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Chemistry and analysis of HNE and other prominent carbonyl-containing lipid oxidation compounds

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Abstract

The process of lipid oxidation generates a diverse array of small aldehydes and carbonyl-containing compounds, which may occur in free form or esterified within phospholipids and cholesterol esters. These aldehydes mostly result from fragmentation of fatty acyl chains following radical oxidation, and the products can be subdivided into alkanals, alkenals (usually α,β-unsaturated), γ-substituted alkenals and bis-aldehydes. Isolevuglandins are non-fragmented di-carbonyl compounds derived from H_2-isoprostanes, and oxidation of the ω-3-fatty acid docosahexenoic acid yield analogous 22 carbon neuroketals. Non-radical oxidation by hypochlorous acid can generate α-chlorofatty aldehydes from plasmenyl phospholipids. Most of these compounds are reactive and have generally been considered as toxic products of a deleterious process. The reactivity is especially high for the α,β-unsaturated alkenals, such as acrolein and crotonaldehyde, and for γ-substituted alkenals, of which 4-hydroxy-2-nonenal and 4-oxo-2-nonenal are best

known. Nevertheless, in recent years several previously neglected aldehydes have been investigated and also found to have significant reactivity and biological effects; notable examples are 4-hydroxy-2-hexenal and 4-hydroxy-dodecadienal. This has led to substantial interest in the biological effects of all of these lipid oxidation products and their roles in disease, including proposals that HNE is a second messenger or signalling molecule. However, it is becoming clear that many of the effects elicited by these compounds relate to their propensity for forming adducts with nucleophilic groups on proteins, DNA and specific phospholipids. This emphasizes the need for good analytical methods, not just for free lipid oxidation products but also for the resulting adducts with biomolecules. The most informative methods are those utilizing HPLC separations and mass spectrometry, although analysis of the wide variety of possible adducts is very challenging. Nevertheless, evidence for the occurrence of lipid-derived aldehyde adducts in biological and clinical samples is building, and offers an exciting area of future research.

Abbreviations

Acr-dG, 1,*N*2-propanodeoxyguanosine; AGEs, advanced glycation end-product; ALEs, advanced lipoxidation end product; ApoB100, apolipoprotein B100; ApoE, apolipoprotein E; ARP, aldehyde reactive probe; AspN, endoproteinase AspN (flavastacin); βLG, β-lactoglobulin; BHZ, biotin hydrazide; BN-PAGE, blue native polyacrylamide gel electrophoresis; BNZ, benzaldehyde; CHD, 1,3-cyclohexanedione; CRT, crotonaldehyde; Cys, Cysteine; DDE, 2,4-dodecadienal; DHA, docosahexenoic acid; DMSO, dimethyl sulfoxide; DNPH, 2,4-dinitrophenylhydrazine; ELISA, Enzyme-Linked Immunosorbent Assay; EPA, eicosapentenoic acid; ESI, Electrospray Ionisation; FACS, Fluorescence-activated cell sorting; GC, Gas Chromatography; GC-MS, Gas Chromatography Mass Spectrometry; GluC, endoproteinase GluC (Staphylococcus aureus Protease V8); GO, glyoxal; GPx4, glutathione peroxidase 4; GSH, glutathione; HDDA, 4-hydroxydodecadienoic acid; HDDE, 4-hydroxydodeca-2,6-dienal; HHA, 4-hydroxyhexenoic acid; HHE, 4-hydroxy-2-hexenal; HHP-lysine, N^3[(hept-1-enyl)-4-hexylpyridinium]lysine; HNA, 4-hydroxhonenoic acid; HNE, 4-hydroxy-2-nonenal; HpETE, 15-hydroperoxyeicosatetraenoic acid; HPLC, High performance liquid chromatography; HPNE, hydroperoxynonenal; HXL, hexanal; IsoLG, isolevuglandins; JNK, c-Jun N-terminal kinases; LC, liquid chromatography; LC-MS, liquid chromatography mass spectrometry; LDL, low density lipoprotein; LLOQ, Lower Limit of Quantification; LOD, Limit of detection; LOX, lipoxygenase; Lys, Lysine; mAB, monoclonal antibody; MALDI, Matrix-assisted laser desorption/ionization; MAPK, Mitogen Activated Protein Kinases; MDA, malondialdehyde; MGO, methylglyoxal; MP-lysine, N^{ε}-(3-methylpyridinium)lysine; MRM, multiple reaction monitoring; NFκB, nuclear factor kappa B; NICI, negative-ion chemical

ionization; NRF2, Nuclear factor (erythroid-derived 2)-like 2; OHE, 4-oxo-2-hexenal; ONE, 4-oxo-2-(E)-nonenal; PE, phosphatidylethanolamine; PFBO, pentafluorobenzyl oximes; PGH_2, Prostaglandin H_2; PKC, Protein kinase C; PONPC, 1-palmitoyl-2-(oxononanoyl)-*sn*-glycerophosphatidylcholine; POVPC, 1-palmitoyl-2-(5'-oxovaleroyl)-*sn*-glycero-3-phosphocholine; PUFAs, polyunsaturated fatty acid; Rnase, ribonuclease; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SIM, selected ion monitoring; TBARS, thiobarbituric acid reactive substances; TOF, time of flight; tt-DDE, *trans,trans*-2,4-decadienal; ULOQ, Upper Limit of Quantification.

Keywords

Phospholipid oxidation; α,β-unsaturated aldehydes; lipoxidation; isolevuglandins; 4-hydroxy-2-hexenal; 4-hydroxy-dodecadienal; liquid chromatography mass spectrometry.

1. Introduction

Oxidative metabolism and redox reactions are essential for energy production and cell survival; however, some of the enzymes and processes that utilize oxygen result in the formation of partially reduced oxygen species such as superoxide, hydrogen peroxide and hydroxyl radical [1]. These compounds are often referred to as reactive oxygen species (ROS), although only the hydroxyl radical is indiscriminately reactive and able to initiate a wide variety of radical oxidations by radical addition to, or hydrogen abstraction from biomolecules [2]. Phospholipids, especially those containing di-unsaturated or polyunsaturated fatty acids (PUFAs), are primary targets of attack by hydroxyl radicals or other downstream radicals, and are modified to a wide variety of oxidized products in a process called lipid peroxidation. This was broadly defined as the oxidative deterioration of polyunsaturated lipids [3]; it has long been understood that polyunsaturated oils and fats deteriorate in air, which they take up in large volumes, leading to the development of rancidity. The unpleasant flavours, smells and increased viscosity are mainly due to the formation of aldehydes as fragmentation products of the oxidized lipids, hence this topic has always been of considerable importance in the food industry. However, much early work in defining the mechanism of lipid peroxidation was carried out by researchers at the British Rubber Producers Association, as this material is also prone to deterioration by oxidation [4].

At the time, the relevance of lipid peroxidation to biological systems was not realized, but subsequently it was discovered that production of important lipid signalling mediators of

the isoprostane family, such as prostaglandins and thromboxanes, involved enzymatically catalysed radical processes to peroxidize arachidonic acid [5, 6]. This area of research has expanded hugely, as other PUFAs yield analogous products with a plethora of biological activities. In addition, it is now understood that adventitious oxidation, as well as enzymatic, has important effects in biological systems by altering physical properties of cells as well as cell signalling pathways [7]. Moreover, oxidized phospholipids are also bioactive, but in many cases have different effects to the corresponding free oxidized fatty acid, including both pro-inflammatory and anti-inflammatory effects. Lipid peroxidation has been associated with the physiopathology of several diseases, including atherosclerosis, cancer, diabetes and neurodegenerative disorders, as previously reviewed [8, 9]. It is also important to bear in mind that non-radical lipid oxidation can also occur through the action of hypochlorous acid, which generates chlorinated products that have been reported to have bioactivity [10]. The aldehyde products mentioned above are notable for their high toxicity to cells and other adverse effects, which largely relate to their high chemical reactivity. This topic forms the focus of this review, together with an overview of the analysis of these lipid peroxidation products.

Many reviews previously have focused on a small number of products that are considered the major products or most reactive and damaging products of lipid oxidation. Here, we provide a broader overview of lipid oxidation derived small reactive carbonyl-containing compounds, including discussion of their reactivity, the formation of protein adducts (lipoxidation), and approaches to the analysis of free aldehydes and their protein adducts.

