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Measurement of Lipid Peroxidation

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Lipid peroxidation results in the formation of conjugated dienes, lipid hydroperoxides and degradation products such as alkanes, aldehydes and isoprostanes. The approach to the quantitative assessment of lipid peroxidation depends on whether the samples involve complex biological material obtained *in vivo*, or whether the samples involve relatively simple mixtures obtained *in vitro*. Samples obtained *in vivo* contain a large number of products which themselves may undergo metabolism. The measurement of conjugated diene formation is generally applied as a dynamic quantitation e.g. during the oxidation of LDL, and is not generally applied to samples obtained *in vivo*. Lipid hydroperoxides readily decompose, but can be measured directly and indirectly by a variety of techniques. The measurement of MDA by the TBAR assay is non-specific, and is generally poor when applied to biological samples. More recent assays based on the measurement of MDA or HNE-lysine adducts are likely to be more applicable to biological samples, since adducts of these reactive aldehydes are relatively stable. The discovery of the isoprostanes as lipid peroxidation products which can be measured by gas chromatography mass spectrometry or immunoassay has opened a new avenue by which to quantify lipid peroxidation *in vivo*, and will be discussed in detail.

INTRODUCTION

Polyunsaturated fatty acids are susceptible to free radical attack with abstraction of an allylic hydrogen atom from a reactive methylene group to form a dienyl radical. This is followed by bond rearrangement, the formation of a conjugated diene and uptake of oxygen to form a peroxyl radical which can then abstract a further hydrogen atom from another unsaturated fatty acid with the formation of lipid hydroperoxides. Lipid hydroperoxides then decompose to form alkoxyl and peroxyl radicals which participate in chain propagation reactions, or decompose to form a great variety of aldehydes following β-cleavage of the lipid alkoxyl radicals. β-cleavage leads to two groups of aldehydes, one in which the parent lipid contains an aldehyde residue, and a second group of fragmentation products derived from the methyl end of the fatty acid.[1] Endocyclization of the peroxyl radical followed by further attack by oxygen has recently been identified as a

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pathway by which a group of compounds now termed isoprostanes are formed, and these are being increasingly used in the assessment of lipid peroxidation.[2]

Is lipid peroxidation an interesting consequence of free radical attack on lipids or is it important in the pathophysiology of disease processes? As more and more work proceeds it is becoming increasingly apparent that lipid peroxidation products directly or indirectly affect many functions integral to cellular and organ homeostasis. For example products of lipid peroxidation may evoke an immune response,[3] initiate fibrosis or inflammation,[4] inactive thiol-containing enzymes,[5] or initiate gene transcription[6] or programmed cell death (apoptosis).[7] One of the strengths of Hermann Esterbauer's work was the convincing demonstration that lipid peroxidation can be assessed *in vitro* and *in vivo*, and that by-products may have important biological effects.

Schauenstein and Esterbauer were among the first to recognize the importance of reactive aldehydes, such as malonaldehyde (MDA) and 4-hydroxynonenal, that are formed during lipid peroxidation reactions.[8–10] The subsequent demonstration by the same group that reactive aldehydes may modify or alter cellular function[11,12] has generated a wealth of information on the biological effects of lipid peroxidation reactions, as well as new means to identify such reactions in pathophysiological processes.[13] Since that time it has become recognized that lipid peroxidation gives rise to a wide variety of oxidation products which can be monitored to assess lipid peroxidation, either *in vitro* or *in vivo*, see Figure 1. Various approaches that can be utilized to assess lipid peroxidation can have

