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Lipoxidation products as biomarkers of oxidative damage to proteins during lipid peroxidation reactions

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Abstract. Oxidative stress is implicated in the pathogenesis of numerous disease processes including diabetes mellitus, atherosclerosis, ischaemia reperfusion injury and rheumatoid arthritis. Chemical modification of amino acids in protein during lipid peroxidation results in the forrnation of lipoxidation products which may serve as indicators of oxidative stress *in vivo*. The focus of the studies described here was initially to identify chemical modifications of protein derived exclusively from lipids in order to assess the role of lipid peroxidative damage in the pathogenesis of disease. Malondialdehye (MDA) and 4-hydroxynonenal (HNE) are well characterized oxidation products of polyunsaturated fatty acids on low-density lipoprotein (LDL) and adducts of these compounds have been detected by immunological means in atherosclerotic plaque. Thus, we first developed gas chromatography–mass spectrometry assays for the Schiff base adduct of MDA to lysine, the lysine–MDA–lysine diimine cross-link and the Michael addition product of HNE to lysine. Using these assays, we showed that the concentrations of all three compounds increased significantly in LDL during metal-catalysed oxidation *in vitro*. The concentration of the advanced glycation end-product N^{ε}-(carboxymethyl)lysine (CML) also increased during LDL oxidation, while that of its putative carbohydrate precursor the Amadori compound N^{ε}-(1-deoxyfructose-1-yl)lysine did not change, demonstrating that CML is a marker of both glycoxidation and lipoxidation reactions. These results suggest that MDA and HNE adducts to lysine residues should serve as biomarkers of lipid modification resulting from lipid peroxidation reactions, while CML may serve as a biomarker of general oxidative stress resulting from both carbohydrate and lipid oxidation reactions.

Key words: N^{ε}-(carboxymethyl)lysine; 4-hydroxynonenal; lipid peroxidation; malondialdehyde; metal-catalysed oxidation; oxidative stress; protein chemical modification

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Introduction

The purpose of this article is to review recent work in our laboratory on the identification of a group of compounds which we have named 'lipoxidation products'. The terminology derives from the nomenclature for glycoxidation products [1], which are chemical modifications of protein resulting from both glycation and oxidation reactions. Thus, lipoxidation products are lipid-derived chemical modifications of protein formed during lipid peroxidation reactions. The lipid components are formed from oxidized polyunsaturated fatty acids (PUFA) in triglycerides, glycerophospholipids and cholesterol esters. Prostaglandins and isoprostanes [2] may also be a source of lipoxidation products, but the quantitative role of the various classes of lipids *in vivo* has not been determined. Intermediates in the formation of lipoxidation products may include both peroxides [3] and aldehydes [4,5] formed during lipid peroxidation reactions. Although discrete products of the reaction of lipid peroxides with protein have not been identified, a variety of aldehydes formed during lipid peroxidation reactions are known to react to form Schiff base or Michael addition compounds with nucleophic groups in proteins. Our long-term goal is to identify and develop assays for these lipoxidation products in protein so that they can be measured as indicators of the status of oxidative stress in tissues. Several of these products, including malondialdehyde (MDA) and 4-hydroxynonenal (HNE) adducts to protein, have now been identified in our laboratory and are the focus of the following discussion.

Intermediates and mechanisms of formation of lipoxidation products

As shown in Table 1, several homologous families of aldehydes are formed on decomposition of lipid peroxides during autoxidation of polyunsaturated lipids, including alkanals and activated aldehydes with α-hydroxy, α,β-unsaturated, and 4-hydroxy-unsaturated functional groups [4–7]. These aldehydes vary in chain length, depending on the site of oxidation and the location of the double bonds in the starting lipid, ranging from 9-carbon compounds, such as HNE, to small 2- and 3-carbon reactive compounds, such as MDA, acrolein and glyoxal. Each of these compounds may react with amino groups in protein and even simple aldehydes, such as hexanal, are potent protein cross-linking reagents [8]. Figures 1 and 2 illustrate a variety of products that may be formed in these carbonyl-amine reactions. These compounds react not only with lysine residues but also with amino-terminal amino acids, and histidine and cysteine residues in proteins. Mechanisms of the reaction include Schiff base formation, Amadori and Cannizzaro rearrangements [9–11], and Michael addition reactions [12,13], as well as secondary oxidation reactions involving protein-bound intermediates. Because a broad spectrum of aldehydes are formed and may react with protein by multiple mechanisms at multiple sites, it is essential to focus on the measurement of specific lipid oxidation products in order to gain insight into the quantitative role of lipoxidation reactions in biological systems. These lipid oxidation products are likely to form in red cell membrane proteins, plasma and intracellular membranes of nucleated cells, and probably also in long-lived proteins, such as collagen, which are bathed in extracellular fluids. In addition, they may be carried on plasma proteins and appear in urine as degradation products of modified amino acids and lipids. Thus far, we have focused on the study of model systems, the metal-catalysed oxidation of human low-density lipoprotein (LDL) [14] and the oxidation of fatty acids in the presence of bovine pancreatic ribonuclease A (RNase) [15,16]. The LDL system provides a natural source of unsaturated fatty acids, while the RNase system allows studies on the oxidation of specific PUFA. The results of our work on the formation of MDA, HNE and N^{ε}-(carboxymethyl) (CML) adducts to these proteins are summarized below.

