Quantification of malondialdehyde and 4-hydroxynonenal adducts to lysine residues in native and oxidized human low-density lipoprotein

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Malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are major end-products of oxidation of polyunsaturated fatty acids, and are frequently measured as indicators of lipid peroxidation and oxidative stress *in vivo*. MDA forms Schiff-base adducts with lysine residues and cross-links proteins *in vitro*; HNE also reacts with lysines, primarily via a Michael addition reaction. We have developed methods using NaBH₄ reduction to stabilize these adducts to conditions used for acid hydrolysis of protein, and have prepared reduced forms of lysine-MDA [3-(N^ε-lysino)propan-1-ol (LM)], the lysine-MDA-lysine iminopropene cross-link [1,3-di(N^ε-lysino)propane (LML)] and lysine-HNE [3-(N^ε-lysino)-4-hydroxynonan-1-ol (LHNE)]. Gas chromatography/MS assays have been developed for quantification of the reduced compounds in protein. RNase incubated with MDA or HNE was used as a model for quantification of the adducts by gas chromatography/MS. There was excellent agreement

between measurement of MDA bound to RNase as LM and LML, and as thiobarbituric acid-MDA adducts measured by HPLC; these adducts accounted for 70–80 % of total lysine loss during the reaction with MDA. LM and LML (0.002–0.12 mmol/mol of lysine) were also found in freshly isolated low-density lipoprotein (LDL) from healthy subjects. LHNE was measured in RNase treated with HNE, but was not detectable in native LDL. LM, LML and LHNE increased in concert with the formation of conjugated dienes during the copper-catalysed oxidation of LDL, but accounted for modification of < 1 % of lysine residues in oxidized LDL. These results are the first report of direct chemical measurement of MDA and HNE adducts to lysine residues in LDL. LM, LML and LHNE should be useful as biomarkers of lipid peroxidative modification of protein and of oxidative stress *in vitro* and *in vivo*.

INTRODUCTION

Oxidative modification of low-density lipoprotein (LDL) *in vitro*, by either added metal ions or cells in culture, results in stimulation of LDL uptake by the macrophage scavenger receptor, leading to the intracellular accumulation of lipid [1,2]. Lipid-laden macrophages, or foam cells, are the hallmark of early atherosclerosis, and a current paradigm for atherogenesis invokes oxidized LDL as a major source of the lipid accumulating in these cells. LDL oxidized *in vitro* displays increased electrophoretic mobility and an increased content of lipid peroxides and thiobarbituric acid-reactive substances (TBARS). Oxidized LDL also contains novel epitopes which show reactivity with antibodies prepared against malondialdehyde (MDA)- and 4-hydroxynonenal (HNE)-modified proteins [3], and increased concentrations of MDA-modified LDL have recently been described in the plasma of patients with atherosclerosis [4]. LDL with many of the chemical, physical and immunological properties of oxidized LDL has been isolated from human atherosclerotic lesions [1,2,5], and autoantibodies to oxidized LDL are elevated in the sera of patients with both peripheral atherosclerosis and coronary artery disease [6–8]. Taken together, these observations support a role for modification of LDL protein by products of lipid peroxidation in the underlying pathogenesis of vascular disease.

Oxidation of polyunsaturated fatty acids (PUFA) in lipoproteins leads to formation of hydro- and endo-peroxides, which undergo fragmentation to yield a broad range of reactive

intermediates, including alkanals, alkenals, hydroxyalkenals and MDA [7]. These carbonyl compounds, and possibly their peroxide precursors [9,10], react with nucleophilic groups in proteins, resulting in chemical modification of the protein. MDA and HNE are each estimated to account for about 20 % of the aldehydes formed during *in vitro* oxidation of LDL [7], and both react readily with lysine residues in protein. The uptake of LDL by its normal receptor is critically dependent on free lysine amino groups in the protein. Thus the uptake of LDL, as well as of other proteins, by the scavenger receptor on macrophages can be effected solely by modification of lysine residues by products of PUFA oxidation [7,9,11]. Many of the potentially deleterious effects of MDA and HNE result, therefore, from their ability to react with protein amino groups, causing structural modification and cross-linking, and to generate new epitopes, which in turn may trigger immunological responses and/or altered protein recognition and uptake via scavenger receptors.

Present methods for assessing protein damage resulting from peroxidation reactions are in general indirect, such as the measurement of TBARS (MDA equivalents) or formation of lipid hydroperoxides in plasma or tissues [12,13]. There are methodological problems with the TBA assay, including lack of specificity and generation of artifactual TBARS under various assay conditions [14]. More importantly, these measurements can be affected by changes in prostaglandin or leukotriene metabolites and they provide, in any case, no information on actual damage to proteins resulting from lipoxidation reactions.

Abbreviations used: CML, N^ε-(carboxymethyl)lysine; fLys, N^ε-(formyl)lysine; GC/MS, gas chromatography/MS; HNE, 4-hydroxynonenal; LDL, low-density lipoprotein; LHNE, 3-(N^ε-lysino)-4-hydroxynonan-1-ol; LM, 3-(N^ε-lysino)propan-1-ol; LMCl, 3-(N^ε-lysino)-1-chloropropane; LML, 1,3-di(N^ε-lysino)propane; MDA, malondialdehyde; PUFA, polyunsaturated fatty acids; TBA, thiobarbituric acid; TBA-MDA, TBA-MDA adduct; TBARS, TBA-reactive substances; TNBS, trinitrobenzenesulphonic acid.

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Antibodies are available for measuring MDA and HNE epitopes on plasma and tissue proteins, but these assays provide only qualitative information on the extent of protein modification by lipoxidation products. The measurement of changes in circulating and tissue isoprostanes [15] provides an alternative means of detecting products of non-enzymic lipid peroxidation reactions, but again does not measure direct protein modification. Thus only partial information has been obtained on the chemical nature and quantitative extent of protein modification by products of PUFA oxidation.