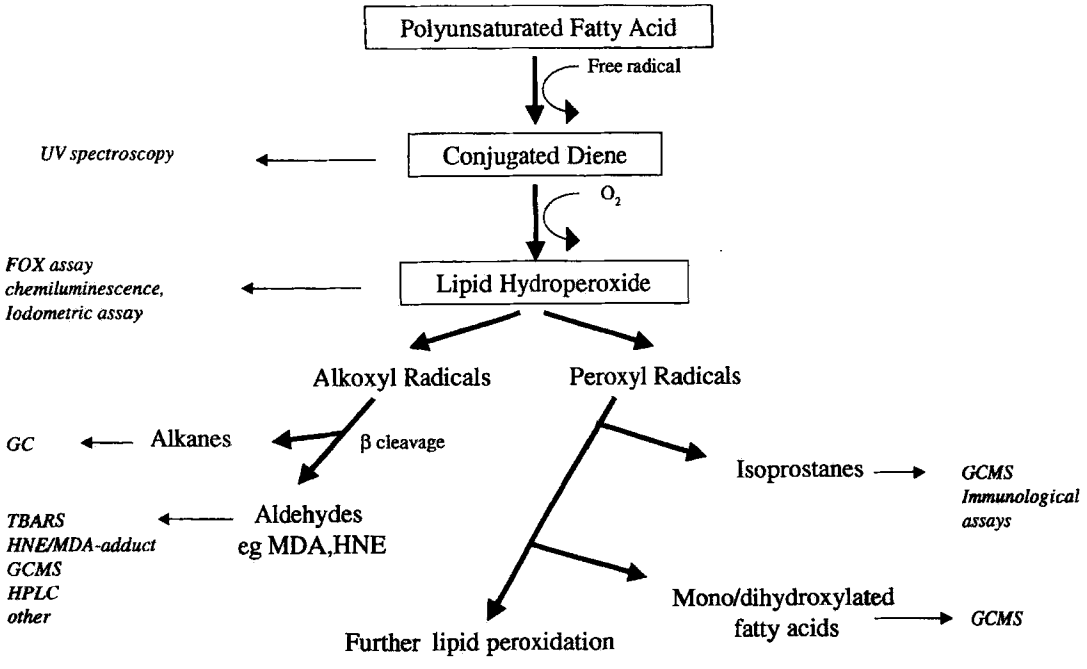

FIGURE 1 An outline of the formation of lipid peroxidation products which can be measured to assess lipid peroxidation products *in vitro* or *in vivo*.

distinct advantages/disadvantages under different circumstances and employing a combination of techniques may be complementary.

MALONALDEHYDE AND 4-HYDROXYNONENAL

(i) TBARS

The thiobarbituric acid reacting substances (TBARS) test, which was first introduced by Kohn and Liversedge in 1944,[14] is still the most frequently used test to assess lipid peroxidation.[15] The TBARS test is easy to perform and is inexpensive. When applied to well-defined simple systems, e.g. peroxidizing microsomes, it can provide useful information but significant shortcomings emerge when it is used to assess lipid peroxidation in complex biological fluids or tissues.[16] The test, in its simplest form, involves heating the material with TBA under acidic conditions and the intensity of a pink chromophore is measured by UV at 532 nm or by fluorescence at 553 nm.[15] One of the problems with this method resides in the reactivity of MDA which readily forms derivatives, and ideally fresh MDA has to be produced for each calibration curve by hydrolysis of 1,1,3,3-tetramethoxypropane or the equivalent ethoxy compound. The extinction coefficient for MDA–TBA complex is 1.5×10^5, and is frequently used to determine the concentration of MDA in the final mixture.[15] The problems relating to the TBA test are firstly that it is non-specific; chromogens are formed with many other aldehydes other than MDA, with carbohydrates, e.g. deoxyribose and deoxyglucose, ascorbate, and amino acids, e.g. arginine, proline, glutamic acid and homocysteine, as well as with certain antibiotics, antifungals and even DNA.[15] These problems of specificity are compounded further when applied to biological fluids. For example even urine, which is relatively devoid of lipids or lipid peroxides, contains many compounds which react with TBA. Secondly, up to 98% of MDA that reacts with TBA is formed artefactually during the incubation stage of the assay, presumably from decomposition of lipid peroxides with the generation of further radicals which amplify the signal. In this sense it can be argued that this reflects the oxidizability of the sample. Thus the presence of chain-breaking antioxidants to terminate reactions (e.g. butylated hydroxytoluene) will dampen or ablate the MDA signal. Thirdly, the presence of free metal ions is necessary to enable color development, presumably because the metal ions are required to enhance MDA formation during the incubation phase of the assay.[15] Thus studies in which metal ions or ion chelators are incorporated in the reaction mixture will yield differing results.

The specificity of the assay is improved by measurement of the TBA–MDA adducts following HPLC.[16] In this method samples are deproteinized in TCA, and the protein free supernatant heated with TBA. Following extraction and neutralization the sample is subjected to HPLC to separate the TBA–MDA chromophore from other compounds which also absorb at 532 nm. The sensitivity can then be improved by fluorescence detection at 553 nm. Nevertheless, whilst such methods clearly enhance the specificity of the assay it should be appreciated that many of the above interfering compounds also form genuine MDA–TBA adducts, which are indistinguishable from MDA formed during lipid peroxidation. Finally MDA is not a specific product of lipid peroxidation; it is also formed as a by-product of the thromboxane synthesis in the cyclo-oxygenase cascade.[17] This problem becomes particularly significant when blood is obtained for the preparation of serum and plasma samples since variable degree of platelet activation and thus thromboxane generation occurs. For example, reports have been published suggesting that lipid peroxidation occurs during coronary surgery, as evidenced by increased

levels of MDA in coronary sinus blood plasma.[18] Whereas that conclusion may or may not be correct, it is well recognized that thromboxane generation is markedly increased during angioplasty, thus confounding an interpretation that increased plasma concentrations of MDA reflect the occurrence of non-enzymatic lipid peroxidation.