Table 1. Aldehyde products of oxidation of polyunsaturated fatty acids[a]

Class of compound	Example
Alkanal	*n*-hexanal
Alkenal	2,4-hexadienal, acrolein
α-Hydroxyalkenal	2-hydroxyheptanal
4-Hydroxyalkenal	4-hydroxynonenal
dicarbonyl compounds	malondialdehyde, glyoxal

[a]See references 4–7.

Malondialdehyde

MDA is one of the most readily assayed end-products of both enzymatic and non-enzymatic lipid peroxidation reactions. The measurement of MDA as an index of lipid peroxidation was introduced by Sato *et al.* [17] in the form of the thiobarbituric acid (TBA) assay. Later studies indicated that the colour yield in the TBA assay resulted from a number of lipid peroxidation products in addition to MDA [5]; thus the assay is now termed the TBARS assay (for TBA Reactive Substances). However, the specific TBA–MDA chromogen formed in the assay can be measured by reversed phase HPLC, providing a direct measure of MDA in plasma [18]. Because of its high reactivity, most MDA in plasma is protein-bound and the TBA–MDA assay actually measures the quantity of MDA released from plasma proteins under the acidic conditions of the assay.

Thus far, we have identified two adducts of MDA to protein (Figure 1) which are formed in both the LDL

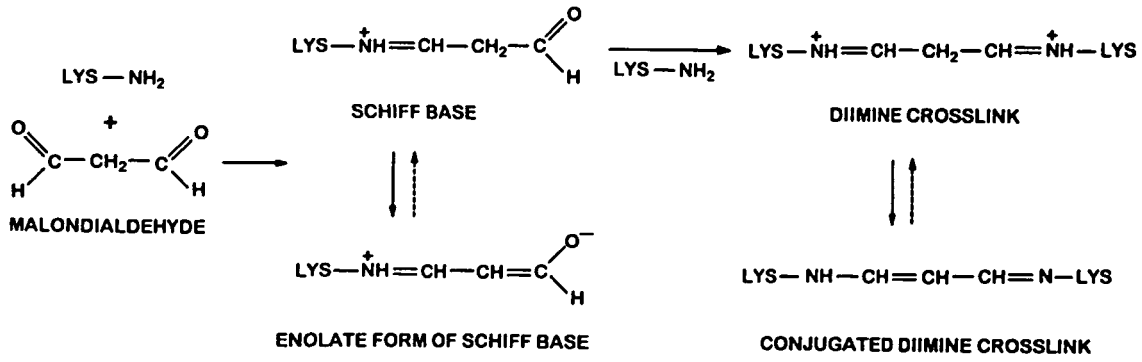

Fig. 1. Malondialdehyde adducts and cross-links with lysine residues in protein. MDA reacts with ε-amino groups of lysine (LYS) residues in protein to form a Schiff base adduct, which is stabilized by equilibration to an enolate. MDA may also react with a second lysine residue to form a diimine cross-link, which is probably also stabilized by conjugation of double bonds.