2. Lipid peroxidation and its primary products

The sequence of reactions in lipid peroxidation has been described previously in many informative reviews [11-18], and will only be outlined briefly here. The essence is that free radical oxidants can abstract a hydrogen atom from bis-allylic carbons in the PUFA chains to form a carbon-centred radical ($L^{\bullet}$). This unstable primary intermediate reacts readily with molecular oxygen (which has a di-radical ground state), resulting in formation of a lipid peroxyl radical ($LOO^{\bullet}$). This is also a reactive radical and in a lipid bilayer is able to abstract a hydrogen atom from an adjacent fatty acyl chain, thus forming a hydroperoxide (LOOH) and propagating the radical chain reaction [16]. Alternatively, the peroxyl radical can attack intramolecularly to yield a dioxetane or an endoperoxide. The position of the double bonds in an unsaturated fatty acid influences the site where hydrogen abstraction occurs, and as the carbon-centred radicals can undergo rearrangement by radical migration, this increases the number of sites at which O_2 can add; for example, in *cis,cis*-9,12-linoleic acid, 9-hydoxyperoxy-octadecadienoate and 13-hydoxyperoxy-octadecadienoate can be formed.

The situation is even more complex in fatty acids with three or more double bonds. Thus a wide variety of distinct products can be generated via lipid peroxidation.

The primary products of peroxidation are lipid hydroperoxides (LOOH); these are relatively stable compared to their radical precursors, at least in the absence of transition metal ions. In appropriately extracted and stored biological samples they can readily be detected by mass spectrometry or HPLC with chemiluminescent detection [19, 20]. Hydroperoxides can be further converted to endoperoxides or isoprostanes and neuroprostanes by intramolecular attack and oxidative cyclisation, which represents a further stabilization of the structure, and explains why isoprostanes are considered good biomarkers of oxidative stress. Additionally, hydroperoxides can be reduced to hydroxy, keto or epoxy species; glutathione peroxidase (GPx4) uses glutathione as a reductant to generate hydroxides (LOH) [21], while thioredoxin reductase (Trx) and selenoprotein P (SelP) also have lipid hydroperoxidase activity [22, 23].

3. Aldehydes are fragmentation products of oxidized lipids

Lipid hydroperoxides or peroxyl radicals can undergo fragmentation by enzymatic or non-enzymatic mechanisms to produce short-chain oxidation products, including a variety of different aldehydes, alkanes and alkenes [13, 16], of which malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE) are by far the most studied [17]. **Table 1** illustrates the wide variety of products that have been detected following oxidative fragmentation of PUFAs. Also, depending on the fatty acid that is oxidized (i.e. its length, and the number and position of the double bonds), the oxidation products vary in length, degree of unsaturation and number of substitutions. In particular, fatty acids can be divided into 2 classes: the ω-3 and ω-6 fatty acids, according to the position of the first double bond from the methyl end, which affects the likely sites of fragmentation and resulting breakdown products. Linoleic and arachidonic acids are both ω-6-polyunsaturated fatty acyl chains, and are good sources of aldehydes by cleavage of a C-C bond. ω-3-PUFAs, including α-linolenic acid, eicosapentenoic acid (EPA) and docosahexenoic acid (DHA) can also undergo fragmentation and give rise to many reactive aldehydes. The non-enzymatic fragmentation mechanism of linoleic acid has been extensively studied, whereas the fragmentation reactions of polyunsaturated acyl chains (containing more than 2 double bonds) are more complicated and less well characterized because of the higher number of possible sites of oxidation [13].

There are three main mechanisms by which a carbon-carbon bond can be cleaved, as described and reviewed in several key publications by researchers at Vanderbilt University and elsewhere [16, 24-27]. The principles have mostly commonly been explored

with linoleic acid and have focused on the formation of 4-hydroxy-2-nonenal (HNE), owing to the fact that it is one of the most abundantly formed lipid peroxidation products and has interesting biological effects [8, 28, 29]. The mechanisms are (i) reduction of hydroperoxide to an alkoxyl radical in the presence of a transition metal followed by β-scission; (ii) Hock rearrangement of a hydroperoxide and migration of a C-C to a C-O bond and cleavage; (iii) cyclization to form a dioxetane and subsequent cleavage. Variations of these mechanisms can arise in the case where additional oxygens are added to the chain; for example if three O_2 are added to the chain to form a hydroxyhydroperoxide intermediate it can undergo β-scission, or with the epoxyhydroperoxide intermediate Hock cleavage can occur. These mechanisms have been reviewed previously [15, 16, 24].

The resulting aldehydes may be classified on the basis of their chemical structure into alkanals, alkenals and γ-substituted-alkenals, which has the advantage of focusing on their different reactivity towards biomolecules.

4. Formation and chemistry of lipid oxidation-derived aldehydes

Alkanals

Alkanals are saturated carbon chains containing an aldehyde group; they are simple and comparatively nonpolar aldehydes. Several alkanal products with chain lengths of 3 to 9 carbons have been identified as lipid peroxidation products from the ω-6 fatty acids linoleic and arachidonic acid (**Table 1**). Identification was performed by comparison of their retention time to aldehyde standards using high-performance liquid chromatography. Wu and Lin [30] listed butyraldehyde, hexanal and nonanal as the major alkanals generated by lipid peroxidation. Interestingly, hexanal was found to be the most abundant aldehyde of all those identified, even compared to 4-hydroxy-2-nonenal, a well-studied aldehyde discussed later in this review. However, there are few studies regarding these aldehydes, which may be due to their lower reactivity and toxicity compared with more complex aldehydes. Their single carbonyl functional group, only allows them to react with amine groups to form Schiff bases (imines), for example with the amino acid residue lysine or potentially the head groups of phosphatidylserine and phosphatidylethanolamine, although this has not yet been demonstrated. This limits their biological reactivity and detrimental effects. However, there have been several studies on the formation and effects of POVPC [31, 32], which has a five carbon chain terminating in an aldehyde moiety esterified at the *sn*-2 position, and thus has structural similarity to pentanal. POVPC has been detected in atherosclerotic plaques by LC-MSMS [33] and can form adducts with ApoB100 on LDL [34], which may have important consequences in disease.

Alkenals

Aldehydes with a double bond in the hydrocarbon chain are called alkenals. Because of the structure of PUFAs, with double bonds separated by 2 single bonds, alkenals derived from lipid peroxidation are often α,β-unsaturated (*trans*-2-alkenals, i.e. a double bond between carbons 2 and 3). An extensive repertoire of alkenals produced by environmental or biological processes exists, but not all of these products may be produced by lipid oxidation [14]. Early work identified several alkenals with chain lengths from 3 to 9 carbon atoms that resulted from lipid peroxidation of ω-6 fatty acids, with acrolein, heptenal, octenal and nonenal appearing in higher amounts than the others [30]. Acrolein, with only three carbons, is the shortest alkenal identified as a lipid peroxidation product. It is also by far the strongest electrophile and therefore the most reactive compound, especially with thiol groups from proteins [35, 36]. Acrolein is a known environmental hazard, as it can be formed by incomplete combustion of organic matter, and occurs at significant levels in wood and tobacco smoke, as well as in processed foods [36]. It can also be formed endogenously by oxidative metabolism of non-lipid precursors such as spermine, spermidine, threonine and methionine, and glycerol [37]. While it is known that acrolein could be formed from over-heating of cooking oils, for some years there was debate over whether it represented a significant product of lipid oxidation *in vivo*. There is now a substantial body of evidence supporting its generation by lipid oxidation [14, 37, 38], although the mechanism for its formation remains unclear. There are reports of it being formed from ω-6 unsaturated fatty acids, but that the yield is higher from ω-3 unsaturated fatty acids and increases with increasing number of double bonds [39]. One possible mechanism involves two β-cleavages at the centre of the arachidonic aliphatic chain [17], although this has been debated recently [14].

Crotonaldehyde (2-butenal) is a simple α,β-unsaturated aldehyde of similar structure to acrolein; although much less toxic, it can still modify proteins and DNA *in vivo* [40]. Crotonaldehyde and the five carbon analogue 2-pentenal come from the oxidative breakdown of ω-3 unsaturated fatty acids such as linolenic acid. In contrast, 2-heptenal, 2-octenal and 2-nonenal are thought to come from the breakdown oxidation of ω-6 unsaturated fatty acids. These longer alkenals are less reactive than acrolein or crotonaldehyde, possibly owing to their lower solubility in aqueous solvents that favour nucleophilic attack; this may explain why they have received less attention as molecules capable of modifying biomolecules. However, using an immunoreactivity assay and high performance liquid chromatography with on-line electrospray ionization tandem mass spectrometry it has been demonstrated that 2-nonenal can cause considerable protein modification, formed preferentially on lysine residues [41].