Another potential drawback of measurements of MDA in biological systems is that free MDA formed during lipid peroxidation can react with the ε-amino groups on lysine residues to form a Schiff base adduct, which will not be detected by the TBA test.[19] Further, MDA is rapidly metabolized or broken down *in vivo*, and this may explain the finding that other measures of lipid peroxidation, e.g. F_2-isoprostanes, provide a much more sensitive index of lipid peroxidation *in vivo*.

Thus, measurement of MDA by the TBARS test can be used with some degree of reliability to assess lipid peroxidation or susceptibility to peroxidation (an indirect measure of the former) in simple systems *in vitro*, but it is subject to many problems related to artefactual and non-specific reactions when applied to more complex biological systems *in vitro* or *in vivo*. This has led to the development of other techniques to measure the aldehydes or their products as outlined below.

(ii) Dinitrophenylhydrazone (DNPH) Method

Aldehydes react with dinitrophenylhydrazine to form the corresponding DNPH derivative. The hydrazone derivatives have an intense yellow color which enables detection with a λ_{max} of ~370 nm and an ε coefficient of 25 000. Following incubation of the aldehydes with DNPH for 2 h the mixture is extracted with dichloromethane and the products separated by TLC, and different zones scraped and analyzed by HPLC or UV spectroscopy. This method is subject to artefact if great care is not taken to prevent contamination of solvents by aldehydes. This method can also be applied to phospholipid bound aldehydes, following their extraction in chloroform : methanol and clean up by silicic acid column chromatography. The DNPH has broad applicability and reasonable sensitivity and selectivity, and the variation of the procedure is 10–15%. It is said to be able to detect levels as low as 1 pmol.[20]

(iii) Cyclohexanedione Method

Aldehydes react with 1,3-cyclohexandione (CHD) to form fluorescent decahydroacridine derivatives, which can then be analyzed by HPLC. Samples are deproteinized by methanol and CHD added to the supernatant under acidic conditions with ammonium sulfate. Following solid phase extraction the sample is separated by HPLC and monitored fluorometrically at 445 nm emission with 380 nm excitation. With standard aldehyde mixtures this method has good reproducibility, and high sensitivity (0.1 pmol). Although this method has been applied to biological tissues, the recovery of aldehydes added to the mixture is highly dependent on the duration of potential reaction time between liberated aldehydes and tissue proteins or thiols.[20] Thus the reproducibility is low for biological samples.

(iv) Fluorescent Lipid Peroxidation Products

Fluorescent chromophores can be formed during the reaction of MDA, or other reactive lipid peroxide products with amino acids to form an N,N′-disubstituted 1-amino-3-iminopropene which will fluoresce. To determine fluorescence of lipid peroxidation products formed *in vivo*, lipids are extracted into chloroform and the fluorescent products separated by TLC. Individual spots are scraped and eluted to remove the age-related fluorescent contaminants. Lipid soluble fluorescent substances are then measured by fluorescence emission at 420–430 nm after excitation at 360 nm. Detailed descriptions of this

method are given by Shimasaki.[21] The major drawback with this approach is one of specificity.

(v) Assays for 4-Hydroxynorenal (HNE)

One of the major cytotoxic products of lipid peroxidation is hydroxynonenal (4-HNE), an α,β-unsaturated aldehyde. This is partly due to its ability to undergo Michael addition reactions to form thioether adducts and Schiff base adducts with free amino acids and with the ε-amino group on lysine residues and the imidazole group on histidine residues of proteins.[22] 4-HNE can be measured by both HPLC and GC/MS.

(a) Determination of HNE by HPLC

HNE has an intense absorption at 223 nm water ($\varepsilon = 13\,750$) or 221 nm in ethanol ($\varepsilon = 13\,100$). Following extraction into dichloromethane, and clean-up by solid phase extraction the sample is then separated by HPLC and detection at 220–223 nm. Whilst we have no experience of this method its application to biological samples must clearly be hampered by the myriad of compounds which will coelute with HNE with a significant absorption at this wavelength. For biological samples the aldehyde has to be quantitatively extracted from the lipid core, and avoid its reaction with SH or amine groups.[13] To circumnavigate some of these problems methods employing GC/MS have been developed.

