Electrospray Ionization Mass Spectrometry Identifies Substrates and Products of Lipoprotein-associated Phospholipase A\textsubscript{2} in Oxidized Human Low Density Lipoprotein\textsuperscript{*}\textsuperscript{S}

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There is increasing evidence that modified phospholipid products of low density lipoprotein (LDL) oxidation mediate inflammatory processes within vulnerable atherosclerotic lesions. Lipoprotein-associated phospholipase A\textsubscript{2} (Lp-PLA\textsubscript{2}) is present in vulnerable plaque regions where it acts on phospholipid oxidation products to generate the pro-inflammatory lysophospholipids and oxidized non-esterified fatty acids. This association together with identification of circulating Lp-PLA\textsubscript{2} levels as an independent predictor of cardiovascular disease provides a rationale for development of Lp-PLA\textsubscript{2} inhibitors as therapy for atherosclerosis. Here we report a systematic analysis of the effects of \textit{in vitro} oxidation in the absence and presence of an Lp-PLA\textsubscript{2} inhibitor on the phosphatidylcholine (PC) composition of human LDL. Mass spectrometry identifies three classes of PC whose concentration is significantly enhanced during LDL oxidation. Of these, a series of molecules, represented by peaks in the \(m/z\) range 594–666 and identified as truncated PC oxidation products by accurate mass measurements using an LTQ Orbitrap mass spectrometer, are the predominant substrates for Lp-PLA\textsubscript{2}. A second series of oxidation products, represented by peaks in the \(m/z\) range 746–830 and identified by LTQ Orbitrap analysis as non-truncated oxidized PCs, are quantitatively more abundant but are less efficient Lp-PLA\textsubscript{2} substrates. The major PC products of Lp-PLA\textsubscript{2}, saturated and mono-unsaturated lyso-PC, constitute the third class. Mass spectrometric analysis confirms the presence of many of these PCs within human atherosclerotic lesions, suggesting that they could potentially be used as \textit{in vivo} markers of atherosclerotic disease progression and response to Lp-PLA\textsubscript{2} inhibitor therapy.

The identification of Lp-PLA\textsubscript{2} as a strong independent predictor of cardiovascular disease (1–4) has raised many questions about possible roles of the lipid substrates and products of the enzyme in the pathogenesis of atherosclerosis. There is increasing evidence indicating a role for oxidized phospholipids in atherosclerotic disease in general and in mediation of inflammatory processes within the lesion in particular (5, 6). Disease development is invariably linked to the formation (and accumulation) of a variety of lipids, including products of LDL oxidation within the plaque, and it has become clear that many phospholipid oxidation products are bioactive species with potential pro-inflammatory actions that may contribute to plaque progression and destabilization (6–9). Both pro- and anti-inflammatory activities have been assigned to glycerophospholipids with a variety of oxidative modifications to polyunsaturated sn-2 fatty acyl substituents (10–19), and some of these species have been identified in both oxidized LDL (20–22) and atherosclerotic lesions (22, 23). Many of these molecules are putative substrates for Lp-PLA\textsubscript{2}. The enzyme predominantly circulates as a constituent of LDL particles (24, 25), and hence, primary products of LDL oxidation may be subject to Lp-PLA\textsubscript{2}-mediated hydrolysis. The products of this reaction, the lysoPCs (LPCs) and oxidized non-esterified fatty acids, have both been demonstrated to mediate potentially proatherogenic processes \textit{in vitro} (26–30). Furthermore, Lp-PLA\textsubscript{2} inhibition during \textit{in vitro} LDL oxidation has been found to reduce activity of the oxidized LDL in the induction of monocyte chemotaxis (29) and macrophage apoptosis (31). These observations along with increasing evidence that the enzyme is present within atherosclerotic lesions and enriched in vulnerable regions (32, 33) have provided a rationale to support development of Lp-PLA\textsubscript{2} inhibitors as possible anti-atherogenic drugs (34–36). Despite this interest, the consequences of Lp-PLA\textsubscript{2} inhibition at the molecular level have not been systematically explored either in

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\textsuperscript{S} The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables 1 and 2.

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\textsuperscript{2} The abbreviations used are: Lp-PLA\textsubscript{2}, lipoprotein-associated phospholipase A\textsubscript{2}; LDL, low density lipoprotein; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; BHT, butylated hydroxytoluene; nLDL, native LDL; oxLDL, oxidized LDL; ox + iLDL, LDL oxidized in the presence of SB222657; HPLC, high performance liquid chromatography; MS, mass spectroscopy.