Complex aldehydes, including γ-substituted alkenals

Lipid peroxidation can also generate complex aldehydes containing additional functional groups, which make them more diverse in their reactivity with biomolecules and therefore more interesting biologically. Substituents at the C4 position are common, and include hydroperoxyl (-OOH), epoxy, hydroxyl (4-hydroxyalkenals), or keto (4-oxo-alkenals) groups, but products with additional double bonds and terminal aldehydic or carboxylic groups are also known. The α,β-substituted-alkenals have additional reactivity over alkenals owing to the susceptibility of the C3 position to nucleophilic attack, allowing the formation of Michael adducts with nucleophilic groups such as thiols and amines. This bi-reactive nature is particularly important, as it enables these compounds to crosslink macromolecules such as proteins, and is likely to contribute to some of the adverse effects in pathologies correlated with lipid peroxidation, such as Alzheimer's disease [18, 42].

The 4-hydroxyalkenals are a well-known type of lipid oxidation products comprising the C6 compound 4-hydroxy-2-hexenal (HHE), the C9 compound 4-hydroxy-2-nonenal (HNE) and the C12 compound 4-hydroxydodeca-2,6-dienal (HDDE). HHE derived from ω-3 PUFAs, whereas HNE is the product of ω-6 PUFAs via 15-hydroperoxyeicosatetraenoic acid (15-HpETE) or 13-hydroperoxy-linoleic acid. HDDE is formed from 12-HpETE, which is thought to be generated enzymatically by 12-lipoxygenase [43]. HNE can also be produced enzymatically by two different 15-lipoxygenases (15-LOX) acting on arachidonate [25]. It is probable that hydroperoxyalkenals, for example hydroperoxynonenal (HPNE), are intermediates on the reaction pathways to hydroxyalkenals. Of these hydroxyalkenals, 4-hydroxy-2-nonenal is without doubt the most studied. Following its discovery in the 1960s [35], it is now classed as one of the major aldehydes formed during lipid peroxidation of ω-6 polyunsaturated fatty acids and it can be considered a useful biomarker of lipid peroxidation [18]. The non-enzymatic processes of HNE generation mainly occur by oxygen radical-dependent reactions and have been extensively studied by several groups as mentioned above; several detailed reviews are also available [15, 16, 24]. The strong interest in 4-hydroxynonenal relates to its high reactivity due to its potential for Michael addictions and Schiff-base formation [44]. Thus its reactivity and biological effects have been the subject of many published articles and it has even been suggested as a signalling molecule, as it has been found to affect several key signalling pathways, including the MAPK pathways JNK and p38, PKC-β and δ, and Nrf2. However, these signalling processes most probably involve its reaction and adduct formation with signalling proteins, thus altering their activity (usually inhibition), rather than reversible ligand binding [45]. Interestingly, it appears that hydroperoxynonenal is not just a reactive intermediate leading to HNE, but also has biological effects in its own right, as it has been reported to modify proteins [46].

The formation of 4-hydroxy-2-hexenal by decomposition of oxidized docosahexaenoic acid (DHA) was first demonstrated by Van Kuijk *et al.*, using liquid

chromatography and mass spectrometry [47]. These authors concluded that it was one of the major lipid peroxidation products of ω-3 PUFAs, and it has been reported as the most prominent hydroxy-alkenal in human plasma, illustrating its potential importance [47, 48]. Although the number of studies focusing on this hydroxyalkenal is limited, there is evidence that it can cause severe peritonitis, retinal or liver damage when injected [49], and has adverse effects via NFkB signalling pathways, including causing apoptosis [48]. 4-Hydroxydodeca-2,6-dienal (HDDE) has also been demonstrated to be present in human plasma samples [50]. Although it was found in lower amounts than HNE and HHE, this finding suggests that HDDE can be produced in vivo and that it deserves to be investigated further. It has a longer aliphatic chain and is more hydrophobic than HNE and HHE, which has previously been suggested to lead to higher reactivity toward less polar biomolecules such as phospholipids. Studies carried out to compare the reactivity of HDDE, HNE and HHE with phosphatidylethanolamine showed that HDDE was the most reactive with the headgroup, resulting in more adduct formation with several different PE molecular species, followed by HNE and the least reactive being HHE [43]. It is also important to note that oxidation of the fatty acyl chains often occurs in phospholipid-esterified forms, and the Hock and β cleavages lead to analogous esterified aldehydes as well as the free forms discussed above. For oxidation of esterified linoleate, the esterified products may be almost mirror images of the free aldehydes [26].

The oxidation and cleavage mechanisms that generate 4-hydroxy-2-alkenals can also generate 4-oxo-2-alkenals, such as 4-oxo-2-(*E*)-nonenal (ONE) [51, 52] and 4-oxo-2-hexenal (OHE) [53, 54]. Several studies on ONE concurred in concluding that it is an even more reactive protein modification and cross-linking agent than HNE [55], although interestingly, its reactivity appears to differ: the rate of Michael adduct formation with cysteine (cys) and histidine (his) is higher but the rate of Schiff base formation is actually slower [56]. Biologically relevant adducts with histones [57] and human serum albumin [58] have been demonstrated recently and have increased attention on this aldehyde as an oxidative stress marker. While OHE was initially reported from studies in vitro and detected in food, there has been interest in its mutagenic properties, which are thought to arise from its reactivity and ability to form adducts with nucleosides [54, 55].

Hydroxy-alkenals and oxoalkenals can be oxidized to the more stable carboxylic acid metabolites 4-hydroxyhexenoic acid (HHA), 4-hydroxynonenoic acid (HNA) and 4-hydroxy dodecadienoic acid (HDDA) by the action of aldehyde dehydrogenases, and these have been found in human urine samples [50]. Since HNE comes from ω-6 fatty acids, HHE from ω-3 fatty acids, and HDDE is a lipoxygenase product, differential increases in their corresponding carboxylic acids in urine could potentially be a marker of the type of peroxidation involved in specific pathophysiological conditions [59].

Bis-aldehydes are another category of important short-chain oxidation products and, like the α,β-substituted-alkenals, they are bifunctional and have the potential to crosslink proteins and DNA [60]. Probably the best known is the 3-carbon molecule malondialdehyde (MDA), as it is a very commonly used marker of lipid peroxidation in biological and clinical samples [61, 62], although it has also been much criticized in this respect. It is generated by the decomposition of oxidised arachidonic acid through enzymatic or non-enzymatic mechanisms. Enzymatically, MDA can be generated as a side product of the synthesis of thromboxane A2, by a well stablished mechanism [63]. The non-enzymatic generation of MDA involves cyclization of peroxyl radicals to form a new free radical that can cyclize again to form bicyclic endoperoxides and undergo cleavage to produce MDA [64]. The reactivity and toxicity of MDA have been well-characterized previously [62, 65]. Glyoxal is the 2 carbon analogue of MDA, but there is less evidence of it as a lipid peroxidation breakdown product; the more common source is the auto-oxidation of glucose, especially in diabetes mellitus; the same is true of methylglyoxal [14, 66]. Other, less well known products include 2,4-decadienal, which has been reported as a product of oxidation of fatty acids, and *trans*-2-butene-1,4-dial, which is produced on further oxidation of the former [53]. In contrast, *cis*-2-butene-1,4-dial is a toxic metabolite of furans, and is thought to contribute to adverse effects of this type of carcinogen [14]. However, whether these aldehydes are produced *in vivo* through lipid peroxidation pathways is not yet clearly established.

Isolevuglandins

The bicyclic endoperoxide H_2-isoprostanes, such as prostaglandin-H_2, can undergo a concerted rearrangement to yield a family of isolevuglandins, which are also known as isoketals or γ-ketoaldehydes [67]. Incubation of PGH_2 in vitro in phosphate buffer resulted in a 22% yield of the isoketal, while in the more organic solvent DMSO the yield was 70%, suggesting that in biological membranes these compounds might be expected to form at appreciable levels under conditions where arachidonate is oxidized. Isolevuglandins are γ-bis-carbonyl compounds, and therefore have the potential to react with amines and crosslink proteins. These compounds were found to be extremely reactive, showing much faster reactions with albumin than HNE (50% depletion of isoketal in seconds, as opposed to > 1hr for HNE). The high reactivity is thought to relate to the fact that isoketals react with amines, but as well as forming Schiff bases, the hemiaminal intermediate can also undergo cyclization to the pyrrolidine form by intramolecular nucleophilic attack on the remaining carbonyl, followed by dehydration (loss of 2 H_2O) to produce the pyrrole as a stable end-product. The dehydration step is essentially irreversible and shifts the equilibrium of the reaction to the pyrrole product. This contrasts with the situation for HNE and other α,β-unsaturated aldehydes, where Michael addition to cysteine residues is favored over the

amine-carbonyl reaction and the corresponding hemiaminal cannot be so readily stabilized [67, 68].

