Chemical modification of proteins during peroxidation of phospholipids

Andrzej S. Januszewski,* Nathan L. Alderson,* Alicia J. Jenkins,† Suzanne R. Thorpe,* and John W. Baynes[1,*,§]

Department of Chemistry and Biochemistry,* University of South Carolina, Columbia, SC 29208; Department of Medicine,† St. Vincent's Hospital, University of Melbourne, Melbourne, Australia; and School of Medicine,§ University of South Carolina, Columbia, SC 29208

Abstract Chemical modification of proteins by advanced glycation and lipoxidation end products is implicated in the pathogenesis of macrovascular disease in aging and diabetes. To identify biomarkers of the lipoxidative modification of protein, we studied the oxidation of phospholipids in the presence of the model protein RNase A and compared protein-bound products formed in these reactions with those formed during oxidation of plasma proteins. Metal-catalyzed oxidation of 1-palmitoyl-2-arachidonoyl-phosphatidylcholine or 1-palmitoyl-2-linoleoyl-phosphatidylcholine in the presence of RNase led to the loss of amino groups in RNase and the incorporation of phosphate, hexanoate, pentanedioate, nonanedioate, and palmitate into protein. Protein-bound palmitate and phosphate correlated strongly with one another, and protein-bound pentanedioate and nonanedioate, derived from arachidonate and linoleate, respectively, accounted for ~20% of the cross-linking of lipid phosphorus to protein. Similar results were obtained on oxidation of total plasma or isolated LDL. We conclude that alkanedioic acids are quantitatively important linkers of oxidized phospholipids to proteins and that measurement of protein-bound phosphate and long-chain fatty acids may be useful for assessing long-term lipid peroxidative damage to proteins in vivo. Analyses of plasma proteins from control and diabetic patients indicated significant increases in lipoxidative modification of protein in diabetic compared with control subjects.—Januszewski, A. S., N. L. Alderson, A. J. Jenkins, S. R. Thorpe, and J. W. Baynes. **Chemical modification of proteins during peroxidation of phospholipids. *J. Lipid Res.* 2005.** 46: **1440–1449.**

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Oxidation of LDL is considered an early event in the conversion of LDL to a proatherogenic form, setting the stage for atherogenesis (1). In support of this hypothesis, products of lipid peroxidation, such as malondialdehyde (MDA) and hydroxynonenal (HNE) adducts to protein, have been detected in plasma of patients with macrovascular disease and in atherosclerotic plaque by both chemical and immunochemical methods (2–5). Oxidized low density lipoprotein (OxLDL) is also recognized by scavenger receptors on macrophages, the cell type in which lipids accumulate during the early stages of atherogenesis (6, 7). Zhang, Yang, and Steinbrecher (8) demonstrated the loss of lysine amino groups during the oxidation of lipoproteins, and Friedman et al. (9) have proposed that the dominant epitope recognized by the major macrophage scavenger receptor, CD36, is a phosphorycholine adduct on oxidized phospholipid linked to lysine residues on LDL as a Schiff base adduct. The linker, pentanoic acid semialdehyde (oxovalerate), was identified as a product of the oxidation of arachidonic acid at the *sn*-2 position of the phospholipid. Podrez et al. (10, 11) have identified a more complex array of compounds, α,β-unsaturated, γ-hydroxy, or oxo acids or aldehydes derived from arachidonate or linoleate, which may also cross-link phospholipids to protein by Schiff base (imine), Michael reactions, and amide linkages. Finally, using immunohistochemical techniques, Osawa and colleagues detected N^{ε}-(hexanoyl)lysine (12) adducts and nonanedioic (azelaic) acid (NDA) cross-links (13) to lysine residues in oxidized lipoproteins. In none of these studies, however, has a dominant lipid adduct to protein been identified, nor have the cross-link structures been quantified in lipoxidized proteins.

In the present study, we have focused on the measurement of two of the characteristic linkages of oxidized

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Abbreviations: CBL, N^{ε}-(carboxybutyl)lysine; CML, N^{ε}-(carboxymethyl)lysine; DTPA, diethylenetriaminepentaacetic acid; HNE, 4-hydroxynonenal; MDA, malondialdehyde; NDA, nonanedioic (azelaic) acid; OxLDL, oxidized low density lipoprotein; PAPC, 1-palmitoyl-2-arachidonoyl-phosphatidylcholine; PDA, pentanedioic (glutaric) acid; PLPC, 1-palmitoyl-2-linoleoyl-phosphatidylcholine; SIM-GC-MS, selected ion monitoring-gas chromatography-mass spectrometry.

[1] To whom correspondence should be addressed.
e-mail: john.baynes@sc.edu

phospholipids to protein, those mediated by the dicarboxylic acids pentanedioic (glutaric) acid (PDA) and NDA, derived from arachidonate and linoleate, respectively (**Fig. 1**). We present preliminary studies on the modification of a model protein, bovine pancreatic RNase A (RNase), by peroxidizing phospholipids containing arachidonate and linoleate and compare the products formed in these model reactions with those formed during Cu^{2+}-catalyzed oxidation of plasma proteins and purified LDLs. Our data indicate that protein-bound phosphate and fatty acids are present in lipoxidized proteins at much higher concentrations than MDA or HNE and that the dicarboxylic acids PDA and NDA may constitute a quantitatively important class of cross-links between oxidized phospholipids and proteins. We conclude that the measurement of protein-bound palmitate and stearate, which are components of the oxidized phospholipids bound to plasma proteins, provides a sensitive technique for assessing lipoxidative damage to proteins and demonstrate that these chemical modifications are increased in total plasma proteins of diabetic compared with control subjects.

MATERIALS AND METHODS

Reagents

Unless indicated otherwise, all reagents were purchased from Sigma-Aldrich (St. Louis, MO), including 1-palmitoyl-2-arachidonoyl-phosphatidylcholine (PAPC), 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PLPC), and RNase (RNase type XII-A). Heavy labeled [$^2H_{11}$]hexanoic acid was purchased from Cambridge Isotope Laboratories (Andover, MA), and [$^2H_4$]palmitic acid and [$^2H_8$]lysine were from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). All solvents were of the highest purity available from Acros Chemicals (Atlanta, GA).