(b) GC/MS Determination of HNE

This method is based on the reaction of aldehyde moiety with pentaflurobenzyl (PFB) hydroxylamine followed by silyation of the hydroxyl group with BSTFA.[23] The PFB group confers electron capturing properties on the derivatized product which can then be analyzed by negative ion chemical ionization (NICI). Deuterated internal standards are required to account for losses during work-up. The ion fragment has a mass of 152, since the PFB group dissociates under the soft ionization conditions. This method is very sensitive but the low mass ($m/z = 152$) of the target ion may cause problems from carryover or interference of larger molecular species, due to the generation of similar target ions with similar retention times. Thus during selective ion monitoring, in which the full spectrum of each peak is not determined, the presence of contaminants in biological samples may be detected and indistinguishable from the HNE derivative. A problem with all methods in which aldehydes are quantitated is the reactivity of the aldehyde group. This problem can be partly circumvented by reducing the aldehydes (HNE or MDA, etc.) to their corresponding alcohol by borohydride or borane triethylamine.[24] Again the target ion m/z is low and subject to similar artefactual peaks in biological samples as outlined above.

(c) 4-HNE-Thioether Derivatives

A detailed description of the method is given in the paper by Uchida and Stadtman.[25] Thioether-linked HNE is reduced by sodium borotritiide, which incorporates tritium into the reduction of the aldehyde to the corresponding alcohol, which is then released from the protein by cleavage (desulfurization) by Raney nickel catalysis, extracted by organic solvent and then quantified by scintillation counting. It will only quantitate thioether linked 4-HNE. It will not measure Schiff base linked HNE. However, experiments with glyceraldehyde 3-phosphate dehydrogenase have questioned whether such assays can be applied to complex protein systems.[25]

(d) 4-HNE-Lysine and 4-HNE-Histidine Adducts

The principle of the assay is based on release of the modified amino acid, stabilized following sodium borohydride reduction, by acid hydrolysis of the protein followed by quantitation by HPLC or GC/MS. The HPLC method relies on formation of the *o*-phthaldialdehyde derivative and detection by fluorescence.[25] The resolution of the chromatogram and formation of isomers renders the method less discriminatory than

that obtained by mass spectrometry. In this the modified amino acid is quantified as the trifluroacetyl methyl ester by electron impact mass spectrometry (EI positive).[19] Similar procedures can be applied to MDA-lysine adducts.[19]

(e) Immunological Techniques

An alternative approach, which is not strictly quantitative but yields semi-quantitative and localizing evidence uses immunohistochemical techniques using antibodies raised against 4-HNE modified proteins. This does not distinguish between modified lysine or histidine residues, and clearly the immunogen has to have reactive lysine and histidine residues, and produce an antibody which recognizes both to be effective.[26] Similar methods have been applied for MDA Modified Protein.[26]

PENTANE

Short chain hydrocarbon gases, e.g. ethane and pentane, are formed as by-products during lipid peroxidation by thermal or metal ion catalyzed decomposition of lipid hydroperoxides, and can be quantified by gas chromatography in exhaled breath.[15] Measurement of exhaled pentane has therefore been applied to assess lipid peroxidation *in vivo*. However, there are several problems with this technique. The formation of pentane can be altered by the local oxygen tension in the tissue. Further, pentane is metabolized by cytochrome p450 enzymes; thus agents which cause induction of p450 enzymes may alter the metabolic clearance of pentane.[27] Also bacteria can produce pentane, which introduces another confounding variable in the setting of bacterial overgrowth or infection. For these reasons, measurement of hydrocarbon gases to assess lipid peroxidation has fallen in disfavor.

LIPID HYDROPEROXIDES

Lipid hydroperoxides are the primary products of lipid peroxidation. These can be measured by HPLC coupled with chemiluminescence, by GC/MS alter reduction to alcohols, by the iodometric assay, by the FOX assay, or by activation of cyclo-oxygenase. Many assays will only detect specific classes or specific hydroperoxides formed during lipid peroxidation. GC/MS or HPLC can be used to measure many hydroperoxides such as the HPODES, HPETES, etc. Sample purification and derivatization is required prior to analysis by GC/MS.

