HDL3-Mediated Inactivation of LDL-Associated Phospholipid Hydroperoxides Is Determined by the Redox Status of Apolipoprotein A-I and HDL Particle Surface Lipid Rigidity

Relevance to Inflammation and Atherogenesis

Amal Zerrad-Saadi, Patrice Therond, Sandrine Chantepie, Martine Couturier, Kerry-Anne Rye, M. John Chapman, Anatol Kontush

Objectives—Small dense HDL3 particles of defined lipidome and proteome potently protect atherogenic LDL against free radical–induced oxidation; the molecular determinants of such antioxidative activity in these atheroprotective, antiinflammatory particles remain indeterminate.

Methods and Results—Formation of redox-active phosphatidylcholine hydroperoxides (PCOOH) and redox-inactive phosphatidylcholine hydroxides (PCOH) was initiated in LDL by free radical–induced oxidation. Human HDL3 inactivated LDL-derived PCOOH (−62%, P<0.01) and enhanced accumulation of PCOH (2.1-fold, P<0.05); in parallel, HDL3 accumulated minor amounts of PCOOH. Enzyme-deficient reconstituted dense HDL potently inactivated PCOOH (−43%, P<0.01). HDL3-mediated reduction of PCOOH to PCOH occurred concomitantly with oxidative damage by free radicals and therefore exerts antiinflammatory effects of HDL are manifested in animal models as attenuated production of reactive oxygen species and diminished expression of endothelial adhesion proteins, and are associated with attenuated atherogenesis.2,3 HDL3 particles are, however, highly heterogeneous in their structure, metabolism, and biological functions.4 Among the major HDL subpopulations, small, dense, protein-rich HDL3 are distinguished by their proteome and lipidome.5,6 Further more, HDL3 exhibit potent capacity to protect LDL from free radical–induced oxidative damage7 and to inhibit oxLDL-induced apoptosis of endothelial cells.8 Atheroprotective properties of small dense HDL3 are consistent with results of clinical and animal studies.9,10 Such antioxidative activity.
appears to derive from both nonenzymatic and enzymatic components of HDL3; however, its precise molecular features remain indeterminate.

Apolipoprotein A1 (apoAI), the major HDL apolipoprotein, may play a central role in HDL-mediated antioxidation, as Met residues 112 and 148 can reduce LOOH into redox-inactive lipid peroxidation.11,12 In addition, apoAI attenuates LDL oxidation by removal of seeding LOOH molecules from LDL.13 Enzymatic components potentially contributing to antioxidative activity of HDL include paraoxonase 1 (PON1), platelet-activating factor-acetyl hydrolase (PAF-AH) or “lipoprotein-associated phospholipase A2”) and lecithin:cholesterol acyltransferase (LCAT),4 all of which were proposed to hydrolyze proinflammatory short-chain oxPL (see 14 for review).14 However, PON1, PAF-AH, and LCAT are weakly reactive toward LOOH.15–18

Recent proteomic studies have demonstrated that small dense HDL3 particles are enriched in apoAI, apoF, apol-I, PLTP, PON1, PAF-AH, and LCAT.5,6 Furthermore, the HDL3 lipidome is deficient in sphingomyelin,6 a structural lipid with positive impact on surface rigidity and negative impact on LCAT activity, thereby implicating surface phospholipids (PL) in the biological activities of HDL. Indeed, the transfer of LOOH from LDL to HDL can occur, either spontaneously or mediated by lipid transfer proteins, directly between lipoprotein PL monolayers.19,20

Our present goal was to dissect the molecular features of the HDL3-mediated protection of LDL from free radical–induced oxidative damage. We reveal that the potent antioxidative activity of HDL3 critically involves the transfer of phospholipid hydroperoxides (PLOOH) from oxLDL to HDL3 in a process dependent on HDL surface lipid rigidity, with subsequent inactivation of LDL-derived PLOOH by Met residues of apoAI.