Modification of RNase by peroxidizing PAPC or PLPC

RNase (0.3 mM, 4.4 mg/ml = 3 mM lysine) was incubated with varying amounts of PAPC or PLPC in the presence of 75 μM $CuSO_4$ for 24 h at 37°C in 0.2 M sodium phosphate buffer, pH 7.4. Aliquots (1 ml) were removed before the addition of $CuSO_4$ and at 24 h after the addition of $CuSO_4$; oxidation was quenched by the addition of diethylenetriaminepentaacetic acid (DTPA) (final concentration = 4 mM). Samples were then dialyzed in 3500 MWCO dialysis tubing (Spectrapor, Rancho Dominguez, CA) against 4 liters of deionized water containing 1 mM DTPA for 24 h at 4°C with four changes of water. After dialysis, the samples were dried in vacuo (Speed-Vac; Savant, Farmington, IN) and resuspended in 1 ml of deionized water. To remove unreacted PAPC or PLPC and free lipid degradation products, samples were extracted with chloroform-methanol (2:1) according to the Folch procedure (14), and the lower organic phase was discarded. The upper aqueous phase and protein at the interface were dried in vacuo and redissolved in 1 ml of deionized water. Aliquots were removed for the measurement of primary amino groups using the trinitrobenzenesulfonic acid assay (15) and protein concentration by the Lowry assay (16). For analysis of protein-bound fatty acids, 20 μl of internal standard solution (4 nmol of [$^2H_{11}$]hexanoic acid and 10 nmol of [$^2H_4$] palmitic acid in methanol) was added to the remaining sample (~4 mg of protein), which was then diluted with an equal volume of concentrated HCl and hydrolyzed for 18 h at 110°C. An aliquot of the hydrolysate was assayed for phosphate using the Bartlett molybdate assay, as modified by Marinetti et al. (17).

Oxidation of plasma

Blood samples were collected from healthy volunteers who were not taking antioxidant vitamins, using protocols approved by the Institutional Review Board of the University of South Carolina. Heparinized plasma was separated by centrifugation at 2,000 rpm for 10–15 min at 4°C. Plasma was oxidized using a final concentration of 5 mM $CuSO_4$ (from a 50 mM $CuSO_4$ stock solution), followed by incubation at 37°C. Aliquots (1 ml) were taken at various times and quenched by the addition of DTPA (final concentration = 10 mM) and stored frozen at −20°C until an-

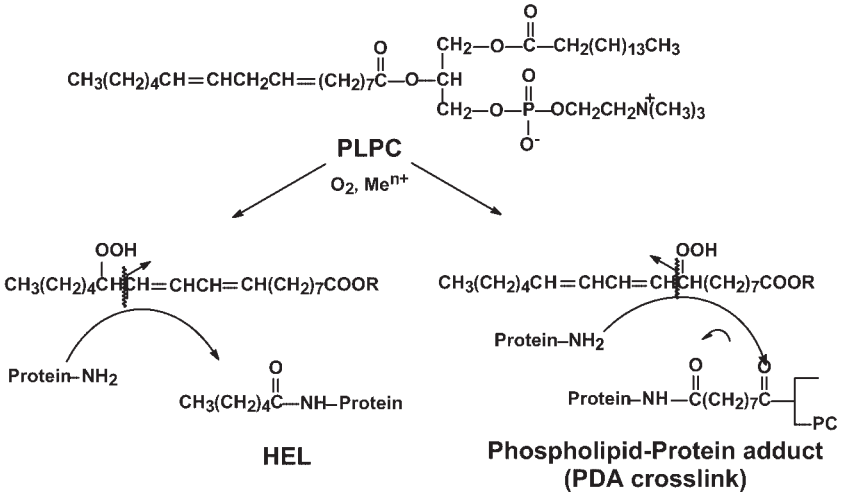

Fig. 1. General scheme for the oxidation of 1-palmitoyl-2-linoleyl-phosphatidylcholine (PLPC) to form N^{ε}-(hexanoyl)lysine and the nonanedioic (azelaic) acid (NDA) cross-link between phospholipid and protein. Asymmetric cleavage of the hydroperoxide, or a decomposition product of the hydroperoxide, yields N^{ε}-(hexanoyl)lysine and the NDA cross-link between phospholipid and protein. 1-Palmitoyl-2-arachidonyl-phosphatidylcholine (PAPC) undergoes similar reactions to form N^{ε}-(hexanoyl)lysine and the pentanedioic (glutaric) acid (PDA) cross-link. The split products of these reactions have not been identified.

alyzed. Plasma incubated in the absence of copper served as a control. The progress of the oxidation reaction was monitored by assay of thiobarbituric acid-reactive substances (18) using MDA as a standard. For analysis of protein-bound products, noncovalently bound lipids were extracted by the addition of 2 ml of methanol-ether (3:1), followed by centrifugation at 2,500 rpm for 10 min at 4°C, and the supernatant was discarded; this procedure was repeated two more times. Preliminary experiments showed that this procedure yielded maximum recovery of protein (~90%) and minimum recovery of free lipid (data not shown). The protein pellet was dissolved in 1 ml of deionized water for the measurement of protein content, and ~4 mg of protein, containing 20 μl of internal standard solution (see above), was hydrolyzed in 2 ml of 6 N HCl, as described above. The phosphate content of the protein was measured after hydrolysis, also as described above.

Oxidation of LDL

Blood samples for lipoprotein isolation were collected from normolipidemic healthy volunteers. LDL was isolated by preparative ultracentrifugation (17 h) from pooled EDTA plasma (n = 3) as described by Lopes-Virella et al. (19). The LDL was dialyzed against 0.15 M NaCl solution containing 300 μM EDTA, pH 8.0, and passed through a 0.22 μm filter for sterilization and removal of aggregates, then stored under nitrogen in the dark at 4°C (final protein concentration ~ 6 mg/ml). After removing EDTA using a PD-10 column (Pharmacia Biotech, Uppsala, Sweden), LDL was oxidized in PBS, pH 7.4, at 37°C for up to 24 h (protein concentration = 1.5 mg/ml; $CuSO_4$ concentration = 40 μM) (19). LDL incubated in the absence of copper served as a control. To monitor the degree of oxidation, aliquots of both LDL preparations, diluted to a protein concentration of 50 μg/ml, were used for continuous monitoring of conjugated diene formation by measuring absorbance at 234 nm. The oxidation was stopped by the addition of DTPA to a final concentration of 1 mM. Samples were then dialyzed in 6000-8000 MWCO dialysis tubing against 4 liters of deionized water containing 1 mM DTPA for 24 h at 4°C with four changes of water. After dialysis, the samples were dried in vacuo and resuspended in 0.4 ml of deionized water. To remove noncovalently bound lipids, samples were extracted with 4 ml of methanol-ether (3:1), as described above, and analysis of protein-bound fatty acids and phosphate was carried out as described above for plasma.

