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Forum Review

Levuglandins and Isolevuglandins: Stealthy Toxins of Oxidative Injury

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ABSTRACT

Inspired by a reaction discovered through basic research on the chemistry of the bicyclic peroxide nucleus of the prostaglandin endoperoxide PGH_2, we postulated that levulinaldehyde derivatives with prostaglandin side chains, levuglandins (LGs), and structurally isomeric analogues, isolevuglandins (iso[n]LGs), would be generated by nonenzymatic rearrangements of prostanoid and isoprostanoid endoperoxides. Two decades of subsequent studies culminated in our discoveries of the LG and isoLG pathways, branches of the cyclooxygenase and isoprostane pathways, respectively. In cells, PGH_2 rearranges nonenzymatically to LGs even in the presence of enzymes that use PGH_2 as a substrate. IsoLGs, also known as isoketals or neuroketals, are generated *in vivo* through free radical-induced autoxidation of polyunsaturated phospholipid esters. Hydrolysis occurs *after* rapid adduction of isoLG phospholipids to proteins. The proclivity of these reactive species to avidly bind covalently with and cross-link proteins and nucleic acids complicated the hunt for LGs and isoLGs *in vivo*. The extraordinary reactivity of these "stealthy toxins" underlies much, if not all, of the biological consequences of LG and isoLG generation. They interfere with protein function and are among the most potent neurotoxic products of lipid oxidation known. Because they can accumulate over the lifetimes of proteins, iso[n]LG-protein adducts represent a convenient dosimeter of oxidative stress. *Antioxid. Redox Signal.* 7, 185–201.

CHEMICAL INSIGHTS AND THE DISCOVERY OF LEVUGLANDINS

IT IS USEFUL TO KNOW the chemical proclivities of biomolecules. This axiom led us to seek an intimate understanding of the chemical idiosyncrasies of the prostaglandin (PG) endoperoxide PGH_2. The unique bicyclic peroxide nucleus of PGH_2 is the reactive locus of this pivotal intermediate in the biosynthesis of PGs, prostacyclins, and thromboxanes. PG endoperoxides are generated through cyclooxygenase (COX)-catalyzed oxidative cyclization of polyunsaturated fatty acids (PUFAs). Nugteren, Vonkeman, and van Dorp discovered that nonenzymatic oxidation of PUFAs can also generate prostanoid endoperoxides and PGs, but the lack of specificity in these autoxidation reactions results in production of stereo and structural isomers of the prostanoids (74). These were subsequently named isoprostanes (isoPs) by Roberts and co-workers (64). A paradigm that presumes minimal or no biological relevance for the nonenzymatic chemistry of biomolecules is epitomized by the fact that these chemical reactions were only considered a nuisance that could contribute artifacts to studies of fatty acid biochemistry. Ignored for two decades after their discovery in 1967, efforts to understand the biological significance of isoPs only began after the seminal observation that isoPs are produced *in vivo* (64).

The present review focuses on biochemistry that is mediated by γ-ketoaldehydes that are generated from PGH_2 and structurally isomeric "isoprostanoid" endoperoxides by a novel nonenzymatic rearrangement. Although the rapid nonenzymatic decomposition of PGH_2 ($t_{1/2}$ = 5 min at 37°C) in the aqueous environment of its biosynthesis was well known (72, 73, 80), γ-ketoaldehydes had never been identified as

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products. In retrospect, the ability of these stealthy compounds to evade detection resulted from their proclivity to react with primary amines, such as the Tris buffer used for *in vitro* experiments. We now know that these γ-ketoaldehydes are extraordinarily reactive electrophiles that covalently modify nucleophilic centers in other biomolecules, including proteins and nucleic acids. Research in this area of lipid biochemistry is emerging as a rich source of insights into important biological processes that are the nonenzymatic sequelae of COX activity and oxidative stress.

Prostanoid endoperoxides rearrange to levulinaldehydes

Our basic research on the bicyclic peroxide nucleus of prostanoid endoperoxides (86) revealed a novel rearrangement to levulinaldehyde (Fig. 1). Our subsequent studies over the next two decades were guided by the unconventional view that the biological ramifications of this unprecedented chemistry demanded further scrutiny. Mechanistic studies showed that the rearrangement involves an intramolecular migration of hydride from a bridgehead to an incipient methyl group with simultaneous cleavage of a C-H, C-C, and O-O bond (Fig. 1) (87, 89). Most interestingly, the rearrangement is especially facile in aqueous solution. This indicates a polarized transition state with an electron-deficient carbon and an electron-rich oxygen that can be stabilized by hydrogen bonding with water. As prostanoid side chains on the ethano bridge are remote from the bonds involved in this process, it was not surprising to find that rearrangement of PGH_2 in aqueous solution proceeds at a similar rate and generates two levulinaldehyde derivatives with prostanoid side chains (Fig. 1) that we named levuglandin (LG) D_2 and E_2 (89).

LGs are chemically sensitive. They readily lose the allylic hydroxyl (46), *e.g.*, to give $AnLGD_2$ (Fig. 1) from LGD_2 (55). Allylic rearrangement delivers isomers, *e.g.*, Δ^9-LGE_2, that are less prone to dehydration (46). To establish unambiguously the structures of the products generated from PGH_2, we developed efficient syntheses that stereoselectively provided the natural enantiomer of LGE_2 (62, 88) and the dehydration product $AnLGD_2$. Characterization of the LGs was achieved by ^1H NMR comparison of the products generated from PGH_2 with authentic samples provided by total syntheses. Absolute configuration was established by optical rotary dispersion comparison of fluorenylidene derivatives with those derived from pure synthetic isomers (62, 85, 88). PGs, the hydration products from thromboxanes, and prostacyclins are all readily isolable from biological sources. In contrast, detecting the generation of LGs *in vivo* is confounded by their exceptional reactivity (*vide infra*) that results in rapid sequestration by covalent adduction to biological nucleophiles. Some evidence suggests that the carbonyl-conjugated dehydration and rearrangement products, *e.g.*, $AnLGD_2$ and Δ^9-LGE_2, are somewhat less reactive (45). Although they too have not yet been isolated from biological sources, they may contribute to PG-like receptor-mediated biological activities of LGs (32–35).

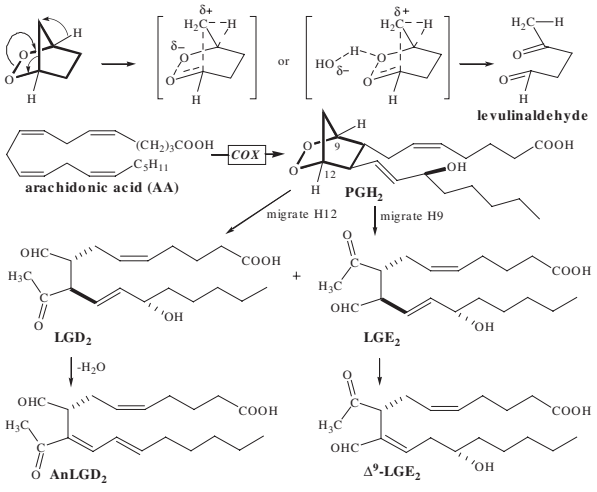

FIG. 1. Rearrangement of prostanoid endoperoxides. Intramolecular hydride shift generates levulinaldehyde derivatives with prostanoid side chains from PGH_2.

LGs bind avidly with proteins

We anticipated that LGs would react rapidly with nucleophilic centers in biological molecules because structurally similar α,β-disubstituted-γ-dicarbonyl compounds were known to participate in irreversible binding to tissues (3, 22, 37). 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 COX-containing tissues, and this binding was not entirely explicable in terms of the known metabolites of AA (2, 27). Exogenous PGH_2 formed such adducts with ram seminal vesicle and guinea pig lung microsomes (18). Incubation of PGH_2 with thromboxane A_2 synthetase affords not only thromboxane A_2, but also malondialdehyde (MDA), both of which form covalent adducts with proteins (28, 103). However, we noted that neither of these metabolites could account for the irreversible association of radioactivity with various proteins or DNA, which occurs upon incubation of 1-^{14}C-AA with COX-containing microsomes in the presence of thromboxane synthetase inhibitors (24). Even if MDA were formed by a non-COX pathway in the presence of this inhibitor, it would not be radioactive if produced from 1-^{14}C-AA. Therefore, we proposed that "levuglandins 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 and DNA" (90).