The aim of the present work was to measure MDA- and HNE-lysine adducts on protein as indicators of direct modification by products of lipid oxidation. We have developed, therefore, a sensitive and specific gas chromatography/MS (GC/MS) assay for the quantification of lysine-MDA, lysine-MDA-lysine and lysine-HNE adducts in protein. The compounds that have been prepared and characterized are the NaBH_4 -reduced derivatives, 3-(N^ϵ -lysino)propan-1-ol (LM), 1,3-di(N^ϵ -lysino)propane (LML) and 3-(N^ϵ -lysino)-4-hydroxynonan-1-ol (LHNE). We report here the measurement of these adducts in a variety of samples prepared from *in vitro* and *in vivo* sources, including native and oxidized LDL.

EXPERIMENTAL

Materials

Poly(L-lysine) (M_r 27000; degree of polymerization 120), bovine pancreatic RNase A (type XII-A), N^ϵ -(methyl)lysine, NaBH_4 , Dowex 50-X2, Bio-Gel P-2 and Sephadex G-75 were purchased from Sigma. MDA dimethylacetal and NaBH_3CN were from Aldrich. N^ϵ -(Formyl)lysine (fLys) was synthesized using either lysine or deuterated lysine ([4,4,5,5- $^2\text{H}_4$]lysine and [3,3,4,4,5,5,6,6- $^2\text{H}_8$]lysine) (MSD Isotopes, Rahway, NJ, U.S.A.) as described previously [16]. HNE was synthesized by the procedure of de Montarby et al. [17] and stored in chloroform under N_2 at -20°C .

Synthesis and isolation of lysine-MDA adducts

MDA was prepared immediately before use by hydrolysis of the dimethylacetal with 1 M HCl for 30 min at 37°C , and quantified by absorbance at 245 nm using $\epsilon = 13700 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [14]. For preparation of LM, fLys (or formyl[$^2\text{H}_8$]lysine) (10 mM) was incubated with an equimolar amount of MDA and an 8-fold excess of NaBH_3CN in 0.2 M phosphate buffer, pH 7.4, overnight at 37°C . The reaction was terminated by raising the pH to 9 using 0.2 M borate buffer, pH 9.2, and adding a 10-fold molar excess of NaBH_4 to reduce any remaining aldehyde. Reduction was carried out overnight at 25°C , followed by deformylation of the product in 2 M HCl at 95°C for 30 min. After drying *in vacuo* (Speed-Vac; Savant, Farmingdale, NY, U.S.A.), the sample was applied to a 3 ml Dowex 50-H⁺cation-exchange column, which was first washed with water to remove salts and then eluted with 0.5 M NH_4OH to remove lysine and derivatives. LM was finally purified by HPLC cation-exchange chromatography using an amino acid analyser (see below).

For large-scale preparation of LM, LML and [$^2\text{H}_8$]LML, poly(L-lysine) (2.2 mg/ml; 2 ml total) was incubated with MDA (lysine/MDA, 1:1, mol/mol) as described for fLys-MDA reactions, except that NaBH_3CN reduction was omitted. The reaction mixture was reduced with NaBH_4 or NaBH_3CN , dialysed against water and dried *in vacuo*. For isolation of LM and LML, the reduced poly(L-lysine)-MDA hydrolysates (see below) were fractionated on a 1 cm $\times$ 83 cm Bio-Gel P-2 column at 4°C using ethanol/water (1:9, v/v) for elution. Fractions of 0.6 ml were

collected and analysed using the trinitrobenzenesulphonic acid (TNBS) assay for primary amino groups [18]. Amine-containing fractions were analysed by amino acid analysis and fractions containing LM or LML were pooled and finally purified from the HPLC amino acid analysis column and desalted as described above.

Synthesis and isolation of LHNE

The HNE concentration in aqueous samples was estimated by absorbance at 223 nm using $\epsilon = 13750 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [19]. Immediately prior to use, an aliquot of HNE in chloroform was dried under N_2 and then applied to a 3 ml C_{18} column (Sep-Pak; Supelco, Bellefonte, PA, U.S.A.) equilibrated with water. The column was washed with 15 ml of water, followed by elution of HNE with 15 ml of 10% (v/v) acetonitrile. The eluates were pooled and extracted twice with chloroform; the chloroform extracts were dried under N_2 and dissolved in phosphate buffer. Equimolar (10 mM) fLys (or formyl[$^2\text{H}_4$]lysine) and HNE were incubated overnight at 37°C in 0.2 M phosphate buffer, pH 7.4. The sample was made 0.1 M in borate, pH 9.0, and was reduced with a final concentration of 100 mM NaBH_4 overnight at room temperature, followed by hydrolysis in 6 M HCl at 110°C for 24 h. After drying, the reaction mixture was dissolved in water and applied to a Dowex 50-H⁺ column; the column was washed with water to remove salts and then eluted with 0.5 M NH_4OH . The base eluate was dried, redissolved in 0.1% heptafluorobutyric acid and applied to a 3 ml Sep-Pak column equilibrated in the same buffer. The column was eluted with 15 ml of 0.1% heptafluorobutyric acid, which removed unreacted lysine, followed by elution with 50% (v/v) acetonitrile, which yielded essentially pure LHNE.

Preparation of MDA- and HNE-modified RNase

RNase (20 mg/ml) in 0.2 M phosphate buffer, pH 7.4, was incubated with MDA (lysine/MDA, 2:1, mol/mol) at 37°C overnight, and the reaction mixture was then dialysed against deionized water to remove unreacted MDA. For preparation of RNase monomer and polymer, an aliquot (14 mg) of the dialysed mixture was fractionated on a 1.5 cm $\times$ 75 cm Sephadex G-75 column at 4°C using PBS, pH 7.4 [20], as the eluting buffer. Fractions (1.7 ml) were collected and monitored for absorbance at 280 nm. Monomer and polymer fractions were pooled, dialysed against water and concentrated *in vacuo*.