Fatty acid analysis by selected ion monitoring-gas chromatography-mass spectrometry

Fatty acids released by acid hydrolysis of delipidated proteins were extracted from the 6 N HCl solution using an equal volume of ether; the procedure was repeated three times. After evaporation under a stream of nitrogen (N-EVAP; Organomation, Berlin, MA), butyl esters were prepared by the addition of 200 μl of 2 M HCl in *n*-butanol, followed by heating at 100°C for 1 h. The esters were extracted into 200 μl of chloroform, and 2 μl was analyzed by selected ion monitoring-gas chromatography-mass spectrometry (SIM-GC-MS). These analyses were carried out on a Hewlett-Packard (Palo Alto, CA) model 6890 gas chromatograph interfaced to an HP 5970 mass selective detector using a 30 m × 0.25 mm Rtx-5 (5% diphenyl, 95% dimethyl polysiloxane) capillary column (Restek, Bellefonte, PA) and helium carrier gas. The injection port was maintained at 275°C and the transfer line at 290°C. The temperature program was as follows: initial temperature of 100°C, hold for 6 min, then 7°C/min ramp to 150°C, 25°C/min ramp to 220°C, 7°C/min ramp to 280°C, and hold for 4 min. Quantification was based on isotope dilution using standard curves constructed from mixtures of a constant amount of heavy labeled internal standards and increasing amounts of nonlabeled fatty acids. The following ions, formed after the loss of butanol, were used to monitor various fatty acids: hexanoic acid, m/z = 99; d_{11}-hexanoic acid, m/z = 110; palmitic acid, m/z = 239; d_4-palmitic acid, m/z = 243; PDA, m/z = 171; NDA, m/z = 227; stearic acid, m/z = 267; oleic acid, m/z = 265; and myristic acid, m/z = 211.

Synthesis of N^{ε}-(carboxybutyl)lysine

In an effort to identify and quantify the oxovalerate cross-link described in oxidized lipoproteins (9), we prepared N^{ε}-(carboxybutyl)lysine (CBL), the product of reduction of the Schiff base cross-link between oxovalerate and protein. CBL was synthesized from glutaric semialdehyde and N^{α}-acetyllysine. Glutaric semialdehyde was prepared by deamination of aminovaleric acid (10 mM in 0.1 M phosphate buffer, pH 8.8) using ~1 g of crude eggshell membrane containing lysyl oxidase, as described by Akagawa, Wako, and Suyama (20). The incubation was carried out for 4 h at 37°C, and the progress of deamination was monitored by HPLC analysis of the 2,4-dinitrophenylhydrazine adduct of glutaric semialdehyde (20). A standard curve prepared from the 2,4-dinitrophenylhydrazine adduct of valeraldehyde was used for quantification and indicated that the yield from the deamination reaction was ~36%. After 4 h, the eggshell membrane was removed using tweezers, and the solution was filtered through a 0.45 μm filter. Solid N^{α}-acetyllysine was added to the filtrate to give a final concentration of 8 mM, and the mixture was then incubated for 1 h at 37°C to allow Schiff base formation. $NaBH_3CN$ was added (100 mM final concentration), and the reduction reaction was carried out for 4 h at room temperature. The reduced sample was dried in vacuo and then deacetylated in 6 N HCl for 3 h at 110°C. Aliquots of the hydrolyzed sample were dried and then converted to the trifluoroacetyl methyl ester derivatives (21) for analysis by SIM-GC-MS, as described below. The mass spectrum of the synthetic CBL (4% overall yield, based on aminovaleric acid) showed the molecular ion at m/z 466 (7.5%) and major ions at m/z 365 (100%) and 240 (77%), representing cleavage of C-C bonds α,β to the secondary amino group, and 369 (95%; M-CF_3CO).

Analysis of CBL in oxidized protein by SIM-GC-MS

For CBL analysis, oxidized and control samples were reduced either with 250 mM $NaBH_4$ in 0.1 M sodium borate buffer, pH 9.2, or with 250 mM $NaBH_3CN$ in 0.1 M sodium phosphate buffer, pH 7.4, for 4 h at room temperature. Protein was then precipitated with an equal volume of 20% trichloroacetic acid and centrifuged for 10 min at 2,000 rpm at 4°C, and the supernatant was discarded. Unbound lipids were extracted with 2 ml of methanol-ether (3:1) solution as described above. For analysis of protein-bound CBL, 20 μl of internal standard solution [1.59 nmol of $[^2H_4]N^{\varepsilon}$-(carboxymethyl)lysine, synthesized according to Knecht et al. (22), and 119 nmol of $[^2H_8]$lysine] was added to the extracted sample, which was hydrolyzed as described above. After drying, the hydrolysates were dissolved in 3 ml of 20% methanol containing 1% trifluoroacetic acid and applied to 3 ml Sep-Pak columns (Supelco, Bellefonte, PA) to remove brown and lipophilic materials before derivatization for GC-MS. The eluates were again dried and then derivatized for measurement of CBL, N^{ε}-(carboxymethyl)lysine (CML), and lysine as their trifluoroacetyl methyl ester derivatives by SIM-GC-MS (21). The injection port was maintained at 275°C and the transfer line at 290°C. The temperature program was as follows: initial temperature of 130°C, hold for 4 min, then 4°C/min ramp to 180°C, 5°C/min ramp to 240°C, 15°C/min ramp to 290°C, and hold for 4 min. Quantification was based on isotope dilution using standard curves constructed from mixtures of a constant amount of heavy labeled internal standards and increasing amounts of nonlabeled CBL and lysine. The following ions were monitored: CBL, m/z = 435; d_4-CME, m/z = 396; lysine, m/z = 180; and d_8-lysine,

TABLE 1. Clinical characteristics of diabetic and control subjects

Variable	Control Subjects	Diabetic Subjects
Number (female/male)	20 (10/10)	20 (10/10)
Age (years)	39.5 ± 11.1	39.3 ± 13.6
Diabetes duration (years)		24.3 ± 11.9
Body mass index (kg/m^2)	24.8 ± 4.61	24.6 ± 3.7
HbA_{1c} (%)	5.31 ± 0.37	7.63 ± 1.14[a]
Total cholesterol (mmol/l)	4.69 ± 0.50	4.66 ± 0.96
Triglycerides (mmol/l)	0.82 ± 0.31	0.83 ± 0.54
LDL-cholesterol (mmol/l)	2.77 ± 0.51	2.61 ± 0.89
HDL-cholesterol (mmol/l)	1.59 ± 0.31	1.59 ± 0.55

Data shown are means ± SD.
[a] $P < 0.00001$ for diabetic versus control subjects.

m/z = 188. CBL was normalized to the lysine content of the protein; [$^2H_4$]CML was used as the heavy labeled internal standard for the measurement of CBL.