In recent years there has been a flurry of activity in this field. There is mounting evidence that isolevuglandins form covalent adducts with proteins in various biological milieux, such as in normal and fibrotic lung tissue [69], human retina [70], and dendritic cells [71]. The mechanisms of protein crossing-linking by isolevuglandins have been investigated, and found to occur both by aminal and pyrrole-pyrrole cross-links [72]. Treatment of hepatic stellate cells with isolevuglandins caused their activation, and was thought to depend on formation of protein adducts, although this was not explicitly demonstrated [73]. Isolevuglandins have been found to react with the head group of phosphatidylethanolamine, and the resulting adducts can be detoxified by an *N*-acyl phosphatidylethanolamine-hydrolyzing phospholipase D [74]. Isolevuglandin-PE contributes to an NFkB-dependent inflammatory response in macrophages exposed to isolevuglandins by a mechanism involving binding to the receptor for advanced glycation end-products (RAGE). Moreover, isoLG adducts of PE were increased in hyperlipidemic and inflammatory conditions, such familial hypercholesterolemia in humans and high-fat diet induced obesity and hepatosteatosis in mice [75]. Analogous compounds called neuroketals can be formed from DHA via H_4-neuroprostanes; these have been detected in brain tissue and age-related increases in protein adducts observed [76]. The emerging evidence on the role of isolevuglandins has been reviewed recently by Salomon [68].

Alpha-chlorofatty aldehydes

Phospholipids containing fatty alcohols esterified by a vinyl ether bond (plasmalogens) are susceptible to oxidative damage by myeloperoxidase and its product hypochlorous acid. Attack by this oxidant leads to cleavage of the vinyl ether by a 2-electron mechanisms to yield an α-chlorofatty aldehyde and a lysolipid [77]. α-chlorohexadecanal and α-chlorooctadecanal have both been detected as plasmalogen oxidation products in activated neutrophils [78], and have been found to be elevated significantly in atherosclerotic lesions in cardiovascular disease [79], and in mouse brain following systemic injection of endotoxin [80]. The formation and biological effects of these α-halogenated aldehydes have been reviewed recently [81], but as yet their reactivity is not very well established compared to the other lipid oxidation products described here.

5. Methods of analysing aldehydic oxidation products.

A key aspect in understanding the production and biological significance of lipid oxidation products is the availability of reliable and sensitive methods of identifying and

quantifying them. Unlike markers of lipid oxidation such as isoprostanes, which are relatively stable, the aldehydes described in this review have additional challenges owing to their higher reactivity. Most lipid oxidation-derived aldehydes can be identified in the free form in vitro, in the absence of nucleophilic biomolecules, but the reactive alkenals, bis-carbonyls and isolevuglandins are more likely to be present in biological samples as covalent adducts with proteins, DNA or aminophospholipids. Thus attempting to quantify free aldehydes in complex biological or clinical samples can be fraught with difficulties, and may not reflect the actual extent of their formation. On the other hand, while adduct formation can effectively stabilize and trap the aldehydes, analysis of these secondary products presents a considerable challenge owing to the plethora of possible products. The choice of analyte should therefore be defined by the research question, as in some cases information about the exact aldehydes formed and their specific targets is required, and in others a general assessment of the extent of oxidative lipid damage may be sufficient.

Analysis of free aldehydic oxidation products and their metabolites

Methods for the detection of free alkanals, 2-alkenals, 2,4-alkadienals, 4-hydroxyalkenals and their quantification include spectrophotometric methods and gas or liquid chromatography coupled to mass spectrometry [82, 83]. The simplest approaches depend on direct spectrophotometric measurements; for example, HNE absorbs in the UV range at 220 nm, and it can be detected free in samples after separation from other aldehydes by HPLC [65]. However, a better approach is to take advantage of the intrinsic reactivity of the aldehydes by using aldehyde-reactive probes, as this allows more specific and usually more sensitive detection, and has the further advantage that it converts the aldehyde to a more stable form. Probably the best known (and most infamous) assay is the TBARS assay for malondialdehyde; it is extensively used owing to its simplicity and low cost, and for specific applications has been found to be a robust method [84], but even when combined with HPLC it has poor specificity and other limitations [61, 62]. A chromophore that reacts with a broader range of aldehydes is 1,3-cyclohexanedione (CHD) [85, 86], although it has declined in popularity in favor of other reagents. 2,4-dinitrophenylhydrazine (DNPH) is a very commonly used probe that reacts to give a dinitrophenylhydrazone product with an absorbance at 380 nm [87]; it can be used to quantify free aldehydes, although it is also often used to detect carbonyls on proteins by ELISA or western blotting, as described below. Recently, a number of new methods and probes have been developed as improved methods to measure free lipid peroxidation derived aldehydes, and applied to the analysis of human plasma, serum or urine samples, as detailed in **Table 2**. In most cases the probes do not distinguish between different aldehydes in a complex mixture, so chromatographic separation is required with either pre-column or post-column derivatization. For chromogenic

or fluorescence assays, separation is usually achieved by reverse phase HPLC, often with a C18 stationary phase [88].

Gas chromatography can also be used to separate different aldehydes, but it requires derivatization of the sample to obtain a volatile analyte, and is usually coupled to mass spectrometry as a detection method. For example, GC coupled to electron-capture negative-ion chemical ionization (NICI) MS has recently been used to analyse HNE in patients with thyroid dysfunction or migraines [89]. Free HNE was converted to pentafluorobenzyl oximes (PFBO) by reaction with pentafluorobenzyl hydroxylamine, followed by formation of trimethylsilyl ethers; the ions of m/z 152 and 333 were chosen for selected ion monitoring (SIM) of PFBO-TMS derivatives of HNE. The LOD and LLOQ were 2.0 and 2.5 nM respectively. A more recent study used a similar approach with GC-MSMS to analyse MDA and HNE in human serum samples. The derivatised aldehydes were quantified by selected reaction monitoring for relevant mass transitions (m/z 442 > 243 for MDA, m/z 403 > 283 for HNE) [83]. Analysis of transitions resulting from specific fragmentations of the analytes should offer greater selectivity over SIM. The concentrations of the aldehydes ranged from 0.2-2.5 μM in the serum of patients with coronary or peripheral artery disease, but MDA levels tended to be 2-3-fold higher than the HNE. Advances in GC-MS and LC-MS methods for measuring MDA and comparison with traditional derivatization methods have been reviewed by Giera *et al*. [65]. Gas chromatography coupled to NICI–MS is also the preferred method for detection of α-chloroaldehydes such as 2-chlorohexadecanal [90], as they are less polar than many other aldehydes and relatively unstable under the conditions required for liquid chromatography. The α-chloroaldehydes are analysed as the corresponding PFBO derivatives; using SIM, a structurally-relevant ion fragment is produced identifying the aldehyde [91]. For example, the PFBO of 2-chlorohexadecanal is detected by the ion at m/z 288. For quantitative analysis it is recommended to use a stable internal standard; the most commonly used is 2-chloro-[d4-7,7,8,8]-hexadecanal (2-Cl-[d4]HDA), which can be detected at m/z 292.

In contrast to GC-MS, LC-MS requires minimal sample manipulation other than extraction prior to analysis [83], and consequently can be considered to have an advantage, although overall it is less used. Several studies have demonstrated the value of LC-MS or LC-MSMS approaches to analyse lipid oxidation derived aldehydes, often following reaction with DNPH to stabilize them [92]. For example, Douny et al. [93] used DNPH derivatization to measure eight different aldehydes in animal feed: malondialdehyde (MDA), crotonaldehyde (CRT), benzaldehyde (BNZ), hexanal (HXL), 4-hydroxy-2-hexenal (4-HHE), 4-hydroxy-2-nonenal (4-HNE), 2,4-nonadienal and 2,4-decadienal. The first 6 could be readily quantified in a single run, whereas the latter 2 showed a more limited linear range,

especially for the upper limit of quantification (ULOQ). MSMS transition for all the aldehydes were reported.