Reduction of MDA-modified RNase was carried out using various amounts of NaBH_4 (noted in legends to Figures) in 0.1 M borate buffer, pH 9.2, containing 1 mM diethylenetriaminepenta-acetic acid and 1–2 drops of hexanol as an anti-foam reagent. Reduction was conducted for 4 h at room temperature and then overnight at 4°C . Reduced samples were dialysed against deionized water, using 6000–8000- M_r -cut-off dialysis tubing; the final reduced protein was essentially colourless. RNase (10.3 mg/ml; 7.5 mM lysine) and HNE (1.5 or 0.75 mM) were incubated and reduced as described for RNase-MDA. These ratios of HNE to RNase were chosen to avoid precipitation of the protein and resulted in only trace amounts of cross-linked protein, as judged by SDS/PAGE under reducing conditions (results not shown).

Preparation and oxidation of LDL

Blood was collected from healthy volunteers following a 12 h fast. Plasma was obtained by centrifugation at 4°C and LDL was isolated by single vertical-spin centrifugation as previously described [21]. Experiments were carried out on LDL pooled

from three individuals. Each pool was judged to be pure based on agarose-gel electrophoresis using a Paragon Electrophoresis System (Beckman Instruments). LDL was stored at 4 °C under nitrogen and used within 24 h of preparation. Oxidation studies were carried out at 32 °C on LDL diluted to ~ 200 µg/ml in PBS and made 5 µM in copper, as described [21]; copper was omitted from control samples. Aliquots (~ 1 mg of protein) were removed at the desired times and the pH was adjusted to 9.2 with 0.1 vol. of 1 M borate buffer, pH 9.2. Diethylenetriaminepenta-acetic acid and NaBH₄ were added to final concentrations of 1 mM and 1 M respectively, and reduction was carried out as described above for MDA-RNase. Reduced and dialysed protein was dried *in vacuo*, 1 ml of water was added and the sample was delipidated using methanol/diethyl ether (3:1, v/v) [22]; precipitated protein was dried under a stream of nitrogen.

Protein hydrolysis, amino acid and GC/MS analyses

Isotopically labelled internal standards ([²H₄]lysine, [²H₈]LM, [²H₂]LML and [²H₄]LHNE) were added to protein samples prior to acid hydrolysis. Samples of 1–2 mg of protein/ml were hydrolysed at 110 °C for 24 h in 6 M HCl, then dried *in vacuo*. Amino acid analysis of protein hydrolysates was carried out by cation-exchange chromatography [23]. The colour factor for *N*-(methyl)lysine was used for quantification of LM and LHNE, and twice this value for estimation of LML.

GC/MS analyses were carried out on a Hewlett–Packard model 6890 gas chromatograph equipped with a 30 m HP-5MS capillary column coupled to a Hewlett–Packard model 6890 mass selective detector. The injection port was maintained at 300 °C; the temperature programme was 2 min at 150 °C, then 5 °C/min to 225 °C, then 25 °C/min to 300 °C, and finally hold at 300 °C for 5 min. Hydrolysed samples were derivatized as their *N,O*-trifluoroacetyl methyl esters for GC/MS analyses [24]. Quantification was by external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analyses were carried out by selected ion monitoring GC/MS, monitoring two ions for each compound, and similar results were obtained using both ions. The ions used were: lysine and [²H₄]lysine, *m/z* 180, 320 and 184, 324 respectively; LM and [²H₈]LM, *m/z* 333, 474 and 340, 482 respectively; LML and [²H₂]LML, *m/z* 393, 518 and 395, 520 respectively; LHNE and [²H₄]LHNE, *m/z* 295, 323 and 299, 327 respectively.

Because of the low levels of LML in native and oxidized LDL, analyses of LML in LDL were carried out on a Hewlett–Packard model 5890 gas chromatograph equipped with a 30 m RTX 5 (5% phenyl) (Restek Corp., Bellefonte, PA, U.S.A.) coupled to a VG 70 SQ magnetic sector mass spectrometer (Fisons, Manchester, U.K.). The injection port was maintained at 90 °C; the temperature programme was 10 °C/min to 140 °C, then 4.5 °C/min to 220 °C, then 10 °C/min to 300 °C, and finally hold for 10 min. Quantification of LML was based *m/z* 518, 675 and 520, 677 for LML and [²H₂]LML respectively.

Other analytical procedures

The TBA assay for MDA (TBA-MDA) was carried out using a modification of an HPLC assay for MDA in plasma [25]. Briefly, samples (~ 25 µg of protein) were placed in screw-cap tubes, mixed with 250 µl each of 1.22 M phosphoric acid and 0.23 % TBA, and diluted to 1 ml with deionized water. After heating at 100 °C for 1 h, the samples were cooled and extracted with an equal volume of *n*-butanol. An aliquot (0.8 ml) of the organic layer was evaporated *in vacuo*. Samples were dissolved in 25 mM phosphate buffer, pH 6.4, containing 10 % methanol, and

analysed by HPLC on a 4.6 mm × 150 mm Vydac C-18 reverse-phase column (The Separations Group, Hesperia, CA, U.S.A.) using a linear gradient from 10 to 90 % methanol in 25 mM phosphate buffer, pH 6.4, over 10 min; the TBA-MDA adduct was detected fluorimetrically (λ_{ex} = 532 nm, λ_{em} = 553 nm) and quantified by comparison with a standard curve prepared by reacting MDA dimethylacetal with TBA as described above.

Protein was measured by the Lowry procedure [26].