Human subjects

Healthy volunteers (n = 20) who had no evidence of vascular disease and were not on medications (other than the oral contraceptive pill) and age- and gender-matched type 1 diabetic subjects (n = 20) were recruited from the Diabetes Clinics at St. Vincent's Hospital (Melbourne, Australia). The study was approved by the St. Vincent's Hospital Ethics Committee, and each subject gave written informed consent. A patient history was recorded, and medical records were reviewed and verified. Patients had at least 15 years of diabetes, with no preproliferative or proliferative retinopathy, normal renal function, and no evidence of macrovascular disease (**Table 1**).

Subjects were evaluated after an overnight fast before any medication, including insulin. Blood was collected by venipuncture, and a spot urine sample was collected. Fasting blood glucose, HbA_{1c}, renal and liver function tests, and lipids were measured by the St. Vincent's Clinical Chemistry Department, and results are shown in Table 1. Plasma and serum were prepared by centrifugation (2,000 rpm, 10 min, 4°C) and stored at −70°C until further analysis, using methods described above.

Statistical analyses

Unless indicated otherwise, data are expressed as means ± SD of three or more replicate experiments. Statistical analyses were performed using the Student's *t*-test.

RESULTS

Modification of RNase by products of phospholipid oxidation

To model the reaction of protein with products of lipid peroxidation, RNase was incubated with phospholipids (PAPC or PLPC) in phosphate buffer at physiological pH for 24 h. We assessed the extent of modification of the protein by phospholipids through measurements of protein-bound

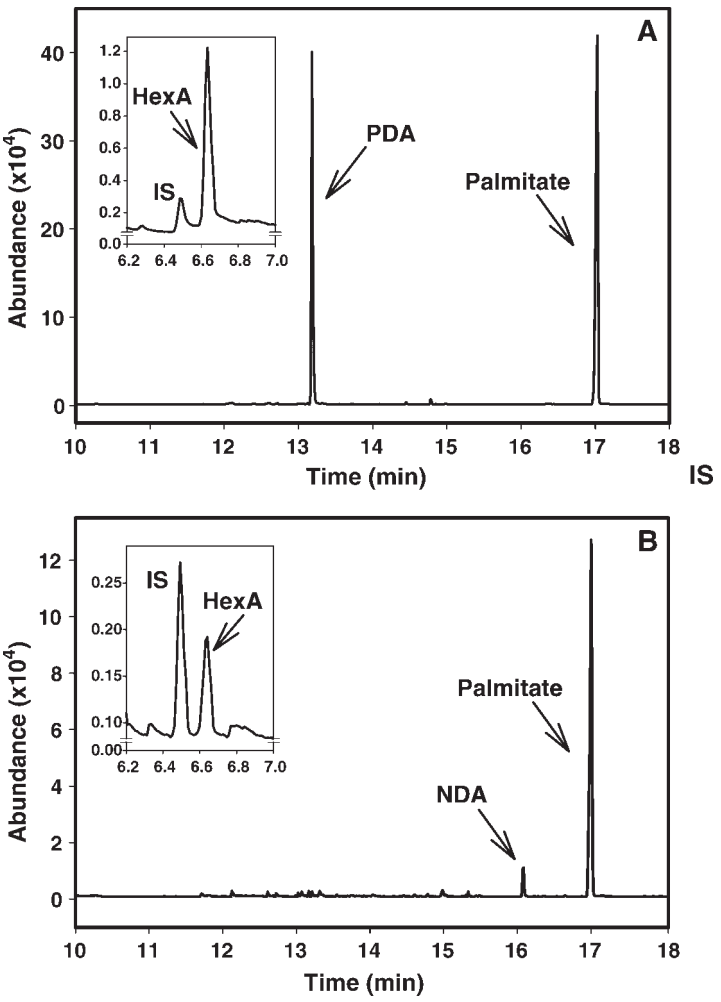

Fig. 2. Typical selected ion monitoring-gas chromatography-mass spectrometry (SIM-GC-MS) chromatograms of fatty acids recovered from RNase modified by PAPC (A) or PLPC (B) at 24 h. Protein-phospholipid incubations were processed for analysis of fatty acids as described in Materials and Methods. No fatty acids were detected in the native protein (data not shown). Note the difference in scales, reflecting the greater extent of derivatization of protein from PAPC compared with PLPC. HexA, hexanoic acid. The peak at 6.5 min is the internal standard (IS), d_{11}-hexanoic acid.

TABLE 2. Loss of amino groups and increase in palmitate and phosphate content in RNase incubated with peroxidizing phospholipids

Phospholipid-Lysine	Amino Groups Modified	Palmitate Bound	Phosphate Bound
mol/mol		*mol/mol RNase*	
PAPC, 0.5:1	0.27 ± 0.01	0.25 ± 0.04	0.27 ± 0.02
PAPC, 1:1	0.56 ± 0.04	0.44 ± 0.01	0.40 ± 0.03
PAPC, 2:1	0.92 ± 0.09	0.89 ± 0.07	0.77 ± 0.03
PLPC, 0.5:1	0.22 ± 0.01	0.041 ± 0.001	0.039 ± 0.002
PLPC, 2:1	0.85 ± 0.02	0.110 ± 0.003	0.085 ± 0.005

PAPC, 1-palmitoyl-2-arachidonyl-phosphatidylcholine; PLPC, 1-palmitoyl-2-linoleyl-phosphatidylcholine. Data shown are averages ± range of two independent experiments analyzed together.