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vitro or in vivo. Here we have used electrospray ionization mass spectrometry to characterize the (quantitative) changes observed in phosphatidylcholine (PC) molecular species during in vitro oxidation of purified human LDL and the further modifications caused by the presence of an irreversible Lp-PLA₂ inhibitor, SB222657 (29) during oxidation. Our results distinguish two classes of PC products of LDL oxidation on the basis of greater or lesser efficiency of hydrolysis by Lp-PLA₂ and confirm that saturated and monounsaturated LPCs are the predominant PC products of the reaction. Mass spectrometric analysis of lipid extracts from human carotid endarterectomy tissue confirms the presence of many of these molecules in human atherosclerotic lesions.

**EXPERIMENTAL PROCEDURES**

**Isolation and Oxidation of LDL**—LDL was isolated from normal human plasma (obtained from volunteers with informed consent and stored at −70 °C) by ultracentrifugation in successive KBr gradients (37). Each preparation was from plasma from a single donor. Briefly, thawed plasma (8 × 4.8 ml in 8 Beckman 355646 10-ml polycarbonate tubes) was adjusted to 1.019 g/ml by the addition of 0.2 ml per tube of 1.34 g/ml KBr solution (containing 1 mM EDTA) and centrifuged at 75,000 rpm (Beckman OptimaMax ultracentrifuge, MLA.80 rotor) at 4 °C for 14 h. The top 1 ml from each tube (containing very low density lipoprotein) was removed and discarded; the remaining from each tube were pooled, mixed thoroughly, and 3.25 ml was pipetted into each of eight fresh polycarbonate tubes. The density was adjusted to 1.063 g/ml by the addition of 0.595 ml of 1.34 g/ml KBr solution (containing 1 mM EDTA), and volume was adjusted to 5 ml by the addition of 1.063 g/ml KBr (containing 1 mM EDTA). Tubes were centrifuged again (as before), and the top 1 ml from each (containing LDL) was pooled and dialyzed overnight in a Pierce Slide-a-lyser cassette (3–12 ml, 3500 molecular weight cut off) against 2 changes of phosphate-buffered saline (4 liter each) containing 1 mM EDTA. Dialysate was sterilized by filtration through a 0.2-μm unit (Minisart) and stored at 4 °C for a maximum of 4 weeks before use. Before oxidation, LDL samples were dialyzed (as above) into phosphate-buffered saline without EDTA and pipetted into 4 ml of chloroform and 1 ml water followed by vigorous shaking and centrifugation at 1000 rpm in an Allegra 6 bench-top centrifuge using GH3.8 swingout rotor (Beckman). The lower (chloroform-rich) phase was carefully recovered into a glass vial, dried at 40 °C under a stream of nitrogen gas, and stored at −20 °C for a maximum of 7 days before MS analysis. Using a comprehensive range of synthetic standards, we determined that recovery of PC was 86.5%, and that of lysoPC was 78.1%. Consequently, the use of the internal recovery standards PC14:0/14:0 and lysoPC17:0 provided a quantitative estimation of PC and lysoPC concentrations, respectively (data not shown).

**Lipid Extraction of Carotid Artery Plaque Samples**—Human carotid artery plaques were obtained with informed consent at endarterectomy. The plaques were segmented, snap-frozen, and stored at −80 °C. Frozen segments of plaque (~200 mg each) were pulverized using a ball-grinding method (Mikro-Dismembrator, Sartorius). Approximately 100 mg of frozen ground artery powder was added to 2 ml of chloroform and 1 ml of methanol together with internal standards dimyristoyl PC (40 nmol), heptadecanoyl LPC (4 nmol), and butyland hydroxytoluene (50 μg/ml). Samples were then homogenized with an Ultraturrax homogenizer for 2 min with cooling in an ice bath. After further addition of 3 ml of methanol and 1.6 ml of 0.9% (wt:vol) NaCl, extracts were vigorously shaken to obtain a single phase. Chloroform (2 ml) and water (2 ml) were then added with further shaking to form two phases which were then separated by centrifugation at 400 × g for 10 min. The lower chloroform layer was decanted, dried under nitrogen gas, and stored as above.

