Cross-linking modifications of HDL apoproteins by oxidized phospholipids: structural characterization, in vivo detection, and functional implications

Received for publication, March 14, 2019, and in revised form, December 16, 2019. Published, Papers in Press, January 6, 2020, DOI 10.1074/jbc.RA119.008445

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Edited by George M. Carman

Apolipoprotein A-I (apoA-I) is cross-linked and dysfunctional in human atheroma. Although multiple mechanisms of apoA-I cross-linking have been demonstrated in vitro, the in vivo mechanisms of cross-linking are not well-established. We have recently demonstrated the highly selective and efficient modification of high-density lipoprotein (HDL) apoproteins by endogenous oxidized phospholipids (oxPLs), including γ-ketoalkenal phospholipids. In the current study, we report that γ-ketoalkenal phospholipids effectively cross-link apo-proteins in HDL. We further demonstrate that cross-linking impairs the cholesterol efflux mediated by apoA-I or HDL3 in vitro and in vivo. Using LC-MS/MS analysis, we analyzed the pattern of apoprotein cross-linking in isolated human HDL either by synthetic γ-ketoalkenal phospholipids or by oxPLs generated during HDL oxidation in plasma by the physiologically relevant MPO-H2O2-NO2 system. We found that five histidine residues in helices 5–8 of apoA-I are preferably cross-linked by oxPLs, forming stable pyrrole adducts with lysine residues in the helices 3–4 of another apoA-I or in the central domain of apoA-II. We also identified cross-links of apoA-I and apoA-II with two minor HDL apoproteins, apoA-IV and apoE. We detected a similar pattern of apoprotein cross-linking in oxidized murine HDL. We further detected oxPL cross-link adducts of HDL apoproteins in plasma and aorta of hyperlipidemic LDLR−/− mice, including cross-link adducts of apoA-I His-165–apoA-I Lys-93, apoA-I His-154–apoA-I Lys-105, apoA-I His-154–apoA-IV Lys-149, and apoA-II Lys-30–apoE His-227. These findings suggest an important mechanism that contributes to the loss of HDL’s atheroprotective function in vivo.

This was supported in part by National Institutes of Health Grants HL145536 (to T. V. B.) and HL077213, HL073311, and HL126738 (to E. A. P.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains Figs. S1–S7 and Tables S1–S7.

The Raw files, MGF files, and search engine files have been deposited to MassIVE with the dataset identifier MSV000084689 (http://massive.ucsd.edu) and ProteomeXchange with the dataset identifier PXD016752 (http://www.proteomexchange.org/).

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High density lipoprotein (HDL) is one of the five major groups of lipoproteins circulating in the blood. HDL has multiple functions, including anti-oxidant, anti-inflammatory, anti-apoptotic, anti-thrombotic, and vasodilatory properties (1–4). HDL is also known as a key player in the atheroprotective reverse cholesterol transport where HDL promotes cholesterol efflux from cells of peripheral tissues, carries cholesterol in the circulation, and delivers it to the liver for reutilization or excretion (5). This function of HDL is believed to be central to its atheroprotective role. It is also known to be compromised via a number of mechanisms (6–9).

HDL consists of a hydrophobic core (cholesteryl esters and triglycerides) surrounded by a monolayer of phospholipids embedded with free cholesterol and apoproteins. The apoproteins of HDL include apoA-I and apoA-II, the most and second most abundant apoproteins in HDL, and minor apoproteins, such as apoA-IV, apoCs, apoE, and others (4). The cholesterol efflux from cells is most effectively carried out by small, dense, and lipid-poor HDL3 particles (10). HDL3 picks up cholesterol via the interaction of apoA-I with the ABC transporter A1 (ABCA1) expressed on the cell surface (5). Lecithin-cholesterol acyltransferase (LCAT) binds to the amino acid 121–186 domain of apoA-I and catalyzes the conversion of HDL-free cholesterol into cholesteryl esters, which migrate to the core of HDL3, causing the particles to change from discoidal to spherical and promoting the maturation of HDL3 into HDL2. HDL2 transports the cholesterols to liver, either directly via hepatic SR-BI receptor or indirectly via transfer to LDL mediated by cholesteryl ester transfer protein. The critical role of apoA-I in reverse cholesterol transport process and protection against atherosclerosis development is demonstrated by the increased atherosclerosis in apoA-I–deficient mice (11).

The oxidative modification of HDL apoproteins by reactive radicals or by short chain aldehydes impairs the atheroprotective
oxPL cross-linking impairs cholesterol efflux by HDL/apoA-I

ative function of HDL (6, 7, 12, 13). Chlorination of tyrosine and oxidation of Trp-72 of apoA-I are associated with the impairment of the cholesterol efflux to apoA-I (9, 14–16). Oxidation of Met-148 of apoA-I impairs the ability of apoA-I to activate LCAT (8). Interestingly, studies on the effects of short chain reactive aldehydes have demonstrated that only the modification leading to apoA-I cross-linking impaired the capacity of apoA-I to promote cholesterol efflux (7). Several other studies also have shown the negative effect of apoA-I cross-linking on the cholesterol efflux (17). Furthermore, ApoA-I isolated from human atheroma is cross-linked and dysfunctional (9).

Short chain aldehydes are generated from the oxidative truncation of the polyunsaturated fatty acid chain at the sn-2 position of phospholipids. Oxidized phospholipids concurrently generated by the oxidative truncation of fatty acid residue in sn-2 position contain reactive aldehyde moieties attached to the glycerol backbone of oxPLs via ester bond at the sn-2 position (18). Although studies on the modification of various proteins, including HDL apoproteins, by short chain aldehydes are numerous (7, 19, 20), the studies of protein modification by oxPLs are very limited. Using a novel enrichment method (21), we recently demonstrated that HDL apoproteins in human plasma are modified by aldehydic oxPLs with high efficiency and selectivity (22). The aldehydic oxPLs preferentially modified the multifunctional domain of apoA-I spanning helices 5–8 and the central domain of apoA-II, the very domains critical for the cholesterol efflux to HDL.