RESULTS

Characterization of lysine adducts by amino acid analysis and GC/MS

Since lysine-MDA Schiff-base adducts are labile to even mild acid hydrolysis, it is necessary to first stabilize them by reduction. Reduction of fLys-MDA reactions resulted in the formation of a single major new product, which was eventually identified as LM (results not shown). In contrast, incubation of poly(L-lysine) with MDA resulted in the formation of several products that were detected by both GC/MS and amino acid analysis (Figure 1A). The full-scan spectra and structures of the derivatized compounds identified as LM and LML are shown in Figures 2(A) and 2(B) respectively; LMCl [3-(*N*^ε-lysino)-1-chloropropane] is an artifact of protein hydrolysis in HCl (see below). Two other compounds, eluting at about 7.5–8.0 min on the GC column, were also noted. These compounds were identified as 3-(*N*^ε-lysino)-1-propene and 3-(*N*^ε-lysino)propane respectively, based on electron-impact and chemical-ion MS (results not shown). In initial experiments these compounds were detected only when exogenous MDA was added to proteins *in vitro*, and were not found in native or oxidized LDL, and thus will not be described further.

Both Schiff-base and Michael addition adducts of HNE to lysine residues are labile to acid hydrolysis, so that incubation mixtures of fLys and HNE were also reduced with borohydride prior to analysis. The total ion current and amino acid chromatograms of a 1:1 fLys/HNE reaction mixture are shown in Figure 1(B). The major compounds recovered were lysine and a late-eluting peak identified by GC/MS as LHNE (Figure 2C). No compound consistent with the predicted structure of the reduced Schiff-base adduct of HNE to lysine was detected by GC/MS.

Routes to the formation of LM, LMCl and LML, as well as resonance forms of unreduced MDA-lysine adducts, are shown in Scheme 1. LM is the expected reduced form of the imino-propene (Schiff base) formed between MDA and lysine. LMCl is a substitution artifact resulting from protein hydrolysis in HCl at 110 °C. Thus, when purified LM was hydrolysed under standard hydrolysis conditions (6 M HCl, 110 °C, 24 h), LMCl appeared in the amino acid chromatogram; however, when fLys/MDA reaction mixtures were deformedylated by heating at 95 °C for 30 min in 2 M HCl, no LMCl was formed (results not shown). LML, the reduced form of the aminoiminopropene cross-link between MDA and two lysine residues, was produced in incubations of poly(L-lysine) or RNase with MDA, but was not formed in incubations of fLys with MDA.

Scheme 2 shows the route to the formation of the Michael addition product between lysine and HNE. While HNE has the potential to react via either Schiff-base or Michael addition reactions, no compound corresponding to the Schiff-base adduct was detected under the reaction conditions used in these experiments, consistent with the results reported by Szweda et al. [27]. Although the predicted structure of the reduced Michael addition product is a linear compound, the mass spectrum of the final derivatized product is consistent with the cyclic structure shown

Figure 1 Total-ion chromatograms of reaction mixtures used to prepare MDA- and HNE-lysine adducts *in vitro*

(A) Poly(L-lysine) was incubated with MDA, reduced with NaBH_4 , hydrolysed and analysed by GC/MS as described in the Experimental section. Identification of LM, LMCI and LML was based on isolation of pure compounds by HPLC (inset shows amino acid chromatogram) and structural characterization by GC/MS. (B) HNE and fLys were incubated, reduced with borohydride and analysed by GC/MS as described in the Experimental section (inset shows amino acid chromatogram). LHNE was identified following chromatographic purification from the reaction mixture, followed by structural characterization by GC/MS. Only lysine was detected in the starting poly(L-lysine) or fLys, and in incubations not reduced with NaBH_4 (not shown).

in Scheme 2. Anhydro-LHNE is formed following dehydration of LHNE under the acidic conditions used for protein hydrolysis.

Stabilization of MDA and HNE adducts in RNase

In initial experiments, MDA adducts to RNase were reduced by treatment with 25 mM NaBH_4 . However, as shown in Table 1, these conditions resulted in the loss of only about 30 % of total TBA-MDA, i.e. the majority of MDA on the protein was still released under the mild acid conditions used in the TBA assay,

Figure 2 Full-scan spectra of MDA- and HNE-lysine derivatives

Spectra and structures of *N,O*-trifluoroacetyl methyl ester derivatives of LM, LML and LHNE are shown in (A), (B) and (C) respectively. The molecular ion of each compound (LM, m/z 506; LML, m/z 744; LHNE, m/z 492) was confirmed by chemical-ionization MS. Assignments for

Scheme 1 Formation of lysine-MDA adducts in protein

MDA is a dicarbonyl compound which can form both mono- and di-Schiff-base adducts with lysine residues in protein [28–30], as well as enolate anions and other resonance structures. Protein is reduced with NaBH_4 to stabilize these adducts to acid hydrolysis. LMCI is a substitution product formed from LM during acid hydrolysis in 6 M HCl. The wavy line represents the protein chain. For convenience, all amine and imine structures are shown in unprotonated form.

and only 10–15% of maximal LM and LML were recoverable by GC/MS analysis. Table 1 also shows that, with increasing concentrations of NaBH_4 up to 1 M, there was a decreasing yield of TBA-MDA and increasing yields of LM and LML. A variety of other reductants and reduction conditions, including NaBH_3CN , dimethylamineborane and Raney Nickel, each at pH 4–9, were also tested, but none was as effective as 1 M borohydride at pH 9.2. The resistance of MDA-lysine Schiff bases to reduction by borohydride is likely to result from equilibration of LM to the enolate form and from resonance stabilization of LML, as shown in Scheme 1 [28–30]. The yield of LHNE in RNase was less sensitive to the excess of borohydride used, and the LHNE content in RNase was essentially unchanged when the reductant concentration was increased from 25 mM to 0.5 M borohydride (see below).