Chemiluminescence-Based HPLC Detection

The HPLC chemiluminescence assay is based on light emission during hydroperoxide-induced oxidation of isoluminol catalyzed by metal ions. The method is very sensitive (detects 0.1 pmol), and interference by biological antioxidants is avoided since these are separated by HPLC. This method has been applied to plasma samples, and levels of cholesteryl ester hydroperoxides are detectable. Phospholipid hydroperoxides are generally much less stable, being reduced by glutathione peroxidase. Lipids are extracted into hexane or chloroform : methanol, and the lower lipid-containing fraction dried under nitrogen. The HPLC system uses a normal phase. Two methods are described in detail as referenced. This technique can be applied to tissue.[28,29]

Iodometric Assay

The iodometric assay is particularly useful in biological system where the complex array of biological intermediates formed may not be readily detected by more specific or limited assays. The principle of the iodometric method of assay involves the reaction of lipid hydroperoxides with iodide in acid to form iodine as shown below:

$$ROOH + 2H^+ + 2I^- \rightarrow ROH + H_2O + I_2$$

The assay described by Jessup and colleagues is based on conversion of iodine to tri-iodide has

a detection limit of < 1 nmol hydroperoxide, and is therefore considerably less sensitive than the chemiluminescence-based assays, which can be < 1 pmol. There are several methods available to detect tri-iodide, including a spectrophotometric assay, an anaerobic assay, a cadmium-based assay, or an HPLC-based system. The system requires that no compounds are present which react with iodine, iodide or tri-iodide. Oxygen, ascorbate and mercaptans can all interfere, as well as metal ions, polyunsaturated lipids and tyrosine-containing proteins. Thus, as with many other methods used to assess lipid peroxidation, the iodometric assay lacks specificity.[30]

FOX Assay

The FOX assay, pioneered by Dr Simon Wolff is based on "Ferrous Oxidation of Xylenol". The FOX assay can applied to hydroperoxides present in the aqueous or lipid phases. It is based on the fact that hydroperoxides oxidize ferrous (iron(II)) to ferric (iron(III)), and the resulting ferric ions can then be detected using ferric sensitive dyes. Xylenol orange binds ferric ion with high selectivity to produce a colored (blue purple complex) with a strong absorbance at 560 nm.

This method is said to give highly reproducible signals for biological samples. It is also said to outperform the iodometric assay, and other assays in terms of simplicity and reproducibility. Details of this assays are given in the paper by Wolff.[31]

CONJUGATED DIENES

The term conjugated diene refers to two double bonds separated by a single bond, which does not normally occur in unsaturated fatty acids. The formation of conjugated dienes is generally accepted as evidence for lipid peroxidation, and is due to re-arrangement of the double bond to the conjugated diene structure, which in the presence of oxygen can form hydroperoxides.[15] Conjugated dienes can be detected spectrophotometrically since they have a characteristic absorption at 234 nm. However, simply measuring absorbance at 234 nm can be very problematic because many biological compounds will have significant absorbance at this wavelength. Further, because of the high absorbance of many solvents at low wavelengths, a peak at 234 nm representing conjugated dienes often presents itself as a shoulder. Second derivative spectrometry relies on both the minima and maxima wavelengths of absorption which confers greater specificity on a background of high lipid content.[32] Details on this method are provided in the reference cited. In general, the primary application of measurement of conjugated diene formation by UV absorbance is to monitor changes in absorbance in dynamic experiments rather than absolute quantification.

ISOPROSTANES

Isoprostanes (IsoPs) are prostaglandin (PG)-like compounds that are produced independently of the cyclo-oxygenase enzyme by free radical catalyzed peroxidation of arachidonic acid, and similar products are also formed during oxidation of higher or lower carbon-based unsaturated fatty acids such as docahexaenoic acid or eicosapentaenoic acid. Their generation and actions have recently been extensively reviewed.[33] The notion that PG-like compounds could be generated non-enzymatically as products of autoxidation of fatty acids was first demonstrated *in vitro* over 20 years ago.[34] However, this interesting observation was never carried further. The discovery of the F_2-IsoPs occurred during the course of attempting to detect isomeric F-ring metabolites of PGD_2 in human plasma and urine using GC/MS. In freshly obtained plasma samples from normal volunteers that were processed and analyzed immediately, a series of peaks were detected with characteristics of F-ring PGs. However, when plasma that had been stored for

several months at −20°C was analyzed, the same peaks were detected as in fresh plasma but at levels approximately 1000-fold higher.[35] A series of investigations undertaken in an attempt to explain this phenomenon led to the discovery that these compounds were PGF_2-like compounds that were generated non-enzymatically by autoxidation of plasma arachidonic acid during storage.[35]

The mechanism involved in the formation of these compounds is shown in Figure 2. Four regioisomers are formed, each of which is theoretically comprised of eight racemic diastereomers, for a total or 64 compounds. Because these compounds contain the F-type prostane ring and are isomeric to $PGF_{2\alpha}$ derived from the cyclo-oxygenase enzyme, they have been termed F_2-IsoPs.