We observed that incubation of ram seminal vesicle microsomes (2 mg/ml) with 5,6-^{3}H-LGE_2 (44 m*M*) resulted in rapid binding of 2 nmol/mg of microsomes within 2 min (90). We presumed that *in vivo* LGs would be rapidly sequestered by covalent adduction, *inter alia*, with proteins. Therefore, to demonstrate the formation of such oxidized lipids *in vivo*, we focused on detecting their protein adducts. Upon incubation of bovine serum albumin (BSA) with an excess of 5,6-^{3}H-LGE_2, we found that 10 molecules of LGE_2 irreversibly bind to BSA within 1 min (90). Our conclusion that LGE_2 is rapidly sequestered by protein adduction was recently con-

firmed by monitoring the disappearance of the LG upon incubation with a 3 molar excess of BSA. More than 50% was adducted within 20 s (14, 82). These results supported the hypothesis that LGs are the reactive electrophiles responsible for much (if not all) of the COX-dependent binding of AA metabolites. Another recent study provided mass spectrometric evidence confirming the formation of LG-derived modifications of lysyl residues in prostaglandin H-synthetases (PGHSs) (~9 adducts per molecule of PGHS) upon bioconversion of AA (11). Furthermore, when the bioconversion was conducted in the presence of ubiquitin or histones, LG-derived modifications of those proteins were generated. A very recent study demonstrated that LG-protein adducts are formed by a PGHS-dependent pathway after platelet activation (13). The formation of these adducts is inhibited by the PGHS inhibitor indomethacin, and is enhanced by an inhibitor of thromboxane synthase. This seminal study allows three especially important conclusions. It provides the first evidence that (a) LG-protein adducts can be formed in a cell as a consequence of oxygenation of AA by a PGHS; (b) a physiological stimulus is sufficient to lead to protein modification through the LG pathway in human platelets; and (c) LGE_2 is formed in platelets and adducts platelet proteins even in the presence of an enzyme that uses PGH_2 as a substrate. It is tempting to speculate that the ability of nonenzymatic rearrangement to compete in a cellular environment with enzymatic conversion to PGs, prostacyclins, or thromboxanes may be influenced by proteins, *e.g.*, albumins that are known to catalyze the decomposition of PGH_2 (41, 72). Furthermore, high concentrations of AA, which are typical during ischemia, inhibit PGE isomerase (29).

LGE_2 was expected to react with the ϵ-amino group of a protein lysyl residue to generate an aromatic pyrrole derivative (Fig. 2). This transformation, a Paal–Knoor synthesis of pyrroles, proceeds through a Schiff base and its enamine tautomer. The production of protein-based LGE_2-derived pyrroles was quantitatively detected by treatment of the adduct

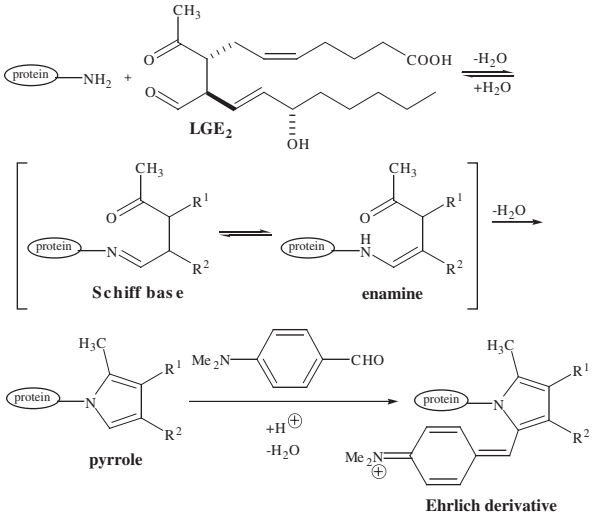

FIG. 2. Reaction of LGs with proteins. Rapid covalent adduction of LGs with proteins delivers Schiff bases that cyclize to pyrroles detectable as Ehrlich derivatives.

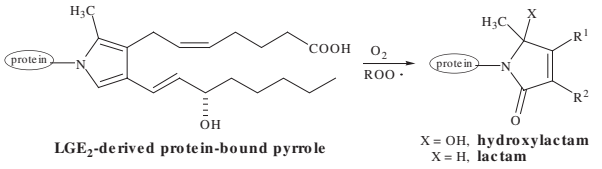

FIG. 3. Oxidation of an LGE_2-derived pyrrole. Lactams and hydroxylactams are stable end products formed from protein-bound pyrroles.

with a reagent that generates a characteristic Ehrlich chromophore (λ_{max} = 610 nm), *p*-dimethylaminobenzaldehyde and acid (47). A subsequent study confirmed the rapid initial formation of lysine-LGE_2 Schiff base adducts by mass spectrometric analysis (10).

An additional transformation was uncovered that further complicated the identification of LG-protein adducts and, consequently, the detection of LG formation *in vivo*. Because they are electron-rich, LG-derived pyrroles are highly susceptible to oxidation. Using mass spectrometric analysis of protein digests, Brame *et al.* discovered that these pyrroles are readily oxidized by air to relatively stable end products, lactams and hydroxylactams (Fig. 3) (14, 82).

Immunoassays for LGE_2-protein adducts

Although protein-bound, LG-derived pyrroles can be detected with the Ehrlich reagent, the resulting chromophore is not sensitive enough to detect LG-protein adducts *in vivo* where submicromolar concentrations were expected. Immunoassy provided a more sensitive analytical method. Because LGE_2-derived pyrroles are unstable, we prepared a pyrazole isostere (Fig. 4) of LGE_2-derived protein-bound pyrrole to serve as stable well defined antigen (52). As expected, immunoreactivity detectable with antibodies raised against the isostere was generated upon reaction of LGE_2 with proteins (25). In spite of the known instability of LGE_2-derived pyrroles, the level of immunoreactivity remained unexpectedly constant over 6 weeks. In hindsight, this is probably because the pyrazole isostere is structurally analogous not only to the pyrrole, but also to the derived lactam and hydroxylactam end products. All of these structures contain a common

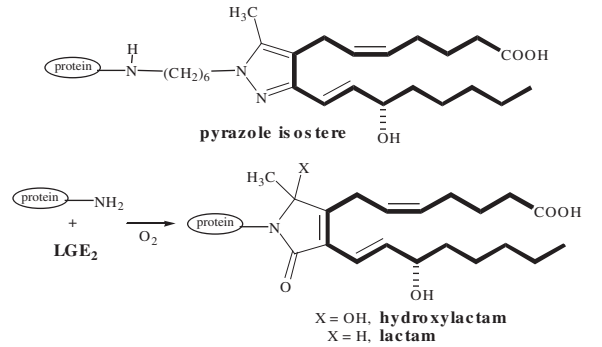

FIG. 4. A pyrazole isostere of the LGE_2-derived pyrrole. Lactam and hydroxylactam end products are structurally analogous to a pyrazole isostere.

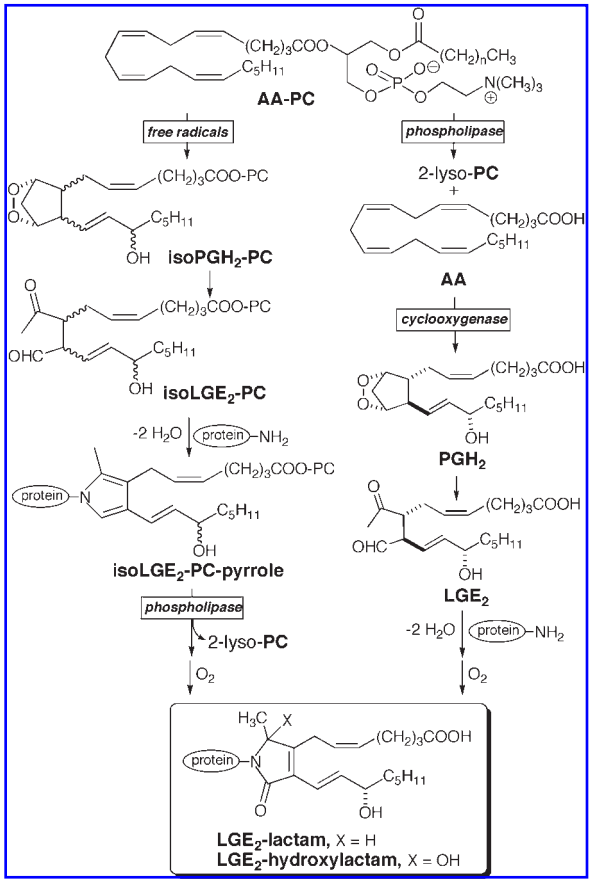

FIG. 5. The COX and isoP pathways to LGs. The same LGE_2-lactam and hydroxylactam protein modifications are generated through the COX and isoP pathways.

motif, the coplanar appendage of two prostanoid side chains on a five-membered ring, and thus likely are similarly recognized by antibodies (see Figs. 3 and 4).

