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Characterization of covalent modifications of HDL apoproteins by endogenous oxidized phospholipids

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Abstract

High density lipoprotein (HDL) is cardioprotective, unless it is pathologically modified under oxidative stress. Covalent modifications of lipid-free apoA-I, the most abundant apoprotein in HDL, compromise its atheroprotective functions. HDL is enriched in oxidized phospholipids (oxPL) *in vivo* in oxidative stress. Furthermore, oxidized phospholipids can covalently modify HDL apoproteins. We have now carried out a systematic analysis of modifications of HDL apoproteins by endogenous oxPL. Human HDL or plasma were oxidized using a physiologically relevant MPO-H₂O₂-NO₂⁻ system or AIPH, or were exposed to synthetic oxPL. Protein adduction by oxPL was assessed using LC-MS/MS and MALDI-TOF MS. The pattern of HDL apoprotein modification by oxPL was independent of the oxidation systems used. ApoA-I and apoA-II were the major modification targets. OxPL with a γ -hydroxy (or oxo)-alkenal were mostly responsible for modifications, and the Michael adduct was the most abundant adduct. Histidines and lysines in helices 5–8 of apoA-I were highly susceptible to oxPL modifications, while lysines in helices 1, 2, 4 and 10 were resistant to modification by oxPL. In plasma exposed to oxidation or synthetic oxPL, oxPL modification was highly selective, and four histidines (H155, H162, H193 and H199) in helices 6–8 of apoA-I were the main modification target. H710 and H3613 in apoB-100 of LDL and K190 of human serum albumin were also modified by oxPL but to a lesser extent. Comparison of oxPL with short chain aldehyde HNE using MALDI-TOF MS demonstrated high selectivity and efficiency of oxPL in the modification of HDL apoproteins. These findings provide a novel insight into a potential mechanism of the loss of atheroprotective function of HDL in conditions of oxidative stress.

Graphical abstract

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Keywords

HDL; apoA-I; oxidized phospholipids; oxidized phospholipid-apoprotein adduct

Introduction

HDL is one of the major carriers of cholesterol in blood. In contrast to other lipoproteins, many physiological functions of HDL influence the cardiovascular system in favorable ways unless HDL is pathologically modified. Atheroprotective functions of HDL include its anti-inflammatory, anti-oxidant, antiapoptotic, antithrombotic, and vasodilatory properties [1–3].

HDL consists of a phospholipid shell and a hydrophobic lipid core comprised of triglycerides and cholesterol esters. The phospholipid shell is stabilized by apoproteins, such as apoA-I and apoA-II, the most and second most abundant apoproteins in HDL. Several minor apoproteins, including apoA-IV, apoCs, apoE, and others are also found in HDL [4]. Under proinflammatory pathological conditions, multiple changes to HDL properties are reported to be proatherogenic in nature. These include the attenuation of the anti-oxidant capacity of HDL, proinflammatory activity, a decrease in HDL-associated enzymes, alterations in HDL protein composition, and changes in lipid composition [5–8]. Such altered HDL has a reduced capacity to support reverse cholesterol transport and has been termed ‘dysfunctional HDL’ [5–8]. HDL isolated from human atherosclerotic lesions is dysfunctional [9–11].

Multiple *in vitro* studies have reported that when HDL is exposed to various oxidation systems, including oxidation by transition metal ions, hypochloric acid, physiologically relevant myeloperoxidase (MPO)–H₂O₂–Cl[–] and MPO–H₂O₂–NO₂[–] system, it loses its atheroprotective activity [10, 12–16]. The oxidative modification of HDL apoproteins has been proposed as a molecular mechanism leading to impairment of the HDL atheroprotective function *in vivo*. Tyr-192 and Tyr-166 of apoA-I are reported to be the preferred sites for nitration and chlorination, while only chlorination of lipid free apoA-I strongly impairs ABCA1-mediated cholesterol efflux [16–18]. Other studies have demonstrated that tyrosine modification in apoA-I is not required for the loss of function of cholesterol efflux, although it impairs the activation of lecithin–cholesterol acyltransferase (LCAT) [19, 20]. Studies on the tryptophan modifications have demonstrated that tryptophan residues of apoA-I are critical for cholesterol efflux function, and site specific oxidation of try72 impairs the cholesterol efflux function [21, 22]. Although the oxidation of Met86 and

Met112 has little effect on the ability of activation of LCAT and lipid binding [23], oxidation of Met148 has been found to impair the ability of apoA-I to activate LCAT [24].

Aldehydic lipids are major products of lipid peroxidation produced via the oxidative fragmentation of the polyunsaturated fatty acid chains of phospholipids, the major component of biological membrane and outer shells of lipoprotein [25, 26]. Modifications of apoA-I by highly reactive short chain aldehydic lipids, such as 4-hydroxy-2-nonenal (HNE) or malondialdehyde (MDA), as well as by the reactive oxygen and nitrogen species, have been the main focus of multiple studies [1, 27, 28]. oxPL capable of covalent interaction with proteins were found in circulation in hyperlipidemia and in atherosclerotic lesions [29, 30]. They possess multiple biological activities [31–35] and their presence is controlled by the innate immune system [36]. Studies on the covalent modification of HDL apoproteins by oxPL are very limited, mainly due to the multiple technical challenges in the identification of the oxPL protein adducts using LC-MS/MS [37, 38]. Previous studies using human plasma supplemented with biotinylated phospholipid probes have demonstrated that apoA-I is one of the major modification targets by biotinylated oxPL. We have recently developed a high efficiency enrichment method for the oxPL–peptide adducts [38], which enabled us to study in detail the covalent modifications of proteins by various endogenous oxPL. We have now applied it to studies of HDL apoprotein modification in isolated human HDL or plasma oxidized by either biologically relevant MPO–H₂O₂–NO₂[−] system or AIPH, a widely used hydrophilic radical initiator which generates free radicals at a controlled and constant rate [39].

We now show that apoA-I is the major modification target of endogenous oxPL in isolated human HDL and in human plasma. We characterized in detail the modification of HDL apoproteins by the endogenous oxPL, and demonstrated high selectivity and efficiency of oxPL in the modification of HDL apoproteins. We demonstrated the presence of domains in apoA-I that are susceptible or resistant to modification. We demonstrated that helices 5–8 of apoA-I and the central domain of apoA-II are intensively modified by oxPL with γ -hydroxy (or oxo)-alkenal in the form of several types of adducts. Four histidines in helices 6–8 of apoA-I are particularly susceptible to modification. We also found that H710 and H3613 in apoB-100 of LDL and K190 of human serum albumin (HSA) were modified by oxPL but to a lesser extent.