The formation of F_2-IsoPs was firmly established to occur *in vivo* by demonstrating that levels could be readily detected in fresh plasma and urine and levels were shown to increase by as much as 200-fold in animal models of oxidant injury, e.g. administration of CCl_4 or diquat to selenium-deficient rats.[2,36] Since the F_2-IsoPs are formed as a result of reduction of the IsoP endoperoxide intermediates. In addition to reduction, it has been shown that the IsoP endoperoxides also undergo rearrangement to form PGD_2-like (D_2-IsoPs), PGE_2-like (E_2-IsoPs), and thromboxane-like (isothromboxanes) *in vivo*.[37,38] Free E/D ring IsoPs are undetectable in normal plasma, but esterified forms are readily measurable and present in tissue. In tissue the F-ring compounds predominate, but in oxidized lipids the E/D ring IsoPs are formed in abundance.

Levels of F_2-IsoPs in normal human plasma and urine exceed levels of cyclo-oxygenase-derived PGs and thromboxane by more than an order of magnitude, and these are not suppressed

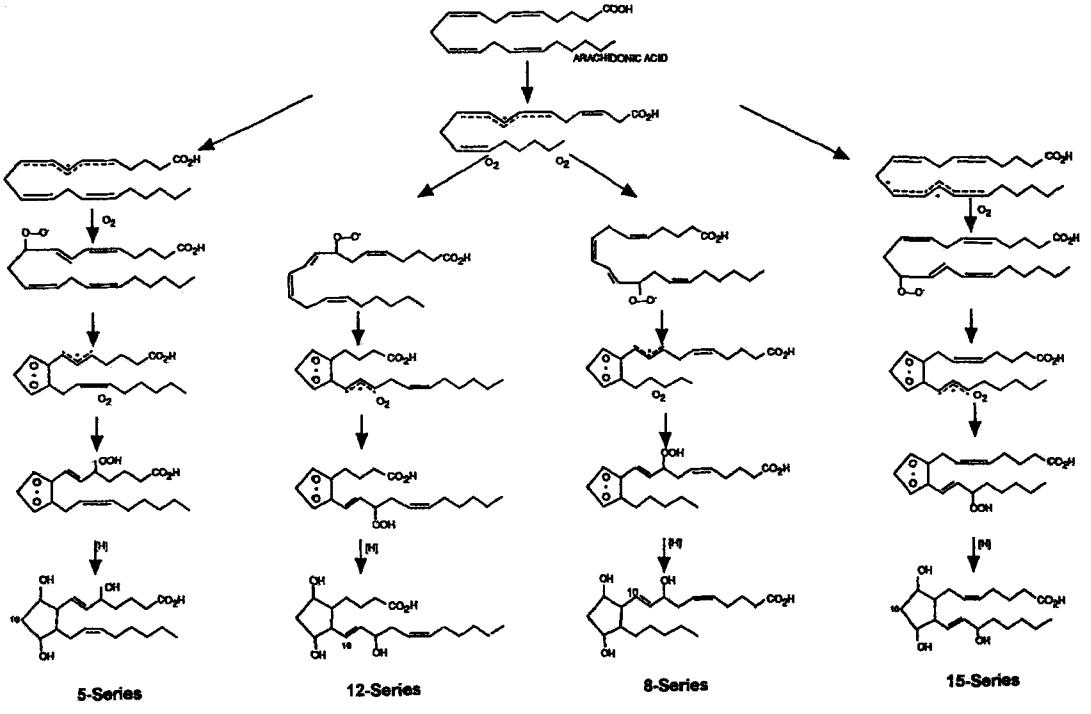

FIGURE 2 Pathways for the formation of F_2-IsoPs during the oxidation of arachidonic acid. Four regioisomers can be formed which can comprise eight (8) racemic diastereomers. The numeral for each series are designated by the position of the side chain hydroxyl as described in Ref. [43]. Reprinted with kind permission from © PNAS 1990 87: 9383.

by cyclo-oxygenase inhibition. Although this clearly indicates that the formation of IsoPs is a major pathway of arachidonic acid disposition, it is important to consider the implications of this finding in the context of ongoing lipid peroxidation in the normal state. Previously, using other methods to assess lipid peroxidation, there had been little concrete evidence obtained indicating that lipid peroxidation occurs *in vivo* except under abnormal circumstances of marked oxidative stress. However, the finding of detectable levels of F_2-IsoPs in all normal human and animal biological fluids indicates that there is certain level of ongoing lipid peroxidation that is incompletely suppressed by the elaborate array of antioxidant defenses that have evolved to prevent the deleterious effects of free radical production.