Subsequently, we raised antibodies against an LGE_2 adduct of keyhole limpet hemocyanin (KLH). These LGE_2-KLH antibodies were expected to be effective for detecting not only protein adducts derived from LGE_2, but also protein adducts of LGE_2 isomers that we predicted (52) would be generated nonenzymatically through free radical-induced oxidation of arachidonyl phospholipids.

A nonenzymatic route to LG isomers from polyunsaturated lipids

As the prostanoid side chains exert little or no influence on the proclivity of the PG endoperoxide nucleus to undergo rearrangement to levulinaldehydes, it was a foregone conclusion that the stereoisomeric mixture of isoP endoperoxides ($isoPGH_2$), generated by free radical-induced lipid oxidation, would also rearrange to stereoisomers of LGs. We now refer to these LG isomers as isolevuglandins (*e.g.*, $isoLGE_2$) in analogy with designation of the corresponding PG isomers as isoPs (*e.g.*, E2-isoP). An alternative nomenclature that refers to LG isomers as "isoketals" (9) is misleading because these compounds are not ketals. Because two stereocenters are abolished during protein adduction of $isoLGE_2$, the same lactam and hydroxylactam end product would be produced through the isoP (free radical-induced oxidation) and COX pathways (Fig. 5). Thus, LGE_2-KLH antibodies could detect isoLGs. However, unambiguous evidence for the *in vivo* occurrence of our postulated free radical-induced pathway to LG isomers (52) would not emerge for years.

To use the LGE_2-KLH antibodies to demonstrate isoLG formation upon oxidation of low density lipoprotein (LDL), it would be necessary to hydrolyze the phospholipid esters formed initially because AA is present mainly esterified in phospholipids in LDL. As expected, our LGE_2-KLH antibodies do not detect protein adducts of the LGE_2 ester of 2-lyso-phosphatidylcholine (PC) because the LG moiety is heavily camouflaged, sandwiched between the protein and lyso-PC units. The first experimental evidence that LG isomers could be generated by free radical-promoted oxidation of arachidonyl phospholipids was provided by the observation that *in vitro* nonenzymatic free radical-induced oxidation of LDL generates LGE_2-protein adduct immunoreactivity (Fig. 6) (92). Phospholipase activity associated with the LDL particle may hydrolyze $isoLGE_2$-PC or its protein adducts, exposing the cryptic epitopes. Hydrolytic release of lyso-PC is known to accompany oxidation of LDL (102).

To corroborate our discovery that isoLG-derived protein adducts are generated during free radical-induced oxidation of LDL, we initiated a collaboration with L.J. Roberts II to characterize the $isoLGE_2$-protein adducts in oxidized LDL (oxLDL) mass spectroscopically. Those studies demonstrated the formation of LGE_2 isomers upon free radical-induced *in vitro* oxidation of AA and of $isoLGE_2$-derived lysine-based lactams and hydroxylactams upon *in vitro* oxidation of LDL (14, 82). However, the detection of protein adducts of LGE_2 isomers can not provide unambiguous evidence for the operation of an "isolevuglandin pathway" *in vivo* because it does not distinguish between protein adducts generated through the COX and isoP pathways. Importantly, our isoLG hypothesis (92) predicted that $isoLGE_2$ is only one of eight structurally isomeric isoLGs that can be generated from AA-PC, each of which can exist as eight stereoisomers (Fig. 7). The bracketed numerals [n] in the names of the structural isomers of LGE_2 and LGD_2 indicate the lengths of the carboxylic side chains. Most importantly, these structural isomers and their protein adducts can only arise through the isoP pathway.

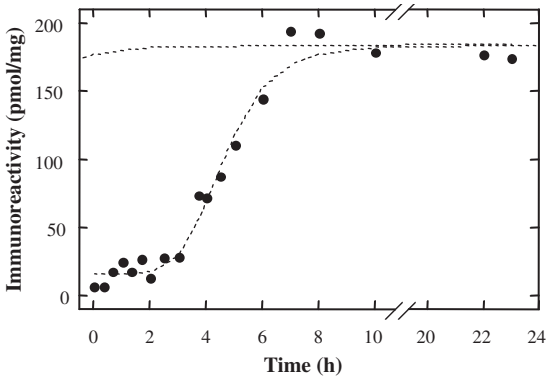

FIG. 6. Oxidation of LDL generates isoLGs. $IsoLGE_2$-protein adduct epitopes are generated upon oxidation of LDL.

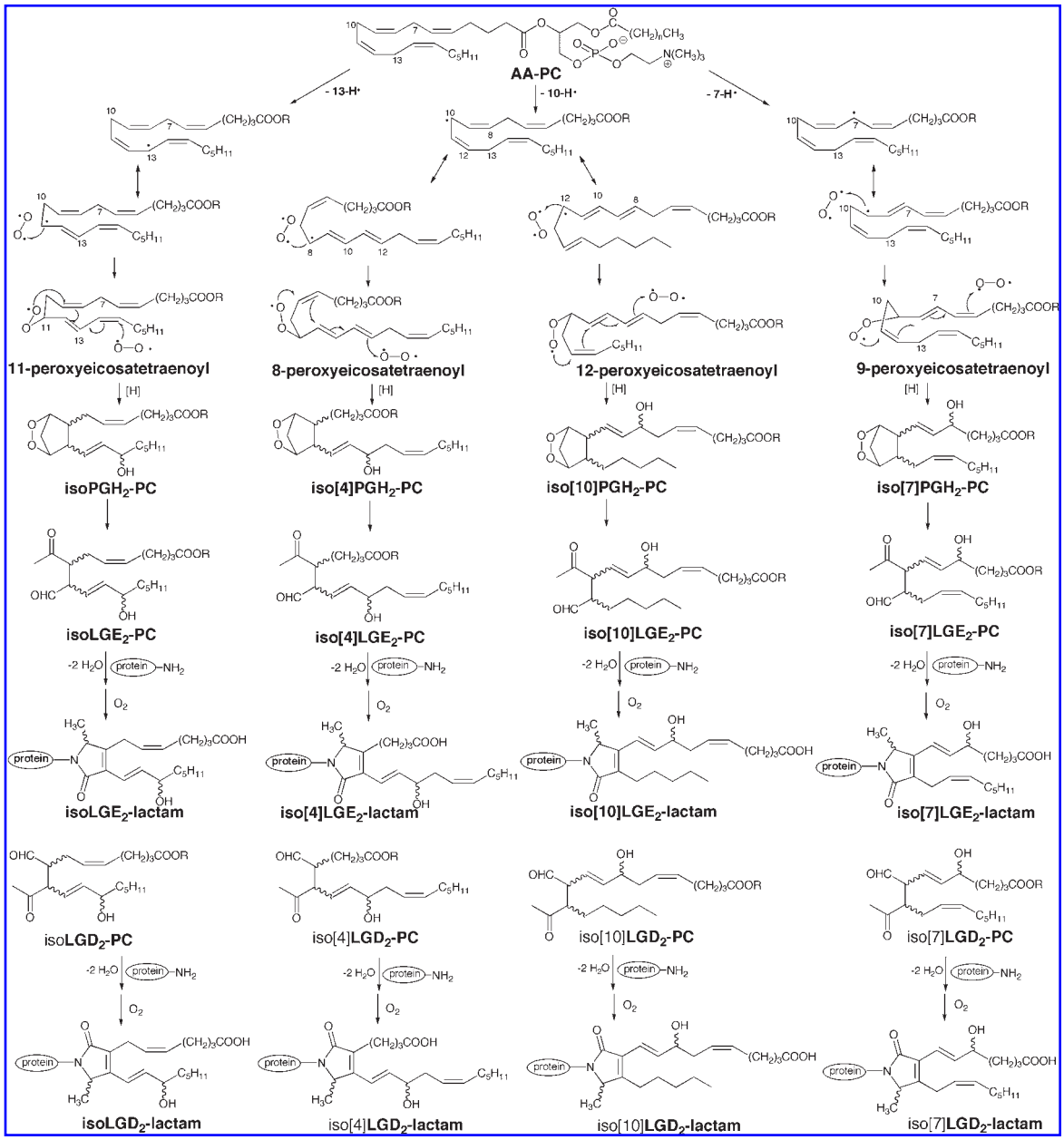

FIG. 7. The isoP pathway generates LGs together with stereo and structural isomers. Free radical-induced autoxidation of arachidonyl phosphatidylcholine (AA-PC) generates phospholipid esters of LGE_2 and LGD_2, as well as stereo (isoLGs) and structural isomers (iso[n]LGs, where the bracketed integers indicate the length of the carboxylic side chain). The iso[n]LGs and their protein adducts (iso[n]LG-lactams) are unique products of the isoLG pathway.

