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Products of the isoprostane pathway: unique bioactive compounds and markers of lipid peroxidation

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Abstract. We previously reported the discovery of prostaglandin F_2-like compounds (F_2-isoprostanes) formed by nonenzymatic free-radical-induced peroxidation of arachidonic acid. Quantification of F_2-isoprostanes has proven to be a major advance in assessing oxidative stress status in vivo. Central in the pathway of formation of isoprostanes are prostaglandin H_2-like endoperoxides, which also undergo rearrangement in vivo to form E-ring, D-ring, and thromboxane-ring compounds. E_2- and D_2-isoprostanes also undergo dehydration in vivo to form reactive cyclopentenone A_2- and J_2-isoprostanes, which are susceptible to Michael addition reactions with thiols. Recently, we described the formation of highly reactive γ-ketoaldehydes (now termed isoketals) as products of isoprostane endoperoxide rearrangement which readily adduct to lysine residues on proteins and induce cross-links at rates that far exceed other aldehyde products of lipid peroxidation. Isoprostane-like compounds (neuroprostanes) and isoketal-like compounds (neuroketals) are formed from oxidation of docosahexaenoic acid, which is enriched in the brain, and measurement of neuroprostanes may provide a unique marker of oxidative neuronal injury.

Key words. Isoprostanes; lipid peroxidation; free radicals; protein adducts; isolevuglandins; mass spectrometry.

Biochemistry of the formation of isoprostanes

One of the major targets of free radicals are polyunsaturated lipids, which undergo peroxidation reactions. A plethora of products are produced by free-radical-induced lipid peroxidation [1, 2]. In 1990, we reported the discovery that a series of novel prostaglandin (PG) F_2-like compounds are produced by free-radical-induced peroxidation of arachidonic acid in vivo [3]. The mechanism by which these compounds are formed is shown in figure 1. Because these compounds are isomeric to $PGF_{2\alpha}$ derived from the cyclooxygenase, they have been termed F_2-isoprostanes (F_2-IsoPs). A unique aspect of the generation of IsoPs is that they are initially formed in situ on phospholipids and then released preformed by phospholipases [4].

As noted in figure 1, three arachidonyl radicals give rise to four F_2-IsoP regioisomers. Each of these regioisomers comprise eight racemic diastereomers. Each regioisomer is designated by the carbon number on which the side-chain hydroxyl group is located; the carboxyl carbon is designated C-1. This is accordance with the official nomenclature system established for IsoPs that has been approved by the Eicosanoid Nomenclature Committee, sanctioned by the Joint Commission of Biochemical Nomenclature (JBCN) of the International Union of Pure and Applied Chemistry (IUPAC) [5]. Studies carried out to establish the relative abundance of the different F_2-IsoP regioisomers indicate that there is no preferential formation of regiosomers or isomeric compounds within a regioisomer group than what would be predicted from a nonenzymatic mechanism [6].

Central in the pathway of formation of IsoPs are PGH_2-like endoperoxides. The H_2- IsoP endoperoxides can be reduced to form F-ring IsoPs (fig. 1). Recently, we reported the discovery that glutathione is an important effector of the reduction of IsoP endoperoxides to F_2-IsoPs [7]. PGH_2 is an unstable molecule in aqueous solution which under-

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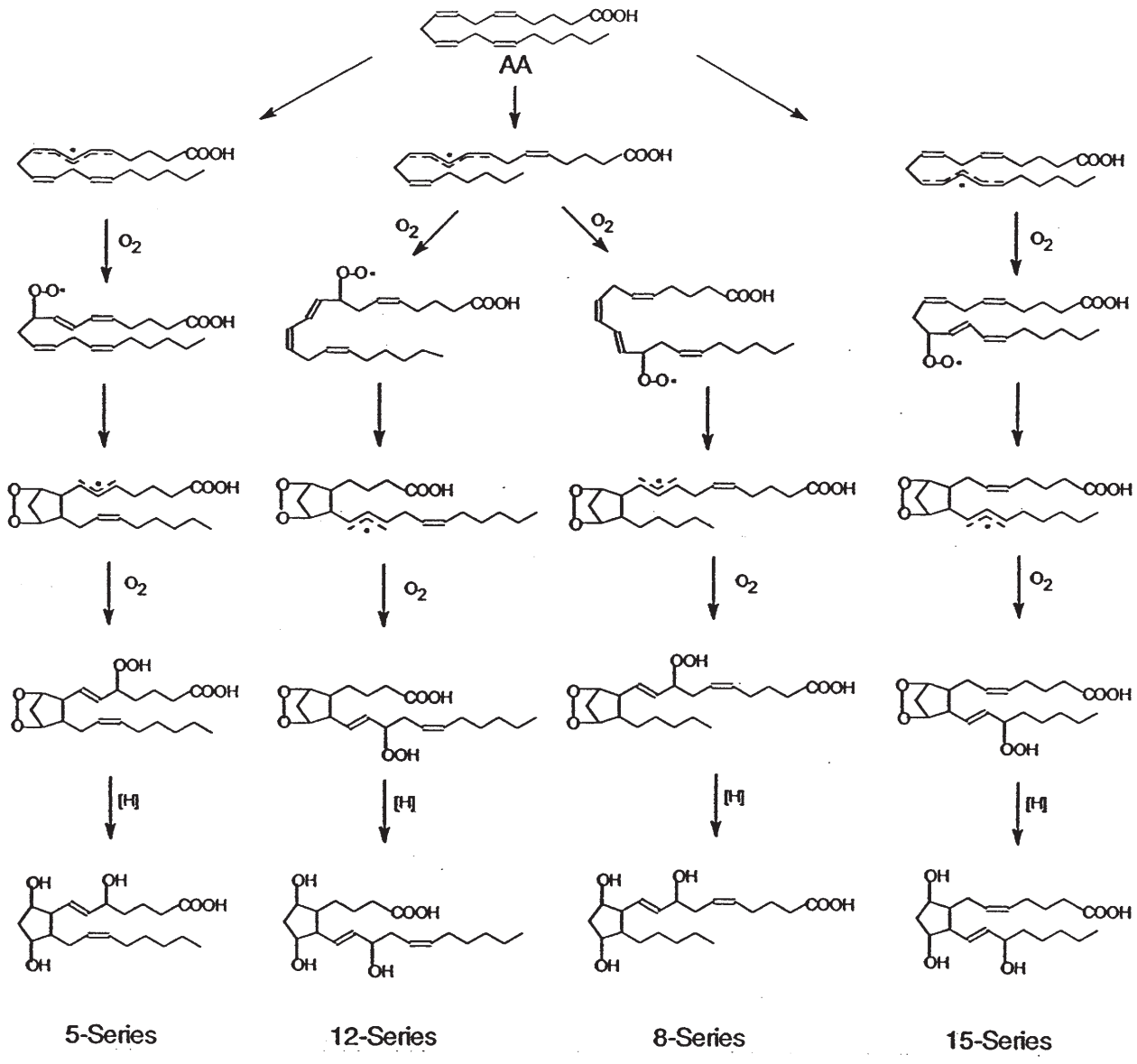