Experimental Procedures

Reagents

Myeloperoxidase from human polymorphonuclear leukocytes was purchased from EMD Millipore (Billerica, MA). 2, 2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (AIPH) were obtained from Wako Chemicals USA (Richmond, VA). Sequencing-grade modified trypsin was purchased from Promega (WI, USA). HODA-PC (9-hydroxy-12-oxo-10-dodecenoic acid ester of 2-lysophosphocholine) was synthesized as described previously [40]. All other chemicals were obtained from Sigma or Fisher Scientific Company unless otherwise specified.

Isolation of human plasma and HDL

Fresh human blood was drawn into EDTA solution (final concentration 5 mM). Human plasma was isolated by centrifugation at 2500 RPM for 15 min. Human HDL (density 1.063–1.210 g/ml) was isolated from human plasma by sequential density gradient ultracentrifugation as described elsewhere [41].

Oxidation of human HDL by MPO–H₂O₂–NO₂[−] [42] and AIPH

HDL (0.5 mg/ml) was oxidized by incubation at 37°C in 67 mM phosphate buffer, pH 7.4, 200 μM DTPA, 500 μM NaNO₂, and 30 nM MPO. H₂O₂ was injected into solution to a final concentration of 100 μM, followed by one hour incubation, followed by addition of another dose of H₂O₂ (100 μM) and one hour incubation. For oxidation using AIPH, HDL (0.5 mg/ml in 67 mM phosphate buffer, pH 7.4) was incubated in the presence of 10 mM AIPH at 37 °C for two hours. Oxidation reactions were terminated by addition of 40 μM butylated hydroxytoluene (BHT). NaBH₄ (100 mM) was added to stabilize reversible oxPL adducts. Preparations were transferred into 100 mM TRIS buffer (pH 7.8) and subjected to tryptic digestion.

Oxidation of human plasma by MPO–H₂O₂–NO₂[−] and sample preparation for LC-MS/MS analysis

Fresh human plasma samples were dialyzed against 67 mM phosphate buffer (pH 7.4) before the oxidation. The oxidation and treatment of plasma was performed similar to oxidation of isolated HDL. Control (not oxidized) plasma samples were treated with NaBH₄ similar to oxidized samples. Urea was added to the plasma samples to a final concentration of 8 M, followed by DTT reduction, IAA alkylation and tryptic digestion. A synthetic oxPL-peptide adduct was added to each sample as an internal standard, followed by enrichment of oxPL-peptide adducts as we described previously [38].

Modification of plasma proteins by HODA-PC

Human plasma was incubated with increasing concentrations of HODA-PC (5, 15, 50 and 100 μM) at 37 °C for 1.5 h in the presence of 20 μM BHT with gentle rocking under the protection of argon. The reaction was terminated by 100 mM NaBH₄ (1 h at room temperature).

Modification of HDL by HODA-PC or HNE and sample preparation for Matrix-assisted Laser Desorption Ionization-Time-of-Flight-Mass Spectrometry (MALDI-TOF MS)

HDL (0.5 mg/ml) was incubated for 6 h with increasing concentration of HODA-PC or HNE (15, 30, 45 and 75 μM) in the presence of 10 μM BHT at 37 °C with gentle rocking under the protection of argon. The reaction was terminated by 100 mM NaBH₄ (room temperature for 1 h). pH of samples was adjusted to neutral using acetic acid (50 %). Melatonin (35 μM) [43] and 10 mM EDTA were added to prevent apoprotein oxidation during the isolation procedure. Then urea solid was added to a final concentration of 6 M. Two ml of methanol-chloroform mixture (2:1, containing 40 μM BHT) was added to 545 μl of HDL solution and vortexed for 30 seconds. Then 0.8 ml of HPLC grade H₂O was added, and vortexed for 30 sec, followed by addition of 2 ml of chloroform (containing 40 μM BHT) and vortexing for

another 30 seconds. The samples were centrifuged at 2000 g for 10 min, and the bottom layer (chloroform) was removed. Chloroform (2 ml) was added to the remaining solution, and the procedure was repeated two more times. The aqueous phase was desalted using centrifugation at 4000g for 10 min with centrifugal filter (3 K cutoff).

Mass spectrometry and data processing

Chromatographic separation of the peptide samples was performed by UltiMate 3000 RSLCnano LC system equipped with a reversed-phase capillary chromatography column (Dionex-Acclaim Pepmap C18, 15 cm $\times$ 75 μ m i.d., 2 μ m, 100 Å). An elution gradient was used by mixing mobile phase A (0.1% formic acid in water) with solvent B (0.1% formic acid in acetonitrile), as follows: isocratic elution with 2% B from 0 to 5 min; increasing to 40% B from 5 to 76 min; increasing to 70% B from 76 to 78 min; isocratic elution with 70% B from 78 to 85 min; decreasing to 2 % B from 85 to 86 min; isocratic elution with 2% B from 86 to 100 min. ESI mass spectrometry was performed with a Thermo Scientific LTQ-Orbitrap-Elite MS in the positive ion mode. Five microliter of the samples was injected. The peptides eluted from the column at a flow rate of 0.25 μ L/min were introduced into the source of the mass spectrometer on-line. The nanoelectrospray ion source is operated at 2.5 kV. The inlet capillary temperature was maintained at 200°C. The samples were analyzed by either a data dependent acquisition (DDA) mode, or a multiple reaction monitoring (MRM) mode in which specific peptides were targeted over the entire course of the LC experiments. For the DDA mode, the dynamic exclusion option was enabled after three repeat acquisitions within 20 s duration, and the exclusion duration was set at 90 s. The MS/MS collision energy was set to 35%. The obtained MS/MS spectra were searched against the Uniprot/Swiss-Prot human protein database. Common modifications in the form of various types of adducts formed between proteins and oxPL, including Schiff base adduct, Michael adduct, Ketoamide adduct, Cyclic hemiacetal adduct, and other previously reported adducts [26], were used for database search. The corresponding mass shifts were shown in Table S1 (Supporting Information). The search parameters used were two missed cleavage sites, a mass tolerance of 10 ppm for the parent ion and 1.2 Da for the fragment ion.