Cyclooxygenation is possible for any 1,4,7-octratriene

Because docosahexaenoic acid (DHA) incorporates a 1,4,7-octatriene functional array, it is expected to be susceptible to cyclooxygenation leading to endoperoxides. However, DHA is not a substrate for mammalian COX, and it inhibits cyclooxygenation of AA (8, 17, 26, 50, 79). In contrast, a piscine COX enzyme (from rainbow trout) does not discriminate against DHA. Incubation of DHA with trout gill homogenate generates a 22-carbon analogue of $PGF_{2\alpha}$, which was called C22-$PGF_{4\alpha}$ (58). Autoxidation of DHA-containing lipids generates endoperoxides, and these rearrange to levulinaldehydes, which have been called isoLGE_4s (98) or neuroketals (9). In theory, a plethora of 256 different isoLG_4s can be generated through autoxidation of DHA. A comparison of typical eicosanoid and docosanoid endoperoxides and representative rearrangement products is presented in Fig. 8. Enzymatic cyclooxygenation of eicosapolyenoic acids involves regioselective participation of C=C bonds at positions 8, 11, and 14 and favors the production of a single enantiomerically pure stereoisomer, *e.g.*, PGH_2, with a prostanoic acid carbon

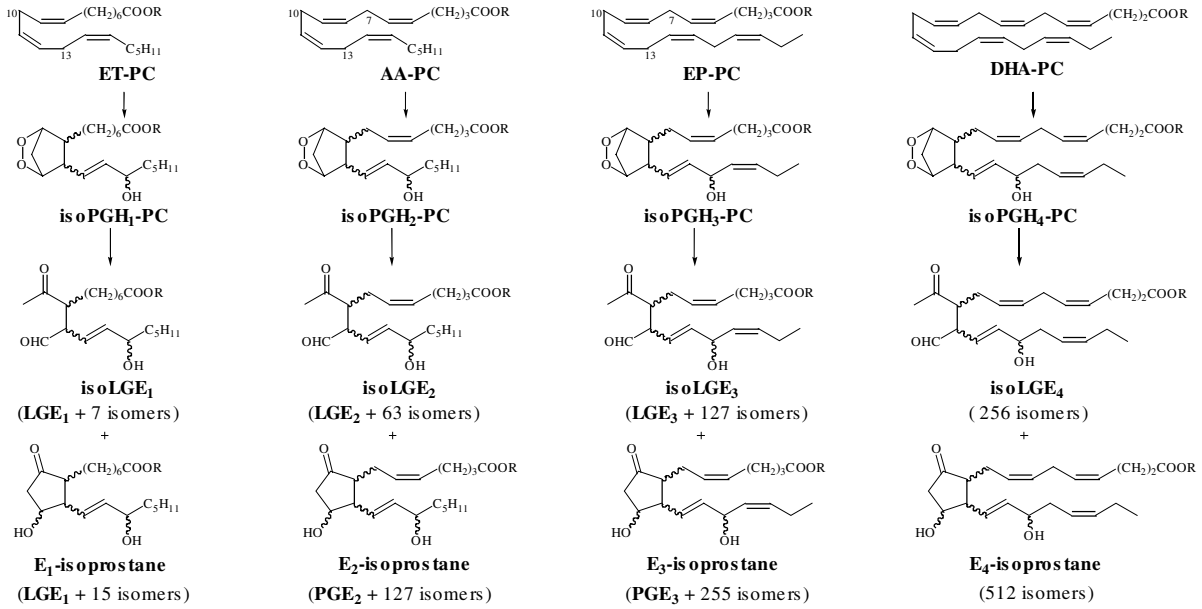

FIG. 8. Cyclooxygenation leading to LGs or isoLGs is possible for a variety of polyunsaturated fatty acids or esters. Cyclooxygenation of various polyunsaturated fatty acids or esters that contain a 1,4,7-octatriene array can generate LGs or isoLGs together with the corresponding PGs through alternative rearrangements of endoperoxide intermediates with one to four C=C bonds (designated by subscripts).

skeleton. The endoperoxides that are generated through enzymatic cyclooxygenation can also be generated nonenzymatically, but they are *racemic* and are accompanied by a complex mixture of stereoisomers. Nonenzymatic cyclooxygenation also produces structural isomers with nonprostanoic acid carbon skeletons (*e.g.*, see Fig. 7), each of which is also a complex mixture of stereoisomers. Each stereoisomeric mixture is designated collectively as a specific "type" of isoP endoperoxide. Two different rearrangements of the isoP endoperoxides lead to levulinaldehyde and hydroxycyclopentanone derivatives that are referred to collectively as isoLGs and isoPs, respectively. It is important to recognize that PGs and LGs are a subset of the isoPs and isoLGs, respectively. In other words, PGs and LGs are products of both the COX and isoP pathways. Furthermore, the PGHS-dependent generation of stereoisomeric PGs *in vivo* is also known (77). Therefore, stereoisomeric PGs, such as 8-epiPGF$_{2\alpha}$, should not be presumed to be products of free radical-induced nonenzymatic oxidation through the isoP pathway (81), unless it is established that they are racemic. In contrast, PGHS does not generate eicosanoid structural isomers with nonprostanoic acid carbon skeletons. Therefore, such structural isomers unambiguously indicate operation of the isoP pathway.

LG- AND isoLG-PROTEIN ADDUCTS *IN VIVO*

LGs and isoLGs are adducted to proteins in vivo

Enzyme-linked imunosorbant assays with LGE$_2$-KLH antibodies revealed LGE$_2$-protein adduct immunoreactivity in human plasma (91). That the production of LGE$_2$-protein adducts might be associated with oxidative injury was suggested by the finding of a severalfold elevation of the mean level in individuals with atherosclerosis compared with healthy controls (91). Immunocytochemical experiments revealed LGE$_2$-protein adduct immunoreactivity in the walls of the major cerebral vessels of the hippocampus (Fig. 9). The presence of protein adducts in the major vessels was not surprising because they often show a high level of other posttranslational modification, *e.g.*, glycation, owing to slow turnover of the abundant extracellular matrix.

Thus, the first evidence for the generation of LG isomers through nonenzymatic rearrangement of prostanoid endoperoxides *in vivo* was finally secured two decades after our call to investigate "the biological ramifications of such transformations" (86). Although the detection of LGE$_2$-protein

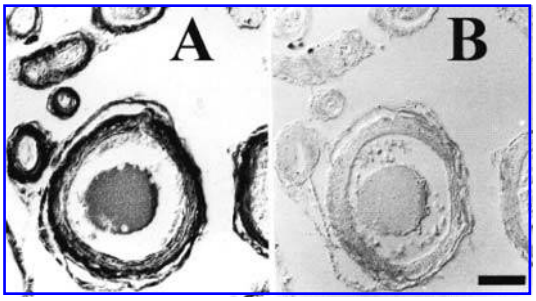

FIG. 9. LGE$_2$-protein adducts in cerebral vessels. (**A**) LGE$_2$-KLH immunoreactivity in vessels of a 77-year-old individual and (**B**) competitive absorption with LGE$_2$-HSA on subjacent thin slice demonstrate antibody specificity. Scale bar = 100 μm.

adducts *in vivo* was a milestone in our efforts to demonstrate the biological consequences of the nonenzymatic rearrangement of prostanoid endoperoxides to levulinaldehyde derivatives, the question remained, are these adducts products of the COX or isoP pathway? To facilitate detecting the *in vivo* occurrence of products that unambiguously reveal the operation of the isoLG pathway, we raised antibodies against an adduct of iso[4]LGE_2 with KLH (94). As expected, immunoreactivity diagnostic for the isoP pathway, detected using antibodies raised against iso[4]LGE_2-KLH, was generated upon nonenzymatic free radical-induced oxidation of LDL *in vitro* (93). Definitive evidence for the generation of isoLGs through the isoP pathway *in vivo* was provided by the detection of iso[4]LGE_2-protein immunoreactivity in human plasma (94). That the epitopes in human blood are protein adducts was supported by the facts that they were removed by ultrafiltration and that Western blot analysis of a polyacrylamide gel with iso[4]LGE_2-KLH antibodies detects several immunoreactive components that comigrate with various plasma proteins, including human serum albumin (HSA), fibrin, fibrinogen, and IgG. A very recent study confirmed the presence of isoLG-protein adducts in human plasma by mass spectroscopic detection of isoLG-modified lysine (referred to as "IsoK lactam adducts") in digests of human serum proteins (15).