**Electrospray Ionization Mass Spectrometry**—Dried lipid extracts were dissolved in solvent (methanol:chloroform:water: ammonia, 70:20:8:2, vol:vol, 500 μl) and introduced by direct infusion at a flow rate of 10 μl/min into the electrospray ionization interface of a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Wythenshaw, UK). Collision gas-induced dissociation of all protonated PC, oxidized PC, and LPC species generated a diagnostic phosphorylcholine fragment of m/z = 184 (39). Consequently, diagnostic precursor scans of m/z = 184 were used to quantify PC species. Because previous reports suggest that the ionization efficiency of polyunsaturated PC species was concentration-dependent compared with monounsaturated species (40), we evaluated this possible differential ionization effect under our experimental conditions. The ionization response of PC16:0/20:4 was identical to those of PC16:0/16:0, PC18:1/18:1, and PC16:0/18:2 over more than 2 orders of magnitude, confirming that the analysis of different molecular species was quantitative within the concentration range of samples analyzed in this study (data not shown). Identities of fatty acyl compositions of resolved PC species were determined by product ion scans under conditions of negative ionization. Protonated PC species do not readily generate negative ions, and consequently [M−15]⁻ species formed by loss of a methyl group from the choline head group were selected for fragmentation. Mass spectrometric data were obtained at unit resolution and processed using MassLynx software (Micromass). After conversion to centroid format, ion intensities were
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FIGURE 1. Changes in PC species following LDL oxidation in vitro. 104 precursor spectra over m/z range 475–850 of lipid extracts from native (A) and oxidized (B) LDL. Region m/z 580–670 shown in 36× magnification highlighting low molecular mass oxidized PC species (m/z 622–664). Int std, internal standard.

corrected for the 13C effect and the progressive decreasing formation of the diagnostic ion with increasing molecular mass (39).

Further Characterization of PC Species by Exact Mass Measurement—Exact mass measurement was performed on a hybrid linear ion trap-orbitrap mass spectrometer (LTQ Orbitrap™, Thermo Electron, Bremen, Germany) used in both positive and negative ion modes. The sample was introduced via infusion at 5 μl/min using an integrated syringe pump and ionized in a standard electrospray source with sheath gas flow of 10 units, capillary temperature of 275 °C, and spray voltage of 4.5 kV. The capillary and tube lens voltages for positive mode analysis were set to 50 and 120 V, respectively. In the negative mode the capillary voltage was −50 V; the tube lens voltage was set to −250 V for most analyses and to −189 V when a chlorinated adduct was a preferred species. The linear ion trap operated with a target value 3 × 10⁶; the corresponding target value for the orbitrap mass analyzer was 2 × 10⁵. The instrument was calibrated externally according to the instructions of the manufacturer. The spectra were acquired under operator control with the resolving power settings of 100,000 at m/z 400 (full width at half-maximum) corresponding to the acquisition time period of 1.5 s/scan. The mass accuracy for all of the species of oxidized PC detected in this study was better than 1.5 ppm.

Statistics—Absolute measured PC concentrations for all LDL samples were expressed relative to total PC concentration (as a percentage) in the corresponding non-oxidized LDL (native) sample. The figures shown all represent the mean ± S.D. of six LDL samples. Levels of PC subclasses in native LDL (nLDL), oxidized LDL (oxLDL), and LDL oxidized in the presence of SB222657 (ox + iLDL) were compared by t test (assuming unequal variances) using Microsoft Excel data analysis tools.

RESULTS

Oxidation of LDL Phosphatidylcholine—Incubation with 40 μM CuSO₄ (in the absence of EDTA and BHT) for 20 h caused substantial and reproducible alterations to the PC molecular species composition of LDL. Oxidation under the conditions described for 20 h was assumed to have proceeded to completion on the basis that no further enhancements of thiobarbituric acid reactive substances or lipid hydroperoxides were detected for oxidation reactions extended beyond this time (data not shown). Representative mass spectra of LDL PC incubated with and without BHT/EDTA (in addition to CuSO₄) are shown in Fig. 1, A and B, respectively, as diagnostic precursor scans of the m/z = 184 phosphorylcholine fragment ion in positive ionization. These spectra are displayed at equivalent ion signal responses, normalized to the base peak (m/z 758) for the native unoxidized sample. They demonstrate that oxidation (i.e. incubation with CuSO₄ in the absence of BHT and EDTA) decreased ion intensities of LDL PC species at m/z = 758, 782, 786, 806, and 810 and increased intensities of ions at m/z = 772, 774, 788, 790, 800, 802, 816, 818, 828, 830, 842, and 848. Supplemental Table 1 summarizes the exact masses and elemental formulae of the native non-oxidized PC and LPC species, determined by LTQ Orbitrap mass spectrometry, together with compositions calculated from P184 scans and molecular species identified from the fatty acyl ions generated by collision-induced dissociation fragmentation of the [M−15]⁻ precursor ions in negative ionization. Oxidation also increased the ion abundances of LPC species at m/z = 496 and 524 and of a series of ions from m/z = 594 to 666 (see Fig. 1, region in 36× magnification). Identities of the oxidation products are discussed below.