MALDI-TOF-MS and data processing

Calibration of the 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems/MDS Sciex) was carried out using a protein mixture consisting of insulin (m/z 5734.590), thioredoxin (m/z 11674.480) and apomyoglobin (m/z 16952.560). Desalted protein samples were mixed with saturated sinapic acid solution (in 30% acetonitrile, containing 0.1% trifluoroacetic acid) at a ratio of 1/5 (v/v), and 1 μ l of the resulting solution was applied to target plate for MALDI-TOF analysis. MALDI-TOF-MS data was acquired over a mass range of 25–35 KDa in the positive linear mode using fixed laser intensity for 1000 shots/spectrum. Data were processed and analyzed with Data Explorer software (Applied Biosystems). For the calculation of average number of HODA-PC covalently adducted to apoA-I, the data were plotted using Origin 8.1 software. The multiplet peaks were fitted with a Lorentzian line shape function, and the area of fitted peaks (FP) was integrated using Origin software. The percentage of each fitted peak (FP%) was calculated as following: $FP\% = 100 \times \text{Area of each FP} / \text{sum of area of all FP}$. The average number of HODA-PC / apoA-I = $\text{sum of } (n \times FP\% \text{ of peak with } n \text{ HODA-PC modification}) / 100$, where n is the number of HODA-PC

adducted to apoA-I. The average number of HNE /apoA-I = (the peak mass of HNE modified apoA-I – the peak mass of native apoA-I) /158. The percentage of HODA-PC or HNE adducted to apoA-I = $100 \times (\text{the average number of HODA-PC or HNE adducted to apoA-I} / \text{ratio of (HODA-PC or HNE : apoA-I)})$ used for specific experiment).

Statistical analysis

Results represent means $\pm$ S.D., and the statistical significance was evaluated using a two-tailed unpaired Student's test. Results were considered statistically significant with p values <0.05 .

Results

Identification of oxPL-protein adducts in oxidized human HDL

Isolated human HDL was mildly oxidized using either physiologically relevant MPO–H₂O₂–NO₂[–] system or AIPH, processed and analyzed by LC-MS/MS, as described in Experimental Procedures. The MS/MS spectra were searched against the Uniprot/Swiss-Prot human protein database using Proteome Discoverer software. The database search covered common adducts formed between nucleophilic amino acids of proteins and oxPL, including Schiff base adduct, Michael adduct, ketoamide adduct, cyclic hemiacetal adduct, and furan adduct (Figure S1, Supporting Information). Nucleophilic amino acids analyzed included lysine, histidine, and cysteine, and oxPL searched included aldehydic oxidized phospholipids shown to be present *in vivo* in dyslipidemia [29] (Table S2, Supporting Information).

The protein modifications in HDL oxidized by the MPO–H₂O₂–NO₂[–] system or by AIPH were detected almost exclusively in apoA-I and apoA-II, two major apoproteins in HDL. In the HDL oxidized by the MPO–H₂O₂–NO₂[–] system, we detected covalent modifications of 14 amino acid residues of apoA-I, and five amino acid residues of apoA-II. Modifications were by a variety of oxPL generated mostly from the more abundant phospholipids in the form of several types of adducts (Table 1 and Table S3, Supporting Information). Both apoA-I and apoA-II were mainly modified by oxPL with a γ -hydroxy (or oxo)-alkenal at the *sn*-2 position, such as HODA / KODA-PL and HOOA / KOOA-PL. In addition, apoA-I and apoA-II were also modified by ON-PL and OV-PL (Table S2, Supporting Information), oxPL with a terminal aldehyde at the *sn*-2 position [44]. These oxidative products are derived from the most abundant HDL phospholipids that contain a linoleic or arachidonic acid at the *sn*-2 position, respectively [44]. We also detected a few modifications of apoproteins by HOHA / KOHA-PL, two γ -hydroxy (or oxo)-alkenal containing oxPL that are derived from less abundant phospholipids containing docosahexaenoic acid at the *sn*-2 chain (Table S2, Supporting Information).

γ -hydroxy (or oxo)-alkenal containing oxPL formed mostly Michael adducts with histidine residues of apoproteins (Figure S1, Supporting Information). These oxPL also formed the Michael adduct and Schiff base adduct with protein lysine residues (Figure S1, Supporting Information). Cyclic hemiacetal adducts (for HODA/HOOA/HOHA-PL) and ketoamide adducts (for KODA/KOOA/KOHA-PL), two stabilized forms derived from the Michael

adduct, were also detected (Figure S1, Supporting Information). In addition, a number of Schiff base adducts of lysine residues with ON-PL and OV-PL were also identified (Table 1 and Table S3, Supporting Information). In samples of HDL oxidized by AIPH, the oxPL involved, the adduct types, and residues that were modified were very similar to those that were detected in the HDL oxidized by the MPO-H₂O₂-NO₂⁻ system (Table S4, Supporting Information), indicating that the mechanism of initiation of oxidation is not critical for the pattern of modification.

The number of peptide spectrum matches (PSM) identified by the database search engine correlates well with the abundance of individual modified peptide [45]. To roughly determine the preference of oxPL modification among all the apoprotein amino acid residues, we compared the total number of PSM for each modified amino acid residues of apoA-I as shown in Figure 1. PSM numbers from analysis of HDL oxidized by copper ion [38] were also calculated and plotted in the same graph for comparison (Figure 1). The region spanning helices 5–8 of apoA-I was found to be more extensively modified by oxPL when either method of oxidation was used. Interestingly, none of the ten lysine residues in helices 1, 2, 4 and 10 were modified, while all six lysine residues in helices 5–8 were modified (Figure 1). All five histidine residues of apoA-I were modified by oxPL, and all five are located in the region spanning helices 5–8, where six out of the ten modified lysine residues are also located (Figure 1). Among the modified residues on apoA-I, H162 in helix 6 has the highest PSM number detected using either method of oxidation. In addition, H193 and H199 in helix 8 have a relatively high PSM number in all three methods (Figure 1). These results suggest that the nucleophilic histidine and lysine residues in the region spanning helices 5–8 of apoA-I are highly susceptible for oxPL modification. In addition, K12 and K23, two lysine residues in N-terminal, and K96 in helix 3, were also modified by oxPL in either method of oxidation. Five out of the nine lysine residues were modified in apoA-II of HDL oxidized by either method. These residues are located in the middle region of apoA-II (K28-K46) (Table 1 and Table S3, Supporting Information).