A clinical pilot study (93) found higher levels of both iso[4]LGE_2-protein and LGE_2-protein adduct immunoreactivity in blood from continuous ambulatory peritoneal dialysis patients who have end-stage renal disease (RD) or atherosclerosis (AS) patients who have undergone coronary artery bypass surgery than in blood from two populations of healthy individuals (N) of average age 33 or 62 years (Table 1).

There is a strong linear correlation ($p < 0.0002$) between blood levels of total cholesterol and apolipoprotein (apo) B (Table 1), the protein component of LDL particles that are cholesterol-laden building blocks of foam cells, the progenitors of atherosclerotic plaques. Besides showing that the isoP pathway generates isoLGs *in vivo*, our clinical pilot study provided presumptive evidence that levels of oxidative injury, as measured by isoLG-protein immunoreactivity, represent a risk factor for cardiovascular disease that is independent of the classical risk factors, total cholesterol or LDL. The data in Table 1 paradoxically show that cholesterol is significantly elevated in older individuals (N[62]) who do not have AS compared with AS patients (AS[63]) of similar average age. This probably reflects the success of therapeutic interventions, including diet or drugs, which reduce total cholesterol levels, but apparently not isoLG levels, for the AS patients. Elevated isoLG-protein levels indicate an independent defect that results in an abnormally high level of oxidative injury that is associated with AS but not with total cholesterol levels.

LG-based modifications of LDL promote binding to and uptake by macrophages

Nonenzymatic oxidation of LDL to an oxidized form (oxLDL) is accompanied by the formation of lipid-based modifications of apoB, and fosters massive unregulated endocytosis by a class of macrophage receptors that recognize the lipid-based protein modifications, but not native LDL (31). This uptake, and impaired processing, lead 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. Hoppe *et al.* found that modification of LDL and other proteins by LGE_2 is orders of magnitude more efficient than modification by other reactive aldehydic products of lipid oxidation studied previously, *i.e.*, MDA or 4-hydroxynon-2-enal (43). Therefore, they examined the effect of LDL modification by LGE_2 on recognition and uptake by macrophage cells. LGE_2-modified LDL binds with and is degraded after receptor-mediated endocytosis by macrophage cells. Both binding and uptake, leading to degradation, are blocked by an excess of oxLDL, suggesting that LGE_2-derived modifications generated upon oxidation of LDL contribute to binding and uptake of oxLDL by macrophages.

Most protein modification by isoLGs in blood does not involve apoB

Recent work on the etiology of AS has focused on the oxidation of LDL within the arterial wall that leads to foam cell formation (106, 107). However, as noted above, iso[4]LGE_2-protein adducts in blood are associated with a variety of plasma proteins, including HSA, fibrin, fibrinogen, and IgG. Furthermore, there is only a 20–22% decrease in iso[4]LGE_2-protein adduct immunoreactivity after immunoprecipitation of apoB from the plasma samples (94). Hence, most of the isoLGs are *not* associated with LDL or very-low-density lipoprotein. The involvement of isoLGs in the etiology of car-

TABLE 1. LEVELS (MEANS ± SD) OF ISOLGS, APOB, AND TOTAL CHOLESTEROL

Cohort [average age]	*N[33]*	*RD[54]*	*AS[63]*	*N[62]*
Ages	33 ± 3	54 ± 5	63 ± 12	62 ± 7
Population	15	8	16	10
LGE_2-protein (pmol/ml)	132 ± 47	306 ± 77	287 ± 62	121 ± 42
Iso[4]LGE_2-protein (pmol/ml)	1,441 ± 144	2,387 ± 699	2,224 ± 439	1,691 ± 252
ApoB (mg/L)	724 ± 180	1,135 ± 225	937 ± 206	1,046 ± 370
Total cholesterol (mmol/L)	4.16 ± 0.87	4.56 ± 1.04	4.58 ± 0.59	5.78 ± 1.42

AS, atherosclerosis patients; N, healthy individuals; RD, renal disease patients.

diovascular disease almost certainly is more extensive than its role in promoting receptor-mediated endocytosis of oxLDL by macrophage cells (see above). Although an understanding of the involvements of isoLGs in the etiology of human disease is just burgeoning, a myriad of pathologic effects of these stealthy toxins are anticipated to result, *inter alia*, from the damage caused by their promiscuous covalent modification of many different proteins.

LG-based modifications of Lp(a), but not of LDL, are elevated in renal disease

Lp(a) is a lipoprotein complex assembled from LDL and a large hydrophilic glycoprotein, apo(a), linked by a disulfide bond to apoB. Elevated levels of Lp(a) are correlated with premature coronary heart disease (101). As end-stage RD is associated with oxidative stress and mortality primarily associated with AS, and as plasma isoLG–protein levels are elevated in RD patients (Table 1), Podrez *et al.* investigated the prevalence of LGE_2-based modifications in Lp(a) (75). As expected, both LDL and Lp(a) levels were elevated in RD patients undergoing continuous ambulatory peritoneal dialysis therapy compared with an age-, race-, and gender-matched group of control subjects with no history of RD. Especially noteworthy was the finding that Lp(a) levels of LGE_2-protein immunoreactivity were 2.4-fold higher in the RD cohort than in the control group ($p < 0.05$). Even after normalization for the higher Lp(a) concentrations in the RD cohort, a 1.6-fold elevation of LGE_2-protein modification ($p < 0.05$) was detected. In contrast, no elevation in LGE_2-protein modification level was found for LDL after normalization. Possibly the greater level of oxidative modification present in Lp(a) from RD patients is a consequence of lower plasma clearance of Lp(a) than of LDL (51), giving more time to accumulate oxidative modifications. Because oxidation enhances the binding of Lp(a) to the plasminogen receptor, it may inhibit fibrinolysis (40) and, thus, promote thrombogenesis.

ENZYME-PROMOTED isoLG PRODUCTION *IN VIVO*

Both enzymatic and nonenzymatic processes contribute to isoLG production

Living systems evolved in an anaerobic environment. The advent of photosynthetic organisms provided advantages, but also challenges associated with the ease with which oxygen participates in nonenzymatic oxidative degradation of biomolecules. Chemical and enzymatic defenses evolved to ameliorate the problems engendered by an aerobic environment. Nevertheless, oxidative injury remains a major challenge to human health, owing in large measure to failures in those defenses. The contribution of oxidative injury to a host of disease processes makes it imperative to seek an understanding of the complex interconnections between the enzymatic and nonenzymatic biochemistry involved. Autoxidation of lipids can be initiated by redox-active metal ions that promote the formation of hydroxyl and alkoxyl radicals from hydrogen peroxide or organic hydroperoxides. As enzyme action—*e.g.*, superoxide dismutase, COX, or lipoxygenase—can generate hydrogen peroxide and hydroperoxides, initiation of free radical-induced autoxidation of lipids can result from enzyme-dependent generation of radical precursors coupled with nonenzymatic generation of the radicals from these precursors.

IsoLGs can be generated *in vitro* by copper or iron ion-promoted nonenzymatic oxidation of AA or arachidonate-containing phospholipids, *e.g.*, AA-PC (14, 76). Metal ions also promote *in vitro* oxidation of LDL, resulting in the generation of isoLG-protein modifications on apoB (92). Therefore, the Fe(II)-Fe(III) and Cu(I)-Cu(II) redox couples may be important contributors to oxidative injury in some circumstances, *e.g.*, iron overload or metallothionine deficiency. However, serum proteins chelate free transition metal ions and thereby inhibit lipid peroxidation initiated by free iron or copper ion-dependent mechanisms (20).

Myeloperoxidase (MPO) promotes the generation of isoLGs in vitro

An enzyme-promoted alternative pathway for initiating free radical-induced oxidation of lipids is provided by MPO. This enzyme serves as a catalyst that initiates free radical-induced oxidation *in vivo* at sites of inflammation (109) to inflict "oxidative injury" on infecting organisms. Thus, MPO-promoted free radical-induced oxidation is a powerful defensive weapon for human health, but it also has pathological potential. Poliakov *et al.* explored the possible involvement of MPO in initiating free radical-induced oxidation of lipids leading to the production of isoLGs (76). Both $isoLGE_2$-protein and $iso[4]LGE_2$-protein adducts were generated upon MPO-promoted *in vitro* autoxidation of LDL. In contrast with copper ion-catalyzed oxidation of LDL, which is prevented by the presence of only 10% of lipoprotein-deficient serum, MPO-promoted formation of isoLG-protein adducts was only modestly attenuated by the presence of serum.