Altered concentrations of PC species after copper-induced oxidation are shown in Table 1 (columns 1 and 3) as the means of six LDL preparations, stratified by degree of unsaturation and lipid class. For diradyl PC species, the extent of the oxidation-induced decrease in concentration correlated well with the degree of fatty acyl unsaturation. Thus, there was loss of most PC species containing arachidonate (20:4) or docosahexaenoate (22:6), significant loss of di- and tri-unsaturated fatty acid-containing species, and negligible change in observed concentration of the major mono-unsaturated species, 16:0/18:1 PC. The concentration of measurable total PC after oxidation was around 20% lower than in the native sample, suggesting that a proportion of the PC had degraded (during the oxidation reaction) to a point where it no longer contained a choline head group or had formed species (possibly protein adducts) that were either no longer extractable or were not detectable in the range analyzed.

Oxidation increased the concentrations (quantified as the percentage of the total PC measured in the corresponding native LDL) of three separate categories of PC that are likely to be the result of oxidative modification of diradyl PC species.
The largest observed increases were to species detected over a range between \( m/z \) 594–666 region of the 184 precursor spectrum, whose levels were increased by oxidation, were all further enhanced by at least 2-fold in the presence of inhibitor (Fig. 2, center panel; total 0.49% in oxLDL and 1.19% in ox + iLDL; \( p < 0.005 \)). This result strongly suggested that all of these ion peaks represent (at least in part) molecules that are substrates of Lp-PLA₂. The inhibitor also further enhanced concentrations of the higher mass PC species characteristic of LDL oxidation, although the proportional increase in these species was considerably less than that observed for the lower \( m/z \) products (total in oxLDL 28.8%; ox + iLDL 31.9%; not significant; Fig. 2, lower panel), suggesting that species constituting the bulk of the PC detected in this region are less efficiently hydrolyzed by Lp-PLA₂ during LDL oxidation.

**Identification of PC Species in Oxidized LDL by LTQ Orbitrap Mass Spectrometry**—Direct infusion analysis by tandem MS/MS as precursor scans of 184 cannot distinguish between endogenous PC species and oxidized PC species with the same nominal mass. However, species containing additional oxygen atoms have fractionally lower molecular mass than isobaric endogenous PC species because they contain fewer hydrogen atoms. Consequently, we were able to investigate the identities of endogenous PC and the products of LDL oxidation further by accurate mass measurements of positively ionized species using the LTQ Orbitrap mass spectrometer. As an example of this approach for the short chain oxidized PC series, Fig. 3 shows accurate mass analysis of peaks in the range \( m/z \) 650.3–650.6. Of the many peaks resolved, the most abundant (\( m/z = 650.4384 \)) had an elemental formula \( C_{33}H_{66}O_{24}N_p \), compatible with the diunsaturated LPC species. Importantly, the PC species in the \( m/z \) 594–666 region of the 184 precursor spectrum, whose levels were increased by oxidation, were all further enhanced by at least 2-fold in the presence of inhibitor (Fig. 2, center panel; total 0.49% in oxLDL and 1.19% in ox + iLDL; \( p < 0.005 \)). This result strongly suggested that all of these ion peaks represent (at least in part) molecules that are substrates of Lp-PLA₂. The inhibitor also further enhanced concentrations of the higher mass PC species characteristic of LDL oxidation, although the proportional increase in these species was considerably less than that observed for the lower \( m/z \) products (total in oxLDL 28.8%; ox + iLDL 31.9%; not significant; Fig. 2, lower panel), suggesting that species constituting the bulk of the PC detected in this region are less efficiently hydrolyzed by Lp-PLA₂ during LDL oxidation.

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TABLE 2
Identities and compositions of oxidized phosphatidylcholine species in oxidized LDL