It is important to bear in mind that as well as reacting with macromolecules, free aldehydes may be metabolized and detoxified, so analysis of the metabolite may represent a good approach to detecting formation of the aldehyde in vivo. It is well known that HNE can be detoxified by conjugation to glutathione, catalysed by glutathione-S-transferases [94], and metabolized further to yield 1,4-dihydroxynonane-mercapturic acid, which can be detected in the urine together with other metabolites [95]. *Trans,trans*-2,4-decadienal (tt-DDE), a lipid peroxidation product of linoleic acid, can be oxidized to the two metabolites 2,4-decadienoic acid and cysteine-conjugated 2,4-decadien-1-ol. These have been found in cell culture models and urine of mice gavaged with tt-DDE, using liquid chromatography coupled to tandem mass spectrometry to identify the structures [96]. Carboxylic acid metabolites of HHE (4-hydroxy-2*E*-hexenoic acid; 4-HHA), HNE (4-hydroxy-*2E*-nonenoic acid; 4-HNA) and HDDE (4-hydroxy-2*E*,6*Z*-dodecadienoic acid; 4-HDDA) were detected in human urine using NICI-MS, and occurred at higher concentrations in aging and diabetes [50]. Similarly, under oxidative conditions *in vivo*, α-chloroaldehydes can be converted to the corresponding acid form. These can be analysed by conversion to the PFB ester, and the derivative analysed using GC with NICI–MS [97]. Alternatively, direct analysis by liquid chromatography mass spectrometry (LC-MS) using 2-Cl-[d4]HA as an internal standard can be carried out [98]. Moreover, it has recently been reported that α-chloro fatty aldehydes may form adducts with GSH, and these can be detected by LC-ESI-MS using selected reaction monitoring [99].

Analysis of aldehydic oxidation products as adducts with macromolecules.

As mentioned above, the analysis of free aldehydes has several disadvantages for understanding the extent of their production in vivo, as their high reactivity means that they readily form adducts with a variety of biological molecules, including small antioxidants such as glutathione, proteins and nucleic acid. Thus a large proportion of the aldehydes formed are likely to exist sequestered as adducts and the free aldehyde level depends on the reversibility, or equilibria, of these reactions. Consequently, a full understanding of the levels and roles of lipid oxidation derived aldehydes depends on analysis of the adducts formed through Schiff base reactions and Michael additions or rearrangement of these products. Although adducts with DNA bases are known to occur and are thought to contribute to the mutagenicity of acrolein and crotonaldehyde, most attention has focused on detecting the formation of adducts with proteins (lipoxidation). The structures of a substantial number of protein adducts have been elucidated, and examples are shown in **Figure 1**. To this end, two main approaches exist: antibody-dependent techniques, and proteomic methods

including proteins identification and sequencing by mass spectrometry. These two approaches may also be combined.

Use of antibody-based methods

Since the realization in the 1990s that many lipid peroxidation products have interesting biological effects, there have been significant efforts to develop specific antibodies against the adducts formed from MDA, HNE and several other aldehydes, which have subsequently been tested and validated for use in ELISAs, western blotting, and immunocytochemistry and immunohistochemistry. Antibodies against MDA- and HNE-modified proteins have been available for many years [100, 101] and polyclonal sera are now commercially available from several suppliers. The antibodies and antisera available for HNE have been reviewed previously [15]. A monoclonal antibody against acrolein adducts with proteins was also developed and tested around the same time [102, 103]; subsequently other researchers produced an antibody against the same immunogen, and showed that it recognized acrolein-modified albumin [104]. Using this antiserum they developed an ELISA and reported limited cross-reactivity with adducts of formaldehyde, malondialdehyde, or 4-hydroxynonenal with albumin. Salomon's group raised polyclonal antibodies against 15-E2-IsoK or 12-E2-IsoK adducts with keyhole limpet hemocyanin, and used them in an ELISA to measure the levels of these isolevuglandin adducts in the plasma from patients with atherosclerosis and with endstage renal disease [105]. A goat anti-neuroketal antibody is now commercially available and has been used to show increased levels of neuroketal adducts in elderly brain [76]. Recently, a monoclonal (mAb 27Q4) has been raised against nonenal-modified keyhole limpet hemocyanin, and used to investigate the presence of protein-bound 2-nonenal in vivo; it was found that the antibody recognises *cis*- and *trans*-N^{ε}-3-[(hept-1-enyl)-4-hexylpyridinium]lysine (HHP-lysine), a novel nonenal-lysine adduct [41], formed via the addition of two equivalents of nonenal. Monoclonal antibodies have also been produced against the acrolein-derived cyclic DNA adduct 1,N^2-propanodeoxyguanosine (Acr-dG) which were only weakly cross-reactive against crotonaldehyde- and HNE-deoxyguanosine adducts; a FACS-based assay and two ELISAs were developed based on these antibodies and tested in experiments with HT29 cells [106].

The antibodies described above have proved invaluable in demonstrating the occurrence of adducts of lipid oxidation derived aldehydes in tissues, and have provided novel information on the structure of some adducts. Some of them, most notably anti-HNE antibodies, have been applied to western blotting to identify lipoxidized proteins [107]. Anti-DNP antibodies are very widely used in western blotting (oxyblotting) and ELISAs for carbonyl-containing proteins [108], but it is important to realize that protein carbonyls can arise from other oxidative modifications and that reactions of lipid peroxidation products with proteins will only result in free carbonyls for bis-carbonyls or bi-reactive compounds that can

form adducts by Michael addition. Although most of the monoclonal antibodies are reported to show high specificity, several of the commercially available antibodies are polyclonal antisera, and likely to have higher cross-reactivity. Ultimately, a potential limitation of all work with antibodies is a dependence on their specificity, and the possibility that the epitope may be hidden or not recognized in some target proteins through altered folding or aggregation.

Protein analysis by mass spectrometry

In order to identify the protein targets of reactive carbonyl compounds that have been detected by western blotting on one- or two-dimensional SDS-polyacrylamide gels, mass spectrometry analysis of the gel spot or band on an equivalent Coomassie-stained gel is often carried out [109, 110], as mass spectrometry is the most informative and reliable technique for this purpose. The spots of interest are excised from the gel and digested to peptides, an enzymatic process usually carried out using trypsin, but other proteases such as AspN or GluC can be used [111]. The resulting peptide mixture can be analysed directly by MALDI-TOF, and the peptide mass fingerprinting used to identify the proteins present, which involves matching the observed peptide mases to theoretical masses of possible peptides in a database [112]. This is the most common method for peptide mass fingerprinting, but the same data can also be achieved by LC-MS with electrospray ionization. In both cases, proteins present in the sample are identified based on the identification of two or more unique peptides; however, this does not demonstrate that the modification was present in that protein unless a peptide with increased mass corresponding exactly to the adduct is observed. In practice, the probability of finding the modified peptide is low, so many studies have reported the identity of the proteins that were identified in spots corresponding to the location of those that reacted in the western blot, and assumed that these are the proteins modified by the aldehyde of interest, without firm evidence for it. For example, using MALDI-TOF specific targets of 4-hydroxy-2-nonenal modifications were identified in Down syndrome cases, supporting similarities with Alzheimer disease. Western blotting after 2D electrophoresis was carried out with a mouse monoclonal anti-HNE antibody, proteins in the corresponding digested spots were identified by MALDI-TOF peptide mass fingerprinting, and the data were searched using the Mascot search engine [113]. A similar method was also successfully applied in the identification of complex I subunits modified by HNE in mitochondria from diabetic kidney. HNE-modified proteins were first identified by western blotting, but then two different types of electrophoresis were performed: blue native polyacrylamide gel electrophoresis (BN-PAGE) to isolate the whole complex I, followed by SDS-PAGE to resolve individual complex I subunits. After in-gel digestion, nano-liquid chromatography mass spectrometry was performed for peptide mapping and protein identification [114]. As the complexes were separated through

additional electrophoresis steps and only 1 protein was identified in each band, the evidence for HNE modification in this case is stronger. In other studies, the presence of several resolved spots, i.e. at slightly different pIs and molecular weights, but with the same provided support for the existence of post-translational modifications [115]. Generally, the same approach has been used when investigating other types of lipoxidation, such as the occurrence of neuroketal adducts in brain of middle-aged and old individuals [76].