Figure 1. Pathway of formation of F_2-isoprostanes.

goes rearrangement to form PGE_2 and PGD_2 with a $t^1/_2$ of approximately 5 min [8]. In this regard, we explored the possibility that the reduction of the H_2-IsoP endoperoxides to F_2-IsoPs may not be completely efficient and found that abundant quantities of E_2/D_2-IsoPs are also produced in vivo [9]. In addition, PGH_2 may also undergo rearrangement to form a thromboxane ring, and we have found that isothromboxanes are also produced in vivo [10].

Measurement of F_2-IsoPs as a marker of lipid peroxidation in vivo

Although the initial discovery of F_2-IsoPs was a curious finding that elucidated new products of lipid peroxidation, the importance of this discovery has evolved considerably over the last few years and encompasses several aspects of the biology and biochemistry of IsoPs. Free radicals have been hypothesized to play an important role in the pathogenesis of a wide variety of disease processes [11, 12]. However, a major impediment in translating these hypotheses to fact has been the lack of a reliable noninvasive approach to assess oxidative stress status in vivo in humans [13]. In this regard, quantification of F_2-IsoPs has proven to be major advance in this area [14–17].

The method we routinely use for measurement of F_2-IsoPs is gas chromatography (GC) negative-ion chemical ionization (NICI) mass spectrometry (MS). This method is very sensitive (lower limit of detection ~ 10 femtomol) and has a high degree of precision (± 6%) and accuracy

(96%) [18]. However, it requires expensive instrumentation, and sample processing is relatively labor intensive. Immunoassay kits for one of the F_2-IsoPs, 15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$), are available commercially from several vendors. However, interfering substances can be problematic, as has been recognized with immunoassays for other prostanoids for many years [19]. More often than not, samples have to be subjected to some degree of purification prior to performing the immunoassay. In some instances, extraction of samples without additional purification by thin-layer chromatography (TLC) or high-pressure liquid chromatography (HPLC) can actually concentrate interfering substances. Recently, a direct comparison of GC/MS and enzyme immunoassay (EIA) for F_2-IsoPs revealed significant inconsistencies between the two methods [20]. Thus, MS remains the most reliable and accurate method for analysis of IsoPs.

To illustrate the comparative advantage of measuring F_2-IsoPs over other measures of lipid peroxidation to assess oxidant injury, we have directly compared measurements of F_2-IsoPs with both malondialdehyde (MDA) and lipid hydroperoxides, two of the most commonly used markers of lipid peroxidation, both in vitro and in vivo. The time-course of formation of F_2-IsoPs during oxidation of liver microsomes correlated very well with the formation of MDA and with the formation of lipid hydroperoxides during oxidation of low density lipoprotein in vitro [21, 22]. However, the fold increase above baseline in F_2-IsoP levels that occurred in vivo in liver following administration of CCl_4 to rats to induce an intense oxidant injury greatly exceeded that of MDA and lipid hydroperoxides by as much as 26-fold [22, 23]. This indicates that measurement of F_2-IsoPs is far superior to measurement of MDA and lipid hydroperoxides as an index of oxidative stress in vivo, notwithstanding the fact that MDA is not a specific marker of lipid peroxidation [13, 24].

Measurement of free F_2 -IsoPs in plasma can provide an index of total body production of IsoPs, whereas measurement of levels of F_2-IsoPs esterified in tissues can localize oxidant injury directly to specific sites of interest. Although sampling of tissues for analysis is primarily limited to experimental animals or post-mortem human tissue samples, analysis of levels esterified in lipoproteins in plasma has been utilized to obtain valuable information regarding the low density lipoprotein (LDL) oxidation hypothesis of atherogenesis in humans [25–28]. One of the potential problems with analysis of IsoPs in plasma is that blood drawing is somewhat invasive and inconvenient, and artifactual generation of IsoPs can occur unless special precautions are used to prevent it [18]. In addition, because the circulating $t^1/_2$ of F_2-IsoPs in the circulation is only approximately 16 min [20], there is also a potential drawback with measuring plasma concentrations of F_2-IsoPs in that it only provides an index of IsoP production at a single limited point in time. This may be problematic in certain disease states in which there may be significant intraday fluctuations in the magnitude of IsoP production. Analysis of urine for unmetabolized F_2-IsoPs also has some limitations as an index of systemic production of IsoPs, because there appears to be some contribution to free levels of unmetabolized F_2-IsoPs in urine from local formation of IsoPs in the kidney which are excreted directly into the urine [14].

