the generation of isoLGE$_2$-PE adducts is almost certainly an important component of the therapeutic benefit of low-dose aspirin for reducing the risk of AS.

NAPE-PLD prevents accumulation of inflammatory and cytotoxic isoLGE$_2$-PEs

Preventing the accumulation of bioactive proinflammatory isoLGE$_2$-PEs is important for protecting cells against inflammation and oxidant injury. N-acyl phosphatidylethanolamine-

hydrolyzing phospholipase D (NAPE-PLD) was recently shown to serve as a promiscuous mammalian PLD for N-modified PEs that metabolizes isoLGE$_2$-PEs (37). Blocking the expression of this enzyme with siRNA specific for NAPE-PLD transfected into HEK293 cells abrogated their ability to hydrolyze isoLGE$_2$-PEs to isoLG-modified ethanolamine that is an order of magnitude less inflammatory than isoLGE$_2$-PE. NAPE-PLD is widely expressed, with the highest expression in tissues such as brain, kidney, and testis that are particularly sensitive to oxidative injury (58). In brain, NAPE-PLD localizes to a variety of brain cells, including neurons, astrocytes, cerebral endothelial cells, and microglia (101). As noted earlier, isoLGE$_2$-PE is rapidly trafficked to the ER (35). Consequently, it is especially noteworthy that NAPE-PLD is preferentially localized to the cisternae of smooth ER in hippocampal mossy fibers (56). NAPE-PLD expression is acutely upregulated in response to spinal cord contusion in rats (27) as would be expected if it has a role in ameliorating the pathological consequences of oxidative damage, for example, by promoting clearance of isoLGE$_2$-PEs.

Conclusions and Prospects

When we reported the discovery that the bicyclic peroxide core of the prostaglandin endoperoxide spontaneously rearranges to levulinaldehyde, we noted that ''the biological ramifications of such transformations demand scrutiny'' (69). It is now apparent that this biological chemistry of lipids is emerging as a rich source of molecular level insights into some pathological consequences of COX activity and oxidative stress. We discovered that a diverse family of lipid-derived levulinaldehydes, isoLGs, is produced through both COX and free radical-induced cyclooxygenation of polyunsaturated fatty acids and their PL esters. IsoLGs have been associated with (i) inhibition of tubulin function, (ii) modification of histones and disruption of their nucleosomal complex with DNA, (iii) cross-linking proteins with DNA, (iv) impairment of mitochondrial function through modification of cytochrome c, (v) disruption of the blood brain barrier, (vi) promotion of Alzheimer's disease through COX-associated isoLG-induced oligomerization of A$\beta_{1\text{-}42}$, (vii) promotion of neurodegeneration and impairment of aqueous outflow in the eye contributing to glaucoma, (viii) inhibition of proteosomal processing of proteins that could impair its regulatory functions, (ix) accumulation of cholesterol that contributes to AMD owing to modification of an oxidase, CYP27A1, (x) cardiac arrythmia owing to disruption of ion channels through modification by isoLGs, and (xi) promotion of AS by converting PEs into proinflammatory isoLGE$_2$-PE agonists and fostering monocyte adhesion to endothelial cells. The activity of these inflammatory mediators is diminished or eliminated through NAPE-PLD-mediated metabolism. Trapping isoLGs with sacrificial primary amines, with PM and analogues being uniquely effective, can be used to prevent the formation of isoLG adducts, for example, with proteins or ethanolamine PLs, *in vivo*. Nonsteroidal anti-inflammatory drugs can be used to inhibit the COX-promoted generation of the stereoisomerically pure isoLGs, for example, LGE$_2$ or LGD$_2$, which have the same absolute configuration as prostaglandins.

Although isoLGs are structurally diverse, their common core renders their biological chemistry predictable although by no means simple. Besides this structural diversity, additional complexity is engendered by an extraordinary proclivity to form adducts with the enormously diverse universe of proteins, ethanolamine PLs, and nucleic acids, altering their function and cross-linking them into cryptic conglomerations. It is also important to note that the formation of isoLGs through free radical-induced cyclooxygenation is accompanied by the cogeneration of many other lipid oxidation products and derived protein modifications. Deconvoluting the relative biological importance of this plethora of molecules remains a redoubtable challenge.