In our current studies, we investigated the effect of various oxPLs on the cholesterol efflux function mediated by apoA-I and HDL3. We found that oxPLs that cross-link HDL apoproteins (γ-ketoalkenal phospholipids) strongly inhibited the cholesterol efflux, whereas oxPLs lacking cross-linking capacity had no significant effect. We analyzed the apoprotein cross-linking using LC-MS/MS analysis and found that all histidine residues in helices 5–8 of apoA-I are prone to cross-linking to the lysine residues, either in helices 3 and 4 of another apoA-I or lysine residues in the central domain of apoA-II, by γ-ketoalkenal phospholipids. To determine whether the oxPL apoprotein adducts are generated during atherogenesis, we analyzed plasma samples and aortas of LDLR<sup>−/−</sup> mice fed with a high-fat, high-cholesterol diet, and detected several oxPL cross-link adducts with HDL apoproteins. To the best of our knowledge, this is the first report on the detection of endogenous oxPL-protein cross-linking adducts and demonstration of their presence in vivo. These findings elucidate a novel additional mechanism for in vivo generation of dysfunctional HDL.

Results

OxPLs impair cholesterol efflux and promote retention of cholesteryl esters in macrophages in vitro

Based on our recent studies (22), we hypothesized that oxPL modification of apoA-I can interfere with the cholesterol efflux from cholesterol-loaded cells mediated by apoA-I or HDL. Thioglycollate-induced murine peritoneal macrophages (MPM) were isolated, prelabeled with <sup>3H</sup>cholesterol tracer as described under “Experimental procedures,” and subjected to cholesterol efflux assay using either control unmodified apoA-I or HDL3, or these cholesterol acceptors modified by preincubation with oxPLs as described under “Experimental procedures.” Individual synthetic oxPL: KODA-PC and KOOA-PC, two phospholipids that exhibited effective modification of apoA-I (22), significantly inhibited cholesterol efflux mediated by either apoA-I or HDL3 (Fig. 1, a and b). Control unoxidized phospholipids such as PLPC and POPC had no such effect as anticipated (Fig. 1, a and b). Thus, oxPLs can affect cholesterol efflux in macrophages by compromising apoA-I and HDL3 function.

We next tested whether oxPLs suppress removal of cholesteryl ester from cholesteryl ester-loaded macrophages. MPM were incubated with AcLDL in the presence of <sup>14</sup>C-labeled oleic acid to induce the accumulation of <sup>14</sup>C-labeled cholesteryl esters. Subsequently, cells were transferred into medium that contained no cholesterol acceptors or into medium containing unmodified apoA-I or apoA-I modified by KODA-PC or KOOA-PC, or apoA-I incubated with PLPC as a control. Experiments using HDL3 as cholesterol acceptor were carried out in a similar fashion. Presence of apoA-I or HDL3 in the cell culture media resulted in rapid loss of <sup>14</sup>C-labeled cholesteryl esters from MPM, as anticipated (Fig. 1, c and d). This process was suppressed when apoA-I and HDL3 were modified by the synthetic KODA-PC or KOOA-PC, resulting in significant retention of cellular <sup>14</sup>C-cholesteryl ester in MPM. Incubation of apoA-I or HDL3 with control phospholipids PLPC or POPC had no such effect (Fig. 1, c and d). To demonstrate that the suppression of cholesteryl ester loss is due to the modification of apoA-I by oxPLs, but not other factors, we performed an intracellular cholesteryl ester loss assay adding apoA-I and KODA-PC to cells concomitantly. The effect of KODA-PC in these settings was significantly reduced (Fig. S3) supporting our conclusion that apoA-I/HDL3 modification by oxPLs during co-incubation is responsible for the observed effects. In additional control experiments we found that the indicated oxPLs did not alter significantly the surface expression and distribution of the major cholesterol transporters ABCA1, ABCG1, and SR-BI on MPM (data not shown). Also we found no signs of cytotoxicity of oxPLs at concentrations used in this experiment using MTT viability assay (not shown).

OxPLs inhibits reverse cholesterol transport and promotes retention of cholesteryl esters in vivo

We next assessed the effect of oxPLs on reverse cholesterol transport in vivo as described earlier (23). Intraperitoneal injection of cholesterol-loaded J774A.1 macrophage cells labeled with <sup>3H</sup>cholesterol into ApoE<sup>−/−</sup> mice on a chow diet resulted in appearance of a significant amount of <sup>3H</sup>cholesterol tracer in plasma after 24 h and in feces. Parallel intraperitoneal injection of oxPLs (KODA-PC) resulted in significant reduction in the amount of <sup>3H</sup>tracer in the circulation (Fig. 2a) and in fecal lipid extract of animals (Fig. 2b). Injection of native unoxidized phospholipid PAPC had no significant effect.

We also tested the effect of oxPLs on the ability of macrophages to dispose of cholesteryl esters in in vivo settings. Thioglycollate-elicited cholesteryl ester-loaded macrophages were isolated from apoE<sup>−/−</sup> mice fed a Western diet and labeled with the cell tracking dye CM-Dil. CM-Dil-labeled macrophages...
were then injected into the peritoneal cavities of apoE<sup>−/−</sup> mice fed a chow diet. Cells were re-isolated by peritoneal lavage 48 h later and analyzed. Based on FACS analysis and immunofluorescence microscopy, we estimated that 60–80% of the injected cells were re-isolated (the total number of CM-DiI–positive cells re-isolated as percentage of injected cells) and that between 10 and 20% of all cells retrieved (percentage of CM-DiI–negative cells) were of the host origin. FACS analysis using Nile Red staining for neutral lipids demonstrated that there was a significant loss of intracellular neutral lipids in the re-isolated macrophages as compared with original injected macrophages (Fig. 2<sup>c</sup>). This is further confirmed by immunofluorescence analysis, which showed that almost all the re-isolated donor macrophages lose neutral lipids dramatically (Fig. 2<sup>d</sup>). However, the loss of intracellular neutral lipids was ineffective (Fig. 2<sup>c</sup>) and more than 90% of the re-isolated donor macrophages still have neutral lipid inclusions (Fig. 2<sup>d</sup>) if recipient mice were additionally injected intraperitoneally with oxPLs (KODA-PC), as compared with PAPC or control buffer alone (Fig. 2, <sup>c</sup> and <sup>d</sup>, and data not shown). Taken together, these results extend our <i>in vitro</i> findings into <i>in vivo</i> settings and support a hypothesis that oxPLs impair the cholesterol efflux and cholesteryl ester elimination process in macrophages.