Quantification of lysine adducts in RNase modified *in vitro*

RNase monomer and polymer fractions were isolated from incubations of RNase with MDA, in order to validate the GC/MS assay for lysine-MDA adducts. When RNase incubated

with MDA (lysine/MDA, 2:1; not reduced with NaBH_4) was applied to a Sephadex G-75 column, fractions corresponding to RNase monomer and to higher- M_r polymers were observed (Figure 3), in agreement with the results of Chio and Tappel [29]. Approx. 70% of the absorbance at 280 nm was recovered in fractions corresponding to higher- M_r species of RNase; however, because modification of proteins with MDA results in an increase in both absorbance and fluorescence [29,30], the pools were also analysed using the Lowry method [26]. Based on this method, only about 30% of the RNase was actually in the higher- M_r region of the column, while total protein recovery from the column was around 100%. The stability of the MDA adducts to protein is emphasized by the observation that the recovery of total LM and LML in the isolated fractions, after chromatography of the unreduced reaction mixture, was nearly identical to that measured in the unfractionated mixture (see Table 2). Furthermore, SDS/PAGE analysis of borohydride-reduced and non-reduced aliquots of the total reaction mixture showed approximately equal amounts of monomer and polymer bands (results not shown).

Table 2 compares the yields of modified amino acids in the RNase monomer and polymer fractions using several different analytical techniques. Amino acid analysis of total, monomer and polymer fractions showed a decrease of about 2.1–2.6 mol of lysine per mol of RNase, whereas the loss of primary amino groups, based on the TNBS assay, was about 3.5–4.0 mol/mol of RNase. The greater loss detected by TNBS may have resulted

ions used for quantification are as follows. LM: m/z 333 ($M^+ - \text{OCOCF}_3 - \text{COOCH}_3$); m/z 474 ($M^+ - \text{CH}_3\text{OH}$). LML: m/z 393 [$M^+ - \text{CH}_3\text{OOC}(\text{CH})(\text{CH}_2)_4\text{NOCOCF}_3$]; m/z 518 [$M^+ - \text{CF}_3\text{CONH}(\text{CH})(\text{CH}_2)_3\text{COOCH}_3$]; m/z 675 ($M^+ - \text{CF}_3$). LHNE: m/z 295 ($M^+ - \text{COCF}_3 - \text{OCH}_3 - \text{CF}_3$); m/z 323 [$M^+ - \text{CH}_3(\text{CH}_2)_4 - \text{COCF}_3 - \text{H}^+$].

Scheme 2 Formation of a Michael addition product between lysine and HNE

The initial Michael adduct, containing a terminal aldehyde residue at C-1, yields LHNE after reduction with NaBH_4 . Following acid hydrolysis, LHNE is measured as its anhydro derivative (see Figure 2C).

from acid-labile lysine adducts which were not detected by differences in amino acid analysis. The total lysine modification in the unfractionated reaction mixture was 1.54 mol of lysine/mol of RNase, based on the sum of that in LM and LML (1.14 mol of lysine in LM plus 0.4 mol of lysine in the LML cross-link). The corresponding values for total modification in the monomer and polymer fractions were 1.30 and 1.48 mol of lysine/mol of RNase respectively. Assuming 80% recoveries of LM and LML (see Table 1), the LM and LML on protein account for 70–80% of lysine loss measured by amino acid analysis. There was excellent agreement between the sum of LM and LML in all fractions, taken as a direct measure of protein-bound MDA, and TBA-MDA measurements by HPLC (Table 2). These results indicate that LM and LML represent the majority of the reducible, TBA-reactive MDA adducts to lysine. Finally, com-

Table 1 Effect of increasing NaBH_4 concentrations on the yield of MDA adducts in RNase

RNase was incubated with MDA (lysine/MDA, 2:1, mol/mol), dialysed and reduced with the indicated concentrations of NaBH_4 , as described in the Experimental section. Values are expressed as a percentage of the maximum value found for each analysis, which were as follows: TBA-MDA, 1.37 ± 0.06 mol/mol of RNase; LM and LML, 1.14 ± 0.2 and 0.2 ± 0.2 mol/mol of RNase monomer respectively. Data are means $\pm$ S.D. of triplicate analyses of a single incubation mixture. Results are typical of 2–3 replicate experiments.

BH_4 (mM)	Adduct formation (% of maximum)		
	TBARS	LM	LML
0	100.0 $\pm$ 4.1	0	0
25	68.8 $\pm$ 8.5	15.5 $\pm$ 0.6	17.8 $\pm$ 0.5
250	39.9 $\pm$ 5.5	21.1 $\pm$ 1.0	65.3 $\pm$ 2.7
500	33.8 $\pm$ 1.8	53.8 $\pm$ 4.8	100.4 $\pm$ 6.0
1000	20.0 $\pm$ 0.7	100.0 $\pm$ 13.4	100.0 $\pm$ 4.6

Figure 3 Fractionation of MDA-modified RNase by Sephadex G-75 gel-permeation chromatography

RNase was incubated with MDA, dialysed and applied to the G-75 column as described in the Experimental section. M and P indicate regions pooled as monomer and polymer fractions respectively; V_0 is void volume; 'RNase' indicates the elution position of the RNase monomer.

Table 2 Composition of RNase after incubation with MDA

RNase was incubated with MDA (MDA/lysine, 1:2, mol/mol) and then fractionated on Sephadex G-75, as described in the Experimental section. Aliquots of the total (unfractionated) reaction mixture, as well as of the monomer and polymer fractions, were reduced with 1 M NaBH_4 and then analysed for their content of lysine, LM and LML; TBA-MDA and TNBS were measured in unreduced protein. Data are expressed per mol of RNase monomer, as measured by amino acid analysis based on valine content (8 valine/mol of RNase). Lysine was measured by amino acid analysis, LM and LML by GC/MS, TBA-MDA by HPLC and TNBS colorimetrically, as described in the Experimental section. Values are means $\pm$ S.D. for triplicate analyses of a single reaction mixture, except for TNBS analyses which were done in duplicate. Starting values in native RNase for lysine and TNBS were 10.1 ± 0.2 and 11.6 ± 0.2 mol/mol of RNase respectively. LM and LML were not detected in native RNase.