Some simplifying principles are also emerging. Although proteins can become covalently linked to many molecules of isoLGs, as little as one isoLG molecule can suffice to destroy protein function owing to preferential modification of a specific protein lysyl residue. IsoLG modification of proteins or ethanolamine PLs can also result in gain of function. Nothing is known about the dependence on those new biological activities on the structures of the adducted isoLGs. Just as protein adducts of the structurally isomeric isoLGE$_2$ and iso[4]LGE$_2$ can be readily distinguished by antibodies, it is reasonable to anticipate that receptor recognition of these protein adducts or the corresponding PE adducts can vary with isoLG structure. It seems likely that some of the gain-of-function biological activities are receptor mediated and, therefore, may depend on isoLG structural isomerism. It is premature to categorically conclude that for adducts of all isoLGs the ''biological activity

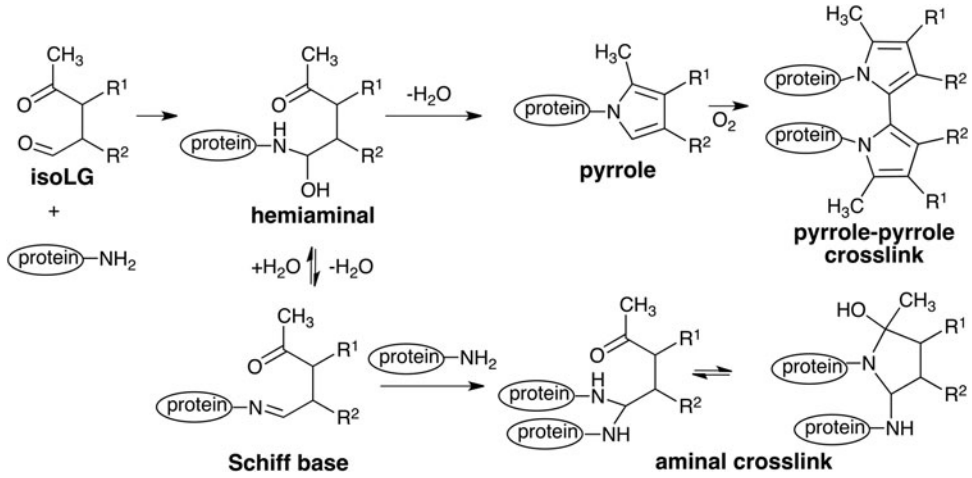

FIG. 8. Structures postulated for protein–protein crosslinks caused by isoLGs.

of all of the isomers would be expected to be nearly identical'' (2). This hypothesis can only be tested by studying the biological responses to adducts of pure isoLG structural isomers that are only readily available by chemical synthesis.

Although protein–protein cross-linking accompanies COX-promoted oligomerization of amyloid peptide as well as a reaction of calpain-1 with isoLGs, non cross-linked adducts are also formed. So the relative pathological importance of cross-linked versus non cross-linked adducts is not yet known. Two different structures have been proposed for protein–protein cross-links caused by isoLGs, that is, a bisaminal and a pyrrole-pyrrole dimmer (Fig. 8). No direct evidence is available to support either of these hypotheses.

Owing to the complexity of the task, gaining a comprehensive understanding of the ''biological ramifications'' of isoLG formation and adduction with biomolecules demands a rare combination of skills in organic synthesis, mass spectrometry, biochemistry, and cell and animal biology.

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Abbreviations Used

AA = arachidonic acid
AD = Alzheimer's disease
AMD = age-related macular degeneration
AS = atherosclerosis
COX = cyclooxygenase
CYP = cytochrome P
ER = endoplasmic reticulum
HDL = high-density lipoprotein
HUVEC = human umbilical vein endothelial cell
isoketal (isoK) = erroneous alternative nomenclature for isolevuglandins (isoLGs)
isoLGE$_2$ = collectively all stereoisomers of LGE$_2$
LDL = low-density lipoprotein
LG = levuglandin
MPO = myeloperoxidase
NAPE-PD = N-acyl phosphatidylethanolamine-hydrolyzing phospholipase D
PC = phosphatidylcholine
PE = phosphatidylethanolamine
PL = phospholipid
PM = pyridoxamine
RD = renal disease
ScFv = single-chain variable fragment antibody
TM = trabecular meshwork
TNF = tumor necrosis factor