ApoA-I is the major protein target for oxPL modification in human plasma exposed to MPO-H₂O₂-NO₂⁻ system

We next tested whether modification of HDL apoproteins occurs in a complex protein milieu of plasma, where radical scavengers and antioxidants are present. We carried out oxidation of fresh human plasma samples using MPO-H₂O₂-NO₂⁻ system and processed samples as described above for oxidized isolated HDL. A synthetic oxPL-peptide adduct was added as an internal standard before the enrichment procedure to allow relative quantification of oxPL-adducts. Samples were subjected to LC-MS/MS analysis, first using a data dependent acquisition (DDA) mode for identification of modified peptides, followed by multiple reaction monitoring (MRM) mode for relative quantification. The MS/MS spectra obtained were searched against a human protein database.

We detected a total of eleven peptides of plasma proteins modified with oxPL. Eight of the eleven oxPL-peptide adducts detected were adducts of apoA-I (Table 2). We also detected two adducts of apoB-100, a major apoprotein of LDL, and one adduct of HSA. All adducts detected are in the form of the Michael adduct, the most abundant adduct type detected in

oxidized HDL. Similar to our findings using isolated HDL, the major oxPL that modified the proteins in plasma were oxPL with a γ -hydroxy (or oxo)-alkenal. Ten out of the 11 adducts were the Michael adduct of histidine, and only one is the Michael adduct of lysine, which was detected in HSA. All modified residues in apoA-I – H155, H162, H193, and H199 are located in the region spanning helices 6–8, again demonstrating the high susceptibility of this region for modification. No lysine adducts in apoA-I were detected, most likely due to the high reversibility of these adducts and, as a consequence, the low abundance of lysine adducts as compared to histidine adducts. Two histidine residues (H710 and H3613) in apoB-100 and one lysine residue (K190) in HSA were modified by oxPL.

We then used a relative quantification of peptides based on the internal standard to assess the time dependence of adduct formation in human plasma exposed to $\text{MPO-H}_2\text{O}_2\text{-NO}_2^-$ system. The level of oxPL-peptide adducts in plasma steadily increased with the increasing time of oxidation without reaching saturation after 7 hours of oxidation (Figure 2). Peptides containing H162, H193, and H199 were more abundant, suggesting that these histidine residues are particularly susceptible to modification by oxPL, in agreement with our analysis of isolated oxidized HDL. Comparison of the relative peak area of modified peptides and the total number of adduct types and modified amino acid residues in apoA-I, apoB-100 and HSA demonstrated that the extent of modification of apoA-I in plasma is significantly higher than that of HSA and apoB-100. These results suggest that apoA-I in human plasma is the main target for oxPL modification in conditions of oxidative stress. Considering the high complexity of human plasma with high concentrations of major proteins and the comparatively low abundance of apoA-I, these results strongly suggest that the oxPL modification of proteins in plasma is highly selective.

ApoA-I is the major target of modification during human plasma exposure to synthetic oxPL

The high selectivity of oxPL modification for apoA-I may simply be due to the preferential formation of oxPL in HDL particles and subsequent modification of proteins that are in close proximity. If this is the case, the oxPL generated outside HDL (e.g., in cells or other lipoproteins) will be less selective in protein modification. To test this hypothesis, human plasma was incubated with increasing concentration of synthetic HODA-PC (Table S2, Supporting Information), one of the major oxPL that, as we found, covalently modifies proteins in human plasma. The plasma samples were then processed and analyzed as described above for oxidized isolated HDL. The total number of PSM for each modified residue was summarized in Table S5 (Supporting Information). In plasma samples treated with 5 μM and 15 μM of HODA-PC, H162 and H193 of apoA-I were the only two modified residues detected (Table S5, Supporting Information), indicating the susceptibility of these two residues of apoA-I for the modification by HODA-PC. At a higher concentration of HODA-PC (50 μM and 100 μM), we detected modifications of four histidine residues of apoA-I in HDL (H155, H162, H193, and H199), one histidine residue in apoB-100 (H710) and two residues in HSA (K212 and H535) (Table S5, Supporting Information). In addition, at 100 μM of HODA-PC, modification of K46 in apoA-II was detected. All detected modifications were in the form of Michael adducts. In general, the PSM numbers for HODA-PC modified residues increased gradually with an increasing concentration of

HODA-PC (Table S5, Supporting Information), indicating an increasing extent of modifications by HODA-PC at higher concentrations. To evaluate the selectivity of modifications, the total numbers of PSM for all modified residues in each modified protein were calculated and compared (Figure 3). At high concentrations of HODA-PC (50 μ M and 100 μ M), the total number of PSM for HODA-PC modified residues from apoA-I is about 50, 50 and 10 times higher than that from apoB-100, apoA-II and HSA, respectively. These results indicate that apoA-I is a primary target for covalent modification by oxPL, even when oxPL is not generated in the lipoprotein. The experiments further confirmed that the four histidine residues located in the helices 6–8 of apoA-I are the most susceptible for modification by oxPL.

oxPL with γ -hydroxyalkenal group modify ApoA-I in HDL rapidly and efficiently

Previous studies have demonstrated strong modification of apoA-I by HNE, a highly reactive product of lipid peroxidation. We now investigated the reactivity of oxPL toward apoA-I using isolated HDL since 90–95% of apoA-I in plasma exists as HDL particles. We used HODA-PC because it shares the reactive group (γ -hydroxyalkenal) with HNE.