These potential limitations with measurements of unmetabolized F_2-IsoPs can be overcome by measurement of the urinary excretion of a metabolite of F_2-IsoPs. This is because (i) metabolism of prostanoids occurs predominantly in extrarenal tissues, (ii) urine collected over several hours can provide an integrated index of IsoP production over time, (iii) collection of urine for analysis is noninvasive, and (iv) IsoP metabolites cannot be generated artifactually *ex vivo* by autoxidation. Toward this goal, we recently reported the identification of the major urinary metabolite of the F_2-IsoP, 15-F_{2t}-IsoP, in humans as 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP (fig. 2) [29] and recently developed a highly accurate stable isotope dilution GC/NICI/MS assay for this metabolite [30]. It is anticipated that the measurement of the urinary excretion of this metabolite will prove to be an important advance in assessing oxidative stress status in vivo in humans that will be applicable to large clinical studies.

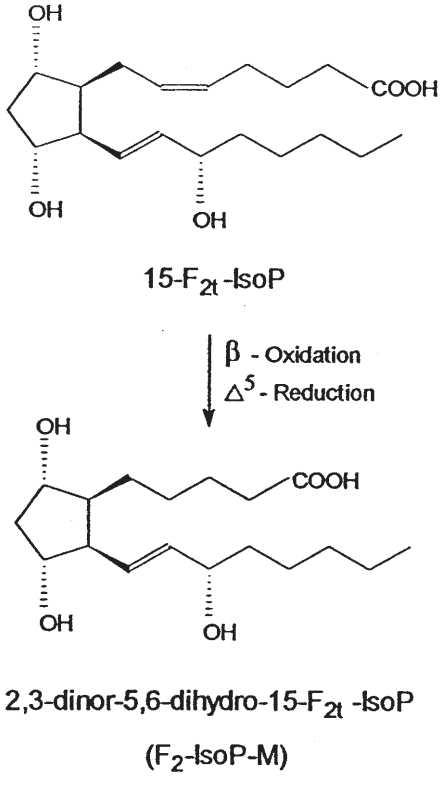

Figure 2. Formation of the major urinary metabolite of 15-F_{2t}-IsoP by one step of β-oxidation and reduction of the Δ^5 double bond.

Measurements of endogenous F_2-IsoP production have provided compelling evidence for a role for free radicals, often for the first time, in the pathogenesis of a remarkably large number of diverse disease processes. These range from cigarette smoking [28, 31], renal failure of rhabdomyolysis [32, 33], renal failure associated with aging [34], hepatorenal syndrome [35], scleroderma [36], CCL_4-induced hepatoxicity [3, 37], diquat-induced hepatotoxcity [3, 38], oxidative modification of LDL and atherogenesis [22, 25–28, 39–41], vitamin E deficiency [42], selenium deficiency [42], halothane-induced hepatotoxcity [43], organophosphate poisoning [44], Alzheimer's disease [45–47], Huntington's disease [48], hyperhomocysteinemia [49], acute Cr (VI) poisoning [50], cisplatin renal injury [51], diabetes [52–54], ischemia/reperfusion injury [25, 55–58], alcoholic liver disease [59, 60], acute cholestasis [61, 62], adult respiratory distress syndrome [63] in addition to data suggesting that F_2-IsoP production has predictive value for survival in this syndrome [unpublished], chronic obstructive lung disease [64], interstitial lung diseases [65], cold preservation of transplantable kidneys [66] and allergic asthma [67]. The reader is referred to the original articles for specifics regarding the data obtained.

Isoprostanes as bioeffectors of oxidant injury

In addition to being reliable markers of lipid peroxidation in vivo, IsoPs can also exert potent biological activity and potentially mediate some of the adverse effects of oxidant injury. First, as mentioned, IsoPs are initially formed esterified on phospholipids. Molecular modelling of IsoP-containing phospholipids reveals them to be remarkably distorted molecules [4]. Thus, the formation of these abnormal phospholipids would be expected to exert profound effects on membrane fluidity and integrity, well-know sequellae of oxidant injury.

Receptor-mediated biological actions

One of the unique characteristics of IsoPs that contrasts with prostaglandins formed by the cyclooxygenase enzyme is that the side chains are predominantly oriented *cis* in relation to the cyclopentane ring [68]. Two IsoPs that have been available for biological testing are 15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$) and 15-E_{2t}-IsoP (8-iso-PGE_2), which differ from their respective counterparts derived from the cyclooxygenase by inversion of the upper side chain stereochemistry. We have previously shown that both 15-F_{2t}-IsoP and 15-E_{2t}-IsoP are produced in vivo [69, 70]. Both of these IsoPs have been shown to be potent vasoconstrictors in a variety of vascular beds, including the kidney [3, 9, 71, 72], lung [73, 74], heart [75], retina [76], portal vein [77], brain [78] and also lymphatics [79]. In addition, 15-F_{2t}-IsoP induces endothelin release and proliferation of vascular smooth muscle cells [80, 81]. Results from initial experiments suggested that the vascular effects of both of these IsoPs may result from an interaction with thromboxane receptors based on the finding that the vasoconstriction could be abrogated by thromboxane receptor antagonists [71]. However, a number lines of evidence obtained subsequently suggests that these IsoPs may not interact with thromboxane receptors [72, 76, 82–85]. Whether these IsoPs mediate their effects by interaction with some other known receptor(s) or a novel 'IsoP receptor(s)' remains to be determined. Interestingly, in cerebral and retinal vasculature, we recently found that 15-F_2-IsoP induces the formation of thromboxane, which mediates its contractile effects in these vascular beds [86, 87]. Induction of thromboxane formation by 15-F_{2c}-IsoP has not been found in other vascular beds [71]. (12-iso-$PGF_{2\alpha}$) has also become available for biological testing, and has been found to activate the $PGF_{2\alpha}$ receptor at relatively high concentrations and to induce hypertrophy of cardiac smooth muscle cells [88, 89]. Metabolism of prostanoids is important mechanism for bioinactivation. However, of considerable interest was our recent novel fining that the major urinary metabolite of 15-F_{2t}-IsoP, 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP, is also a potent vasoconstrictor [90]. This is also interesting from another perspective in that this metabolite can also be formed by direct oxidation of γ-linolenic acid. The forthcoming availability of additional compounds for biological testing will likely contribute in a valuable way to our understanding of receptor-mediated actions of IsoPs as effectors of oxidant injury.