MPO promotes the generation of IsoLGs in vivo

MPO uses hydrogen peroxide together with NO_2^- to generate nitrogen dioxide, a free radical capable of initiating lipid peroxidation. MPO knockout mice are more susceptible than wild-type mice to *Candida albicans* infection (5), making the *Candida* sepsis model a useful tool for studying the role of MPO in inflammation. Both wild-type and MPO knockout mice exhibited comparable mean plasma levels, 34 and 38 pmol/mg protein, respectively, of $iso[4]LGE_2$-protein adducts. Intraperitoneal injection with *C. albicans* induced substantial increases to 184 and 278 pmol/mg protein. These findings strongly support the view that isoLG-protein adducts are good biomarkers of oxidant stress. Notably, these results show a significant 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. This provides presumptive evidence that MPO contributes to the formation of isoLG-protein adducts *in vivo* under inflammatory conditions.

Serum isoLG-protein adducts and F_2-isoPs are fundamentally different markers of oxidative injury

In contrast to isoLG-protein adduct levels, plasma levels of F_2-isoPs were not increased in wild-type or MPO knockout mice after challenge with *C. albicans* (76). This contrast is understandable because F_2-isoPs are cleared from the circulation very rapidly (7, 65), whereas isoLG-protein adducts presumably are not. The exceptional reactivity of LGs and isoLGs toward proteins results in rapid trapping. Because LG-protein and isoLG-protein adducts can accumulate for days or weeks over the lifetimes of proteins (100), they represent a convenient dosimeter that provides a cumulative index of oxidative stress. In this regard, it is noteworthy that LG modification of proteins confers resistance to proteasomal degradation (21) that could extend the lifetimes of some proteins (see below). In contrast, F_2-isoP levels provide a snapshot that may be more suited for monitoring changes in oxidative stress on a shorter timescale. For example, greatly increased levels of isoPs in blood or urine, caused by oxidative stress associated with myocardial reperfusion, drop precipitously within minutes (23).

Schiff base isoLG-protein adducts are generated in vivo *as phospholipid esters*

A very recent study uncovered a surprising dichotomy between Schiff base and lactam adducts of isoLGs found in rat liver after administration of CCl_4. This is a well established model of oxidant injury to the liver that is associated with marked overproduction of isoPs through free radical-induced oxidation of polyunsaturated phospholipids (64, 65). Thus, whereas the lactam adducts were present as free acids, >97% of the Schiff base adducts were apparently esterified to phospholipids because treatment of the proteins with base caused a 42-fold increase in the level of the free acid detected (15). These observations reconfirm that isoLGs are generated *in vivo* through nonenzymatic oxidation of AA-PC. The resulting isoLG-PC esters then bind rapidly to proteins to generate Schiff base adducts. These metastable adducts undergo a slow subsequent conversion, via unstable pyrrole intermediates, into lactam end products. Neither the isoLG-PC esters nor their Schiff base adducts are hydrolyzed appreciably. However, hydrolysis occurs readily after the subsequent slow conversion to lactams (Fig. 10). It is remarkable and not at all obvious why the lactams are so much better substrates for ester hydrolysis than the Schiff base adducts.

Protein adducts of DHA-derived isoLGs are abundant in human brain

DHA is uniquely abundant in neuronal cells, especially in gray matter and the outer segments of photoreceptor rod cells. Neuronal cells are under considerable oxidative stress owing to high energy requirements for maintaining ionic gradients, and a consequently high level of aerobic catabolism. This stress in conjunction with a relative abundance of PUFAs and weak antioxidant defenses compared with other tissues (30) makes the brain especially sensitive to oxidative injury. Because it possesses an unusually high number of doubly allylic methylene groups, sites prone to hydrogen

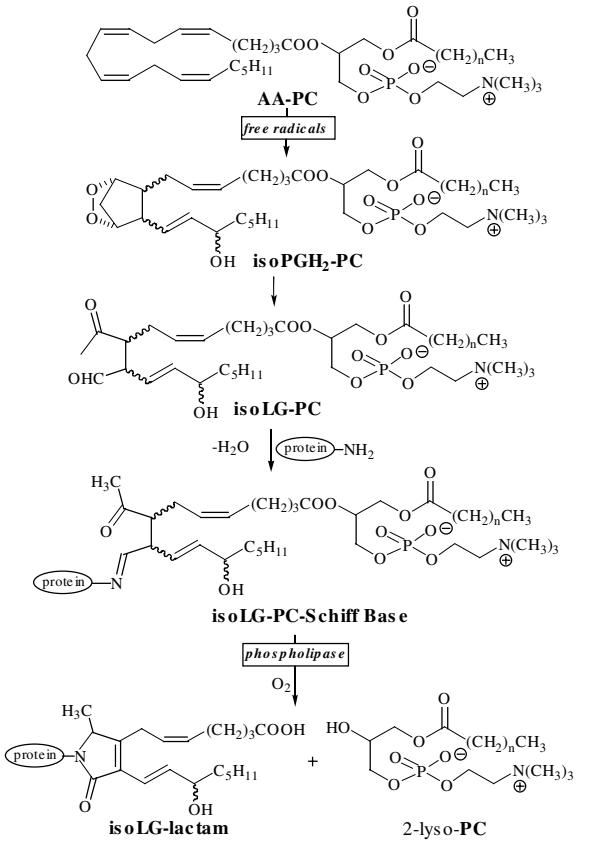

FIG. 10. Proteins trap isoLG-phospholipids as Schiff base adducts. IsoLG-PC generated *in vivo* in rat liver is present as Shiff base adducts, whereas isoLG-lactam adducts are present as free acids.

atom abstraction, DHA is exquisitely reactive toward autoxidation. Autoxidation of DHA-containing lipids generates endoperoxides that rearrange to levulinaldehydes, which have been called isoLGE_4s (98) or neuroketals (9). IsoLG_4-protein adducts are abundant in normal human brain at a level of 9.9 ± 3.7 ng/g of cerebral cortex from individuals who had no known neurological disease (9). Such protein modifications by PUFA-derived γ-ketoaldehydes are expected to be neurotoxic by analogy with the neuropathological effects of γ-diketones (38, 54). Under conditions of elevated oxidative stress associated with Alzheimer's disease (AD) (59), the production of isoLG_4s through a C22 variant of the isoP pathway (which has been dubbed the neuroprostane pathway) would be increased. As expected, concomitantly elevated isoLG_4-protein adduct levels are found in AD brain (21).

CROSS-LINKING OF PROTEINS AND DNA BY LGS AND isoLGS

LGs and isoLGs are highly effective protein cross-linking agents

We chose ovalbumin (OA) as a model substrate to explore the proposition that binding of LGs with proteins could lead

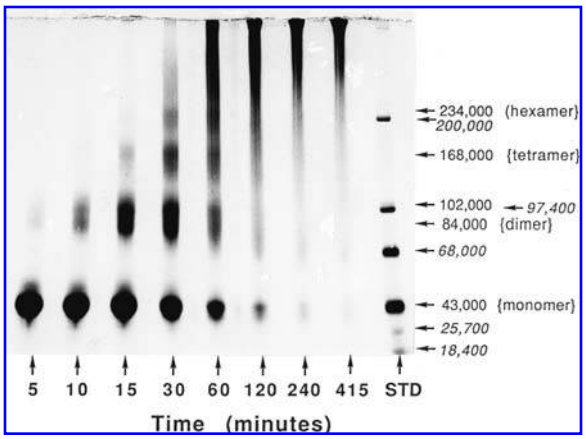

FIG. 11. Time course of the LGE_2-protein reaction. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel (6–12% linear gradient) of products from OA and LGE_2 (15 equivalents) at 37°C for various times is shown. Oligomer molecular weights are indicated at right. STD, standard proteins (molecular weights italicized).