HODA-PC was incubated with HDL at a molar ratio of 3.7 / 1 (HODA-PC / apoA-I) for increasing time at 37 °C and the reaction was terminated by reduction using NaBH₄. HDL and HDL treated with HODA-PC in the presence of NaBH₄ served as control. HDL was treated with HNE in a similar fashion for comparison. Samples were processed as described in Experimental Procedures and analyzed using MALDI-TOF MS. The MALDI-TOF MS spectra of control HDL samples demonstrated a singlet peak of apoA-I around 28.1 K (Figure 4a). In contrast, in HDL incubated with HODA-PC, we observed multiplet peaks with a molecular mass difference of 708 between adjacent peaks. This difference corresponds to the molecular mass gained by HODA-PC adduction in the form of the reduced Michael adduct. The leftmost peak corresponds to the unmodified apoA-I, and the four newly generated peaks represent the apoA-I with 1, 2, 3 and 4 Michael adducts (Figure 4a). These multiplet peaks were fitted with a Lorentzian line shape function, and the area of fitted peaks was integrated using Origin software. The average number of HODA-PC covalently adducted to apoA-I was calculated as described in the Experimental Procedures (Figure 4c). The reaction between HODA-PC and apoA-I in HDL is fast, reaching a plateau by 5 min (Figure 4c). About 2.1 out of 3.7 HODA-PC molecules were adducted to apoA-I, giving a HODA-PC conversion rate of 56.8%. In comparison, the incubation of HNE with apoA-I in HDL for increasing time periods generates singlet peaks with increased m/z value (Figure 4b). We found that less than two molecules of HNE were adducted to apoA-I by 30 min (Figure 4d). The reaction of HNE with apoA-I in HDL reached a plateau after 5 hours, indicating that it is significantly slower than the reaction between HODA-PC and apoA-I in HDL. About 3.2 out of 3.7 HNE molecules were adducted to apoA-I after 5 h incubation, giving a high HNE conversion rate of 86.5%.

Next, we studied the efficiency of HODA-PC adduction to apoA-I in HDL. The average number of HODA-PC adducted to apoA-I increased steadily with the increasing ratio of HODA-PC/apoA-I from 1.1/1 to 5.5/1, generating multiplet peaks shifting toward the high m/z direction in MALDI-TOF MS spectra (Figure 5a and 5c). At the 1.1/1 HODA-PC/apoA-

I ratio, about 0.95 molecule of HODA-PC, on average, was adducted to HDL apoA-I (Figure 5e), giving a high HODA-PC conversion rate of 86.4% and demonstrating the high efficiency of HODA-PC adduction to apoA-I in HDL. The conversion rate of HODA-PC decreased gradually with an increasing HODA-PC/apoA-I ratio (Figure 5e). The incubation of HDL and HNE with the increasing ratio of HNE/apoA-I lead to the formation of apoA-I modified by an increasing number of HNE, as demonstrated by the right shifting of apoA-I peak in MALDI-TOF MS spectra (Figure 5b and 5d). Unlike HODA-PC, the conversion rate of HNE remains high with an increasing ratio of HNE/apoA-I, as shown in Figure 5f. These results indicate that both HODA-PC and HNE have a high conversion rate for apoA-I adduction at a ratio of 1.1/1 (HODA-PC or HNE / apoA-I) whereby their concentration used (15 μ M) is close to a physiologically relevant concentration [29, 46].

HODA-PC is more selective and effective than HNE for the modification of apoA-I in HDL in the presence of high concentration of HSA

The high reaction rate and high selectivity of HODA-PC modification of apoA-I in HDL may be due to the long fatty acid chain at *sn*-1 position of HODA-PC, allowing HODA-PC to stay tethered to the phospholipid shell of HDL particles and increasing the chance to react with the apoA-I. This may result in higher selectivity of oxPL like HODA-PC, as compared to short chain aldehydes like HNE, in the modification of HDL apoA-I in the presence of high concentration of other proteins.

To test this hypothesis, we incubated HODA-PC or HNE with HDL (0.5 mg/ml) at a ratio of 2.2/1 (HODA-PC or HNE / apoA-I) in the absence or presence of high concentration of HSA (20 mg/ml) for 5 h at 37 °C. Samples were then processed and analyzed using MOLDI-TOF MS as above. The average number of HODA-PC modifications per apoA-I was minimally reduced by the presence of a 40 fold excess of HSA (Figure 6a and 6c). In comparison, the presence of high concentration of HSA during the incubation of HNE with HDL lead to a dramatic decrease in the average number of HNE adducted to apoA-I in HDL (Figure 6b and 6c). This experiment demonstrates that oxPL such as HODA-PC are more selective for the modification of apoA-I in HDL in the presence of plasma proteins than highly hydrophilic short chain aldehydes such as HNE.

Discussion

The studies on the modification of HDL apoproteins by endogenous aldehydic oxPL, a major product of lipid peroxidation known to be present *in vivo* at sites of oxidative stress, are very limited [29, 37, 38]. In this study, we carried out a comprehensive characterization of the HDL apoprotein modifications by endogenous oxPL induced by physiologically relevant MPO-H₂O₂-NO₂[−] oxidizing system, and exogenous synthetic oxPL. Our studies demonstrated the highly selective modification of HDL apoproteins and the particularly high susceptibility of residues in the functionally important helix 5–8 of apoA-I and the central domain of apoA-II to modification by various oxPL in the form of several adduct types.

We recently developed a high efficiency enrichment method for peptide adducts with oxPL, which allowed us to apply LC-MS/MS to the identification of the target proteins and detailed characterization of their modification by the endogenous aldehydic oxPL generated

in complex biological systems [38]. Using this approach, we have now studied in detail the modification of apoproteins by endogenous oxPL in human HDL subjected to oxidation by the *in vivo* relevant MPO-H₂O₂-NO₂⁻ system and mild free radical oxidation by AIPH [39, 42]. We have also compared the data with a previous analysis of HDL oxidized by strong oxidation by copper ions [38]. We further supported these data using modification with synthetic oxPL. We have found that the pattern of HDL apoprotein modification by aldehydic oxPL is independent of the method of oxidation. All three oxidation methods produced very similar patterns of apoprotein modification: the major modification targets of oxPL include apoA-I and apoA-II, the most and second most abundant apoproteins in HDL; both apoproteins are mainly modified by oxPL with a γ -hydroxy (or oxo)-alkenal in the form of the Michael adduct; and the helices 5–8 of apoA-I and the central domain of apoA-II are preferentially modified by oxPL. This finding indicates that oxPL species generated and their relative abundance are likely determined by the original fatty acid composition of the lipoprotein phospholipids, but not by the method of oxidation.