Receptor-independent biological effects of compounds generated by the IsoP pathway

Recently we reported the discovery of two new groups of novel compounds that are capable of exerting biological effects due to their inherent chemical reactivity. One group of compounds are cyclopentenone IsoPs [91]. These compounds are formed by dehydration of E_2-isoPs and D_2-IsoPs (fig. 3), analogous to the formation of PGA_2 and PGJ_2 by dehydration of cyclooxygenase-derived PGE_2 and PGD_2, respectively. Therefore, these cyclopentenone IsoPs are termed A_2/J_2-IsoPs. As with the other IsoP series of compounds, four regioisomers of both A-ring and J-ring IsoPs are formed. The unique feature of these compounds is that they are α,β-unsaturated carbonyls, which confers reactivity, in particular rendering them highly susceptible to Michael addition reactions [92–94]. Cyclopentenone prostaglandins derived from the cyclooxygenase have been a subject of considerable interest because of the unique biological actions they exert, which has been attributed to the reactive α,β-unsaturated carbonyl moiety [94]. Specifically, they have

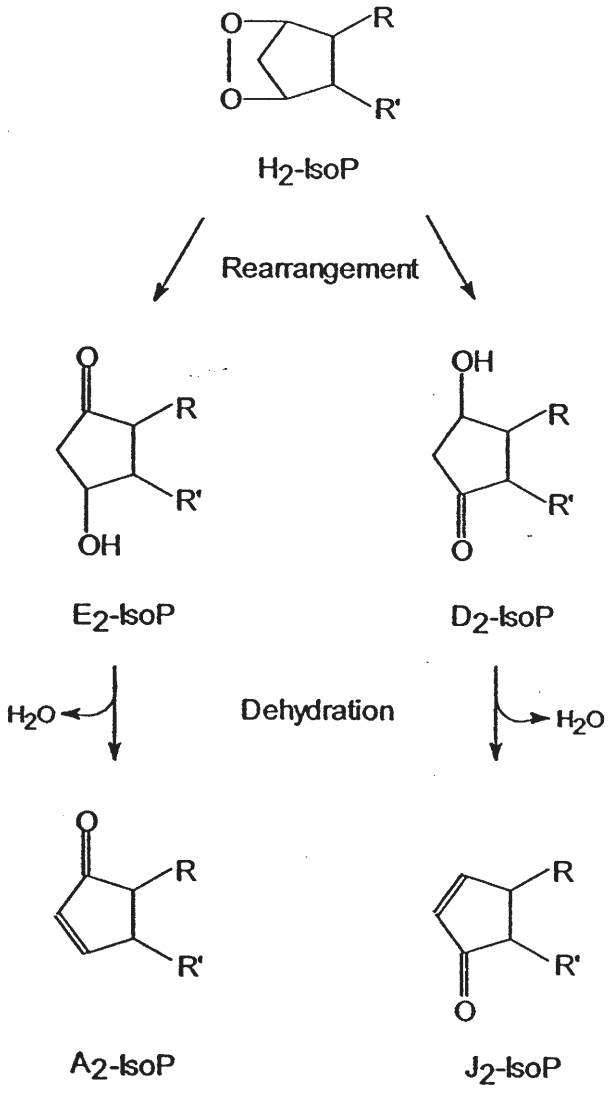

Figure 3. Formation of cyclopentenone A_2-IsoPs and J_2-IsoPs by dehydration of E_2-IsoPs and D_2-IsoPs, respectively.

been shown to inhibit cellular proliferation and induce differentiation, an effect that may be related to their ability to modulate a variety of growth-related and stress-induced genes [95–97]. These cytostatic effects can be reversible, but higher concentrations are cytotoxic and induce apoptosis [96, 98, 99].

Although the biological effects exerted by PGA_2 and PGJ_2 have been extensively investigated for many years, the extent to which these compounds are formed in vivo has been the subject of continuing controversy for over 2 decades [100–102]. Recently, Δ^{12}-PGJ_2 was definitively identified in human urine, but whether this arose from dehydration of PGD_2 in the genitourinary tract prior to voiding or from systemic sources is unclear [91, 103]. Recently, we demonstrated that in normal rat liver, levels A_2/J_2-IsoPs could be detected esterified in phospholipids at a level of 5.1 ± 2.3 ng/g liver [91]. In the same livers, levels of E_2/D_2-IsoPs were 28.0 ± 4.3 ng/g liver. Following administration of CCl_4 to induce an oxidant injury, levels of A_2/J_2-IsoPs and E_2/J_2-IsoPs increased strikingly and to a similar extent, 23.9-fold and 21.2-fold, respectively. One of the A_2-IsoPs, 15-A_{2t}-IsoP, was found to readily undergo Michael addition with glutathione in the presence of glutathione-*S*-tranferase; approximately 70% had conjugated within 2 min, and the conjugation was complete by 8 min (fig. 4A). In addition, 15-A_{2t}-IsoP was demonstrated to form convalent adducts with protein, using albumin as a model (fig. 4B). Interestingly, whereas F_2-, E_2- and D_2-IsoPs reach very high levels in the circulation following administration of CCl_4 [3,9,37], A_2/J_2-IsoPs could not be detected in free form in the circulation, even following administration of CCL_4. This can likely be explained by the finding that almost all the radioactivity excreted into urine following administration of radiolabelled 15-A_2-IsoP to a human volunteer had undergone conjugation to form a polar conjugate, presumably with