**Cross-linking of apoA-I by oxPLs is associated with the inhibition of the cholesterol efflux mediated by apoA-I and HDL3**

Based on the ability to covalently modify proteins, oxPLs can be separated into three groups: oxPLs that do not covalently modify proteins (carboxylic oxPLs such as KDDiA-PC), oxPLs that can covalently modify proteins (such as HOOA-PC and HODA-PC), and oxPLs that covalently modify proteins and, theoretically, can efficiently form protein cross-links (KDDiA-PC and KODA-PC) (Table S1). We next compared these three groups in cholesterol efflux assays using HDL3, preincubated with three representative phospholipids: KODA-PC, KODA-PC, and KDDiA-PC. We observed that KDDiA-PC, a carboxylic oxPLs that binds with strong affinity to class B scavenger receptors via charge-charge interaction (24), but cannot covalently modify proteins, had no effect on cholesterol efflux at concentrations used. Phospholipid with potent cross-linking activity, KODA-PC, strongly inhibited the HDL3-mediated cholesterol efflux from cells (Fig. 3<sup>a</sup>). Interestingly, HODA-PC, a phospholipid that actively modifies HDL apoproteins, but cannot efficiently induce cross-linking, was not an effective inhibitor (Fig. 3<sup>a</sup>). Significant cross-linking of apoA-I by KODA-PC and the lack of cross-linking by HODA-PC were
**oxPL cross-linking impairs cholesterol efflux by HDL/apoA-I**

**Figure 2.** Oxidized phospholipids inhibit reverse cholesterol transport and promote retention of cholesteryl esters in vivo. **a** and **b**, ApoE−/− mice on chow diet were injected with cholesterol-loaded J774A.1 macrophage cells labeled with [3H]cholesterol along with small unilamellar vesicles containing 100 μg of either native phospholipid or oxidized phospholipids as described under “Experimental procedures.” 24 h after injection [3H]tracer levels in plasma (**a**) and in fecal lipid extract (**b**) were determined as described under “Experimental procedures.” **c** and **d**, thioglycollate-elicited macrophages were harvested from apoE−/− female mice fed a Western diet for 16 weeks, labeled with Cell Tracker CM-DiI (red color, donor cells). A subset of donor cells as well as resident macrophages from recipient mice were also stained with Nile Red (green color) and visualized (**d**, left two panels). Donor cells (2 × 10⁶ cells/animal) were injected into the peritoneal cavities of anesthetized apoE−/− female mice fed a chow diet (recipient cells), with small unilamellar vesicles containing 100 μg of either native phospholipid or oxidized phospholipids. After 48 h macrophages were reisolated from apoE−/− mice on chow diet, cultured briefly and stained with Nile Red (green color) and 4′,6-diamidino-2-phenylindole (blue color) and analyzed by flow cytometry (**c**) or fluorescence microscopy (**d**). Scale bar: 20 μm.

**Figure 3.** Cross-linking of apoA-I by oxidized phospholipids (KODA-PC) is associated with the inhibition of the cholesterol efflux mediated by apoA-I and HDL₃. Cholesterol efflux experiment (**a**) and measurement of cellular cholesteryl ester loss (**c** and **d**) were carried out as described in the legend to Fig. 1. Data represent mean of three independent experiments. *, p < 0.05; **, p < 0.01, as compared with apoA-I or HDL₃. **b**, HDL₃ (0.2 mg/ml) was incubated with the indicated oxidized phospholipid (20 μM) at 37 °C for 6 h in 100 mM HEPES buffer (pH 7.4) under the protection of argon. Samples were mixed with loading buffer containing no β-mercaptoethanol, and proteins separated by SDS-PAGE. Spliced lanes on the same SDS-PAGE gel are indicated by black lines.
confirmed by SDS-PAGE (Fig. 3b). Similar results were observed in the intracellular cholesteryl ester loss assay with both HDL3 and apoA-I as cholesterol acceptors (Fig. 3, c and d). These results suggest that the cross-linking of apoproteins by oxPLs could be particularly damaging for the cholesterol efflux mediated by apoA-I and HDL3.

**Cross-linking of apoA-I in HDL3 by oxPLs interferes with reverse cholesterol transport in vivo**

We further investigated the effects of oxPL cross-linking of HDL3 apoA-I in vivo. J774A.1 cells were incubated with [3H]cholesterol-loaded J774A.1 macrophage cells along with buffer alone or HDL3 modified in vitro by either HODA-PC or KODA-PC as described under “Experimental procedures.” Feces were collected continuously from 0 to 36 h and plasma samples were collected 36 h after injection. [3H]Tracer levels in plasma (a) and in fecal lipid extract (b) were then determined. Data represent the mean of three independent experiments. *p < 0.05.