palmitate (*sn*-1 fatty acid) and phosphate to the protein; however, we also searched for the putative dicarboxylic acid cross-links, PDA and NDA (Fig. 1). Typical GC-MS analyses (**Fig. 2**) revealed the presence of protein-bound palmitate, PDA from linoleate in PLPC, and NDA from arachidonate in PAPC. As shown in **Table 2**, the extent of modification of lysine residues in RNase by both PAPC and PLPC increased with the increasing molar ratio of phospholipid to protein. Of the 11 free amino groups per mole of RNase (10 lysine residues plus the α-terminal amino group), ~10% were modified when either PAPC or PLPC was present at a 2-fold molar excess over lysine residues. The amount of protein-bound phosphate agreed with the amount of palmitic acid released during hydrolysis (Table 2), indicating that the glycerol backbone of phospholipids was linked to protein through a structure derived from the PUFA at the *sn*-2 position in the phospholipid. For the PAPC reaction, the amount of phospholipid phosphate and palmitate bound to protein corresponded closely with the extent of amino group modification. For PLPC, however, protein-bound phospholipid and palmitate accounted for only 20% of lysine loss, indicating that in this case products other than phospholipid adducts also contributed to the loss of lysine amino groups. For RNase modified by PAPC or PLPC, the palmitate and phosphate were 87–95% releasable by saponification (1 M KOH in 95% methanol for 1 h at 37°C), consistent with the structures proposed in Fig. 1.

Hexanoate and dicarboxylic acid contents of RNase incubated with peroxidized phospholipids

As with palmitate and phosphate, the concentrations of PDA and NDA also increased with the increasing ratio of phospholipid to protein (**Fig. 3A, B**). Based on data in Fig. 3 and Table 2, PDA accounted for ~20% and 10% of lysine modification and palmitate (or phosphate) adduction in the reactions of PAPC and PLPC with RNase, respectively. The PDA or NDA yield per mole of palmitate (or lysine modification) was relatively independent of the ratio of PAPC or PLPC to lysine (**Table 3**), suggesting that phospholipid degradation in this model system was initiated in the lipid phase, probably at the micelle surface, independent of protein and that polar oxidation products reacted subsequently with protein. Based on these data, dicarboxylic acids may account for10–20% of the cross-linking of phospholipid to protein.

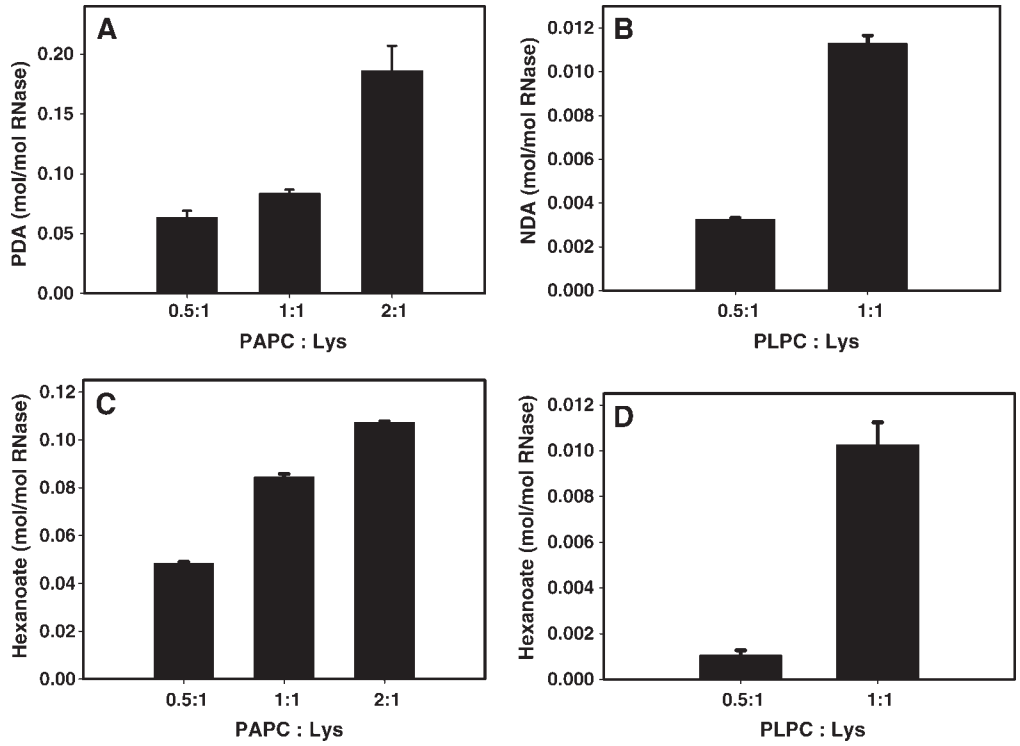

Fig. 3. Measurement of carboxylic acid content of RNase exposed to oxidizing phospholipids. PDA was formed exclusively from PAPC (A), and NDA was formed exclusively from PLPC (B). Hexanoate was formed from both PAPC (C) and PLPC (D). Data (mean ± range; n = 2) are from reaction mixtures described in Table 2.

TABLE 3. Formation of protein-bound phospholipid and dicarboxylic acids in RNase incubated with peroxidizing phospholipids

Phospholipid-Lysine	PAPC-Derived PDA	PLPC-Derived NDA
	mol/mol palmitate	
0.5:1	0.26 ± 0.06	0.11 ± 0.01
1:1	0.19 ± 0.01	—
2:1	0.22 ± 0.02	0.12 ± 0.01

NDA, nonanedioic (azelaic) acid; PDA, pentanedioic (glutaric) acid. Data shown are means ± range from two independent experiments.

Figure 3 also shows that, for both PAPC (Fig. 3C) and PLPC (Fig. 3D), the amount of bound hexanoate increased with the increasing ratio of phospholipid to RNase. The extent of modification of the protein by hexanoate from PLPC was only 10% that observed with PAPC, so that hexanoate appears to be a more sensitive indicator of the modification of the protein by arachidonate compared with linoleate. The similarities in hexanoate and PDA or NDA content of the protein suggest that the hexanoate and dicarboxylic acids may be formed by the same pathway.