| Exact mass | Elemental formula | LDL | LDL + SB222657 | oxLDL | oxLDL + SB222657 |
|------------|------------------|-----|----------------|-------|-----------------|
| **Oxidized lysoPC species** |
| 534.3188   | C_{26}H_{49}O_8NP | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.09 ± 0.02 | 0.08 ± 0.02 |
| 536.3342   | C_{26}H_{49}O_8NP | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.33 ± 0.10 | 0.20 ± 0.06 |
| 538.3499   | C_{26}H_{49}O_8NP | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.23 ± 0.06 | 0.13 ± 0.05 |
| 550.3135   | C_{26}H_{49}O_8NP | 0.02 ± 0.01 | 0.01 ± 0.00 | 0.29 ± 0.05 | 0.20 ± 0.04 |
| 552.3291   | C_{26}H_{49}O_8NP | 0.01 ± 0.01 | 0.01 ± 0.00 | 0.08 ± 0.02 | 0.09 ± 0.02 |
| **Short chain oxidized PC species** |
| 594.3763   | C_{26}H_{49}O_8NP | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.01 ± 0.00 | 0.04 ± 0.02 |
| 606.3691   | C_{26}H_{49}O_8NP | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.01 ± 0.00 | 0.02 ± 0.01 |
| 608.3992   | C_{26}H_{49}O_8NP | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.01 ± 0.00 | 0.04 ± 0.02 |
| 620.3992   | C_{26}H_{49}O_8NP | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.02 ± 0.01 | 0.04 ± 0.01 |
| 622.4076   | C_{26}H_{49}O_8NP | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.05 ± 0.01 | 0.15 ± 0.04 |
| 636.4231   | C_{26}H_{49}O_8NP | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.03 ± 0.01 | 0.12 ± 0.04 |
| 648.4225   | C_{26}H_{49}O_8NP | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.03 ± 0.01 | 0.07 ± 0.02 |
| 650.4384   | C_{26}H_{49}O_8NP | 0.10 ± 0.02 | 0.08 ± 0.03 | 0.23 ± 0.04 | 0.39 ± 0.11 |
| 664.4182   | C_{26}H_{49}O_8NP | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.06 ± 0.02 | 0.15 ± 0.05 |
| 666.4700   | C_{26}H_{49}O_8NP | 0.00 ± 0.00 | 0.01 ± 0.01 | 0.04 ± 0.01 | 0.17 ± 0.11 |
| **Long chain oxidised PC species** |
| 772.5484   | C_{44}H_{85}O_10NP | 0.66 ± 0.11 | 0.64 ± 0.09 | 2.29 ± 0.50 | 2.21 ± 0.43 |
| 774.5640   | C_{44}H_{85}O_10NP | 0.37 ± 0.07 | 0.41 ± 0.09 | 3.37 ± 0.66 | 3.31 ± 0.36 |
| 788.5432   | C_{44}H_{85}O_10NP | 0.00 ± 0.00 | 0.10 ± 0.11 | 6.65 ± 1.23 | 7.02 ± 0.75 |
| 790.5589   | C_{44}H_{85}O_10NP | 0.29 ± 0.11 | 0.29 ± 0.08 | 2.87 ± 0.38 | 3.88 ± 0.58 |
| 800.5796   | C_{44}H_{85}O_10NP | 0.22 ± 0.07 | 0.23 ± 0.09 | 1.63 ± 0.35 | 1.58 ± 0.20 |
| 802.5954   | C_{44}H_{85}O_10NP | 0.15 ± 0.06 | 0.13 ± 0.03 | 1.46 ± 0.28 | 1.54 ± 0.14 |
| 804.6180   | C_{44}H_{85}O_10NP | 0.21 ± 0.03 | 0.22 ± 0.06 | 0.65 ± 0.11 | 0.95 ± 0.19 |
| 814.5588   | C_{44}H_{85}O_10NP | 0.37 ± 0.19 | 1.22 ± 0.30 | 1.42 ± 0.25 |
| 816.5745   | C_{44}H_{85}O_10NP | 0.17 ± 0.11 | 0.17 ± 0.30 | 4.15 ± 0.86 | 4.19 ± 0.55 |
| 818.5900   | C_{44}H_{85}O_10NP | 0.29 ± 0.09 | 0.27 ± 0.09 | 2.10 ± 0.28 | 2.21 ± 0.31 |
| 828.5383   | C_{44}H_{85}O_10NP | 0.10 ± 0.02 | 0.11 ± 0.02 | 0.49 ± 0.12 | 0.97 ± 0.25 |
| 830.5540   | C_{44}H_{85}O_10NP | 0.19 ± 0.04 | 0.17 ± 0.02 | 1.17 ± 0.19 | 1.69 ± 0.28 |
| 842.5899   | C_{44}H_{85}O_10NP | 0.13 ± 0.09 | 0.13 ± 0.09 | 0.47 ± 0.08 | 0.56 ± 0.05 |
| 848.5649   | C_{44}H_{85}O_10NP | 0.15 ± 0.02 | 0.13 ± 0.03 | 0.25 ± 0.09 | 0.40 ± 0.10 |

with an oxidatively truncated PC, and the same elemental formula as a commercially available oxidized PC standard, 1-palmitoyl-2-(9’-oxy-nonanoyl)-sn-glycero-3-phosphocholine (Fig. 3, inset). The ion peak at 650.4742 corresponds to a di-saturated PC species, with an elemental molecular formula of C_{26}H_{49}O_8NP. There were negligible quantities of this ion peak detectable in the nLDL. There was also generation of an oxidized species in the ox + iLDL at 790.5589 (C_{44}H_{85}O_10NP) that was absent from nLDL. Similar mass analysis has allowed us to deduce probable molecular formulae for all the high m/z PC oxidation products quantified in Fig. 2 (lower panel). Exact mass assignments and molecular formulae for these oxidation products are presented in Table 2. In all cases the predominant mass ion observed in the unit mass 184 precursor scan (Fig. 2, lower panel) corresponded to PC species containing 9, 10, or 11 oxygen atoms.