The most easily detected adducts were those formed by oxPL having γ -hydroxy (or oxo)-alkenal with histidine residues in form of Michael adduct. These oxPL also formed the Michael adduct and Schiff base adduct with protein lysine residues. The lysine adducts are not stable and reversible [26], but we were able to detect them due to the stabilization of adducts by NaBH₄ reduction immediately after oxidation procedure. We also detected two stabilized forms derived from the Michael adduct – cyclic hemiacetal adducts (for HODA/HOOA/HOHA-PL) and ketoamide adducts (for KODA/KOOA/KOHA-PL). Schiff base adducts of lysine residues with ON-PL and OV-PL were also identified. We were not able to detect other well-known adduct types, such as furan adduct, probably due to their low abundance. The prevalence of the Michael adducts of HODA / KODA-PL and HOOA / KOOA-PL with histidines of apoA-I that we detected is likely due to the relatively high abundance of HODA / KODA-PL and HOOA / KOOA-PL [29, 30], their high reactivity toward histidine residues leading to fast formation of Michael adduct and relative stability of the Michael adduct with histidine residues at neutral pH [26].

Our studies clearly demonstrate the existence of domains in apoA-I that are highly susceptible or resistant to oxPL modification. In isolated human HDL, the lysine and histidine residues in helices 5–8 of apoA-I are the major targets for modification by various oxPL. In addition, N-terminus, a region involved in apoA-I amyloidosis [47], and helix 3 were also modified. Plasma contains multiple antioxidants, therefore it is not surprising that we detected significantly less modifications of apoA-I in oxidized plasma. Nevertheless, the modifications of four histidine residues (H155, H162, H193, and H199) in helices 6–8 were still detected, indicating that these residues are particularly susceptible for covalent modification. Further experiments using low concentrations of synthetic HODA-PC and short incubation times with human plasma suggested that H162 and H193 are the residues that are most susceptible to covalent modification. These findings are in agreement with previous studies that demonstrated that helices 6–8 of apoA-I in human plasma is susceptible to modification by synthetic HNE [28]. Tyr 166 and Tyr 192, which are close to H162 and H193, respectively, were also reported to be the major sites for chlorination and nitration of apoA-I in HDL exposed to MPO-generated oxidants [17]. The oxidation of HDL using MPO-H₂O₂-NO₂⁻ system could lead to both nitration of tyrosine residues in apoA-I

and to modification of lysine and histidine residues of apoA-I by oxPL. Nevertheless, our experiments have demonstrated that the pattern of HDL apoprotein modification by oxPL is independent of the method of oxidation, suggesting that the nitration of tyrosine residues has little effect on the HDL apoprotein modification by oxPL.

The oxPL modifications in helices 5–7 are of particular interest because this region of apoA-I is critical for LCAT activation [48]. Helix 8 is important for the apoA-I lipidation [48] and is critical for the ABCA1 facilitated binding of apoA-I to the surface of peripheral cells, an event indispensable for ABCA1 mediated cellular cholesterol efflux to apoA-I [49–51]. The covalent modifications of helices 5–8 by oxPL might limit conformational adaptability of apoA-I and its ability to interact with ABCA1 and cell surface, thus impairing its ability to pick up cholesterol from peripheral cells. Similarly, these modifications might also limit the ability of apoA-I to interact with and activate LCAT, thus impeding the HDL maturation process.

In contrast to histidines, the lysine residues on apoA-I are clearly split into two groups – those susceptible to modification and those that are resistant. While all six lysine residues in helices 5–8 were modified, we found no modifications of the ten lysine residues in helices 1, 2, 4 and 10. Even when HDL was oxidized to a high degree by copper ions, these residues were not modified. The mechanism of protection is not clear and needs further investigation.

Our studies also demonstrated that five out of nine lysine residues of apoA-II in HDL were modified by various oxPL. They are located in the central domain of apoA-II (K28-K46). Previous studies have shown that the three lysine residues in the central domain of apoA-II can be modified by HNE [28]. The highly hydrophilic central domain of apoA-II contains eight out of nine nucleophilic amino acid residues of apoA-II, explaining the preference of oxPL modification for this domain. The central domain of apoA-II is indispensable for ABCA1 mediated cholesterol efflux from cells, suggesting that modifications can have consequences for the function of apoA-II [52].

One of our most intriguing findings is the high selectivity of the apoA-I modification by oxPL in oxidized human plasma. We found that apoproteins in HDL are by far the major targets for oxPL modification, even though their concentrations in plasma are much lower than that of major plasma proteins. Taking into account the following, i) the rate of modification of HSA and apoA-I shown in Fig 2, ii) molar concentrations of apoA-I and HSA in plasma, and iii) assuming that peptides of apoA-I and albumin are detected with similar sensitivity, the apoA-I is at least 100 times more likely to be modified by oxPL than HSA. The simplest explanation for these observations is that oxPL, generated in lipoprotein during plasma oxidation, may preferentially stay incorporated in the phospholipid shell of lipoprotein via *sn*-1 long hydrophobic chain [53], making reaction with the surrounding apoproteins preferable. This is in contrast to the previously reported modification pattern of plasma proteins by the hydrophilic short chain aldehydes [28], where multiple non-lipoprotein related proteins were modified. Direct comparison of the oxPL (HODA-PC) and short chain aldehyde (HNE) revealed that both have a high conversion rate for adduction to apoA-I in HDL at a physiologically relevant concentration. However, the reaction of apoA-I in HDL with oxPL is much faster compared to HNE. Furthermore, while the adduction of

HNE to apoA-I was dramatically suppressed by the presence of albumin, the adduction of HODA-PC to apoA-I was affected only slightly, suggesting that short chain aldehydes like HNE do not discriminate between lipoprotein and non-lipoprotein targets [28, 54]. These results suggest that short chain aldehydes and oxPL may have different targets *in vivo*. oxPL may play a more critical role than short chain aldehydes in the modification of targets associated with cell membrane or lipoproteins including HDL apoproteins.

Another interesting result is the relative lack of oxidative modifications of apoB-100 of LDL. Theoretically, this observation may be explained by preferential formation of oxPL in HDL in plasma. Indeed, it has been shown that LDL is less susceptible to oxidation than HDL [55, 56] due to LDL having more antioxidant protection [57]. It has been also proposed that apoA-I may catalyze formation of aldehydic phospholipids from phospholipid hydroperoxides, leading to selective accumulation of such phospholipids in HDL [58]. However, we observed that oxPL preferentially modify HDL apoproteins even when synthetic oxPL is added to plasma, a condition that excludes the effect of antioxidants or apoproteins on generation of oxPL adducts. Thus, a different mechanism is needed to explain the preferential modification of HDL apoA-I by oxPL. The preferential association of oxPL with HDL particles independent of the site of oxPL formation may be such a mechanism. Such association can be due to high affinity of apoA-I to oxPL, or simply due to preferential incorporation of conical-shaped lipids like oxPL into HDL high curvature lipid shell [59–62]. Further studies are needed to test this hypothesis.