**Identification of cross-link adducts by oxPLs in isolated human HDL and human plasma exposed to the MPO-H₂O₂-NO₂⁻ system**

We next analyzed the cross-linking of HDL apoproteins by oxPLs generated in isolated human HDL exposed to oxidation by the physiologically relevant MPO-H₂O₂-NO₂⁻ system and detected four apoA-I/apoA-II cross-link adducts and three apoA-I/apoA-II cross-link adducts (Table 2, Table S5, and Figs. S3, and S5). They included 12 intra and inter-protein cross-link adducts of apoA-I. Out of ten apoA-I inter-protein cross-links, five cross-links are in agreement with the 5/5 or 5/4 registry arrangement of apoA-I monomers (27), whereas the other five (shown in italics) are not. Although we cannot exclude that the latter represent inter HDL particle cross-links, it should be noted that we were able to detect these modifications even when reaction was performed at low protein concentrations (0.15 mg/ml) of HDL. We also identified four cross-link adducts of apoA-I with apoA-II, and 10 adducts of minor apoproteins in HDL with apoA-I or apoA-II (Table 1). Several of the latter were less abundant and detected only in 33–50% of analyzed samples.

The central domain of apoA-I of HDL3 was particularly susceptible to cross-linking. Two intra protein cross-links were identified in helix 8: H193–K195 and H199–K195 (Fig. 5). Three histidine residues located at helix 5 and 6 of apoA-I: His-135, His-155, and His-162, were found to form likely inter-protein cross-link adducts with lysine residues located in helix 3 and 4 of apoA-I (Fig. 5 and Table 1). To determine whether these cross-link adducts are intra or inter–apoA-I cross-links, we assessed the distance between each pair of cross-linked residues in various HDL models, including previously described discoidal models of HDL and model of spherical HDL (28, 29) (Table S4). Based on the length of the cross-linker and the distance between each cross-linked residues in published HDL models, it is likely that these cross-links link two antiparallel apoA-I chains (inter–apoA-I cross-links). In addition, we found that apoA-I is cross-linked with apoA-II via three histidine residues in helix 5, 6, and 8 of apoA-I and three lysine residues in the middle region of apoA-II (Fig. 5 and Table 1). Thus, the central domain of apoA-I in HDL3 is a subject to heavy cross-linking by the representative γ-ketoalkenal phospholipid, KODA-PC, either with another apoA-I molecule or with the central domain of apoA-II (Fig. 5). Based on our in vitro experiments (Fig. 3), it is highly likely that these cross-links lead to the loss of cholesterol efflux function of HDL3.
oxPL cross-linking impairs cholesterol efflux by HDL/apoA-I

Table 1

Oxidized phospholipid-apoprotein cross-link adducts identified in HDL3 treated with KOOA-PC

HDL3 (1.5 mg/ml) was incubated with KOOA-PC (10 μM) at 37 °C under the protection of argon. After 4 h incubation, samples were subjected to NaBH₄ reduction, trypsic digestion, peptide enrichment, and LC-MS/MS analysis. The MS/MS spectra obtained were searched against a protein sequence database containing major and minor apoproteins of human HDL using MassMatrix search engine to identify oxPL–peptide cross-link adducts as described under “Experimental procedures.”

| HDL apoproteins cross-linked by oxPLs | Cross-linked residues | Peptides cross-linked by oxPLs | n
|-------------------------------------|----------------------|-----------------------------|---
| ApoA-I–apoA-I                        | AI H135–AI K107      | KSWQEOEMELYRQK⁶               | 3
|                                     | AI H155–AI K106      | AHSVDAIRTHLAPSDELR           | 6
|                                     | AI H162–AI K94       | DLEYVKSXAKVQYLDFFQQK         | 6
|                                     | AI H162–AI K96       | AKSVQYLDFFQQK                | 6
|                                     | AI H162–AI K106      | AHSVDAIRTHLAPSDELR           | 6
|                                     | AI H193–AI K195      | LAEHYSKAKATELHSTLSEK         | 4
|                                     | AI H199–AI K195      | LAEHYSKAKATELHSTLSEK         | 4
|                                     | AI H115–AI K182      | QRLAARLEALKSSEGNAELGAHYAK    | 6
|                                     | AI H115–AI K12       | ARAHSVDAIRTHLAPSDELR         | 4
|                                     | AI H119–AI K133      | ATELQEGARQKSLHEQKLPSLLEEMR  | 4
|                                     | AI H119–AI K140      | ATELHSTLSEK                  | 3
|                                     | AI H193–AI K208      | LEALKENGARLAEHYSHKATELHSTLSEK | 4

| ApoA-I–apoA-II                        | AI H135–AI K46       | AELQEGARQKSLHEQKLPSLLEEMR   | 6
|                                     | AI H135–AI K30       | AELQEGARQKSLHEQKLPSLLEEMR   | 6
|                                     | AI H155–AI K28       | AHSVDAIRTHLAPSDELRQR        | 6
|                                     | AI H193–AI K46       | SYFEKSSQEQLTPLIK            | 5

| Minor apoproteins–apoA-I/apoA-II      | AI K140–AI K73       | QLHELQELKSLPSLLEEMR         | 6
|                                     | AI H162–AI K73       | LAKSYDEKLSKELIGK            | 4
|                                     | AI H162–apoE K233    | TRDRLDEKevinVQAEVK          | 6
|                                     | AI K46–AI K96        | SYFEKSSQEQLTPLIK            | 4
|                                     | AI K44–AI K227       | SYFEKSSQEQLTPLIK            | 4
|                                     | AI K46–apoE K157     | DADDLQKSLRLAVYQAGAR         | 6
|                                     | AI H162–CI K48       | THSALAPSDLR                 | 6
|                                     | AI H162–CI K48       | THSALAPSDLR                 | 6
|                                     | AI K06–CIV K26       | WSLVRGMKSDELLETVVNR         | 6
|                                     | AI K30–CIV K81       | DLMKSVPSLEQAEAK             | 6

n represents number of experiments in which specific cross-link adduct was detected.

H and K represent histidine and lysine residues cross-linked by oxPLs. AI, AII, AIV, CI, and CIV represent apoA-I, apoA-II, apoA-IV, apoC-I, and apoC-IV, respectively.