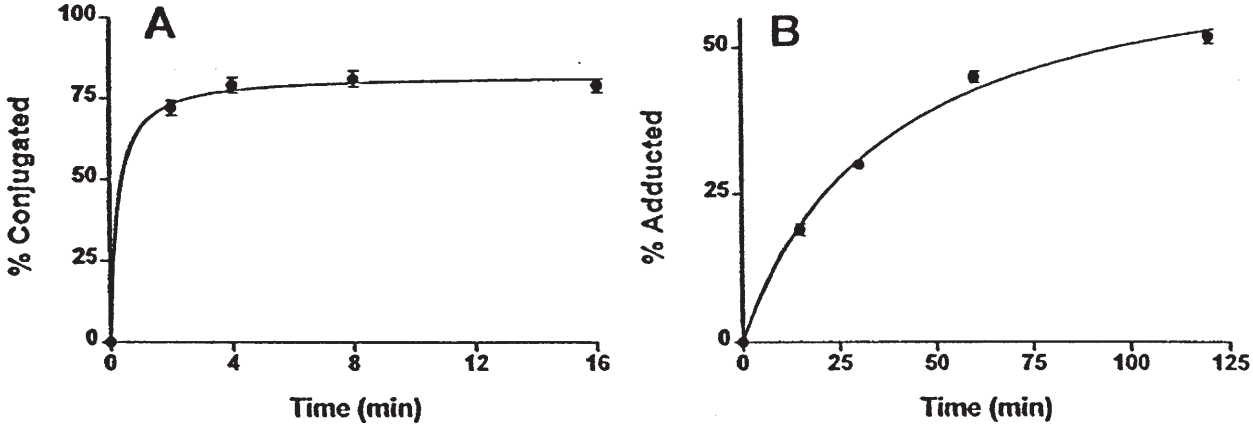

Figure 4. (*A*) Rate of conjugation of 15-A_{2t}-IsoP with glutathione in the presence of glutathione-*S*-transferase. (*B*) Rate of covalent adduction of of 15-A_{2t}-IsoP with albumin.

glutathione [91]. These data are consistent with our previous findings indicating that formation of polar conjugates is a major pathway of metabolic disposition of Δ^{12}-PGJ_2 in the rat [104]. In summary, these studies have elucidated a new class of reactive compounds formed as products of the IsoP pathway that are capable of exerting biological effects relevant to the pathogenesis of oxidant injury.

We recently reported the discovery of another class of even more highly reactive compounds, acyclic γ-ketoaldehydes, that are formed as products of the IsoP pathway [105]. In 1985, Salomon and colleagues described the formation of γ-ketoaldehydes from rearrangement of PGH_2 derived from the cylooxygenase in vitro [106]. Because of the structural similarities to levulinaldehyde, these compounds were termed levuglandins (LGs) E_2 and D_2. Thus, we explored the hypothesis that analogous γ-ketoaldehydes, which we now term E_2- and D_2- isoketals (IsoKs) to distinguish them from LGs formed by the cyclooxygenase, may also be formed by rearrangement of H_2-IsoP endoperoxides (fig. 5). Because, as shown in figure 1, there are four H_2-IsoP endoperoxide regioisomers, four regioisomers of both E_2- and D_2-IsoKs are also formed.

Oxidation of arachidonic acid in vitro yielded a series of compounds that were confirmed to be IsoKs using a number of mass spectrometric approaches, including electron impact mass spectral analysis. Interestingly, the amounts IsoKs formed in vitro were found to be intermediate between the amount of F_2-IsoPs and E_2/D_2-IsoPs formed, indicating that these compounds are formed in relevant quantities compared with other IsoPs. Nonetheless, we could not detect their formation in biological systems in vitro, i.e. oxidation of liver microsomes and LDLs, nor could we detect them in plasma, urine, or in the circulation or liver following administration of CCl_4 to rats. We hypothesized that this may be due to very rapid adduction to proteins. In this regard, it should be mentioned that other reactive aldehydes that are generated as products of lipid peroxidation, e.g. 4-hydroxynonenal and malondialdehyde, can be detected in their free nonadducted form in biological fluids and tissues [107]. To obtain support for this hypothesis, we assessed the rate of adduction of E_2-IsoK to protein, using albumin as a model, and compared this with the rate of adduction of 4-hydroxynonenal. The results obtained were most informative. Rate of adduction was determined by assessing the percent decline in free levels of compounds measured in aliquots removed during incubations with albumin over time. As shown in figure 6, E_2-IsoK underwent adduction with extreme rapidity; 60% had adducted within the first 20 s of incubation. In striking contrast, approximately 50% of 4-hydroxynonenal still remained unadducted after 1 h. Of note is that the free level of E_2-IsoK does not decline to completely zero but plateaus near zero. This is likely due to the presence of some E_2-IsoK in which the double bond on the lower side chain has migrated from the Δ^{10} to the Δ^9 position, rendering the molecule less reactive [108]. These data indicate that LGs adduct to protein at a rate that exceeds that of 4-hydroxynonenal, which is considered one of the most reactive aldehydes formed as a product of lipid peroxidation, by several orders of magnitude.