While most of the modifications we found were in apoproteins A-I and A-II, we also detected the Michael adduct of oxPL with K190 of albumin when human plasma was oxidized using the MPO–H₂O₂–NO₂[−] system. An additional two modified residues of HSA, K212 and H535, were identified when human plasma was incubated with synthetic HODA-PC. These residues on albumin are prone to covalent modification. They have been shown to be covalently modified by a wide range of molecules including sugars in the glycation process [63], organophosphates [64], covalent drugs [65, 66], and base propenals [67]. K190 and K212 are located around the fatty acid binding domain IIA, and H535 is located in the fatty acid binding domain IIIB [68]. There is a possibility that fatty acid binding domains may bind the oxPL, shifting the equilibrium toward the formation of covalent adducts.

In conclusion, we present the first systematic investigation of HDL apoprotein modification by endogenous oxPL induced *in vitro* by a variety of methods, in isolated HDL and in plasma. Our studies demonstrate that endogenous oxPL selectively and efficiently target HDL apoproteins in plasma and that apoproteins are preferentially modified by oxPL containing a γ -hydroxy (or oxo)-alkenal group at *sn*-2 position. The functionally important helices 5–8 of apoA-I, and particularly H162 and H193, are preferentially modified by oxPL. The central domain of apoA-II, which is important for the ABCA1 mediated cholesterol efflux, is also extensively modified by oxPL. Taken together, these findings provide a novel insight into a potential mechanism of the loss of atheroprotective function of HDL *in vivo* in conditions of oxidative stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

HNE	4-hydroxy-2-nonenal
MDA	malondialdehyde
MPO	myeloperoxidase
LCAT	lecithin–cholesterol acyltransferase
AIPH	2, 2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride
BHT	butylated hydroxytoluene
MALDI-TOF MS	Matrix-assisted Laser Desorption Ionization-Time-of-Flight-Mass Spectrometry
PSM	peptide spectrum matches
MA	Michael adduct
CH	cyclic hemiacetal adduct
KA	ketoamide adduct
SB	Schiff base adduct
HODA-PL	HOOA-PL and HOHA-PL, the 9-hydroxy-12-oxo-10-dodecenoic acid, 5-hydroxy-8-oxo-6-octenoic acid and 4-hydroxy-7-oxo-5-heptenoic acid ester of lysophospholipid
KODA-PL	KOOA-PL and KOHA-PL, the 9-keto-12-oxo-10-dodecenoic acid, 5-keto-8-oxo-6-octenoic acid and 4-keto-7-oxo-5-heptenoic acid esters of lysophospholipid
OB-PL	OV-PL and ON-PL, the 4-oxobutyric acid, 5-oxovaleric acid and 9-oxononanoic acid esters of lysophospholipid
oxPL	oxidized phospholipids
PC	Phosphatidylcholines

PAPC	1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphatidylcholine
PLPC	1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphatidylcholine
PDPC	1-palmitoyl-2-docosaheptaenoyl-sn-glycero-3-phosphatidylcholine

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Highlights

- Modification sites on HDL apoproteins, adduct types and oxPL involved are identified
- Modification of HDL apoproteins by oxPL is fast, efficient and highly selective
- The pattern of HDL apoprotein modification is independent of the HDL oxidation method
- HDL apoA-I residues in helices 5–8 are highly susceptible to modification by oxPL
- Michael adduct of γ -hydroxy/oxo alkenal PL and apoA-I histidine is prevalent in oxHDL

Figure 1. The region spanning helix 5–8 of apoA-I was extensively modified by various oxPL in human HDL oxidized by either MPO

–H₂O₂–NO₂[–] system, AIPH or copper ion. For oxidation using MPO–H₂O₂–NO₂[–] system, HDL (0.5 mg/ml) was incubated with MPO (30 nM), NaNO₂ (500 μM), H₂O₂ (added hourly, 100 μM for each addition) and DTPA (200 μM) at 37 °C for 2 h. For oxidation using AIPH, HDL (0.5 mg/ml) was incubated with AIPH (10 mM) at 37 °C for 2 h. Samples of oxidized HDL were subjected to NaBH₄ reduction, tryptic digestion and LC-MS/MS analysis. Total numbers of peptides spectrum matches (PSM) for each modified residue were calculated and plotted against the residue number. The open triangles, filled triangles and filled arrows indicate unmodified lysines, modified lysines and modified histidines, respectively. The PSM number from copper oxidized HDL was normalized by dividing by 2 and plotted in the same graph for comparison.

Figure 2. ApoA-I is the major protein target for oxPL modification in oxidized human plasma Human plasma was oxidized using physiologically relevant MPO–H₂O₂–NO₂[−] system as described in Figure 1 for indicated time (0, 2, 4, and 7h). Plasma samples were then subjected to NaBH₄ reduction, tryptic digestion and peptide enrichment as described in Experimental Procedures. OxPL-peptide adducts were analyzed using LC-MS/MS with MRM mode. The peak area of oxPL-peptide adducts were normalized to that of internal standard (IS) and plotted against oxidation time. a, oxPL-peptide adducts of apoA-I; b, oxPL-peptide adducts of apoB-100 and HSA.

Figure 3. ApoA-I remains the major target of modification by externally added oxPL in human plasma

Human plasma was incubated with various concentration of HODA-PC (5, 15, 50 and 100 μM) at 37 °C for 1.5 h. Plasma samples were then processed and analyzed using LC-MS/MS as described in Table 1. Total numbers of PSM for all oxPL-modified peptides identified in each protein was calculated and plotted against HODA-PC concentration. Data represent mean of three independent experiments and statistical significance was determined by t-test. * $P < 0.05$, *** $P < 0.001$.