Figure 5. The central domain of ApoA-I in HDL3 is extensively cross-linked by γ-ketoalkenal phospholipids. LC-MS/MS analysis was used to identify the cross-link adducts of apoproteins in HDL3 incubated with KOOA-PC as described in Table 1.

apoA-I in a fashion similar to HDL3 apoprotein cross-linking by synthetic KOOA-PC. The His-135 and His-155 were cross-linked with Lys-107 in apoA-I and Lys-28 of apoA-II, respectively. The apoA-I (His-135)–apoA-I (Lys-107) adduct was also identified in oxidized HDL, and the adduct of apoA-I (His-155)–apoA-II (Lys-28) also has its similar counterpart apoA-I (His-155)–apoA-II (Lys-30) identified in oxidized HDL. Thus, consistent with the cross-linking induced by individual synthetic γ-ketoalkenal phospholipids, the central domain of apoA-I is preferentially cross-linked by γ-ketoalkenal phospholipids both in oxidized HDL and in oxidized plasma.

Identification of oxPL-protein adducts generated in murine HDL

Samples of murine HDL were subjected to oxidation and analyzed as described above for human HDL but using the Uniprot/Swiss-Prot murine protein database. Eleven amino acid residues of apoA-I and four of apoA-II were covalently modified by a variety of oxPLs in the form of various types of covalent adducts, whereas single modifications of apoC-I and apoE were detected (Table S6). The oxPLs involved and the adduct types detected were very similar to that detected in oxidized human HDL (22). Similar to human apoA-I and apoA-II, the helix 5–8 of murine apoA-I and the central domain of murine apoA-II were preferentially modified by oxPLs (Table S6 and Fig. S4) (22). Analysis of cross-link adducts identified six cross-link adducts of apoA-I, including four apoA-I/apoA-II and two apoA-I/apoA-IV cross-link adducts (Table S7). Two apoA-II/apoA-II adducts and one apoA-II/apoE adduct were also identified in oxidized murine HDL. The central domain of murine apoA-I is the preferred target for cross-linking by oxPLs, similar to human apoA-I.
To NaBH₄ reduction, tryptic digestion, peptide enrichment, and LC-MS/MS analysis. Experiments using human plasma were carried out similarly. The MS/MS spectra obtained were searched against a protein sequence database containing major and minor apoproteins of human HDL using Massmatrix search engine to identify oxPL-peptide cross-link adducts as described under "Experimental procedures."

**OxPL protein adducts are present in murine plasma and aorta**

We analyzed plasma and aorta isolated from LDLR⁻/⁻ mice fed proinflammatory high-cholesterol Western diet (WD) or chow diet. Three apoprotein cross-link adducts were identified in plasma of LDLR⁻/⁻ mice fed WD, including apoA-I/apoA-I, apoA-I/apoA-IV, and apoA-II/apoE adducts (Table S7). The His-165 and Lys-93 at the central domain of apoA-I were cross-linked by KOOA-PC (Fig. 6a), which is very likely to be inter-apoA-I cross-link adduct based on the structure of human apoA-I in HDL. His-154 at the central domain of apoA-I was cross-linked with Lys-149 on apoA-IV (Fig. 6b), and Lys-30 at the central domain of apoA-II was cross-linked with His-227 of apoE (Fig. 6c). We also identified one apoA-I (His-154)–apoA-I (Lys-105) cross-link adduct in aorta of LDLR⁻/⁻ mice fed high-cholesterol WD (Fig. 6d). The cross-link adducts that we detected in vivo have their counterparts or similar counterparts identified in oxidized murine HDL (Table S7). No cross-link adducts were found in the control plasma or aorta samples collected from LDLR⁻/⁻ mice fed a chow diet, when analyzed by LC-MS/MS using both data-dependent acquisition and a more sensitive multiple reaction monitoring mode.

**Discussion**

Our current in vivo and in vitro studies have demonstrated that exposure of HDL3 and apoA-I to γ-ketoalkenal phospholipids that, theoretically, could induce cross-linking of apoproteins, significantly inhibited the cholesterol efflux and reverse cholesterol transport. Using LC-MS/MS analysis, we identified a number of intra- and inter-protein cross-links formed in the central domain of HDL apoprotein apoA-I by γ-ketoalkenal phospholipids in vitro. We also identified cross-links of apoA-I and apoA-II with ApoA-IV and apoE. We further demonstrated the presence of such apoprotein cross-links in plasma and aorta of hyperlipidemic LDLR⁻/⁻ mice.

Several studies have shown that ApoA-I in human atheroma is cross-linked and dysfunctional (30, 31), demonstrating that ApoA-I cross-linking has significant relevance to human pathology. HDL apoprotein cross-linking during oxidation or by selected products of lipid peroxidation is well-documented (32–36). To the best of our knowledge, our study is the first to demonstrate the molecular mechanism of apoprotein cross-linking by oxidized phospholipids: the formation of nonreversible pyrrole adduct.

Approtein cross-linking by γ-ketoalkenal phospholipids is fast and highly efficient. The cross-link adducts of apoproteins can be easily observed on SDS-PAGE after only 10 min of incubation of HDL3 with KOOA-PC. More than 90% of apoA-I in HDL3 is cross-linked by KOOA-PC at a 1:2.5 molar ratio of apoA-I to KOOA-PC, demonstrating high efficiency of the reaction. Most of the detected cross-links in HDL include apoA-I, we detected only four cross-links not involving ApoA-I (Table 1). About one-third of all identified cross-links in human HDL3 involve apoA-II. We detected no apoA-II/apoA-II cross-links in human samples, likely due to the absence of histidine residues in human apoA-II. Murine apoA-II has a histidine residue in position 46, correspondingly histidine-lysine apoA-II/apoA-II cross-links were detected in oxidized murine HDL. The cross-links that we identified are almost exclusively between histidines and lysines. Lys-Lys cross-links were rare, likely reflecting higher reactivity of γ-ketoalkenal phospholipids toward histidine residues. All five histidine residues in apoA-I are involved in cross-linking. Three histidine residues of helices 5 and 6 (His-135, His-155, and His-162) form cross-links with proximally located lysine residues on helices 3 and 4 of antiparallel apoA-I chain (Fig. 5 and Table 1). Histidine residues His-193 and His-199 of helix 8, on the other hand, form intra-protein cross-links with the Lys-195 of the same apoA-I chain: His-193–Lys-195 and His-199–Lys-195. Thus the central domain of apoA-I in HDL is a subject to heavy cross-linking by the γ-ketoalkenal phospholipids, either with another apoA-I or with the central domain of apoA-II.