and RNase systems: (i) the MDA Schiff base adduct to the N^{ε}-amino group of lysine residues in protein; and (ii) the bis-Schiff base diimine cross-link formed by reaction of MDA with two lysine residues in protein, resulting in either intra- or intermolecular cross-linking of the protein. Both of these compounds have been described previously in model organic synthetic systems [19], but they have not yet been identified in proteins. We recently identified these adducts after reduction of MDA-modified protein with $NaBH_4$. Since the Schiff base adducts are labile to the acidic conditions needed to release amino acids from protein, they are first stabilized by reduction. The Schiff base adduct of MDA to lysine is unusually resistant to reduction by $NaBH_4$, suggesting that it is stabilized in the form of the enolate salt of the Schiff base adduct shown in Figure 1. Similarly, the lysine–MDA–lysine adduct is also naturally stabilized by conjugation of double bonds and by the apposition of lysine amino groups which are cross-linked in the protein. While aliphatic Schiff bases are not normally stable compounds, both the MDA Schiff base to lysine and the diimine cross-link are stable to dialysis of protein at 4°C and pH 7.4, consistent with resonance stabilization, and suggesting that these adducts may have significant half-lives on tissue proteins. The reduced form of the MDA adduct, 3-(N^{ε}-lysino)propanol (LM), and of the cross-link 1,3- bis-(N^{ε}-lysino)propane (LML), were first isolated from model reactions of poly-L-lysine and MDA, and their structures confirmed by chemical-ion mass spectrometry of the trifluoroacetyl methyl ester (TFAME) derivatives. Following development of gas chromatography–mass spectrometry (GC/MS) assays for LM and LML we measured these compounds in native and oxidized human LDL (J. R. Requena *et al.*, in preparation).

Table 2 summarizes preliminary results of analyses of the LM and LML content of LDL, before and after copper-catalysed oxidation. It is not clear at present whether the small amounts of LM and LML found in freshly isolated LDL are formed by oxidation of PUFA on the LDL itself or if LDL (and other plasma proteins) is serving as a carrier of MDA released into plasma from tissue metabolic or autoxidative processes. Since MDA is such a potent protein cross-linking agent [16,19], it is possible that the ageing of lipoxidized proteins may lead to the gradual conversion of LM adducts to LML cross-links in protein. We have not yet studied the effects of atherosclerosis, diabetes mellitus and renal failure on LM and LML in plasma proteins or in proteins other than LDL, but our results suggest that these compounds may be steady-state indices of ambient oxidative stress, i.e. they may be slowly reversible adducts under physiological conditions and their levels on plasma proteins may vary with the recent rate of lipid peroxidation reactions. Differences in the distribution of protein-bound MDA between LM and LML may also be important for understanding the effects of lipoxidation reactions on the cross-linking of protein during oxidative stress. N^{α}-Acetyl-N^{ε}-(2-propenal)lysine has been measured in rat urine [20], and the measurement of urinary LM and LML may also provide insight into systemic oxidative stress and whole body formation of lipoxidation products.

Table 2. Concentration (mmol/mol lysine) of lipoxidation products and fructoselysine in native and copper-oxidized LDL[a]

Type of LDL	LM[b]	LML[b]	LHNE[b]	CML	FL
Native	0.05	0.002	<0.001	0.03	0.88
Oxidized	0.32	0.12	0.41	0.68	0.88

[a]Data are based on analysis of oxidation of two separate pools of LDL, each prepared from three healthy donors.

[b]Preliminary data obtained after reduction of LDL using 25 mM $NaBH_4$, which may not give quantitative recovery of all of these lipoxidation products.