Figure 4. oxPL containing γ -hydroxyalkenal group modify ApoA-I in HDL more rapidly than HNE

(a) HODA-PC (50 μ M) or (b) HNE (50 μ M) were incubated with human HDL (0.5 mg/ml) at 37 $^{\circ}$ C for increasing time. The reaction was terminated by reduction with NaBH₄. HDL samples were then processed as described in the Experimental Procedures and analyzed using MALDI-TOF MS. The average number of HODA-PC (c) or HNE (d) covalently adducted to apoA-I was calculated as described in the Experimental Procedures and plotted against the incubation time. Results represent two independent experiments.

Figure 5. Efficiency of modification of ApoA-I in HDL by oxPL containing γ -hydroxyalkenal group and HNE

(a) HODA-PC or (b) HNE was incubated with human HDL (0.5 mg/ml) at an increasing ratio of HODA-PC or HNE to apoA-I at 37 °C for 6 h. The samples were processed and analyzed as described in Figure 4. The average numbers of HODA-PC (c) or HNE (d) covalently adducted to apoA-I were plotted against the ratio of HODA-PC / apoA-I or HNE / apoA-I respectively. The percentage of HODA-PC (e) or HNE (f) adducted to apoA-I was calculated as described in the Experimental Procedures and plotted against the ratio of HODA-PC / apoA-I or HNE / apoA-I respectively. Results represent two independent experiments.

Figure 6. oxPL with γ -hydroxyalkenal group are more selective and effective than HNE for the modification of apoA-I in HDL in the presence of high concentration of HSA

(a) HODA-PC (30 μ M) or (b) HNE (30 μ M) were incubated with human HDL (0.5 mg/ml) in the absence or presence of HSA (20 mg/ml) at 37 $^{\circ}$ C for 6 h. The samples were processed and analyzed as described in Figure 4. (c) The average number of HODA-PC or HNE covalently adducted to apoA-I was calculated as described in the Experimental Procedures and plotted as column bars. Data represent mean of three independent experiments. Statistical significance was determined by t-test. *P < 0.05, **P < 0.01.

Table 1

OxPL-peptide adducts detected in human HDL exposed to MPO–H₂O₂–NO₂[−] system.

Proteins	Modified residues	Adduct types	OxPL types
ApoA-I	K208	MA	KOOA/HOOA-PL*, KODA/HODA-PL
	H199	MA, CH	KOHA/HOHA-PL, KOOA/HOOA-PL, KODA/HODA-PL
	K195	SB	KODA-PL, ON-PL
	H193	MA, CH	KOOA/HOOA-PL, KODA/HODA-PL
	K182	CH or KA*	HOHA-PL
	H162	MA, CH	KOHA/HOHA-PL, KOOA/HOOA-PL, KODA/HODA-PL
	H155	MA	KOHA/HOHA-PL, KODA/HODA-PL
	K140	MA, SB	KOOA/HOOA-PL, KODA/HODA-PL, ON-PL
	H135	MA, CH	KODA/HODA-PL, HOHA-PL
	K133	MA, SB	KOOA/HOOA-PL, ON-PL
	K96	SB	KODA/HODA-PL, ON-PL, OV-PL
	K94	MA, SB	KOOA/HOOA-PL, ON-PL
	K23	MA, SB	KOOA/HOOA-PL, KODA/HODA-PL, ON-PL
	K12	MA, SB	KOOA/HOOA-PL, KODA/HODA-PL, ON-PL
ApoA-II	K46	CH or KA, MA, SB	KOOA/HOOA-PL, KODA/HODA-PL, HOHA-PL, ON-PL, OV-PL
	K44	MA, SB	KOOA/HOOA-PL, KODA/HODA-PL, ON-PL
	K39	MA, SB	KOOA/HOOA-PL, KODA/HODA-PL, ON-PL, OV-PL
	K30	MA, SB	KOOA/HOOA-PL, KODA/HODA-PL, ON-PL
	K28	MA, SB, CH or KA	KOOA/HOOA-PL, ON-PL

HDL (0.5 mg/ml) was incubated with MPO (30 nM), NaNO₂ (500 μM), H₂O₂ (added to 100 μM / hour) and DTPA (200 μM) at 37 °C. After for 2 h incubation samples were subjected to NaBH₄ reduction, tryptic digestion, peptide enrichment procedure and LC-MS/MS analysis. The MS/MS spectra obtained were searched against a human protein database to identify oxPL modified peptides as described in Experimental Procedures. MA, CH, KA and SB represents Michael adduct, cyclic hemiacetal, ketoamide and Schiff base adduct respectively. KOOA/HOOA-PL* represents either KOOA-PL or HOOA-PL. In case of the reduction of MA and SB adduct by NaBH₄, KOOA-PL and HOOA-PL moieties of these adducts were converted into the same structure with same molecular mass and not be differentiated by LC-MS/MS. CH or KA*, the CH adduct and KA adduct (reduced form) have the same molecular mass which could not be differentiated by LC-MS/MS.

Table 2

OxPL-peptide adducts detected in human plasma exposed to physiologically relevant MPO-H₂O₂-NO₂⁻ system.

Protein	oxPL-modified peptides	OxPL (Adduct types)
Apo-AI	ATEH(199)LSTLSEK	KOOA/HOOA-PL (MA) *
		KODA/HODA-PL (MA)
	LAIEYH(193)AK	KOOA/HOOA-PL (MA)
		KODA/HODA-PL (MA)
	TH(162)LAPYSDELRL	KOHA/HOHA-PL (MA)
		KOOA/HOOA-PL (MA)
		KODA/HODA-PL (MA)
	AH(155)VDALR	KOOA/HOOA-PL (MA)
ApoB-100	VLVDH(710)FGYTKDDK	KOOA/HOOA-PL (MA)
	IH(3613)SGSFQSQVELSNDQEK	KOOA/HOOA-PL (MA)
HSA	DEGK(190)ASSAK	KODA/HODA-PL (MA)

Human plasma was subjected to mild oxidation using MPO-H₂O₂-NO₂⁻ system followed by sample preparation and analysis using LC-MS/MS as described in Table 1. The MS/MS spectra obtained were searched against a human protein database to identify oxPL modified peptides in the form of various types of adducts as described in Experimental Procedures. MA represents Michael adduct and HSA denotes human serum albumin. KOOA/HOOA-PL (MA)

* represents MA adduct of peptide with either KOOA-PL or HOOA-PL.