Metal-catalyzed oxidation of plasma

To evaluate the role of PDA and NDA in the linkage of oxidized phospholipids to plasma proteins, plasma was exposed to metal-catalyzed oxidation and protein modifications were measured (**Fig. 4**). High concentrations of Cu^{2+} (5 mM) were used to overcome the inhibitory effect of albumin and endogenous antioxidants. The kinetics of the increase in MDA and protein-bound phosphate (**Fig. 5A**, **B**, respectively) were similar, and both reached a plateau after ~12 h of oxidation. The yield of MDA, a characteristic product of lipid peroxidation (23), was ~10% that of phosphate bound to protein. The fatty acids derived from the *sn*-1 position of plasma phospholipids (myristate, palmitate, oleate, and stearate) increased with similar kinetics. The total content of long-chain fatty acid bound to oxidized plasma proteins (Fig. 5C) was comparable to the phosphate content of the proteins (Fig. 5B), suggesting that phospholipids, rather than cholesterol or triglycerides in plasma, were the primary source of chemical modifications in the oxidized proteins.

Hexanoate, PDA, and NDA concentrations also increased in concert with the increase in the phosphate content of the protein (**Fig. 6A**). As observed with RNase, the protein content of PDA and NDA accounted for cross-linking of ~20% of the lipid phosphorous to proteins in oxidized plasma. The ratio of PDA to NDA was greater than unity during the first 4 h of oxidation but decreased with time, suggesting that arachidonate, despite its lower abundance in plasma, was oxidized more rapidly than linoleate. However, the final concentration of protein-bound NDA was ~2-fold higher than that of PDA, reflecting the higher abundance of linoleate versus arachidonate in plasma phospholipids (24).

To evaluate the involvement of 5-oxovalerate in the modification of amino acid residues in proteins, we measured the concentration of protein-bound CBL. Regardless of the reducing agent used to stabilize double bonds ($NaBH_4$ or $NaBH_3CN$), we were unable to detect quantifiable amounts of CBL either in protein samples from copper oxidation of plasma or in samples of RNase incubated with PAPC (data not shown). Measurements of synthesized CBL showed that we were able to detect and quantify levels of 0.7 pmol of CBL per milligram of protein, which is ~1% of the lowest values of PDA concentration in copper-oxidized plasma samples.

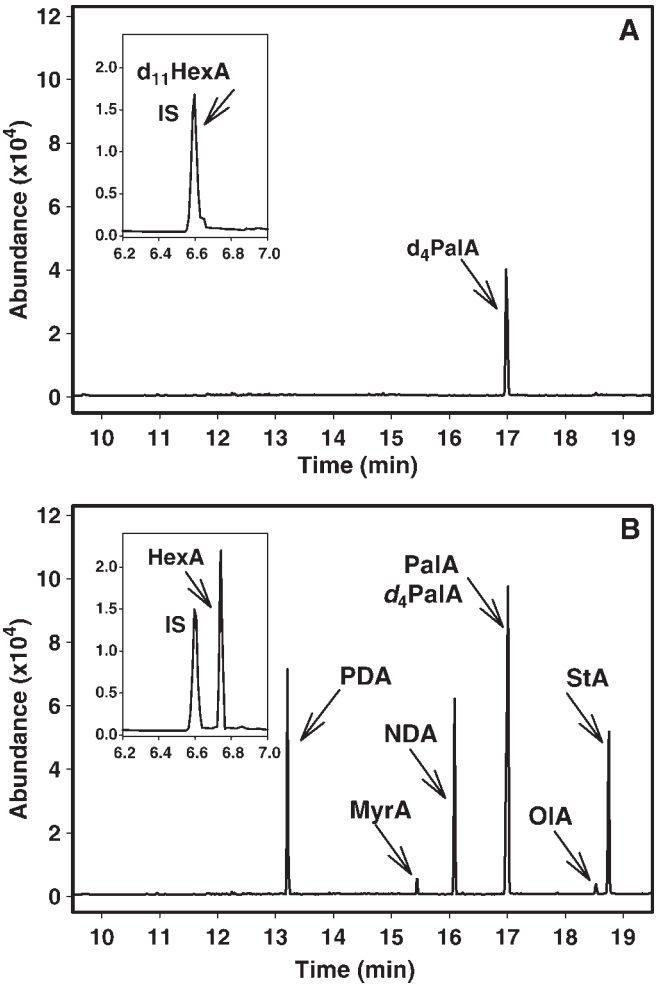

Fig. 4. Typical SIM-GC-MS analysis of plasma proteins at time zero (A) and after metal-catalyzed oxidation (B) (t = 24 h). Plasma was oxidized with 5 mM $CuSO_4$ for 24 h at 37°C. Samples were quenched at the indicated times with diethylenetriaminepentaacetic acid (DTPA), as described in Materials and Methods. HexA, hexanoic acid; MyrA, myristic acid; OlA, oleic acid; PalA, palmitic acid; StA, stearic acid.

Metal-catalyzed oxidation of LDL

To further evaluate the role of PDA and NDA in the modification of oxidized lipoproteins, we measured the formation of these compounds during the Cu^{2+}-catalyzed oxidation of LDL. The kinetics of the increase in conjugated dienes (absorbance at 234 nm) in OxLDL reached a plateau after ~18 h of oxidation (data not shown). As shown in **Table 4**, the fatty acids palmitate, oleate, and stearate increased in concert with the phosphate content