Sample	Content (mol/mol of RNase)				
	Lysine	TNBS	LM	LML	TBA-MDA
Total	7.7 $\pm$ 0.6	7.0 $\pm$ 0.12	1.14 $\pm$ 0.2	0.20 $\pm$ 0.02	1.37 $\pm$ 0.06
Monomer	7.9 $\pm$ 0.2	7.1 $\pm$ 0.27	1.18 $\pm$ 0.07	0.06 $\pm$ 0.01	1.32 $\pm$ 0.11
Polymer	7.4 $\pm$ 0.2	7.5 $\pm$ 0.06	0.86 $\pm$ 0.03	0.31 $\pm$ 0.01	0.99 $\pm$ 0.06

Figure 4 Formation of MDA- and HNE-lysine adducts during copper-catalysed oxidation of LDL

LDL (two independent pools) was incubated in PBS at 32 °C with (○, □) or without (●, ■) copper, as described in the Experimental section. (A) The formation of conjugated dienes, an indicator of LDL oxidation, was monitored by measurement of absorbance at 234 nm. The kinetics of formation of LM (B), LML (C) and LHNE (D) were measured by GC/MS using aliquots of protein removed at the indicated times and reduced with 1 M NaBH₄; at 24 h, aliquots of copper-treated samples (◇, ○) were also analysed after reduction with 25 mM NaBH₄. Data are typical of analyses for four separate LDL pools.

pared with the monomer, the RNase polymer fraction showed a somewhat lower LM content, but about five times more LML cross-links, consistent with the presence of inter- as well as intra-molecular cross-links. However, since there must be at least 1 mol of LML/mol of polymer, there should be 0.5 mol of LML/mol of RNase if LML accounts for all of the polymer cross-links. The observed value of 0.3 mol of LML/mol of RNase suggests that there may be other cross-link structures present in the protein.

The yield of LHNE adducts in RNase was lower than that of

MDA derivatives. When the ratio of lysine to HNE was 10:1 or 5:1 (mol/mol), the amount of LHNE, based on GC/MS analysis, was 7.0 ± 1.6 and 20.0 ± 1.4 mmol/mol of lysine (mean $\pm$ S.D., $n = 3$) respectively when reduction was carried out using 25 mM borohydride, and did not increase on using 0.5 M reductant. The lower yield of LHNE compared with LM and LML resulted in part from the higher ratio of lysine to HNE compared with that of lysine to MDA. At ratios of lysine to HNE comparable with those used in the MDA experiments, there was substantial precipitation of protein. Unlike MDA, HNE also reacts with histidine residues, and is more reactive towards histidine than lysine, so that histidine is a preferred site of modification in protein [27]. Measurement of HNE-histidine adducts will be reported elsewhere (J. R. Requena, M. U. Ahmed, M.-X. Fu, A. J. Jenkins, T. J. Lyons, J. W. Baynes and S. R. Thorpe, unpublished work). Accurate measurements of lysine loss by amino acid analysis of HNE-modified RNase were not possible because of the low levels of lysine modification, i.e. 0.07–0.20 mol/mol of RNase.

Formation of LM, LML and LHNE in copper-oxidized LDL

Figure 4 compares the kinetics of formation of conjugated dienes during the metal-catalysed oxidation of LDL with the kinetics of formation of LM, LML and LHNE. After an initial lag phase of approx. 40 min, conjugated dienes increased rapidly during the propagation phase of the oxidation reaction and then more slowly thereafter (Figure 4A). An increase in LM (Figure 4B) was unexpectedly observed during the lag phase of the oxidation reaction, and LM levels also appeared to increase somewhat faster than those of conjugated dienes during the propagation phase, but began to plateau in a similar fashion to conjugated dienes between 2 and 4 h of oxidation. As noted below, the absence of an increase in conjugated dienes and LM in samples incubated in copper-free buffer suggests that the source of LM measured during the lag phase was not preformed lipid peroxides on the starting LDL. The rise in LML (Figure 4C) generally tracked the increase in conjugated dienes during the lag and propagation phases of the oxidation reaction. There was only a limited increase in both LM and LML after 4 h of oxidation. From an initial value of ~ 0.12 mmol of LM/mol of lysine, the concentration of LM rose 30-fold to a maximum value of ~ 3.6 mmol of LM/mol of lysine. LML was present at trace levels in the starting LDL, and rose to a maximum concentration of ~ 0.04 mmol/mol of lysine. LM and LML concentrations in seven individual pools of freshly isolated LDL were 0.12 ± 0.02 and 0.002 ± 0.001 mmol/mol of lysine respectively (mean $\pm$ S.D.). The ◇ symbols in Figures 4(C) and 4(D) show the recovery of LM and LML when reduction was carried out using 25 mM rather than 1 M borohydride. The yields of the MDA adducts were increased about 3-fold under the more vigorous reduction conditions, a more modest increase than that found during reduction of MDA-RNase (Table 1), indicating that the efficiency of reduction may be influenced by protein structure.

The rise in the LHNE concentration during oxidation of LDL (Figure 4D) was more gradual than that of either LM or LML during the early part of the reaction. However, LHNE continued to increase for about 12 h and remained constant thereafter. LHNE was not detectable in native LDL, but became measurable after ~ 2 h of oxidation and reached a final value of nearly 0.8 mmol/mol of lysine. As shown by the ◇ symbols in Figure 4(D), the yield of LHNE was only marginally affected by changing the reduction conditions from 25 mM to 1 M borohydride, similar to the results of experiments with HNE-RNase. Control samples, incubated in the absence of copper, showed

minimal changes in conjugated dienes or content of lipoxidation products throughout the course of the experiment (Figures 4A–4D, closed symbols).

Analysis of lipoxidation products in human skin collagen and lens protein

Lens crystallins and tissue collagens become increasingly brown, fluorescent and cross-linked with age [31]. Since these are also characteristics of proteins incubated with MDA and HNE, we examined samples of insoluble skin collagen from 27- and 72-year-old individuals [32], and aqueous lens protein extracts [33] from individuals aged 1, 10, 40, 67 and 74 years, for the presence of LM, LML and LHNE. None of these samples contained detectable MDA- or HNE-lysine adducts, although *N*^ε-(carboxymethyl)lysine (CML), which may be formed from either carbohydrate or lipid precursors [21], was readily measured (results not shown). HPLC analysis of the lens samples using the MDA-TBA assay were also negative. Because of the acetic acid extraction step used for preparation of insoluble collagen [32], the MDA-TBA assay was not applied to the collagen samples. Overall, these results indicate that MDA and HNE adducts do not accumulate on long-lived protein, although maturation of these adducts to other structures cannot be excluded.