Based on our in vitro experiments, it is highly likely that the cross-linking underlies the loss of cholesterol efflux activity of HDL3 when exposed to γ-ketoalkenal phospholipids. ApoA-I cross-linking and, in general, covalent modifications of apoA-I are, with very few exceptions (7), associated with an impairment of HDL-mediated cholesterol efflux (7, 12, 13, 37). Mechanistically cross-linking has been shown to induce the inhibition of HDL recognition by HDL receptors, protein unfolding, dissociation, lipoprotein fusion, and changes in apoA-I conformation and conformational adaptability (38–40). We found that the γ-hydroxyalkenal phospholipids (such as HODA-PC), whereas inducing multiple covalent modification (but no cross-linking) of helices 5–8 of apoA-I (22), have no significant impact on the cholesterol efflux mediated by HDL3 or apoA-I, at least at low micromolar concentrations used in this work.

### Table 2

| Cross-linked apoproteins | Cross-linked residues (oxPL type) | Peptides cross-linked by oxPLs | Human HDL | Human plasma |
|-------------------------|---------------------------------|------------------------------|-----------|-------------|
| ApoA-I–apoA-I           |                                 |                              |           |             |
| ApoA-I H135–apoA-I K107 (KODA-PL) | LHELQEKSLPQGEERH | KSWIQEMLYR | ✓ | - |
| ApoA-I H162–apoA-I K194 (KOHA-PL) | DELVEKSAKYQVYLDQKF | TKSHLAPSDLR | ✓ | - |
| ApoA-I H199–apoA-I K195 (KOPLA-PL) | THSLAPYSLMRL | QK$VEPLR | ✓ | - |
| ApoA-I–apoA-II          |                                 |                              |           |             |
| ApoA-I H135–apoA-II K46 (KOAA-PL) | LHELQEK | SKSEQILTPK | ✓ | - |
| ApoA-I H155–apoA-II K28 (KOHA-PL) | AHSVDALR | DLMK$SVK | ✓ | - |
| ApoA-I H155–apoA-II K30 (KOPLA-PL) | DLMK$VKSPELQAEKSYFEK | DRAH$VDALR | ✓ | - |
| ApoA-I–apoA-II K46 (KOAA-PL) | AHSVDALR | SKSEQILTPK | ✓ | - |

* HS and KS represent histidine and lysine residues cross-linked by oxPLs.
likely due to the reversible nature of many modifications. At the same time, even relatively low levels of apoA-I cross-linking by the \( \gamma \)-ketoalkenal phospholipids are sufficient to induce a noticeable inhibitory effect. Specific effect of cross-linking on HDL function was reproduced in vivo in a reverse cholesterol transport experiment, where mice were injected with HDL3 modified either by noncross-linking HODA-PC or cross-linking KODA-PC. Importantly, to avoid potential non-HDL-related effects of reactive phospholipids, after modification both preparations of HDL were subjected to NaBH\(_4\) reduction to convert HODA-PC or KODA-PC into the same nonreactive derivative. The results further demonstrated that the apoprotein cross-linking by oxPLs interferes with HDL3-mediated reverse cholesterol transport in vivo.

We identified several cross-link adducts of HDL apoproteins generated by \( \gamma \)-ketoalkenal phospholipids in vivo in the plasma and aorta of LDLR\(^{-/-}\) mice further demonstrating the in vivo relevance of our findings. We were not able to detect other types of adducts, including the Michael adduct abundant in oxidized isolated HDL, probably due to the reversibility of this adduct (20, 41). Although the detection of high molecular weight apoA-I bands in human aorta has been reported before (9, 31, 42), whether some of these are generated by oxPL cross-linking needs to be determined.

Our studies suggest that \( \gamma \)-ketoalkenal phospholipids may play an important role in compromising the atheroprotective function of HDL in vivo. Several lines of evidence support this hypothesis. The \( \gamma \)-ketoalkenal phospholipids represent a major group of products generated during the oxidative fragmentation of polyunsaturated fatty acid at the sn-2 position of oxPLs and are generated at a faster rate than most of the other oxidized lipids (43). They are significantly more reactive than many other oxPLs toward the nucleophilic amino acid residues (20). Although many oxPLs form unstable and highly reversible adducts with proteins, the pyrrole cross-link adduct, a final product of \( \gamma \)-ketoalkenal phospholipid reaction with proteins, is irreversible, thus the reaction equilibrium is shifted toward the product. Also \( \gamma \)-ketoalkenal phospholipids preferentially react with HDL apoproteins but not other proteins in complex biological systems, in contrast, for example, to short chain reactive aldehydes (44), probably due to preferential incorporation of conical-shaped oxPLs into the high curvature phospholipid shell of HDL (22). The HDL particle provides an ideal condition for the cross-linking of apoproteins by \( \gamma \)-ketoalkenal phospho-
oxPL cross-linking impairs cholesterol efflux by HDL/apoA-I

lipids at least for two reasons. It creates very high local concentrations of HDL apoproteins, and, probably oxPLs, in a phospholipid shell, leading to a significantly increased rate of reaction (45). Also, the closely spaced antiparallel orientation of apoA-I chains in HDL is the perfect target for cross-linking. Finally, the cross-linking seems to be particularly damaging to HDL function as cholesterol acceptor (7).