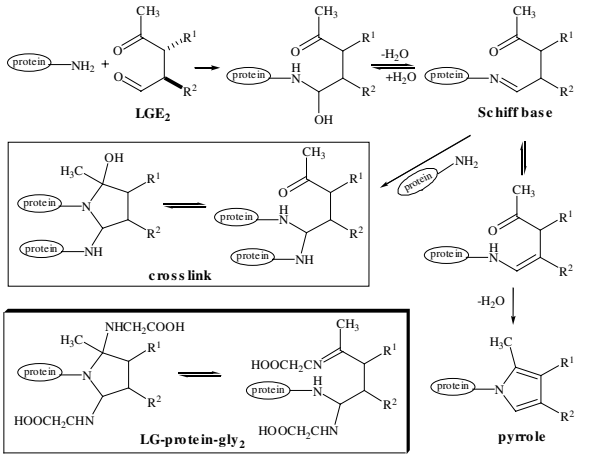

FIG. 12. LGs and isoLGs cross-link proteins. The nascent LGE_2-protein adduct is a reactive electrophilic "activated monomer" that covalently binds lysyl ϵ-amino residues to form protein–protein cross-links. The activated monomer can bind two molecules of glycine.

to protein–protein cross-linking. Incubation of OA with LGE_2 causes extensive oligomerization of the protein (45). Analysis by polyacrylamide gel electrophoresis revealed bands corresponding to various oligomers. The time course of intermolecular cross-linking was conveniently followed by incubating LGE_2 with OA and quenching the oligomerization after various time intervals by addition of excess glycine (Fig. 11). The consumption of monomeric protein by reaction with 15 equivalents of LGE_2 is almost complete within 2 h. Initially, dimer yield rises rapidly, reaching 30% within 25 min, but then drops, and only high-molecular-weight oligomers are present after 4 h. The formation of a "ladder" of various oligomers was readily apparent after 30 min. No discernible oligomerization was caused by PGE_2, PGB_2, PGD_2, PGA_2, $PGF_{2\alpha}$, MDA, or 4-hydroxynon-2-enal under the same conditions. Both MDA and 4-hydroxynon-2-enal bind and cross-link proteins. However, LGE_2 is orders of magnitude more effective in generating protein–protein cross-links than any of these other AA-derived oxidized lipids tested.

Although many molecules of LGE_2 bind within 1 min to each molecule of protein (see above), oligomerization can be completely prevented by adding a large excess of glycine to the reaction mixture 5 min after combining the protein and LG (Fig. 11). Such behavior is expected for a cross-linking mechanism involving rapid generation of a reactive electrophilic adduct between LGE_2 and monomeric protein and cross-linking involving reaction of that "activated monomer" with a free primary amino group in another protein molecule. As the rate of binding is rapid compared with the rate of cross-linking, the ability of glycine to completely quench the reaction is apparently the result of intercepting reactive electrophilic centers on an "activated monomer" rather than the result of intercepting free LGE_2. Using ^{14}C-labeled glycine and ^{3}H-labeled LG, we confirmed this scenario and determined that the nascent LGE_2-protein adduct initially binds nearly 2 equivalents of glycine (48). A possible cross-linking mechanism involves aminal formation (Fig. 12). The 2:1 adduct is probably a cyclic bis aminal. The ability of the LGE_2-protein adduct to bind glycine then slowly decreases, reflecting a competition, *inter alia*, with cross-linking and dehydration driven by the aromatization that accompanies pyrrole formation.

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 as demonstrated with glycine. Another example, reported recently (11), is the LGE_2-promoted covalent linkage of proteins with spermine, a polyamine that is abundant in both the cytosol and nucleus.

There is some evidence to suggest that nonsteroidal antiinflammatory drugs are prophylactic against AD and cognitive decline in patients with AD (16, 44, 83), suggesting that COX activity contributes to the etiopathology of AD. Given the ability of LGs to cross-link proteins, it is plausible that these toxins, generated by the nonenzymatic rearrangement of PGH_2, promote the formation of oligomers of the amyloid peptide $A\beta_{1-42}$ that are abundant in regions of AD brain exhibiting neuronal pathology (60, 84). A molecular basis for a hypothesis linking COX activity to the formation of these oligomers was suggested by the observation that treatment of $A\beta_{1-42}$ with PGH_2 results in the formation of a "ladder" of various oligomers (12). Mass spectral evidence confirmed the formation of LG-based adducts with the peptide. Electron microscopy revealed that treatment of $A\beta_{1-42}$ with PGH_2 caused the formation of small granular particles that are believed to be protofibrillar aggregates generated by LG-induced oligomerization of the amyloid peptide.

LGE_2 cross-links DNA with proteins

We envisioned the possibility that LGs might cause DNA-protein cross-links (DPCs) by analogy with the behavior of

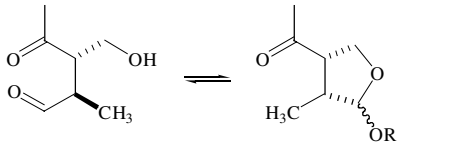

FIG. 13. Botryodiplodin (BD) forms DNA-protein cross-links (DPCs). The levulinaldehyde form of BD, but not its methyl acetal, generates DPCs in eukaryotic cells and blocks DNA synthesis.

botryodiplodin (BD) (6, 63, 96), a mutagenic mycotoxin (67). BD forms DPCs in eukaryotic cells (66, 69) and blocks DNA synthesis in a manner consistent with a DNA-damaging agent (68). BD exists as a hemiacetal in equilibrium with an aldehyde, which is the biologically active form (Fig. 13). Thus, blocking interconversion to the aldehyde form with a methyl group in an acetal derivative abolishes the ability to cause DPCs (69). Thus, the active form of BD contains the same reactive electrophilic γ-ketoaldehyde functionality as found in the LGs.

We found that exposure of V79 Chinese hamster lung fibroblasts to LGE_2 generates repair-resistant DPCs and causes cell death (LD_{50} = 230 n*M*) (71). Repair resistance, which is relevant to cell killing, is also a characteristic of DPCs formed by exposure of cultured cells to ultraviolet radiation or to visible light in the presence of photosensitizers (78). Photogeneration of radicals might initiate autoxidation and the production of isoLGs through the isoP pathway. The fact that photodynamic treatment also causes release of AA metabolites through the COX pathway (78) suggests that LGs also contribute to cell killing in this context. This study demonstrated not only that LGs are able to cross cell membranes and modify intracellular proteins, but also that sequestration by intracellular proteins does not prevent passage of at least some LGs through the cytoplasm and into the nucleus.

PATHOLOGICAL CONSEQUENCES OF THE LG AND isoLG PATHWAYS

LGE_2 damages brain tissue and causes blood-brain barrier opening

It seemed likely that tissue damage would result from covalent binding of LGs to membrane proteins. Schmidley *et al.* found that injection of as little as 100 nmol of LGE_2 in 50 μl of phosphate buffered saline into the brain of a live rat caused lesions that were readily apparent at 175× magnification (97). The cellular infiltrate consisted largely of lipid-laden macrophages. Readily apparent changes were induced in the ependyma and choroid plexus by injection of 0.5 μmol of LGE_2 into the cerebrospinal fluid in the ventricle (Fig. 14). That the LG caused opening of the blood–brain barrier was demonstrated by a 1.5-fold increase ($p < 0.05$) in extravasation of Evans blue (relative to buffer alone) caused by injection of 0.5 μmol of LGE_2.

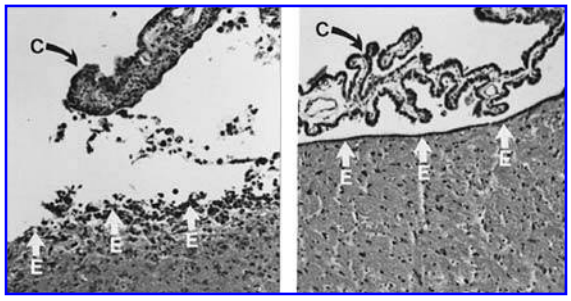

FIG. 14. Brain tissue damage caused by LGE_2. (**Left panel**) 0.5 μmol of LGE_2 injected into the cerebrospinal fluid of the ventricle induces denudation of the ependyma (E) with cellular infiltration of the subjacent neuropol and choroid plexus (C) by cells. (**Right panel**) Normal structures for comparison.

Covalent modification by LGs or isoLGs interferes with protein function

Mounting evidence supports the view that most, if not all, of the biological consequences of LG and isoLG production result from the exceptional reactivity of these γ-ketoaldehydes toward biological nucleophiles, *e.g.,* ε-amino groups of lysyl residues in proteins. The resulting covalent modifications, which can include cross-links, are expected, *inter alia*, to alter or abolish important functions of biomacromolecules. The first evidence for such biological ramifications, noted above, resulted from the ability of LGE_2 to cause DNA-protein cross-linking. Although the identity of the protein(s) involved remain(s) to be established, it is especially noteworthy that histones are lysine-rich. In this regard, it should be reiterated that bioconversion of AA into PGH_2 with PGHS in the presence of histone types II and III resulted in covalent, presumably LG-based, modification of the histones (11). Histones act like positively charged spools around which negatively charged nucleic acids are wound in chromatin. LG-based cross-links between DNA and the associated histones could prevent the obligatory unwinding of DNA that must precede gene expression. Alternatively, the cytotoxicity of LGs may arise because LG-derived DPCs act as permanent roadblocks against replication. Another possibility is that mitosis can be prevented by LG-based damage of the cytoskeletal machinery that is critical for cell division.