Protein sequencing and identification of modifications by LC-MSMS

Ultimately, absolute confirmation of the presence of lipoxidation or other post-translational modifications requires tandem MS to sequence the peptide and demonstrate the specific site and nature of the modification [116]. This is most routinely done by LC-MSMS; this bottom-up approach is advantageous in complex protein mixtures and has the potential to identify low abundance proteins, given the appropriate experimental parameters. However, untargeted or discovery techniques are extremely difficult to implement in complex samples, and have most commonly been applied to individual proteins. Nevertheless, important information can be obtained about the potential modification sites, the nature of the adducts formed, and the different propensities of individual proteins for modification, as shown in some recent studies.

Zhu et al. incubated chymotrypsin, cytochrome c, β-lactoglobulin (β-LG) and RNase A with 2,4-dodecadienal and used a label-free LC-MSMS approach to investigate the sites of modifications [117]. They observed that β-lactoglobulin was far more susceptible to dodecadienal adduct formation than the other proteins. A characteristic fragment ion at *m/z* 286 was observed and deduced to correspond to the protonated pyridine moiety, indicating the presence of a lysyl-pyridinium adduct. These diagnostic fragment ions are extremely useful as they offer potential for semi-targeted (precursor ion scanning) or targeted (single or multiple reaction monitoring) mass spectrometry routines to find particular modifications. Another study focused on rat ApoE protein and using MALDI-TOF/TOF MS demonstrated a series of adducts induced by acrolein, including an aldimine adduct at K149 and K155 (+38 Da); a propanal adduct at K135 and K138 (+56 Da); an N^{ε}-(3-methylpyridinium)lysine (MP-lysine) at K64, K67, and K254 (+76 Da), and an N^{ε}-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine) derivative at position K68 (+94 Da) [118]. (MALDI-TOF/TOF MS). The rat ApoE treated with acrolein was found to have impaired binding to the LDL receptor and heparin, which was thought to result from overall changes in folding of the protein, and illustrates some important biological outcomes of lipoxidation.

Such studies with purified proteins can direct subsequent studies on biological and clinical samples, as demonstrated elegantly by Charvet et al [70] for isolevuglandin adducts of the sterol-metabolizing enzyme CYP27A1. Bottom up LC-MS/MS analysis showed that

CYP27A1 was modified by iso[4]LGE_2 *in vitro* specifically at three Lys residues: Lys134, Lys358, and Lys476. This enabled the generation of an internal standard, ^{15}N-labeled CYP27A1 modified with iso[4]LGE2, and design of transitions for multiple reaction monitoring for the modified peptides AVLK^{358}(-C20H26O3)ETLR and VVLAPETGELK^{476}(-C20H26O3)SVAR, and demonstration of iso[4]LGE_2 adducts in a human retinal sample. More recently, a similar approach was used to study the formation of isoketal-lysine-lactam adducts in dendritic cells by stable isotope dilution multiple reaction monitoring mass spectrometry [71]. The ability to identify isolevuglandin adducts in vivo is important to demonstrate the formation of these lipid oxidation products, as free isolevuglandins as cannot be detected in tissues, owing to their very high reactivity with amines [67].

Is it worth remembering that several esterified chain-shortened products of lipid oxidation contain aldehydes, such as POVPC and PONPC, and that these can also form adducts with proteins. In fact, the E06 antibody that has been used extensively in clinical studies is thought to recognize the phosphocholine headgroup and bind to lipoproteins containing POVPC adducts [119]. The formation of these adducts has now also been demonstrated using a semi-targeted approach, involving mining high resolution LC-MSMS data of Apo-B100 digested with trypsin for the diagnostic fragment at m/z 184 indicative of phosphocholine [34].

Enrichment and labelling approaches to identifying oxidized lipid adducts with proteins

Despite the positive findings described in the section above, a major challenge of label-free MS analysis is that post-translationally modified proteins are generally only present at low stoichiometric levels in a sample protein pool, and thus are difficult to identify amongst proteins thousands of times more abundant. One way to overcome this issue is to apply enrichment tools to bottom-up LC-MS approaches, which can lead significantly increase the sensitivity and specificity [120]. The most common principle is to use a biotin hydrazide to label protein carbonyls and then enrich these proteins by avidin capture on a solid phase. In 2006 Chavez *et al*. introduced a biotin-tagged aldehyde reactive probe (ARP) that is able to react with protein aldehydes present on Michael-type conjugates to form a C=N bond and a stable biotinylated oxime derivative; this can be detected by either western blotting or mass spectrometry, following fragmentation of the labelled peptide [121]. This affinity approach was subsequently applied to lipoxidation adducts in cardiac mitochondria, where adduction by acrolein, β-hydroxyacrolein, crotonaldehyde, 4-hydroxy-2-hexenal, 4-hydroxy-2-nonenal and 4-oxo-2-nonenal was observed [122]. A more recent study used ARP to quantify the susceptibility of proteins from hepatic mitochondria to HNE damage, using magnetic streptavidin beads for enrichment either at the level of modified protein or modified peptide after tryptic digestion [123]. Many protein targets and modification sites

were identified, which may be useful in future targeted assays. An alternative method involving d_0/d_4-succinic anhydride labelling followed by enrichment using hydrazine-functionalized beads (Affi-gel Hz beads) has been developed by the same group for relative quantification of site-specific carbonyl adducts, and it was reported that hydroxy-2-hexenal (HHE) Michael adducts were most abundant, although adducts of acrolein, 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-noneal (ONE) to cysteine, histidine and lysine were also identified [124]. A comparison of 2,4-dinitrophenylhydrazine (DNPH), biotin hydrazide (BHZ) and *O*-(biotinylcarbazoylmethyl) hydroxylamine (ARP) as carbonyl-labeling reagents has been carried out, and it was concluded that all were selective for aliphatic aldehydes and ketones, and that the best method is to digest the proteins first and then label the peptides [125].

Thus the biotin affinity labelling with MS based peptide sequencing can provide detailed information about a variety of protein modifications in biological samples. However, it can also detect carbonyl-containing modifications produced by direct, free radical attack on proteins (e.g. lysine, arginine, proline and threonine residues), and will miss Schiff base adducts formed by mono-aldehydes.

A different and novel approach to determining targets of small reactive aldehydes involves the chemical synthesis of aldehyde analogues with alkyne or azido termini, which allows their adducts to be selectively extracted from biological samples [126]. For example, Vila et al worked with alkynyl analogues of HNE to form adducts that can be label with azido-biotin tags by Cu^+-catalyzed cycloaddition (Click chemistry), and they found that heat shock proteins 70 and 90, and the 78-kDa glucose-regulated protein, were selectively adducted [127]. While clearly a very effective approach to determining targets of HNE, unfortunately it cannot as yet be used to study the physiological occurrence of aldehydes in biological or clinical samples.

Detection of adducts on intact proteins

While bottom-up proteomic approaches to detecting lipoxidation are most common, they have the limitation that they do not provide information on the modification of the whole protein, only on the peptides that can be sequenced. Understanding the extent and kinetics of modification of a protein requires analysis of the intact protein, using a high resolution instrument. As a protein has many residues that can carry surface charge, it can exist in a variety of charged states, each with a different mass-to-charge ratio. Deconvolution software facilitates the conversion of the m/z envelope to the observed mass of the protein. Treatment with a reactive carbonyl compound can result in the formation of multiple adducts, as shown for acrolein adducts of lysozyme in **Figure 2**. This approach has been used to investigate the susceptibility of several proteins to 2,4-dodecadienal (DDE), by monitoring

mass increments of 134 Da corresponding to Lys Schiff bases or Michael adducts at +152 Da. It was found that β–lactoglobulin became much more extensively modified than cytochrome c or RNase A, and the adducts appeared to be more stable over the incubation period [117]. In another study, ubiquitin was selected as a model protein to investigate the formation of AGEs (with methylglyoxal and glyoxal), and ALEs (with malondialdehyde and 4-hydroxy-2-nonenal). The order of reactivity with this protein was found to be MGO > GO > HNE > MDA. The top-down approach was supplemented with typical nano LC-ESI-MS/MS analysis of peptides from a tryptic digest as described above to map the specific sites of modification [128].