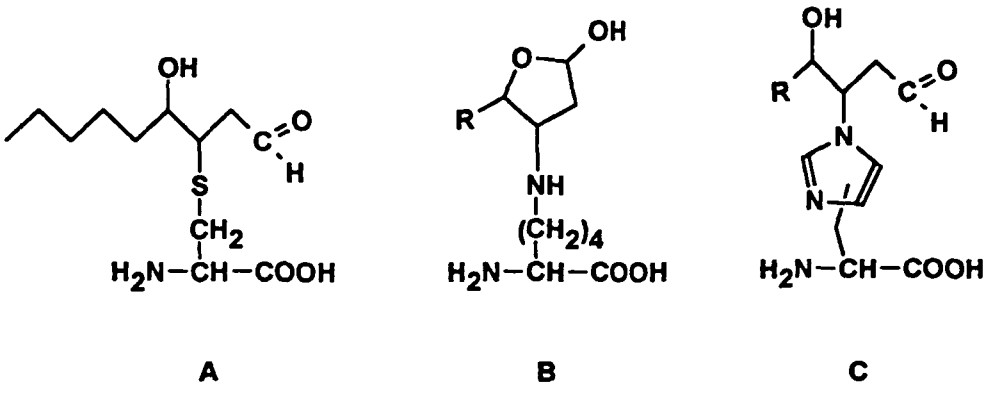

Fig. 2. Products of Michael addition reactions of HNE with (A) cysteine, (B) lysine and (C) histidine residues in protein. The aldehyde functional group remaining after the Michael addition may form a Schiff base with adjacent amino groups, yielding intra- or intermolecular cross-links in protein. The adducts to cysteine and histidine are shown in the reactive, open-chain aldehdye conformation, and the adduct to lysine in the cyclic hemiacetal conformation. $R = CH_3(CH_2)_4$-.

HNE

HNE is a reactive, polar lipid which is cytotoxic to cells in culture [21]. Stadtman and colleagues [12,13] have shown that HNE reacts with protein by both Schiff base formation with amino groups and by Michael addition reactions with cysteine, histidine and lysine residues (Figure 2). Like MDA, HNE can cause cross-linking of protein, but a variety of adducts and cross-links may be formed, for example, by Michael addition to cysteine, histidine or lysine, followed by Schiff base formation with an amino group. HNE adducts to protein have been detected in model systems by both chemical and immunological methods [12,13], but have not yet been measured quantitatively in tissue proteins. We have recently developed a GC/MS assay for the reduced form of the Michael adduct of HNE to lysine (LHNE) (Ahmed *et al.*, in preparation) and have observed trace amounts of LHNE in LDL isolated from healthy volunteers (Table 2). As with MDA adducts, the terminal aldehyde group of HNE must first be reduced with $NaBH_4$ to the alcohol product to stabilize LHNE to hydrolysis of the protein. As shown in Table 2, LHNE also increases during metal-catalysed oxidation of LDL. Unlike the Schiff base linkages in LM and LML, the Michael adduct formed between HNE and lysine is a chemically stable product and a permanent chemical modification of protein. Thus, measurements of LHNE may provide an index of long-term damage to proteins as a result of lipid peroxidation reactions. Because cysteine and histidine residues are strong nucleophiles in protein, there are likely to be higher levels of HNE adducts to these amino acids than to lysine in oxidized LDL [13] and tissue proteins. Thus, we are continuing our studies to identify other products of reaction between HNE and protein.

N^{ε}-(Carboxymethyl)lysine as a lipoxidation product

Oxidation of lipoproteins has been implicated in the pathogenesis of atherosclerosis [22] and diabetic microangiopathy [23]. Evidence that the extent of glycation of lipoproteins is increased by hyperglycaemia in diabetes [24], that Amadori adducts on glycated proteins are a source of reactive oxygen species [23] and oxidative stress, and that glycation enhances the susceptibility of lipoproteins to oxidation [25] suggests a mechanistic link between glycation, lipoprotein oxidation and vascular disease in diabetes. For this reason, we set out to study the effect of glycation on formation of the lipoxidation products LM, LML and LHNE during metal-catalysed oxidation of lipoproteins. To assess the role of the Amadori adduct fructoselysine (FL) as an initiator of peroxidation and potential source of lipoxidation products, we also measured the FL content of the protein and the formation of CML, a glycoxidation product formed on oxidation of FL [1,26]. We observed the expected increases in LM, LML and LHNE during oxidation of LDL (Table 2) and also observed an increase in the CML content of the protein during the oxidation reaction. Surprisingly, however, the FL content of the LDL remained constant (Table 2). We concluded tentatively that CML, which is known as an advanced glycation end-product (AGE) and glycoxidation product [1], was being formed during lipid peroxidation reactions, presumably from products of lipid peroxidation rather than from carbohydrates. In experiments to be reported elsewhere (M.-X. Fu *et al.*, submitted for publication), we have established that the reduction of LDL by $NaBH_4$, which converts FL to the inert N^{ε}-(hexitol)lysine derivative, does not affect the yield of CML during oxidation of LDL. Further, CML is also formed during peroxidation of arachidonate and linoleate in the presence of RNase, a protein which contains neither enzymatically nor non-enzymatically attached carbohydrate. We have concluded from these studies that CML is both a glycoxidation and lipoxidation product, and that it may be useful as a general biomarker of oxidative stress in biological systems.