DISCUSSION

A significant fraction (30–40 %) of the lysine residues in apolipoprotein B are modified during metal-catalysed and cell-mediated oxidation of LDL [7,9]. Aldehydes generated during the peroxidation of PUFA are thought to be responsible for such modifications, by formation of adducts to ϵ -amino groups of lysine residues. In fact, reaction of LDL with PUFA-derived aldehydes *in vitro* yields an LDL particle with many of the properties of oxidized LDL, such as increased electrophoretic mobility, fluorescence and uptake by macrophages [7,11]. Although hexanal is quantitatively (~40 %) the major aldehyde formed during *in vitro* oxidation of LDL [7], Schiff bases (imines) formed between aliphatic aldehydes and amines are relatively labile, and hexanal-lysine adducts dissociate reversibly to the component aldehyde and amine. In contrast, MDA and HNE, the two aldehydes formed at next highest abundance, yield chemically stable adducts to lysine residues, stabilized by either resonance forms or covalent carbon–carbon bonds (Schemes 1 and 2). The presence of MDA and HNE adducts on proteins *in vivo* is confirmed by the observation that monoclonal antibodies prepared against MDA- and HNE-modified proteins not only cross-react with copper-oxidized LDL but also stain atherosclerotic lesions [3]. In addition, the predominant population of autoantibodies to epitopes on oxidized LDL found in human and mouse sera in association with atherosclerosis is specific for MDA-modified protein [4,6,34]. Overall, these results indicate that MDA and HNE form relatively stable, recognizable, adducts to protein and that these adducts are, in part, responsible for the immunological recognition of oxidized lipoproteins, a process which may be involved in immune-mediated mechanisms of atherogenesis [35].

The GC/MS methods described in the present paper provide a means for the specific quantification of MDA-lysine Schiff-base adducts and cross-links, as well as the Michael addition adducts of lysine to HNE, in proteins. Both LM and LML were detected in native LDL, and increased in concert with conjugated dienes during the course of metal-catalysed oxidation. These results represent the first report of chemically characterized lysine-MDA cross-links occurring in native and oxidized LDL. LHNE

was not detectable in the native protein, but increased during oxidation of LDL. Uchida et al. [36] have previously reported the detection of trace amounts of LHNE in copper-oxidized, but not native, LDL by amino acid analysis. The sensitivity of the GC/MS assay used in the present studies permits quantification of LHNE and documents the low level of this adduct in the oxidized protein. The failure to detect LHNE in native LDL and the slower appearance of LHNE, compared with LM and LML, during oxidation is consistent with the observation that formation of MDA somewhat precedes formation of HNE during oxidation of LDL [7], suggesting that LHNE may be a better indicator of extended damage to proteins during lipid peroxidation reactions.

Whereas LM and LML could be measured in native LDL, they were not detectable in total plasma protein ($\leq 2\%$ LDL protein). Thus, compared with other plasma proteins, LDL appears to be enriched in MDA. Based on a concentration of 1 mg of LDL/ml of plasma and a value of ~0.1 mmol of LM + LML/mol of lysine in native LDL, our analyses indicate that the level of MDA bound to plasma proteins would be ~70 nM. This estimate is in reasonable agreement with a study by Yeo et al. [37], which reported a range of 25–38 nM MDA in human plasma, based on GC/MS analysis of free MDA, and suggests that LDL is a major carrier of MDA in plasma. The discrepancy between our calculated value for total plasma MDA and the measurements of Yeo et al. [37] may arise from differences in the amount of MDA released from protein in their procedure, compared with our measurement of protein-bound MDA. In general, however, results from both laboratories confirm that there are only trace amounts of MDA in normal plasma and plasma proteins.

The maximal yields of LM, LML and LHNE in oxidized LDL account for only about 0.5 % (~5 mmol/mol) of lysine residues in the protein (Figure 4). We recently reported that CML is also formed during the oxidation of LDL, and this accounted for modification of another ~0.1 % of lysine residues [21]. Although CML is not likely to be formed directly from any of the quantitatively major aldehydes (hexanal, MDA and HNE) produced during LDL oxidation, it is striking that the concentration of CML reached a value comparable with those of the MDA and HNE adducts. Thus, while more than 100 lysine residues are modified during oxidation of LDL [7,9], only about 1 % of the total can be ascribed presently to chemically characterized and assayable products. Our results suggest that intermediates significantly more reactive than MDA and HNE are formed during lipid peroxidation reactions, but that they may not be detected in the free form because of their rapid reaction with the protein. Such intermediates might include carbon- and oxygen-centred radical intermediates in lipid peroxidation reactions, lipid peroxides [10] or hydroxylaldehyde and dicarbonyl compounds [7,38,39]. Indeed, the dicarbonyl compound glyoxal has recently been detected among the products formed during oxidation of PUFA *in vitro* [21,38,39] and is a known precursor of CML [40]. A large fraction (up to 50 %) of lysine modifications in oxidized LDL are stable to acid hydrolysis [9], suggesting that other acid-stable compounds, analogous to CML, may be formed from related dicarbonyl compounds.

In conclusion, the present work describes, for the first time, the chemical nature and concentration of MDA and HNE adducts to lysine in both native and copper-oxidized human LDL, and presents a method for quantifying these structures in proteins. The quantification of lipoxidation products such as MDA- and HNE-lysine adducts in atherosclerotic plaques and in tissue proteins should help to clarify the role of lipid peroxidation in the pathogenesis of atherosclerosis and other disorders in which oxidative stress is implicated.