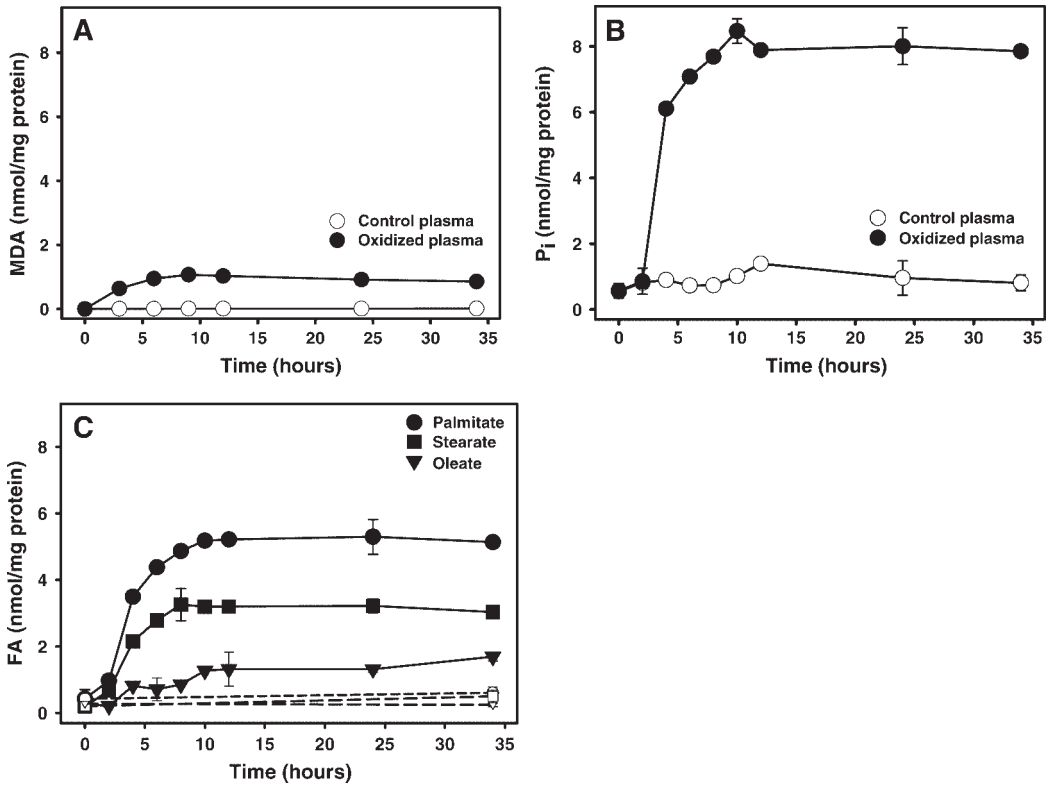

Fig. 5. Kinetics of the formation of malondialdehyde (MDA), protein-bound phosphate (P_i), and long-chain fatty acids during metal-catalyzed oxidation of plasma. Plasma was oxidized with 5 mM $CuSO_4$ for 34 h at 37°C, and samples were quenched at the indicated times with DTPA. A: Kinetics of the formation of MDA, measured by the thiobarbituric acid-reactive substances assay. B: Kinetics of the accumulation of protein-bound phosphate, measured by the Marienetti assay. C: Kinetics of the formation of protein-bound fatty acids released after acid hydrolysis of delipidated plasma, measured by SIM-GS-MS. Myristic acid (not shown) reached ~0.3 nmol/mg protein at 34 h. Each point represents the mean ± SD of duplicate samples from three independent experiments. Open symbols, control plasma; closed symbols, oxidized plasma.

of the OxLDL, in agreement with the results of plasma oxidation experiments (see above). The levels of phosphate, ~1.6 and 68 mol/mol LDL in the starting and oxidized LDL, respectively, were essentially the same as those reported previously in studies leading to the description of the oxovaleraldehyde cross-link (9, 25). Similarly, both PDA and NDA increased significantly during LDL oxidation and corresponded with the increase of hexanoate content. The OxLDL content of PDA and NDA accounted for the cross-linking of ~7% of the lipid phosphorous to the proteins, consistent with the results of experiments on plasma oxidation (Figs. 5, 6).

Analysis of plasma proteins from control and diabetic subjects

Table 5 summarizes the results of the analysis of protein-bound fatty acids and phosphate in total plasma proteins from a group of control and type 1 diabetic patients. Protein-bound fatty acids and phosphate were readily detected in these samples and were increased significantly (mean increase = 40%) in plasma proteins of diabetic patients, even in well-controlled patients with similar lipid profiles (Table 1) and without complications. As in the model studies described above, the total concentration of protein-bound fatty acids agrees reasonably with the phosphate content of the plasma protein. Assuming an average molecular mass of 100,000 Da for plasma proteins, there are 0.1–0.2 mol of protein-bound phosphate per mole of protein in plasma. Although we have no evidence at this point, it seems likely that the phospholipid adducts would be concentrated in the lipoprotein fraction of plasma proteins.

DISCUSSION

We have shown that during the oxidation of PAPC or PLPC in the presence of RNase, the modification of lysine residues is accompanied by increases in the covalently bound phosphate and palmitate contents of the protein. These data confirm previous work describing the binding of largely intact phospholipids to protein during lipoxidation reactions (9–11). We have also quantified hexanoate, PDA, and NDA covalently bound to lipoxidized RNase and oxidized plasma proteins prepared in vitro. For RNase, plasma proteins and LDL, PDA, and NDA explain 5–20% of the cross-linking of phosphate to protein. Our results support the recent work of Kawai et al. (13), identifying

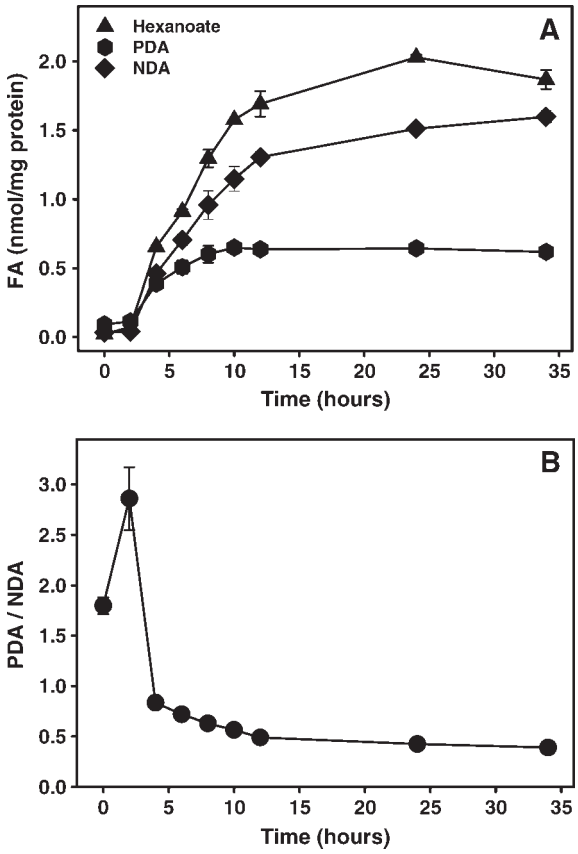

Fig. 6. Kinetics of the formation of protein-bound hexanoate, PDA, and NDA during metal-catalyzed oxidation of plasma. Analyses are for samples described in Figure 5. A: Formation of hexanoate (triangles), PDA (hexagonals), and NDA (diamonds). These fatty acids were not detected in unoxidized plasma and did not increase throughout the plasma incubation. B: Ratio of PDA to NDA versus time.