A novel aspect of the formation of IsoPs is that, unlike cyclo-oxygenase-derived prostaglandins, they have been shown to initially be formed *in situ* esterified to phospholipids and then subsequently released in free form.[39] An interesting question, however, remains regarding the mechanism involved in the hydrolysis of F_2-IsoPs from phospholipids *in vivo*. It is reasonable to assume that the hydrolysis is catalyzed by phospholipases, and bee venom phospholipase A_2 has been found effective in hydrolyzing isoprostanes from lipids *in vitro*, However, the nature of the phospholipase responsible for the hydrolysis of isoprostanes *in vivo* remains to be established.

Importance of F_2-Isoprostanes

One of the most important aspects of the discovery of the F_2-IsoPs is their application as a means of quantifying lipid peroxidation *in vivo*. The lack of a readily and accurately quantifiable marker of lipid peroxidation *in vivo* has hampered research in this area. This is because most methods previously available to assess oxidant stress *in vivo* suffer from a lack of specificity and/or sensitivity and are unreliable. However, a substantial body of evidence has been obtained to indicate that measurement of F_2-IsoPs provides a reliable non-invasive approach to assess lipid peroxidation *in vivo* and, as such, appears to be a major advance in our ability to assess oxidative stress status in humans. F_2-IsoPs are present at levels that are easily detected in free form in all biological fluids and in esterified form in all tissues. Measurement of levels esterified in tissues allows a direct assessment of the extent of oxidant injury in key tissues of interest. Although this approach has its principal application in animal models of oxidant injury, the sensitivity of the mass spectrometric method of analysis is sufficient to quantity levels of F_2-IsoPs in small biopsies of human tissue as well. However, it is important to mention that the formation of IsoPs esterified to tissue lipids and their subsequent release in free form can be a very dynamic process. Thus, the timing of collection of tissue samples for analysis of esterified IsoPs or biological fluids for analysis of free IsoPs can be very critical. As mentioned, in addition to F_2-IsoPs, E_2/D_2-IsoPs and isothromboxanes are also formed. However, because of the fact that F_2-IsoPs are very stable molecules, unlike the E_2/D_2-IsoPs, and the fact E_2/D_2-IsoPs and isothromboxanes cannot be detected in the circulation except under circumstances of severe oxidant injury, the preferred approach to assess oxidative stress status is quantification of F_2-IsoPs.

Studies have clearly demonstrated the advantages of measuring F_2-IsoPs to assess lipid peroxidation *in vivo* compared to other methods, in particular measurement of MDA by the TBARS assay and measurement of monohydroxy derivatives of arachidonic acid.[40,41] In peroxidizing microsomes *in vitro*, the formation of MDA quantitatively far exceeded that of IsoPs. However, the *n*-fold-increases over baseline and the time-course of formation of both MDA and IsoPs were highly correlated. when MDA and IsoPs were compared as indicies of lipid peroxidation *in vivo* by measuring these compounds in the

livers of rats which had been given CCl_4 to induce severe lipid peroxidation, levels of MDA increased less than 3-fold in the livers of CCl_4-treated animals whereas the levels of F_2-IsoPs increased by as much as ~80-fold.[41] The reason for the trivial increases in MDA compared to F_2-IsoPs in these animals is not completely understood but could be explained by rapid metabolism of MDA. Similarly, in another study, the increases in levels of F_2-IsoPs measured in the livers of CCl_4-treated rats greatly exceeded the observed increase in the levels of monohydroxy arachidonic acid derivatives in the liver.[40] Importantly the levels of isoprostanes increased dramatically in plasma but HETEs remained undetectable in the circulation even under the situation of severe oxidative stress.

Thus, the ability to quantify F_2-IsoPs opens up many new avenues for investigation to explore the role of free radicals in the pathophysiology of a wide range of human diseases. It also provides an extremely valuable tool to define the clinical pharmacology of antioxidant agents. In support of this, the formation of F_2-IsoPs increases significantly in animals rendered deficient in vitamin E, even in the absence of the administration of an agent to induce endogenous oxidant injury.[36] In addition, administration of antioxidants has been shown to inhibit the formation of F_2-IsoPs in animal models of oxidant injury as well as in limited studies carried out thus far in humans.[33,42] The ability to define the pharmacodynamic effects of antioxidants to inhibit free radical processes *in vivo* in humans should contribute in a valuable way to long-term studies aimed at exploring the efficacy of antioxidants to prevent the development of pathology, e.g. atherosclerosis.