6. Quantification of aldehydes in human plasma or serum

The physiological serum concentrations of free aldehydes may depend on a number of factors, such as the rate of production versus breakdown or detoxification, as well as the amount of adduction of aldehydes to biomolecules [129]. Moreover, Schiffs base and even Michael adduct formation is reversible unless the adducts are stabilized by reduction or a rearrangement such as cyclization, which may affect quantification. The concentrations of free aldehydes in plasma or serum have been measured in healthy subjects and in a number of disease states using GC, HPLC with a range of different spectrophotometric detection methods, or GC/LC-MS, with the most comprehensive data coming from patients suffering from diabetes, rheumatoid arthritis and lung cancer. A representative sample of these data is summarized in **Table 3**. There appear to be significant levels of most of the aldehydes measured even in healthy subjects, although it is worth noting that there is generally a lack of consensus on plasma concentrations for individual aldehydes between different studies, which may be due to the different techniques being used. For example, in healthy subjects HNE concentrations of 80 to 960 nM are reported, and malondialdehyde varies from 0.36 to 15 μM. This issue has been noted previously: even when a single batch of samples was prepared and distributed to several laboratories for analysis; in this study, malondialdehyde was found to have the lowest inter-laboratory variability compared to HNE and isoprostanes [84]. The analysis and levels of MDA and HNE in clinical samples have been discussed in an extensive recent review [83], and the same author also reported that sample storage time can significantly affect the concentrations [130]. In general, no or only a small (approximately 2-fold) increase in plasma aldehyde concentration is seen in disease, although some of these are statistically significant. Larger increases have been reported for hexanal and heptanal in lung cancer and acrolein in diabetics. However, care needs to be taken in interpreting increases in acrolein concentration as it may come from sources other than lipid oxidation, such as the catabolism of threonine and spermidine or the oxidation of sugars [37].

7. Perspectives

Of the very wide number of different carbonyl-containing and reactive lipid oxidation products known, still a relatively small fraction have been thoroughly investigated, although the area continues to broaden. This may in part be due to the availability of the compounds, which are not all commercially available currently. Specific antibodies to aldehyde-protein adducts have also been a major contributing factor to detecting the formation of adducts and understanding their role in pathology, but again, a fairly limited selection are currently available. Development of antibodies to a wider range of adducts, including those resulting from less commonly studied aldehydes such as OHE and HDDE, will be an important area for future research. However, it is challenging because of the large number of different chemical structures that can result following cyclization or cross-linking reactions, which therefore requires prioritization of the adducts. Data to inform such decisions requires the application of the advanced mass spectrometry analytical methods described above, as this can help to identify the chemical structures that occur *in vivo*, and characterize or confirm the structures of adducts used as antigens. On the other hand, bottom-up LC-MSMS is a very data- and labor-intensive approach to finding lipoxidation adducts, akin to looking for a needle in a haystack, hence there is a substantial benefit to the development of specific enrichment and labelling techniques that reduce the number of proteins that need to be sequenced.

An important message that is emerging from studies of reactive carbonyl-containing compounds is that their reactions with proteins are selective; some proteins are more susceptible to adduction by a particular aldehyde than other proteins and the types of residues modified is different. For example, in one protein HNE might react preferentially with lysine, while in another histidine and cysteine are most modified. Moreover, within a single protein all lysine residues are not equal, and the same applies to cysteine and histidine. This may depend both on solvent accessibility of the residues and its pKa, which can be greatly affected by the local environment within the protein. More comprehensive mapping of modification sites within pathophysiologically relevant proteins will allow improved understanding of the mechanisms by which reactive lipid-derived aldehydes affect signalling pathways and alter cell behaviour. Notwithstanding these challenges, it is already clear that lipoxidation is an important research topic that deserves future attention.

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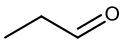
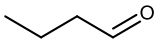
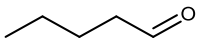
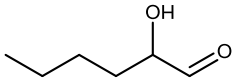
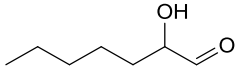
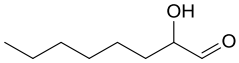
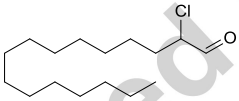
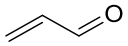
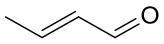
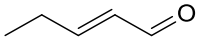
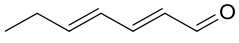
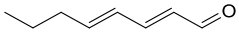
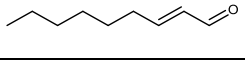
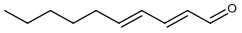
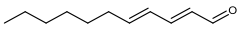
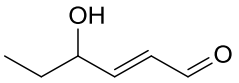
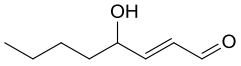
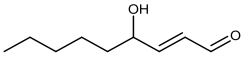
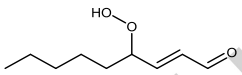
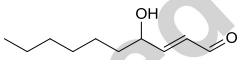
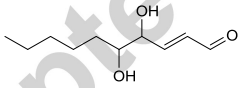
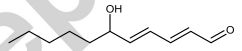
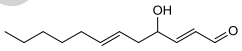
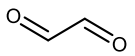
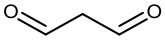
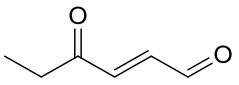
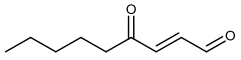
Figure 1. An overview of cyclic products formed by the reaction of aldehyde-containing lipid oxidation products with amino acid side chains. Where reactions depend on Michael additions only the lysine product is shown, but as indicated this can be replaced by cysteine or histidine. Reactions that can form crosslinks between amino acid side chains are identified as such. Reactions are shown for representative members (usually the most abundantly formed) of the class of oxidation product.

Figure 2. Formation of acrolein adducts on lysozyme. Lysozyme (1 mg/mL) was reduced with dithiothreitol, treated with 4 mM acrolein for 2 hours and the adducts stabilized by reduction with $NaBH_4$. Untreated lysozyme solution (A) or acrolein-treated lysozyme (B) were infused into an SCIEX 5600 TripleTOF. The raw MS data containing the charge state envelopes were deconvoluted using Biotools within the PeakView software (Sciex) to give actual mass spectra. Adducts of acrolein correspond to mass additions of 58 Da. Minor peaks in the native protein correspond to salt adducts that were lost on zip-tip purification of the treated sample.

Table 1. Structures of carbonyl-containing lipid oxidation products

Name		Structure	Comments	Ref.
		Alkanals		
Propionaldehyde	C3		Identified as produced from both linoleic and arachidonic acid radical oxidation *in vitro* by GC and LC. Found in rat heart oxidative stress and other conditions.	[30, 37, 131, 132]
Butyraldehyde	C4		Identified as produced from both linoleic and arachidonic acid radical oxidation *in vitro* by GC and LC.	[30, 37]
Valeraldehyde (pentanal)	C5		Identified as produced from both linoleic and arachidonic acid radical oxidation *in vitro* by GC and LC. The analogous alkanals hexanal (C6), heptanal (C7), octanal (C8) and nonanal (C9) were also identified.	[30, 37]
		α-Substituted alkanals		
2-hydroxyhexanal	C6		Derived from peroxidation of n-6 polyunsaturated fatty acids	[133]
2-hydroxyheptanal	C7		Major product from all n-6 polyunsaturated fatty acids peroxidation.	[133-135]
2-hydroxyoctanal	C8		From oleic acid acid hydroperoxide, identified as PFBO derivative by GC from Fe^{2+}/ascorbate oxidation. The analogous substituted alkanals 2-OH-nonanal (C8) and 2-OH-decanal (C10) were also identified.	[133, 134]
α-Chloro-hexadecanal	C16		Generated from reaction of plasmalogens with HOCl	[78]
α-Chloro-octadecanal	C18		Generated from reaction of plasmalogens with HOCl	[78]
		Alkenals		
Acrolein (propenal)	C3		Highly reactive electrophile and most cytotoxic. Formation requires two β-cleavages. 2-alkenal cytotoxicity depends on chain length.	[35, 37, 136]
Crotonaldehyde ((*2E*)-but-2-enal)	C4		Derived from peroxidation of ω-3 polyunsaturated fatty acids (e.g. 15-OOH of linolenic acid)	[37]
2-pentenal	C5		Derived from lipid peroxidation of ω-3 polyunsaturated fatty acids (e.g. linolenic 18:3). 2-heptenal and 2-octenal (C8) were also identified.	[37]
2,4-heptadienal	C7		Derived from lipid peroxidation of ω-3 polyunsaturated fatty acids; identified in oxidized LDL and cooking oils.	[137-139]
2,4-octadienal	C8		Derived from lipid peroxidation of ω-3 polyunsaturated fatty acids; identified in cooking oils.	[140]
2-nonenal	C9		Less reactive than other 2-alkenals, such as acrolein and crotonaldehyde, so has received relatively little attention as an	[41, 136]