**Identification of PC Species in Carotid Artery Plaque Samples**—

Copper oxidation of LDL represents a maximal, non-physiological modification of PC composition. Consequently, we wished to establish whether comparable oxidized species could be quantified in samples of carotid artery plaque. Ion peaks with appropriate nominal masses (m/z) for the majority of oxidized PC species were identified by precursor scans of 184 (results not shown); these were then further identified by exact mass measurement by LTQ Orbitrap mass spectrometry (Fig. 5). Exact
mass measurement detected 38 distinct non-oxidized PC ion peaks in plaque, with a preponderance of 18:2- and 20:4-containing species similar to LDL PC, and none with a mass greater than \( m/z \) 834. A total of 26 distinct ion peaks with masses characteristic of molecular species of oxidized PC were quantified in carotid artery plaque samples by exact mass measurement. There were nine, ten, five, and two masses corresponding to PC species containing respectively 9, 10, 11, and 12 oxygen atoms. There was very little overlap between oxidized and non-oxidized PC species in plaque extracts; only five nominal masses contained both. Four of these contained \( \approx 50\% \) unmodified PC, whereas \( m/z \) 788 comprised less than 10% oxidized PC. Moreover, some of these nominal masses contained multiple oxidized species. For instance, in contrast to oxLDL, the major oxidized plaque PC species in the low mass range \( (m/z \ 650) \) contained molecules with both 9 and 10 oxygen atoms (Fig. 6). Overall there was a wide variation in the composition and concentration of oxidized PC species in these five plaques, and none of the samples contained all of the species detected.

**DISCUSSION**

A possible role for Lp-PLA₂ in development and progression of atherosclerosis has been suggested by studies indicating that circulating levels of the enzyme are a strong independent predictor of cardiovascular disease (1–4) and by recent evidence that the enzyme is expressed in atherosclerotic lesions and indeed may be more strongly expressed in unstable and inflammatory regions of plaques (32, 33). A major proposed action of Lp-PLA₂ in the plaque is hydrolysis of modified phospholipids formed during oxidation of LDL within the intima. The products of this reaction are the LPCs and oxidized non-esterified fatty acids, both of which have been hypothesized to play pro-inflammatory roles within the plaque, thus suggesting a strong rationale for anti-Lp-PLA₂ therapy in atherosclerosis (34, 41). Despite this recent attention, the possible consequences of Lp-PLA₂ activity (and inhibition of this activity) in atherosclerosis at the molecular level have not been systematically explored.

In this study we have used electrospray ionization mass spectrometry to characterize the changes in PC content in LDL after oxidation with Cu²⁺. We have also used an inhibitor of Lp-PLA₂, present during the oxidation reaction, to identify molecular species that are substrates and products of Lp-PLA₂ in oxidized LDL.

We have initially used a precursor scan of \( m/z = 184^+ \) phosphorylcholine fragment ion to identify the changes in PC content of LDL after copper oxidation *in vitro*. These are summarized in Table 1. As expected, we find that oxidation leads to a virtually complete loss of the peaks corresponding to diacyl PC species with polyunsaturated fatty acyl substituents and a substantial loss of those corresponding to PC species with di-unsaturated substituents, whereas the levels of PCs containing only monounsaturated and saturated substituents are essentially unchanged by the reaction. It is clear from the quantitative analysis of the 184 precursor spectrum that a proportion of
the PC that is oxidized during the reaction is not modified into extractable species that contain the intact phosphorylcholine head group. The likely explanation for their complete disappearance from the 184 precursor spectrum is that many of the oxidized molecules have formed covalent adducts with the proteins present in the LDL particle (42), although it is beyond the scope of the current study to determine this definitively. We are, however, able to detect some enhancement of many m/z 184 precursor ion peaks corresponding to three classes of molecules that are likely to be products of PC oxidation.