NDA cross-links between oxidized phospholipids and proteins using immunochemical methods, and extend this work by the identification of the homologous PDA cross-link and by the quantification of both NDA and PDA. At the same time, we were unable to detect the putative oxovalerate cross-link in RNase oxidized in the presence of RNase, or in oxidized plasma protein or LDL, or in human plasma samples. Reduction conditions used for this assay were vigorous, comparable to those used for the reduction and quantification of MDA and HNE adducts to lysine residues in protein (26). We conclude that the PDA and NDA cross-links, although they have yet not been rigorously characterized, may account for up to 20% of the lipid modification of protein and therefore may be quantitatively significant cross-links between oxidized phospholipids and protein.

TABLE 4. Measurement of protein-bound phosphate and carboxylic acid content of LDL and oxidized LDL

Phosphate	Time 0	Time 24 h
	mol/mol LDL	
Hexanoate	0.6 ± 0.1	5.0 ± 0.05
PDA	0.26 ± 0.01	1.8 ± 0.01
NDA	0.24 ± 0.02	2.8 ± 0.2
Palmitate	1.2 ± 0.01	24.9 ± 4.1
Stearate	1.6 ± 0.1	10.6 ± 0.5
Oleate	0.5 ± 0.3	20.6 ± 7.6
Phosphate	1.6 ± 0.1	68.8 ± 1.6

Data shown are means ± range from two independent experiments.

TABLE 5. Phosphate and carboxylic acid adducts in total plasma proteins of control and type 1 diabetic subjects

Analyte	Control Subjects	Diabetic Subjects
Palmitate	1.09 ± 0.28	1.50 ± 0.54[a]
Oleate	0.38 ± 0.18	0.59 ± 0.44
Stearate	0.29 ± 0.08	0.40 ± 0.13[a]
Phosphate	1.54 ± 0.36	1.89 ± 0.49[a]

Data shown are means ± SD and are expressed as nmol per milligram of plasma protein.

[a] $P < 0.02$ for type 1 diabetic versus control subjects.

Oxidation of PUFAs in lipoproteins is known to yield a complex array of products, both protein-bound and free in solution, including isoprostane-derived levuglandin (27, 28) and isoketal (29, 30) derivatives and numerous keto and hydroxy fatty acids (11). A range of smaller, more polar aldehydes, ketoaldehydes, alkenals, and hydroxyalkenals are also released into solution and react with protein to form Schiff bases, Michael adducts, and pyrrole and pyridinium compounds that contribute to the brown color, fluorescence, and cross-linking of oxidized lipoproteins. The quantification of these compounds is complicated by the facts that a wide range of products is formed, many in low yield, and that many of these products are unstable to acid hydrolysis. Others, such as MDA or HNE, may continue to react to form secondary products and cross-links (31). In contrast, protein-bound phosphate and long-chain saturated fatty acids are end products that can be recovered in good yield after acid hydrolysis. As shown in Fig. 5, the levels of protein-bound phosphate and fatty acids in oxidized plasma proteins exceed that of MDA, a commonly measured indicator of lipid peroxidation, by nearly 10-fold. Although not measured in the present work, levels of HNE and CML are comparable to that of MDA in lipoxidized RNase and in oxidized LDL (31), so the assay of protein-bound phosphate and fatty acids provides theoretically greater sensitivity for quantifying the exposure of a protein to lipoxidative stress.

The goal of the present paper is not to present a method for measuring phosphate and fatty acids adducted to oxidized lipoproteins but to demonstrate that measurement of these adducts may be useful for quantifying lipoxidative damage to protein. Although measurement of protein-bound fatty acids by GC-MS provides excellent sensitivity and specificity for detecting lipoxidation reactions, GC-MS assays are inconvenient, requiring derivatization, chromatography, and mass spectrometry. It is conceivable that these assays may be adapted to more convenient LC-MS, ELISA, or receptor-based assays for routine analysis. Another limitation of the current assay is the possible presence of background levels of enzymatically attached phosphate and fatty acids on proteins. Although this background appears to be negligible for plasma pro-

teins, it may limit the usefulness of the assay for the analysis of lipoxidation products on cellular proteins, because protein phosphorylation and lipation are common mechanisms of signal transduction. For cellular proteins, it may be appropriate to focus on protein-bound glycerol or phosphate released by saponification. Glycerol can be measured enzymatically, and organic phosphates, such as glycerylphosphorylcholine, can be measured by variations of the phosphomolybdate assay procedure used here.

Because of the stability of the phospholipid adducts and the relatively long half-life of plasma proteins, assays of covalently bound phosphate and long-chain fatty acids on plasma protein may be useful for integrating oxidative damage to proteins over a period of several days to weeks, depending on the half-life of the major target (modified) proteins in the circulation. Such an assay may complement the measurement of glycated hemoglobin for the integration of blood glucose control and glycative damage to protein in diabetes, providing an independent index of lipid-derived versus sugar-derived damage to protein. This assay may also prove useful for assessing lipoxidative damage to plasma proteins as a result of the consumption of foods rich in lipid peroxidation products. In preliminary studies (Table 5), we detected phosphate and long-chain fatty acids on total plasma proteins from fasting human plasma and found higher concentrations of these adducts in the plasma proteins of even well-controlled, complication-free type 1 diabetic patients. The patients chosen for this study were well matched for their lipid profiles, implicating hyperglycemia, rather than hyperlipidemia, in the modification of proteins. Further studies are planned or in progress to quantify these adducts in total plasma proteins and isolated plasma lipoproteins from both type 1 and type 2 diabetic patients, as a function of the duration and severity of disease, the presence of vascular disease, and other complications in subjects with dyslipidemia but not diabetes, in subjects with metabolic syndrome, and in isolates from atherosclerotic plaque from both diabetic and nondiabetic subjects. In these subjects, the phospholipid adducts may be innocuous indicators of risk for, or the presence of, disease. However, it is also possible that the fatty acids of phospholipids on the surface of lipoxidized proteins may serve as lipid anchors, binding these proteins to cell surfaces and promoting oxidative stress and receptor-independent endocytosis and the accumulation of intracellular lipids.

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