			agent for modification of proteins. 2-alkenal cytotoxicity depends on chain length	
2,4-decadienal	C10		Lipid peroxidation of ω-6 polyunsaturated fatty acids. Found in plant material e.g. peanuts, cooked meat (used as a flavour ingredient), and mammalian cells. Possible intermediate in the formation of HNE.	[50, 53, 96, 137]
2,4-undecadienal	C11		Lipid peroxidation of ω-6 polyunsaturated fatty acids. Found in plant material, cooked meat and peanuts and used as a flavour ingredient.	[141, 142]
		γ-Substituted alkenals		
4-hydroxy-2-hexenal (HHE)	C6		Derived from peroxidation products of ω-3 polyunsaturated fatty acids. A potential mediator of mitochondrial permeability transition.	[49, 143, 144]
4-hydroxy-2-octenal (HOE)	C8		Derived from peroxidation products of ω-6 polyunsaturated fatty acids.	[143]
4-hydroxy-2-nonenal (HNE)	C9		The main aldehyde formed during peroxidation of ω-6 polyunsaturated fatty acids. Formed via 15-hydroperoxy-eicosatetraenoic acid 15-HpETE (from arachidonic acid) and from 13-hydroperoxy-octadecadienoic acid 13-HpODE (from linoleic acid).	[37, 144]
4-hydroperoxy-2-nonenal (HpNE)	C9		Derived from double peroxidation of ω-6 polyunsaturated fatty acids. Equivalent hydroperoxy forms would be expected for other α,β-unsaturated aldehydes and as precursors to hydroxides in this table,	[46]
4-hydroxy-decenal	C10		Derived from peroxidation of ω-6 polyunsaturated FA.	[143]
4,5-Dihydroxydecenal	C10		Identified in NADPH-Fe induced peroxidation of liver microsomes	[138]
6-hydroxy-2,4-undecadienal	C11		Derived from peroxidation of ω-6 polyunsaturated FA.	[133, 134]
4-hydroxy-2,6-dodecadienal (HDDE)	C12		Derived from the breakdown of 12-hydroperoxy-eicosatetraenoic acid 12-HpETE (from arachidonic acid).	[42, 43, 59]
		Bis-aldehydes		
Glyoxal (oxaldehyde)	C2		Oxidation product common to all polyunsaturated fatty acids. Precursor of N^{ε}-(carboxymethyl)lysine (CML). Point of interplay between glycation and lipid peroxidation.	[145, 146]
Malondialdehyde (1,3-propanedial, MDA)	C3		Arachidonic acid is the main precursor of the bicyclic endoperoxides which undergo further reactions to form MDA. Global marker of lipid peroxidation.	[17, 27, 35]
4-oxo-2-hexenal (OHE)	C6		Derived from lipid peroxidation of ω-3 polyunsaturated fatty acids	[147]
4-oxo-2-nonenal (ONE)	C9		From 13-hydroperoxy-octadecadienoic acid 13-HpODE (linoleic acid). Highly reactive aldehyde originating from the peroxidation of n-6 polyunsaturated fatty	[58]

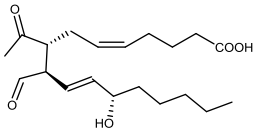
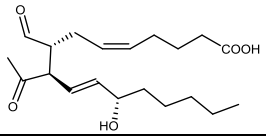
		acid.		
		Levuglandins and Isoketals		
Isoketal (Levuglandin E2)	C20		Formed from the H_2-isoprostane pathway. From the rearrangement of prostaglandin H2.	[67]
Isoketal (Levuglandin D2)	C20		Formed from the H2-isoprostane pathway. From the rearrangement of prostaglandin H_2.	[67]

Table 2. Recently reported probes for analysis of lipid peroxidation derived aldehydes and their application to biological systems.

Aldehyde Reactive Probe	Detection method	Aldehydes analysed	Linear Range	Levels in biological samples	Reference
2,2'-furil (with ammonium acetate)	Fluorescence (250/355 nm)	Glyoxal Acrolein HNE MD	0.10–5.00 μM 0.20–10.0 μM 0.20–40.0 μM 0.40–10.0 μM	0.7-4.0 μM 0.7-9.3 μM 13-40 μM 0.8-2.5 μM (sera)	[148]
4-(N,N-dimethyl-aminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole	Peroxyoxalate-dependent chemi-luminescence	Methylglyoxal, Acrolein, Crotonaldehyde Trans-2-hexenal	20–420 nM 16–320 nM 15–360 nM 20–320 nM	141-310 nM 42-76 nM 57-68 nM 54-71 nM (sera)	[149] and refs therein
Rhodamine B hydrazide (RBH)	Fluorescence (560/580 nm) & LC-MS	MDA	0.8-1500 nM LOD=0.25 nM LOQ=0.80 nM	40-250 nM (plasma) 20-70 nM (urine)	[88] and refs therein
2,4-dinitrophenyl-hydrazine (DNPH)	LC-MSMS ketone gave an m/z 179 ion; aldehyde gave m/z 163.	Acrolein Crotonaldehyde Pentanal Hexanal Heptanal	Not quoted	n/a	[117, 150]

Table 3. Concentrations of various aldehydes in serum or plasma.

Aldehyde	serum/plasma				Detection Method[1]	Ref.	Comments
	Healthy	Diabetic	Rheumatoid Arthritis	Lung cancer			
Glyoxal	1.04 μM	2.75 μM	1.34 μM		A	[148]	$P < 0.05$ for all diseases vs control
Methylglyoxal	146.5 nM	299 nM	145 nM		B	[149]	Significant difference for control vs diabetes
Acetaldehyde	1279 nM				C	[129]	
Propionaldehyde	135 nM				C	[129]	
Acrolein	42.2 nM	68.7 nM	76.3 nM		B	[149]	Significant difference for control vs diabetes and RA
	1.26 μM	6.35 μM	2.92 μM		A	[148]	$P < 0.05$ for all diseases vs control
	5.54-6.56 μM				D	[151]	Good precision, sensitivity and accuracy for all 4 methods. SIM the most accurate.
Malondialdehyde (MDA)	0.36-1.24 μM				E	[152]	Correlation ($P < 0.05$) with smoking and alcohol consumption
	15.1 μM	25.6 μM	37μM		A	[148]	$P < 0.05$ for all diseases vs control
	0.44-0.89 μM				E	[153]	Note an age related increase in concentration
Crotonaldehyde	61.3 nM	63.5 nM	60.9 nM		B	[149]	
Valeraldehyde	7 nM				C	[129]	
	17 nM				C	[129]	
Hexanal	1.04-2.06 μM			2.28-25.3 μM	F	[154]	Results similar to previous studies using a range of measurement methods
2-hexenal	59.5 nM	59.2 nM	61.7 nM		B	[149]	
Heptanal	0.02-0.91 μM			0.16-3.91 μM	F	[154]	Results similar to previous studies using a range of measurement methods
	0.96 μM	1.27 μM	1.78 μM		A	[148]	$P < 0.05$ for all diseases vs control
HNE	82 nM				G	[155]	
	68.9-107 nM				H	[153]	Note an age related increase in concentration
	0.65 ± 0.39 μM				I	[156]	Significant increase noted during reperfusion

[1]**Detection methods**. **A** - HPLC–fluorescence detection of difurylimidazole derivatives; **B** - LC-MS with peroxyoxalate chemiluminescence detection after fluorescence labeling with 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole; **C** - HPLC of fluorescent decahydroacridine derivatives; **D** - HPLC-DAD, HPLC-fluorescence, LC-DAD or LC/MS-SIM; **E** - HPLC analysis of the thiobarbituric acid (TBA) derivative; **F** - Derivatization with 2,4-dinitrophenylhydrazine (DNPH), extraction with DLLME-SFO, analysis by HPLC; **G** - HPLC of the oxime-*bis*-tert-butyldimethylsilyl derivative; **H** - Derivatization with dinitrophenylhydrazine (DNPH), analysis by LC; **I** – DNPH derivitization, SPE of hydrazine and HPLC with detection at 370 nm.

Highlights

- Lipid oxidation and fragmentation generates a wide variety of reactive alkanals, alkenals, bis-aldehydes and α-chlorofatty aldehydes.
- Acrolein, crotonaldehyde, 4-hydroxy-2-hexenal, and 4-hydroxy-2-nonenal are among the most reactive and abundant products.
- There is large inter-study variability in levels of free aldehydes measured in human serum or plasma, possibly reflecting procedural differences.
- Aldehyde cytotoxicity and biological activity depend on their ability to form adducts with cellular proteins and DNA.
- Aldehyde-protein adducts can best be analysing by a combination of enrichment methods and LC-MSMS.