First, the levels of saturated and monounsaturated LPC are significantly enhanced in oxidized, compared with native LDL. Theoretically these LPC species should be the most abundant products of PC hydrolysis by PLA₂ enzymes, as these fatty acid substituents are most abundant at the sn-1 position of PC, suggesting they may be generated by Lp-PLA₂ action on modified PCs formed during LDL oxidation. Lp-PLA₂ specificity is largely determined by substrate solubility since, uniquely among PLA₂s, this enzyme accesses substrate from the aqueous phase (43). Consequently, Lp-PLA₂ has significant activity only against phospholipids with short or polar sn-2 substituents that form substantial monomeric, instead of micellar distributions (44). Phospholipids with oxidatively modified sn-2 substituents are, therefore, much better substrates for Lp-PLA₂ than unmodified PCs, and it is likely that action of Lp-PLA₂ on these oxidation products is a major source of the LPC generated by LDL oxidation. The enzyme is predominantly associated with LDL in human plasma, where it specifically associates with a defined region of apoB100 (24, 25), and it co-purifies with LDL during the centrifugation isolation procedure. We have, therefore, been able to test the hypothesis that LPC is generated by Lp-PLA₂ action during LDL oxidation by including an irreversible Lp-PLA₂ inhibitor during the reaction. In the presence of 300 nM SB222657 the generation of LPC was largely, although incompletely reversed, suggesting that the majority of LPC was indeed formed by Lp-PLA₂ action during oxidation. More complete inhibition of LPC generation by SB222657 (at 100 nM) has previously been reported in LDL subjected to Cu²⁺ oxidation for only 4 h (29). The likely explanation for this difference is that a low rate of LPC generation occurs independently of Lp-PLA₂ during LDL oxidation in vitro, resulting in the observed higher LPC levels at the end of the longer incubation period used here. It is worth noting that, as with diacyl PC, most LPC with di- or polyunsaturated substituents is modified during the oxidation reaction, showing that, as expected, they are not gen-
Concomitant with the reduction in LPC generation caused by the presence of SB222657, significant enhancement in the levels of the second class of PC oxidation products, those species represented by peaks in the 184 precursor spectrum between m/z 594 and 666 (that we have designated short chain-oxidized PC species), was also observed. The changes in LPC and short chain oxidized PC are, in terms of -fold difference, by far the major changes observed between LDL oxidized in the presence and absence of inhibitor and indeed are the only results that were statistically significant changes. This is clear evidence that the 594 – 666 ion peaks, confirmed by LTQ Orbitrap analysis as oxidatively modified polyunsaturated fatty acid-containing PC species, are efficient substrates for Lp-PLA2. This experiment, thus, identifies a class of molecules present in human LDL that represents a major source of Lp-PLA2-generated LPC and, therefore, also of oxidized free fatty acids during LDL oxidation. The presence of high levels of these molecules within atherosclerotic plaques in conjunction with the high activity of Lp-PLA2 may, therefore, be an important factor in determining plaque vulnerability.

A number of previous reports have identified and characterized constituents of a 184 precursor peak at m/z 650 in oxidized LDL (21, 22) and material from atherosclerotic lesions (22, 23). Harrison et al. (21) separated phospholipids isolated from oxidized LDL by reverse phase HPLC and identified components of an m/z 650-184 precursor peak (from a single HPLC fraction) as 1-stearoyl-2-(7-hydroxyhepta-5-enoyl)-sn-glycero-3-phosphocholine and 1-palmitoyl-2-(9-oxo-nonanoyl)-sn-glycero-3-phosphocholine. The molecular formulae of both these is C_{33}H_{65}O_{9}NP; thus, either or both could be major components of the abundant 650.4384 peak detected by the LTQ Orbitrap in oxidized LDL. By contrast Hoff et al. (23), who used selected positive ion monitoring MS/MS to track all 650-184 transitions in an HPLC separation of phospholipids extracted from either oxidized LDL or human atherosclerotic lesions, concluded that a major component of the 184 precursor m/z 650 peak in oxidized LDL and human atherosclerotic lesion was likely to be 1-palmitoyl-2-(5-hydroxy-8-oxo-6-octenoyl)-sn-
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glycerol-3-phosphocholine (C₃₂H₆₅O₁₀NP). Our data suggest that compounds with this molecular formula are at best minor constituents of the 184 precursor m/z 650 peak in oxidized human LDL, although possibly differences in LDL preparations and oxidation conditions could account for this discrepancy. LTQ Orbitrap analysis also indicates that a measurable proportion of the 184 precursor unit mass m/z 650 peak in oXLDL (also present in nLDL; data not shown) corresponds to a native, desaturated PC species (most likely 12:0/14:0).