The mechanism of formation of CML is uncertain, but as shown in Figure 3, it may be formed from carbohydrates by a variety of mechanisms, including autoxidation of glucose [10] or other carbohydrates to glyoxal, followed by reaction of glyoxal with protein, or by oxidative cleavage of carbohydrate adducts to protein, including carbinolamine, Schiff base and Amadori adducts. Similarly, the formation of CML during lipid peroxidation may proceed by multiple mechanisms and intermediates, including glyoxal and α-hydroxyaldehydes, which Spiteller and colleagues [6,7] have identified as products of lipid peroxidation. We observed inhibition of CML formation in RNase in lipoxidation reactions by aminoguanidine concomitant with isolation of 3-aminotriazine, the product of reaction of aminoguanidine with glyoxal. However, glyoxal may not be the only source of CML during lipid peroxidation reactions. Other common intermediates may be formed from both carbohydrates

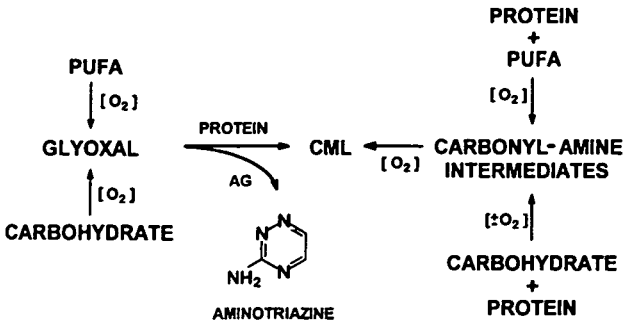

Fig. 3. Proposed routes to formation of CML on protein during glycoxidation and lipoxidation reactions. Glyoxal formed by autoxidation of carbohydrate or PUFA can react directly with lysine residues in protein to form CML. Aminoguanidine (AG), acting as a dicarbonyl trap, can intercept glyoxal, limiting the formation of CML. CML could also be formed by oxidative cleavage of other carbonyl-amine adducts formed during carbohydrate or lipid oxidation.

and lipids during autoxidation reactions, such as α-hydroxyaldehydes or β,γ-unsaturated α-hydroxyaldehydes, which may also yield CML on reaction with lysine under oxidative conditions. Considering the ease of oxidation of PUFAs compared with glucose, it seems likely that a significant fraction of CML, as well as other AGEs and glycoxidation products, detected in biological systems may be derived from lipid, rather than carbohydrate, autoxidation reactions. Indeed, the recent observation of Palinski *et al.* [27] that AGEs are detected immunohistochemically in atheroma of atherosclerotic but normoglycaemic rabbits suggests that the AGEs which accumulate in atheroma may, in fact, be derived from lipid peroxidation reactions.

Conclusion

Although Maillard's original studies focused on browning reactions in model systems containing carbohydrates and amines, the scope of the Maillard or browning reaction now includes reactions of both carbohydrates and lipids with proteins. The formation of reactive carbonyl compounds and their reaction with amines, i.e. carbonyl-amine chemistry, is the central feature relating the browning reactions of lipids and proteins. Carbohydrates are already partially oxidized compared with lipids. Browning reactions of smaller carbohydrates, such as tetroses and trioses, proceed under antioxidative conditions, while browning by hexoses and ascorbate is greatly accelerated under oxidative conditions. In contrast, browning of proteins by lipids is, to the best of our knowledge, absolutely dependent on autoxidation reactions or redox chemistry involving the formation of radical intermediates and the insertion of oxygen. The focus of the studies described here was initially on the identification of chemical modifications of protein derived exclusively from lipids in order to assess the role of lipid peroxidative damage in the pathogenesis of disease. The work began with development of GC/MS assays for LM, LML and LHNE, which could be predicted based on the current understanding of lipid peroxidation reactions and carbonyl-amine chemistry. We learned, however, that one product, CML, an AGE and glycoxidation product, was also formed during lipid peroxidation reactions. These results emphasize the similarity in intermediates formed on oxidation of carbohydrates and lipids in biological systems. It seems likely that other common products will be identified in glycoxidation and lipoxidation reactions and that these may include brown and fluorescent compounds which accumulate in tissue proteins during ageing and diabetes. Through the measurement of unique products of lipoxidation reactions (such as LM, LML and LHNE) and glycoxidation reactions (such as pentosidine [28]), along with products common to these two pathways (such as CML), it may become possible to assess the independent and common roles of carbohydrate and lipid autoxidation in the chemical modification of proteins in ageing and disease.

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