Therefore, we turned to developing methods to detect the formation of IsoKs as protein adducts using liquid chromatography (LC) electrospray ionization (ESI) tandem

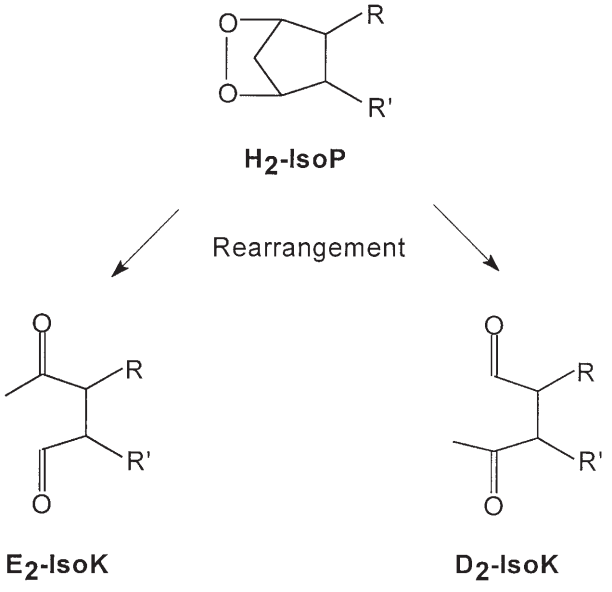

Figure 5. Formation of IsoKs as rearrangement products of IsoP endoperoxides.

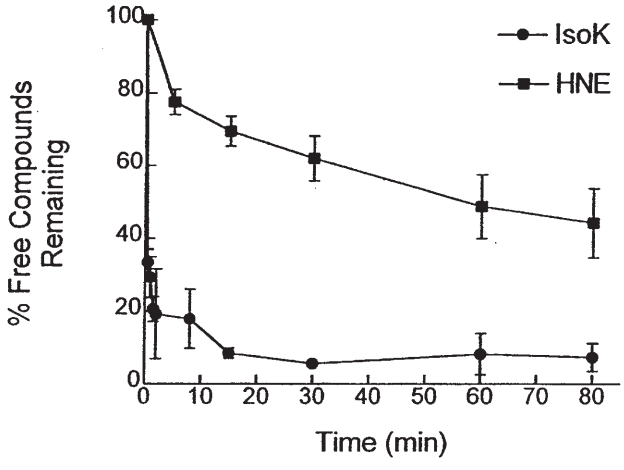

Figure 6. Comparative rates of adduction of E_2-IsoK and 4-hydroxynonenal during incubation with albumin. Formation of covalent adducts was assessed by the decline in levels of free compounds measured in aliquots removed at various times indicated.

mass spectrometry (MS/MS). Salomon and colleagues had obtained evidence that E_2-IsoK forms a pyrrole adduct with lysine residues on proteins [109]. However, LC/ESI/MS analysis following incubation of E_2-IsoK with lysine as a model did not yield evidence for the presence of compounds with the predicted MH^+ ion for a lysyl E_2-IsoK pyrrole adduct. However, full-spectrum scanning analysis revealed major MH^+ ions present 16 and 32 Da higher than the MH^+ ion for the E_2-IsoK lysyl pyrrole. These compounds were consistent with lactam and hydroxylactam adducts formed by facile autoxidation of highly substituted pyrroles [110]. This was confirmed by tandem mass spectrometric analyses of adducts formed with various lysine analogs and [$^{13}C_6$] lysine. The analyses for these adducts were performed following prolonged incubation of E_2-IsoK with lysine. We then analyzed earlier time points, and a new adduct species appeared. This had the appropriate MH^+ ion for a Schiff base adduct. Evidence confirming that this was a lysyl E_2-IsoK Schiff base adduct was obtained by tandem mass spectrometric analysis and the finding that expected products were formed following treatment with NaCN, methoxyamine $^{\bullet}$HCL, and $NaBH_4$ [111].

The time course of formation of these various adducts was then assessed (fig. 7). As noted, the Schiff base adduct is formed very rapidly but is unstable and disappears over time. In contrast, the lactam adducts accumulate much more slowly. The time course of formation of these adducts is consistent with the proposed mechanism of adduct formation depicted in figure 8. E_2-IsoK and lysine are initially converted via an intermediate to a Schiff base adduct, which is reversible. This intermediate also proceeds through an irreversible pathway leading to the formation of a pyrrole adduct, which then undergoes autoxidation to form the lactam and hydroxylactam adducts.

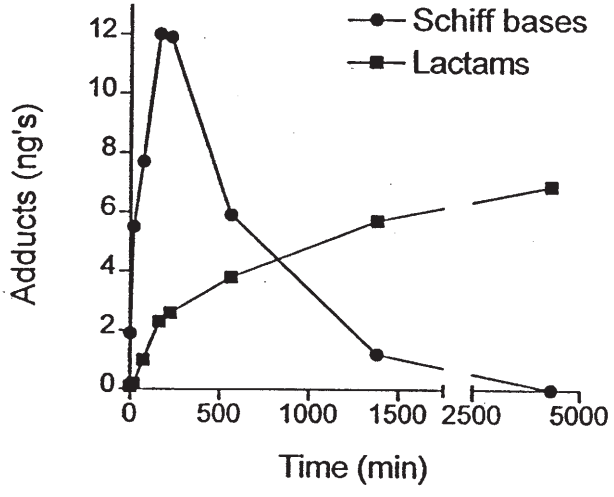

Figure 7. Time course of formation of lactam and Schiff base adducts during incubation of E_2-IsoK with lysine

With this information in hand, we turned to explore whether we could detect the formation of IsoLG adducts on ApoB protein during oxidation of LDL in vitro. In these experiments, the Apo-B protein was enzymatically digested to individual amino acids and then analyzed for lysyl E_2-IsoK lactam adducts. This was considered an important experiment in that previously we could not detect the formation of IsoKs in free form during oxidation of LDL. IsoK lactam adducts could not be detected in native LDL, but intense signals were detected for lysyl lactam and hydroxylactam IsoK adducts on ApoB protein following oxidation of LDL [105]. The key question is whether IsoK adducts are formed in vivo. Data obtained from recent experiments indicate that IsoK adducts (i) can be detected in normal rat liver, (ii) levels in rat liver increase significantly following induction of an oxidant injury by administration of CCl_4 and (iii) can be detected in normal human plasma [unpublished].