Another very important aspect of the discovery of IsoPs has been the finding that at least two IsoPs, now termed 15-E_{2t}-IsoP (8-iso-PGE_2) and 15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$),[43] can exert potent biological activity. Both compounds have been shown to be very potent vasoconstrictors in the renal, portal and other vascular beds, and exert additional biological actions as well.[44–46] Interestingly, evidence suggests that the vasoconstricting actions of these IsoPs may be mediated by a unique receptor.[47] Thus, in addition to being valuable markers for lipid peroxidation *in vivo*, IsoPs may also participate as mediators of oxidant injury.

Method of Analysis of F_2-Isoprostanes

The method most commonly employed for measurement of F_2-IsoPs is a negative chemical ionization GC/MS assay.[48] Various modifications of this method have been used by others. This method is highly sensitive with a lower limit of detection in the low picogram range and is highly accurate (precision = 6%, accuracy = 96%). Measurement of esterified levels of F_2-IsoPs in tissues is accomplished by measurement of free compounds following alkaline hydrolysis of a lipid extract of tissue. However, the mass spectrometric method of assay is labor intensive and the technology is not widely available. However, several groups have developed immunoassays for specific F_2-IsoPs,[49] and used these tools to localize F_2-IsoPs by immunohistochemistry. The availability of these techniques will greatly expand research in this area.

Precautions must be taken to prevent artefactual generation of IsoPs by autoxidation in samples that are analyzed for IsoPs both during storage and during sample processing. Whereas IsoPs are generated by autoxidation in lipid-containing samples during prolonged storage even at −20°C, we have found that autoxidation does not occur in lipid containing samples, e.g. plasma, that are initially snap frozen in liquid nitrogen and stored at −70°C for up to six months. However, once thawed, samples should be assayed immediately and not refrozen and stored. Tissue samples collected for measurement of esterified IsoPs also must either be analyzed immediately or snap frozen in liquid nitrogen and stored at −70°C. Autoxidation is not a problem with urine samples owing to the fact

that urine contains only trivial quantities of lipid; levels of F_2-IsoPs do not increase even when urine is incubated at 37°C for one week.

Measurement of Isoprostanes to Assess Oxidative Stress Status *In Vivo*

The measurement of IsoPs in biological fluids has radically altered the way in which we assess oxidative stress *in vivo*. These compounds can be measured as the free form in plasma or urine, as esterified complexes in tissue lipids (membranes), as esterified complexes in circulating lipid particles or as the urinary excreted metabolite. There are problems with the measurement of IsoPs, apart from the technical side. These compounds are metabolized, and excreted.[50] Thus new assays based on measurement of the dinor and 13,14-dihydro-dinor metabolite are under development. A further potentially confounding issue with regard to measuring tissue levels is the upregulation of endogenous phospholipases. These enzymes cleave endogenously formed isoprostanes, and when tissue phospholipase activity is high, then low tissue levels of isoprostanes may give a false impression that oxidative injury has not occurred. The use of other fluids (plasma and urine) will to some extent overcome these problems as well as determination of the arteriovenous gradient. A further problem is the recognition by some that a small amount of IsoPs formed *in vivo*, may be formed by cyclo-oxygenase.[51] The contribution of this pathway in man is small, and inhibition of cyclo-oxygenase in man has no effect on urinary levels.[2] Thus, measurement of IsoPs seems, at present, to be one of the best markers of oxidative stress *in vivo*.

The discovery of IsoPs as unique products of non-enzymatic lipid peroxidation has opened up many new avenues for investigation. First, the use of quantification of IsoPs as markers of oxidative stress status *in vivo* appears to be a major advance in our ability to explore the role of free radicals in the pathogenesis of human disease. The efforts to develop specific and reliable immunoassay methods for the measurement of IsoPs are likely to lead to an expansion of the use of such assays to assess oxidative stress status *in vivo* both in animal models of oxidative injury and in humans. In addition to being valuable markers of oxidative stress, the IsoPs tested to date possess interesting and potent biological activity. The availability of additional IsoPs in synthetic form should significantly broaden our knowledge concerning the role of these molecules as mediators of oxidant stress. In addition, information regarding the nature of the receptor(s) that mediate the biological actions of IsoPs will be of considerable importance as it contributes to the development of specific antagonists or agonists of the biological actions of these compounds.

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