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REFERENCES

- Witztum, J. L. and Steinberg, D. (1991) *J. Clin. Invest.* **88**, 1785–1792
- Grundey, S. M. (1995) *Clin. Chem.* **41**, 139–146
- Palinski, W., Rosenfeld, M. E., Butler, S., Socher, S. A., Parthasarathy, S., Curtiss, L. K. and Witztum, J. L. (1990) *Arteriosclerosis* **10**, 325–335
- Holvoet, P., Perez, G., Zhao, Z., Brouwers, E., Bernar, H. and Collen, D. (1995) *J. Clin. Invest.* **95**, 2611–2619
- Ylä-Herttuala, S., Palinski, W., Rosenfeld, M. E., Parthasarathy, S., Carew, T. E., Butler, S., Witztum, J. L. and Steinberg, D. (1989) *J. Clin. Invest.* **84**, 1086–1095
- Salonen, J. T., Ylä-Herttuala, S., Yamamoto, R., Butler, S., Korpela, H., Salonen, R., Nyyssönen, K., Palinski, W. and Witztum, J. L. (1992) *Lancet* **339**, 883–887
- Esterbauer, H., Gebicki, J., Puhl, H. and Jürgens, G. (1992) *Free Radicals Biol. Med.* **13**, 341–390
- Maggi, E., Chiesa, R., Melissano, G., Castellano, R., Astore, D., Grossi, A., Finardi, G. and Bellomo, G. (1994) *Arterioscler. Thromb.* **14**, 1892–1899
- Steinbrecher, U. P., Loughheed, M., Kwan, W.-C. and Dirks, M. (1989) *J. Biol. Chem.* **264**, 15216–15223
- Fruebis, J., Parthasarathy, S. and Steinberg, D. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10588–10592
- Steinbrecher, U. P. (1987) *J. Biol. Chem.* **262**, 3603–3608
- El-Saadani, M., Esterbauer, H., El-Sayad, M., Goher, M., Nassar, A. Y. and Jürgens, G. (1989) *J. Lipid Res.* **30**, 627–630
- Wolff, S. P. (1994) *Methods Enzymol.* **233**, 182–189
- Janero, D. R. (1990) *Free Radicals Biol. Med.* **9**, 515–540
- Morrow, J. and Robers, II, L. J. (1991) *Free Radicals Biol. Med.* **10**, 195–200
- Hofmann, K., Stutz, E., Spuhler, G., Yajima, H. and Schwartz, E. T. (1960) *J. Am. Chem. Soc.* **82**, 327–332
- de Montarby, L., Tourbah, H. and Grée, R. (1989) *Bull. Soc. Chim. France* **3**, 419–432
- Spadaro, A. C. C., Draghetta, W., Del Lama, S. N., Camargo, A. C.M and Greene, L. J. (1979) *Anal. Biochem.* **96**, 317–321
- Esterbauer, H., Schaur, R. J. and Zollner, H. (1991) *Free Radicals Biol. Med.* **11**, 81–128
- Delbecco, R. and Voigt, M. S. (1954) *J. Exp. Med.* **98**, 167–173
- Fu, M.-X., Requena, J. R., Jenkins, A. J., Lyons, T. J., Baynes, J. W. and Thorpe, S. R. (1996) *J. Biol. Chem.* **271**, 9982–9986
- Osborne, J. C. (1986) *Methods Enzymol.* **128**, 213–222
- Baker, J. R., Zyzak, D. V., Thorpe, S. R. and Baynes, J. W. (1993) *Clin. Chem.* **39**, 2460–2465
- Knecht, K. J., Dunn, J. A., McFarland, K. F., McCance, D. R., Lyons, T. J., Thorpe, S. R. and Baynes, J. W. (1990) *Diabetes* **40**, 190–196
- Young, I. S. and Trimble, E. R. (1991) *Ann. Clin. Biochem.* **28**, 504–508
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Szweda, L. I., Uchida, K., Tsai, L. and Stadtman, E. R. (1993) *J. Biol. Chem.* **268**, 3342–3347
- Chio, K. S. and Tappel, A. L. (1969) *Biochemistry* **8**, 2821–2827
- Chio, K. S. and Tappel, A. L. (1969) *Biochemistry* **8**, 2827–2833
- Kikugawa, K., Takayagi, K. and Watanabe, S. (1985) *Chem. Pharm. Bull.* **33**, 5437–5444
- Sell, D. R. and Monnier, V. M. (1989) in *Physiology of Aging* (Masero, E. J., ed.), pp. 235–305, Oxford University Press, New York
- Dunn, J. A., McCance, D. R., Thorpe, S. R., Lyons, T. J. and Baynes, J. W. (1991) *Biochemistry* **30**, 1205–1210
- Wells-Knecht, M. C., Huggins, T. G., Dyer, D. G., Thorpe, S. R. and Baynes, J. W. (1993) *J. Biol. Chem.* **268**, 12348–12352
- Palinski, W., Tangirala, R. K., Miller, E., Young, S. G. and Witztum, J. L. (1995) *Arterioscler. Thromb. Vasc. Biol.* **15**, 1569–1576
- Lopes-Virella, M. F. (1994) *Clin. Immunol. Immunopathol.* **73**, 155–167
- Uchida, K., Toyokuni, S., Nishikawa, K., Kawakishi, S., Oda, H., Hiai, H. and Stadtman, E. R. (1994) *Biochemistry* **33**, 12487–12494
- Yeo, H. C., Helbock, H. J., Chyu, D. W. and Ames, B. N. (1994) *Anal. Biochem.* **220**, 391–396
- Loidl-Stahlhofen, A. and Spitteller, G. (1994) *Biochim. Biophys. Acta* **1211**, 156–160
- Mlakar, A. and Spitteller, G. (1994) *Biochim. Biophys. Acta* **1214**, 209–230
- Wells-Knecht, K. J., Zyzak, D. V., Litchfield, J. E., Thorpe, S. R. and Baynes, J. W. (1994) *Biochemistry* **34**, 3702–3709