Isoprostane and IsoK-Like compounds from other fatty acids

The basic requirement for cyclization to occur by oxidation of unsaturated fatty acids is the presence of at least three double bonds. Thus, oxidation of linoleic acid (C18:2) does not generate IsoP-like compounds, whereas oxidation of linolenic acid (C18:3) would generate F_1-IsoPs. However, the relevance of the formation of F_1-IsoPs is dubious because linolenic acid is only normally present in very minor quantities in vivo. F_3-IsoPs have recently been described as products of free-radical-induced peroxidation of eicosapentaenoic acid (C20:5) [112]. Again, however, this is not an abundant fatty acid under normal circumstances. The formation of F_3-IsoPs may be of interest, however, in situations where the ingestion of eicosapentaenoic acid is high, such as high dietary intake of fish and dietary supplementation with fish oil.

Docosahexaenoic acid (DHA) (C22:6) has been a focus of interest because it is present in abundant quantities in the brain, particularly in grey matter, where it comprises up to 25–35% of total fatty acids in aminophospholipids [113, 114]. We recently described the formation of F-ring IsoP-like compounds with four double bonds during free radical-induced peroxidation of DHA in vitro and in vivo [115]. Because DHA is uniquely highly enriched in neurons, we have termed these compounds F_4-neuroprostanes (F_4-NPs). Oxidation of DHA leads to the formation of eight F_4-neuroprostane regioisomers, each of which is theoretically comprised of eight racemic diastereomers for a total of 128 compounds (fig. 9). Recently, we have also found that the NP-endoperoxide intermediates also undergo rearrangement to form E-ring

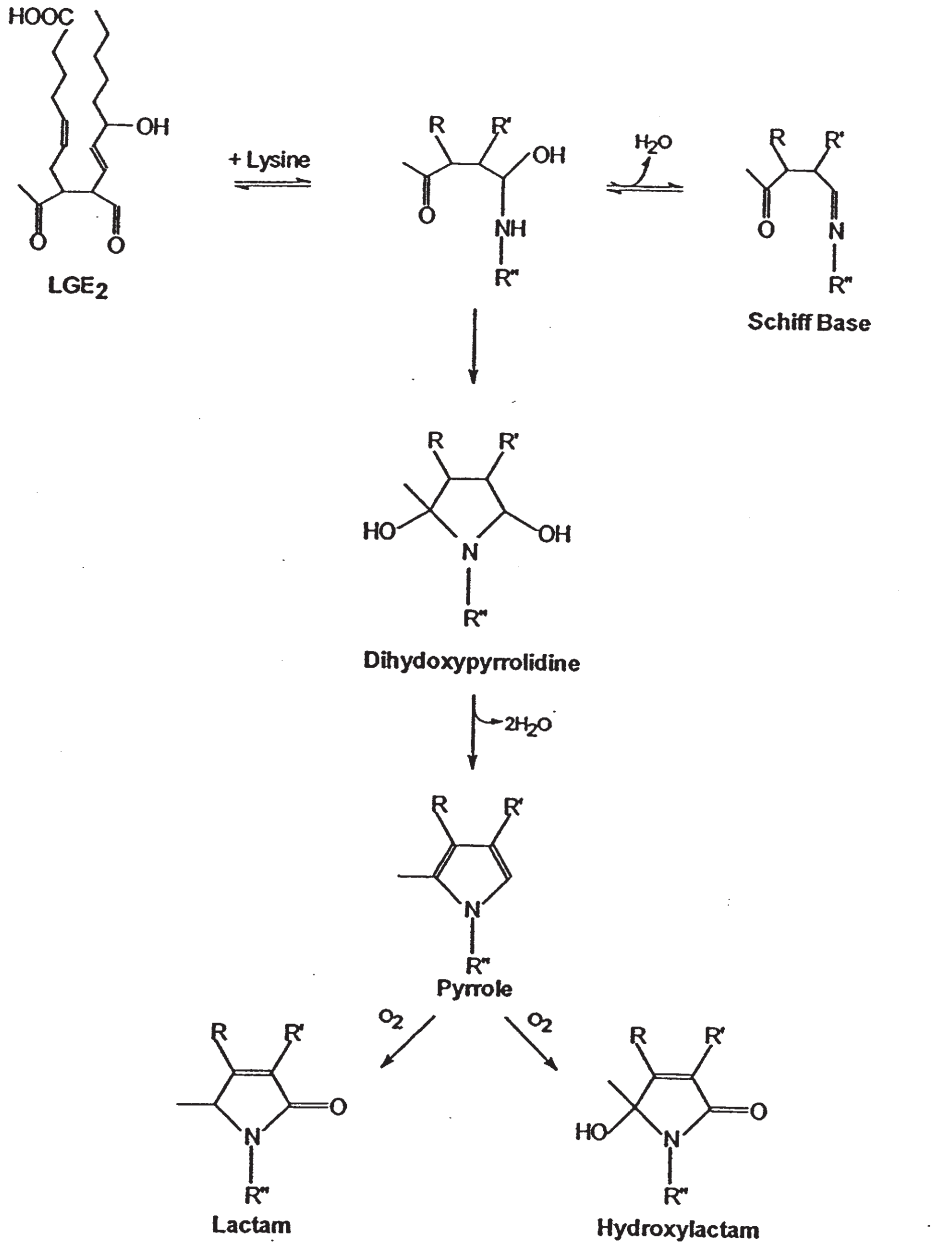

Figure 8. Mechanism of the formation of E_2-IsoK lysyl Schiff base and lactam adducts.