In conclusion, our findings identify a novel mechanism for cross-linking of HDL apoproteins, provide detailed molecular information of the specific apoproteins and apoprotein sites susceptible for modification, and demonstrate the presence of novel modifications in vivo in conditions of hyperlipidemia and inflammation.

**Experimental procedures**

**Reagents**

Myeloperoxidase from human polymorphonuclear leukocytes was purchased from EMD Millipore (Billerica, MA). Sequencing-grade modified trypsin was purchased from Promega (WI). Oxidized phospholipids (Table S1), KOOA-PC (5-keto-8-oxo-6-octenoic acid ester of 2-lyso phosphocholine), KODA-PC (9-keto-12-oxo-10-dodecenolic acid ester of 2-lyso phosphocholine), and HODA-PC (9-hydroxy-12-oxo-10-dodecenolic acid ester of 2-lyso phosphocholine) were synthesized as previously described (46, 47). KDdiA-PC (9-keto-10-dodecenolic acid ester of 2-lyso phosphocholine) was purchased from Avanti Polar Lipids, Inc. All other chemicals were obtained from Sigma or Fisher Scientific Co., unless otherwise specified.

**Isolation of human plasma and HDL**

All experiments done with human blood were approved by the Institutional Review Board of the Cleveland Clinic. Informed consent was obtained in accordance with the Declaration of Helsinki. 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) and HDL3 (density 1.125–1.210 g/ml) were isolated from human plasma by sequential density ultracentrifugation as described elsewhere (48).

**Mice**

Wildtype (WT) C57BL/6J, apoE−/−, and LDLR−/− mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were used for experiments at 8–12 weeks unless otherwise specified. All animal procedures were approved prior to study by the Institutional Animal Care and Use Committee of the Cleveland Clinic. To detect the oxPL-protein adducts generated in vivo in mice, 8-week-old LDLR−/− mice were fed a chow diet or a high-cholesterol Western diet (TD96121; Harlan-Tecklad, Madison, WI) for 12 weeks.

**Collection of murine tissues and preparation of samples for LC-MS/MS analysis**

Multiple precautions were taken to prevent accidental oxidation of plasma and aorta samples. Blood was collected and mixed at a ratio of 2/1 (v/v) with saline containing EDTA (15 mM) and mixture of antioxidants, including butylated hydroxytoluene (10 μM) and melatonin (150 μM). Plasma was immediately separated by centrifugation and treated with NaBH₄ (final concentration 100 mM) to reduce and stabilize the reversible oxPL-protein adducts, followed by the addition of acetyl cysteine (1 mM final concentration) to prevent the ex vivo generation of specific oxPL-protein adducts under study. Preparations were transferred into 100 mM Tris buffer (pH 7.8) containing 8 M urea and stored at −80 °C before use. To harvest aortas, mice were first perfused with saline containing EDTA (5 mM) and the mixture of antioxidants mentioned above. Harvested aortas were homogenized at 4 °C in 100 mM Tris buffer containing 8 M urea, 5 mM EDTA, and a mixture of antioxidants. The aorta homogenate was treated with NaBH₄ and acetylcysteine as described for plasma samples. Next, plasma and aorta homogenates were subjected to DTT reduction, IAA alkylation, and tryptic digestion under the protection of argon, followed by addition of a synthetic peptide-oxPL adduct as an internal standard and enrichment of oxPL-peptide adducts as described (21).

**Oxidation of human HDL by MPO-H₂O₂-NO₂⁻**

HDL (0.5 mg/ml) was oxidized by incubation at 37 °C in 67 mM phosphate buffer (pH 7.4) containing 200 μM diethylenetriamine pentaacetic acid (DTPA), 500 μM NaNO₂, and 30 mM MPO. H₂O₂ was injected into solution to a final concentration of 100 μM, followed by 1 h incubation and then addition of another dose of H₂O₂ and 1 h incubation. Oxidation reactions were terminated by addition of 40 μM butylated hydroxytoluene. NaBH₄ (100 mM) was added to reduce and 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 oxidized against 67 mM phosphate buffer (pH 7.4) before the oxidation. Plasma samples were oxidized and processed as described above for HDL. 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 previously described (21).

**Modification of apoA-I or HDL3 by oxPLs**

Reaction of apoA-I or HDL3 (0.5 mg/ml or the indicated concentration) with KOOA-PC or other oxPLs (50 μM or the indicated concentration) was carried out at 37 °C for 6 h in 100 mM HEPES buffer (pH 7.4) under the protection of argon gas. 50 mM NaBH₄ was then added to stabilize reversible adducts of oxPLs with apoA-I or HDL3. The sample buffer was exchanged to 100 mM Tris buffer (pH 7.8) or other buffer solutions as indicated using repeated centrifugation through a centrifugal filter (10K cutoff, Millipore).

**Cholesterol efflux assay**

Cholesterol efflux from MPM was assessed as previously described (49). Briefly, MPM were incubated with RPMI 1640, 0.2% BSA containing 100 μg/ml of acetylated LDL (AcLDL), 5
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µCi/ml of [3H]cholesterol for 24 h at 37 °C. Cells were equilibrated for 6 h in RPMI 1640, and then cultured in RPMI 1640, 0.2% BSA containing increasing concentrations of HDL3 (or apoA-I) or HDL3 (or apoA-I) preincubated with oxidized phospholipids or native control phospholipids. Radioactivity was quantified in the media and in cells at selected time points.

Measurement of intracellular cholesteryl ester loss

Thioglycollate-induced MPM were cultured in RPMI 1640, 0.2% BSA containing 100 µg/ml of AcLDL, 1.5 µCi/ml of [14C]oleic acid for 24 h at 37 °C. Cells were then washed with PBS and treated with HDL3 (or apoA-I) or with HDL3 (or apoA-I) preincubated with oxidized phospholipids or native control phospholipids. After 24 h incubation at 37 °C, cells were washed with PBS, cholesterol and cholesteryl esters were extracted, separated by TLC, and radioactivity in the cholesteryl ester band was quantified by liquid scintillation counting.