Accurate mass analysis demonstrates that the higher m/z peaks in the 184 precursor spectrum whose levels were increased significantly during LDL oxidation correspond, as expected, to non-fragmented oxidized PCs. It is interesting to note that oxidized PC species with masses suggesting that they correspond to two of the species we observe in oxidized LDL (m/z 788.5441 and 790.5597) have been identified by nanoelectrospray Fourier transform ion cyclotron resonance MS of oxidized PC species extracted from soybean (45). Fatty acid substituents of these oxidized PCs were also identified in this system after in-source collision at high voltage in negative ion mode. The masses of these suggested that each had a palmitoyl substituent (m/z 255.2325) and an oxidized substituent containing two additional oxygen atoms (m/z 309.2073 of 18:3 + 2O and m/z 311.2221 of 18:2 + 2O for 788.5441 and 790.5597, respectively) (45). Preliminary MS/MS analysis of oXLDL peaks 772 and 774 in negative ion mode (M-16 ions for 788 and 790 in positive ion mode) suggests they have substituents of similar masses (data not shown); it, thus, seems likely that the molecules we observe in oxidized LDL are identical to these soybean-oxidized PCs. Overall, the long chain oxidized PCs represent quantitatively the most abundant PC products of LDL oxidation (Fig. 2), although it is clear from the use of SB222657 that they are less efficient as substrates for Lp-PLA₂ than are the short chain oxidized PCs (Fig. 2). Interestingly, it has recently been reported that F₂-isoprostanes are efficiently released from their (precursor) PC esters by the action of Lp-PLA₂ in human plasma (46). Despite a high reported affinity for PC F₂-isoprostane (Kₘ 4.5 nm, compared with 5.2 and 19.3 µm, respectively, for platelet-activating factor and palmitoyl oxyvasoconstrictor), Lp-PLA₂ was found to release the non-esterified F₂-isoprostane at a considerably slower rate than its action on platelet-activating factor and palmitoyl oxyvasoconstrictor in release of acetate or oxoaldehyde. The reason for this discrepancy is not clear, but the results support our findings that non-truncated oxidized PC molecules are subject to hydrolysis by Lp-PLA₂ at a slower rate than the truncated products of PC oxidation (46).

The physiological importance of our identification of Lp-PLA₂ substrates in oxidized LDL is underlined by the results obtained from LTQ Orbitrap mass spectrometric analysis of lipid extracts from human carotid endarterectomy explants. Comparison of the oxidized PC spectra of LDL oxidized in the presence of SB222657 (Table 2) and that of human carotid plaque tissue (Fig. 5, supplemental Table 2) reveals considerable overlap, although some oxidized PC species have been uniquely detected in ox + iLDL (for example C₃₃H₆₅O₁₀NP and C₄₄H₆₅O₁₀NP) or in plaque (for example, C₃₃H₆₅O₁₀NP and C₄₆H₆₅O₁₁NP). In particular some of the more abundant species in ox + iLDL of both the short chain (for example C₃₃H₆₅O₉NP) and long chain (for example C₄₂H₇₉O₁₀NP and C₄₄H₇₉O₁₀NP) oxidized PCs are also major constituents of many of the plaque lipid extracts. It should be emphasized that these identities are at the level of molecular rather than structural formulae; it is possible that many of the accurate mass peaks are detecting more than one species of oxidized PC and, thus, that there are differences (potentially qualitative and quantitative) between the species constituting the peaks in ox + iLDL and those in carotid plaque. Identification of the precise structural formulae of PC species that constitute the oxidized PC peaks detected, and hence, confirmation of the degree of overlap that exists between ox + iLDL and plaque-oxidized PCs will require chromatographic separation of species with identical molecular formulae. This would constitute an important extension of this work, particularly in light of data implicating the oxidized free fatty acids as potentially the most proatherogenic constituents of Lp-PLA₂-mediated oXPC hydrolysis (29).

In summary, we have quantified the PC molecules detectable in human LDL and those present after exhaustive oxidation with copper sulfate both in the presence and absence of an irreversible inhibitor of Lp-PLA₂. As expected, oxidation resulted in the loss of a substantial fraction of di-unsaturated- and polyunsaturated-substituted PC species, compatible with the susceptibility of these molecules to oxidative modification. Although a proportion of these molecules so modified was not subsequently detectable, presumably as a result of protein adduct formation by oxidized PCs, we detected enhanced concentrations of LPC species and two classes of oxidized PC (confirmed by accurate mass measurements using an LTQ Orbitrap Mass Spectrometer) in oxidized LDL. Use of the irreversible Lp-PLA₂ inhibitor, SB222657, during oxidation identified saturated and mono-unsaturated LPCs and short chain oxidized PCs as the major products and substrates, respectively, of the enzyme in oxidized LDL. PCs that had undergone oxidative modification without chain fragmentation appeared to be very much less efficient substrates for Lp-PLA₂ but were quantitatively the most abundant PC products of LDL oxidation and, therefore, possibly major contributors to LPC production by Lp-PLA₂. Human carotid plaque samples were found to contain many oxidized PC species of the same molecular formulae as those identified in LDL oxidized in the presence of Lp-PLA₂ inhibitor (as well as some not detected in ox + iLDL), although the amount and composition of oxidized PC varied considerably between different plaques. In view of the increasing evidence of a contributory role for Lp-PLA₂ in development and progression of atherosclerotic lesions, it would be of great interest to quantify these molecules in vulnerable plaques (and specifically in vulnerable regions of plaques) as well as in the circulation of potentially vulnerable individuals. PC substrates and products of Lp-PLA₂ may, thus, have the potential to provide clinically important information as markers of atherosclerotic disease progression as well as of response to potential Lp-PLA₂ inhibitor therapy.

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