and D-ring NPs in vivo and that the levels of E_4/D_4-NPs esterified in brain of rats slightly exceed the levels of F_4-NPs [116]. We considered the possibility that measurement of F_4-neuroprostanes may represent a novel marker of oxidative neuronal injury. Of considerable interest is that we have found that levels of F_4-NPs are elevated in cerebrospinal fluid from patients with Alzheimer's disease, providing considerable support for a role of free radicals in neuronal injury in this disease [45, 115]. Interestingly, we found an excellent correlation between levels of F_2-IsoPs and F_4-NPs in cerebrospinal fluid from patients with Alzheimer's disease and age-matched normal controls over the range of concentrations present ($r = 0.88$, $P = 0.0003$) [45]. Interestingly, however, only levels of F_4-NPs, but not F_2-IsoPs, esterified in brain lipids were found to be increased in Alzheimer's disease [117, 118]. The reason for these differences between the relative increases in tissue versus cerebrospinal concentrations of F_2-IsoPs and F_4-NPs remains unclear.

We also recently discovered that IsoK-like compounds (neuroketals, NKs) are also formed in vivo from DHA [119]. Our interest in NKs stems from the possibility that these highly reactive compounds may in part be responsible for cross-linking of proteins in neurodegenerative

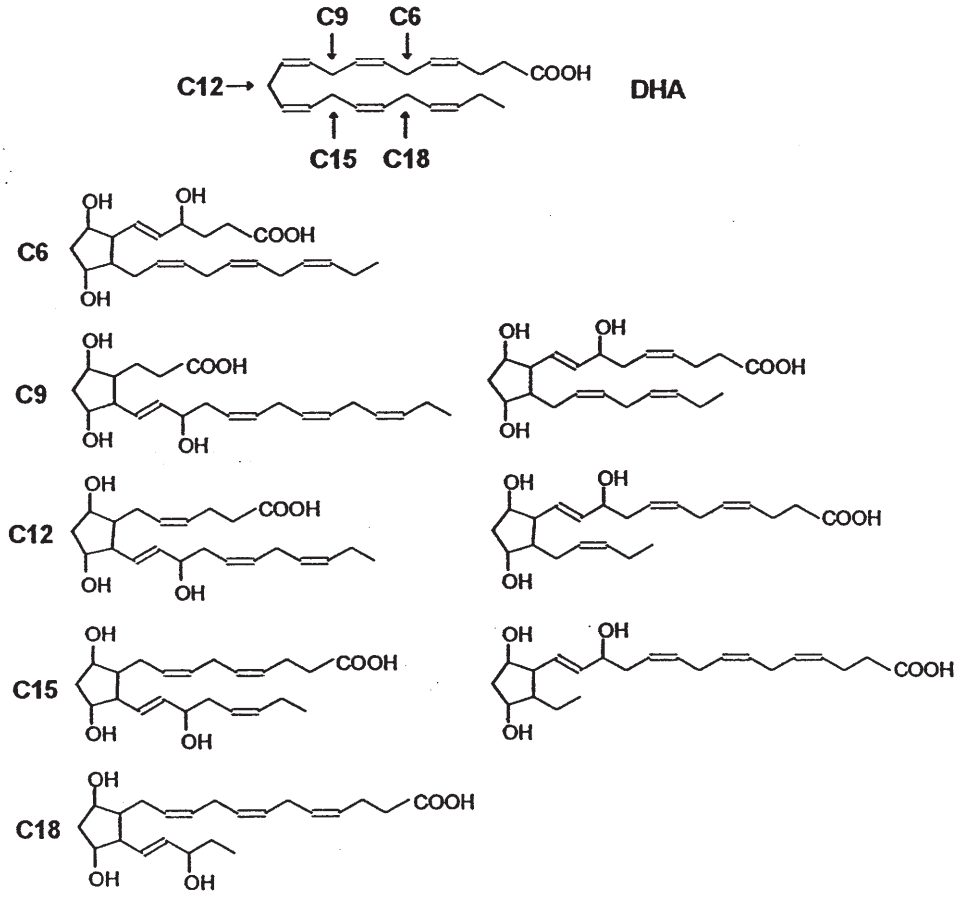

Figure 9. Formation of eight regioisomers of F_4-neuroprostanes from oxidation of DHA. Abstraction of specific allylic hydrogens that result in the formation of the individual regioisomers are designated by carbon atom numbers (C).

diseases, such as amyloid-beta and tau proteins in Alzheimer's disease.

In summary, the initial of discovery of F_2-IsoPs was of biochemical interest, but this has evolved into a discovery of import for a number of reasons. First, quantification of F_2-IsoPs has proven to be a major advance in our ability to reliably assess oxidative stress status in vivo. Many studies utilizing measurements of F_2-IsoPs have been able for the first time to strongly implicate an important role for free radicals in many disease processes. Further, our understanding of the biochemistry of the IsoP pathway has expanded greatly. In addition to F-ring IsoPs, we have found that E-ring, D-ring, A-ring, J-ring, thromboxane-ring compounds and acyclic levuglandin-like compounds are also produced in vivo as products of the IsoP pathway. This is of considerable interest in that almost the entire spectrum of compounds produced by the cyclooxygenase pathway has now been shown to be produced by a nonenzymatic process. In addition, IsoP-like compounds can also be generated from other unsaturated fatty acids and of particular interest in this regard are NPs formed from oxidation of DHA. IsoPs and related compounds produced by the IsoP pathway are also relevant not simply from a biochemical perspective but because they can exert potent biological activity. This involves apparent receptor-mediated actions as well as, in the case of cyclopentenone IsoPs and IsoKs, and NKs, actions attributed to inherent chemical reactivity, which can lead to covalent modification of critical biomolecules, i.e. proteins and DNA. Thus, the current understanding of the IsoP pathway has diverged into a variety of areas of potential biochemical and biological importance that is likely to continue to expand as new avenues for scientific inquiry regarding these unique molecules emerge.

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