Tubulin provided the second example of the ability of LGs to interfere with protein function (70). Tubulin is the major constituent protein of the microtubule, an important component of the eukaryotic cytoskeleton and an integral part of the mitotic spindle. LGE_2 inhibits GTP-induced assembly of bovine microtubule protein (~1.4 mg/ml, ~85% tubulin, and 15% microtubule-associated protein) with an ED_{100} ~80 μ*M*, and inhibited the first synchronous cell division of fertilized sea urchin eggs with a slightly lower ED_{100} of ~25 μ*M*. These data provide presumptive evidence that LGE_2 can enter cells and bind to tubulin, preventing microtubule assembly and inhibiting mitosis. The LG did not cause depolymerization of preassembled tubulin. Although 0.03–33 equivalents of the LG relative to α,β-heterodimer bound essentially quantitatively, complete inhibition of assembly occurred upon bind-

ing of only about two molecules 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.

The effect of isoLGE$_2$-based protein modifications on proteosomal protein degradation provides yet another example of the influence of LG-protein modification on protein function. In this case, however, the effect is not primarily associated with modification of the protease, but rather with modification of its protein substrates. Thus, covalent modification of a model protein OA or an amyloid peptide ($A\beta_{1-40}$) with as little as 1 equivalent of LGE_2 isomers (referred to as isoketals or E_2-isoK) strongly curtailed their subsequent processing by the 20S proteasome (21). Furthermore, both isoLGE$_2$-OA and isoLGE$_2$-$A\beta_{1-40}$ 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. A simple explanation of these findings is that 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 blocks access of nonadducted proteins to the active site. Preincubation of the proteasome with the isoLG does not effectively modify the active site because it is buried within the central cavity of this multienzyme complex, whereas nonspecific binding of the isoLG to other proteasomal lysine residues is not similarly hindered.

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. As direct adduction of LG isomers to the proteosome only caused weak inhibition, a novel mechanism of action may be operative for these stealthy toxins. 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.

It is probable, although as yet unproven, that isoLG-protein adduction would similarly inhibit the ATP-dependent degradation of ubiquinated proteins by the 26S proteasome 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 n*M*) and induced cell death at higher concentrations (LC_{50} = 670 n*M*). As this concentration was 100-fold lower than that reported previously for 4-hydroxynon-2-enal, LGs and isoLGs are "among the most potent neurotoxic products of lipid oxidation heretofore identified" (21).

Oxidative stress is known to affect the function of various membrane proteins, including receptors, enzymes, and ion channels (4, 36, 49, 104, 105). In view of their exceptional reactivity and their production through free radical-induced autoxidation of membrane lipids, it is reasonable to presume that isoLGs mediate these functional alterations. A very recent model study (15) demonstrated that covalent adduction of isoLGE$_2$ impairs the functional integrity of a model integral-membrane K^+ channel that is relevant to oxidative ischemia/reperfusion injury-induced cardiac dysrhythmias (99, 108). Whole patch-clamp experiments revealed a pronounced dose-dependent inhibition (IC_{50} = 2.2 μ*M*) by the isoLG of the rapidly activating delayed rectifier K^+ current. Full inhibition was only achieved slowly, 60 min after addition of the isoLG. As isoLGs bind within seconds to proteins, the involvement of channel protein cross-linking—which is expected to be slow relative to binding—seems likely, although time-dependent diffusion into the membrane cannot be ruled out.

LG- or isoLG-derived protein adducts may trigger autoimmunity

Lipid-derived oxidative protein modifications can foster the appearance of autoantibodies. Elevated levels of antibodies binding MDA-HSA or MDA-LDL are found in uremic patients undergoing peritoneal dialysis (56), patients with coronary artery disease (42, 53, 57, 95), systemic lupus erythematosus (1), scleroderma (1), and rheumatoid arthritis (1, 19). Elevated levels of antibodies binding lipid-derived carboxyethylpyrrole derivatives of HSA are found in the blood of patients with age-related macular degeneration (39). The detection of autoantibodies against isoLG-derived protein adducts in human plasma showed that isoLG-protein adducts generated *in vivo* are immunogenic (94). It also raises the possibility that LG- or isoLG-derived protein modifications may contribute to autoimmune pathologies.

CONCLUSIONS AND PROSPECTS

The last few decades have witnessed a move away from the view that the nonenzymatic chemistry of biological molecules is merely an *in vitro* nuisance that potentially interferes with knowing the biologically important chemistry of living systems. Rather, the nonenzymatic chemistry of biomolecules must be seen as a fact of life that biological systems have evolved to control. This is exemplified by the vast array of natural antioxidants and enzymes that defend against nonenzymatic oxidation of biomolecules. It should also be noted that living systems sometimes exploit nonenzymatic chemistry to their advantage. This is exemplified by the oxidative burst of neutrophils that is a potent weapon in the immunological armamentarium. It remains to be learned whether biologically important beneficial roles exist for the nonenzymatic formation of lipid-derived levulinaldehyde derivatives and their reactions with biological nucleophiles.

Given their ability to avidly bind and cross-link proteins, nucleic acids, and other biological nucleophiles, LGs and isoLGs should be deemed prime suspects to account for the formation of oxidatively modified proteins and protein aggregates that are refractory toward proteolysis, as has been demonstrated for proteosomal degradation. The cytotoxicity of LGs and isoLGs suggests that these oxidized lipids can mediate tissue necrosis, edema, and increased vascular permeabil-

ity, which are characteristics of oxidative reperfusion injury. IsoLGs are expected to be important contributors to pathologies associated with oxidative stress. LGs are expected to be important contributors to pathologies associated with aberrant overproduction of prostanoid endoperoxides through the COX pathway. For example, the generation of abnormally high concentrations of PGH_2—owing to the release of a torrent of free AA resulting in a postischemic burst of COX activity—provides an excellent opportunity for LG production. Unraveling the pathological involvements of the COX and isoP pathways is challenging. In the past, this has been done by examining the effects of putatively selective inhibitors of specific pathways. However, the effects of enzyme inhibitors can be complex. For example, hydroperoxide intermediates produced in enzymatic oxidations through the COX or lipoxygenase pathways may contribute to initiation of nonenzymatic oxidations through the isoP pathway. The quantification of specific molecular species that are diagnostic for the operation of a nonenzymatic pathway, such as iso[n]LGs, can provide unambiguous evidence for its occurrence and prevalence under particular circumstances.

As isoPs and isoLGs arise from alternative nonenzymatic rearrangements of common endoperoxide intermediates, the appearance of isoPs *in vivo* will be accompanied by the coproduction of isoLGs. Although the isoPs may exert biological effects by masquerading as PGs, they are chemically benign compared with the highly reactive isoLGs. Therefore, we suspect that a significant portion of pathological sequelae of the free radical-induced oxidation of polyunsaturated lipids through the isoP pathway will not be mediated by isoPs. Rather they will ensue through the nonenzymatic biochemistry of isoLGs. A similar dichotomy may prevail under certain circumstances with respect to the relative importance of PGs and LGs in mediating pathological sequelae of the aberrant overproduction of prostanoid endoperoxides through the COX pathway. Thus, although a torrent of PG biosynthesis is seen during reperfusion following ischemia, the PGs may be relatively benign "innocent bystanders" that are rapidly cleared from the circulation, whereas LGs are the potent cytotoxins whose protein adducts accumulate and contribute to the pathological sequelae of ischemia/reperfusion injury. It is also interesting to note that the antibiotic BD (see Fig. 13), a simple levulinaldehyde derivative analogous to LGs and isoLGs, causes pigment formation, *i.e.*, "pink stains," when applied to human skin (96). The reaction of proteins with LGs or isoLGs generates an unidentified purple-pink chromophore. This suggests that LGs or isoLGs may be involved in the pigment (lipofuscin) formation that is associated with aging and heart damage (61).

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ABBREVIATIONS

AA, arachidonic acid; AD, Alzheimer's disease; apo, apolipoprotein; AS, atherosclerosis; BD, botryodiplodin; BSA, bovine serum albumin; COX, cyclooxygenase; DHA, docosahexaenoic acid; DPC, DNA-protein cross-link; HSA, human serum albumin; isoLG, isolevuglandin; isoP, isoprostane; KLH, keyhole limpet hemocyanin; LDL, low-density lipoprotein; LG, levuglandin; MDA, malondialdehyde; MPO, myeloperoxidase; OA, ovalbumin; oxLDL, oxidized LDL; PC, phosphatidylcholine; PG, prostaglandin; PGHS, prostaglandin H-synthetase; PUFA, polyunsaturated fatty acid; RD, renal disease.

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