In vivo cholesterol efflux assays

Thioglycollate-elicited macrophages were harvested from apoE−/− female mice fed a high-fat Western diet for 12 weeks, washed with PBS, and labeled with Cell Tracker CM-Dil (20 µM) according to the manufacturer’s instructions. 2 × 10⁷ cells were transferred to the peritoneal cavity of anesthetized apoE−/− female mice on chow diet. Macrophages were re-isolated 48 h later, cultured briefly, fixed, stained with Nile Red, and mounted with Vecta-Shield containing DAPI mounting media (Vector Laboratories). Fluorescence images were processed using Image-Pro Plus software. Alternatively, the cells isolated from mice were washed and analyzed by flow cytometry using FACScan (BD Bioscience) directly or after staining with Nile Red and mounted with Vecta-Shield containing DAPI mounting media. fluorescence images were processed using Image-Pro Plus software.

Reverse cholesterol transport assay

Reverse cholesterol transport assays were performed as previously described with minor modifications (23). Briefly, mouse macrophages cell line J774A.1 (American Type Culture Collection, Manassas, VA) was incubated with 50 µg/ml of AcLDL and 2.5 µCi/ml of [3H]cholesterol in RPMI with 0.5% BSA for 24 or 36 h. [3H]Cholesterol-loaded J774A.1 cells (8 × 10⁶ cells with radioactivity counts of 8.2 × 10⁶ counts per minute (cpm)) were transferred to 0.8 ml of RPMI 1640, 0.25% BSA containing increasing concentrations of HDL3 (or apoA-I, or HDL3 treated by oxPLs) and injected intraperitoneally into the apoE−/− mice. After 24 h (or indicated time), mice were anesthetized, blood was collected, plasma separated, and [3H] radioactivity in plasma assessed by liquid scintillation counting. Feces were collected continuously from 0 to 24 h (or indicated time) and stored at 4 °C before extraction of lipids using Bligh and Dyer method (50) and subsequent quantification of total fecal [3H] count.

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 × 75 µm inner diameter, 2 µm, 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. Electrospray ionization MS was performed with a Thermo Scientific LTQ-Orbitrap-Elite MS in the positive ion mode. Five microliters of samples were injected. The peptides eluted from the column at a flow rate of 0.25 µl/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 mode, in which each full MS scan was acquired in the Orbitrap at a resolution of 60,000 and followed by 15 MS/MS scans, or a multiple reaction monitoring mode in which specific peptides were targeted over the entire course of the LC experiments. For the data-dependent acquisition 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%. Compound lists of the resulting spectra were generated using XCalibur software (version 2.2, Thermo Scientific). The MS/MS spectra obtained were searched against a SwissProt mouse database (25,035 sequences, 2017) using Thermo Proteome Discoverer (version 1.4). Trypsin was set to cleave at Lys and Arg. Fixed modification included carbamidomethylation on cysteine residues (Cys, 57.021 Da). Dynamic modifications included oxidation on methionine (Met, 15.995 Da) and oxPL modifications in the form of various types of adducts with proteins, including Schiff base adduct, Michael adduct, ketoamide adduct, cyclic hemiacetal adduct, and furan adduct (Fig. S1) (20). The corresponding mass shifts were shown in Table S2. The search parameters used were three missed cleavage sites, a mass tolerance of 10 ppm for the parent ion, and 0.6 Da for the fragment ion. The search results were filtered by a false discovery rate of 5% with a decoy database search.

For cross-link adduct analysis, the MS/MS spectra were searched against an apoprotein sequence database of human or murine HDL (apoA-I, apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III, apoC-IV, and apoE) using Mass Matrix Xtreme search engine (version 3.0.9.8 Alpha, 2012), software that allows a search for protein cross-links (25, 26). Trypsin was set to cleave at Lys and Arg. Fixed modification included carbamidomethylation on cysteine residues (Cys, 57.021 Da). Dynamic modifications included oxidation on methionine (Met, 15.995 Da) and oxPL modifications in the form of various types of adducts with proteins, including Schiff base adduct, Michael adduct, ketoamide adduct, cyclic hemiacetal adduct, and furan adduct (Fig. S2). The corresponding mass shifts are shown in Table S2. The search parameters used were four missed cleavage sites, a mass tolerance of 10 ppm for the parent ion, and 1.0 Da for the fragment ion. All identified MS2 spectra were manually checked. Three statistical scores (pp, pp2, p ptag) were used to evaluate the quality of a peptide match. A peptide match with max (pp, pp2) > 2.4 and p ptag > 1.3 is considered to be significant with a p value <0.05.
Statistical analysis

All experiments were conducted two to three times, and representative results are presented. Data are presented as mean ± S.E. The statistical significance of differences was evaluated using the Student’s t test or Mann-Whitney U test. Significance was accepted at the level of p < 0.05. When multiple comparisons were made, a Bonferroni correction was made for each test.

Author contributions—D. G., M. Z. A., and E. A. P. conceptualization; D. G., M. Z. A., L. Z., N. S. K., and E. A. P. data curation; D. G., M. Z. A., L. Z., N. S. K., and E. A. P. formal analysis; D. G., M. Z. A., L. Z., and N. S. K. investigation; D. G., M. Z. A., and E. A. P. methodology; D. G., M. Z. A., and E. A. P. writing—original draft; D. G. and E. A. P. project administration; D. G. and E. A. P. writing—review and editing; T. V. B. and E. A. P. resources; T. V. B. and E. A. P. supervision; T. V. B. and E. A. P. funding acquisition.

Acknowledgments—We thank Jessica Altemus and Miroslava Tisch-enko for technical assistance, Dr. Belinda Willard and Dr. Ling Li for technical assistance on LC-MS/MS analysis, and Emelye Crehore and Jessica Altemus for proofreading of the manuscript. The Fusion Lumos instrument was purchased via National Institutes of Health shared instrument grant 1S10OD023436-01.

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