**Methods**

Our experimental strategy involved (1) isolation of HDL2 (a mixture of HDL2a and 2b subclasses) and HDL3 (a mixture of HDL3b and 3c subclasses) particles by density gradient ultracentrifugation, (2) cooxidation of HDL and LDL by 2,2′-azobisis(2-amidinopropane) hydrochloride (AAPH), an azo-initiator of lipid peroxidation,21 and (3) coincubation of LDL with HDL preoxidized by AAPH. Details of this strategy, and of: (1) reagents and chemicals, (2) blood samples, (3) fractionation and characterization of plasma lipoproteins, (4) preparation of reconstituted HDL (rHDL) and liposomes, (5) determination of phosphatidylcholine hydroperoxides (PCOOH), phosphatidylcholine hydroxides (PCOH), cholesteryl ester hydroperoxides (CEOOH), PL, cholesteryl esters (CE), lysophosphatidylcholine (lysoPC), free fatty acids, and oxidized Met residues of apoAI by HPLC, (6) the capacity of HDL to protect LDL from oxidative damage, (7) the rigidity of liposomal PL monolayers, and (8) the activities of HDL-associated enzymes, are available in the supplemental materials (available online at http://atvb.ahajournals.org). Data presented are means±SD of at least 3 independent experiments with at least 3 independent LDL samples.

**Results**

**Inactivation of PCOOH and CEOOH During LDL+HDL3 Oxidation**

**PCOOH**

Small dense HDL3 attenuated accumulation of PCOOH (the sum of the 18:2/16:0, 20:4/16:0, and 22:6/16:0 molecular species; supplemental Table I) during LDL oxidation by AAPH. Indeed, levels of PCOOH tended to be lower (−58%) in LDL+HDL3 mixtures as compared to LDL oxidized alone after 2-hour incubation and were significantly decreased at 6 hours (−48%, P<0.05; supplemental Figure 1A). Furthermore, PCOOH levels in LDL+HDL3 mixtures were reduced as compared to those in LDL and HDL3 oxidized separately (supplemental Table II). In parallel, reduction in PCOOH levels was observed in LDL reisolated from LDL+HDL3 mixtures after oxidation for 2 hours (−65%) and 6 hours (−44%, P<0.05; supplemental Figure 1B). During oxidation, small amounts of PCOOH accumulated in reisolated HDL3 (supplemental Figure 1C).

Large light HDL2 tended to attenuate levels of PCOOH in LDL+HDL3 mixtures after 2-hour and 6-hour oxidation (−50% and −44% as compared to LDL oxidized alone respectively; n=4, P=ns). In LDL reisolated from LDL+HDL2 mixtures, PCOOH concentrations tended to fall (−62% and −26% at 2 hours and 6 hours, respectively; n=4, P=ns). Accumulation of PCOOH was significantly more pronounced in reisolated HDL2 as compared to that in reisolated HDL3 (6.6-fold at 2 hours and 2.8-fold, P<0.05, at 6 hours; n=4), consistent with greater rates of oxidation in LDL+HDL2 versus LDL+HDL3 mixtures.

**CEOOH**

CEOOH levels in LDL (the sum of the 18:2 and 20:4 molecular species; supplemental Table I) were diminished in the presence of HDL3. Indeed, CEOOH levels tended to diminish in LDL+HDL3 mixtures as compared to LDL oxidized alone after both 2-hour and 6-hour oxidation (−38% and −26% respectively; supplemental Figure 1A). A marked reduction in CEOOH content in the presence of HDL3 (−50%) was noted in LDL reisolated from mixtures with HDL3 after 2 hours (supplemental Figure 1B). Overall, the effect of HDL3 on CEOOH accumulation in LDL was less potent than that on PCOOH. In parallel, accumulation of CEOOH was detected in reisolated HDL3 (supplemental Figure 1C).

Similarly, CEOOH concentrations tended to decrease in the presence of HDL2 both in LDL+HDL2 mixtures (−35% and −29% after 2 hours and 6 hours, respectively; n=4, P=ns) and in reisolated LDL (−48% after 2 hours; n=4, P=ns). CEOOH accumulation was markedly higher in reisolated HDL2 as compared to reisolated HDL3 (4.0-fold and 4.7-fold difference; n=4) after oxidation for 2 hours and 6 hours (P<0.05), respectively.

**Inactivation of PLOOH and CEOOH in Preoxidized LDL**

**PCOOH**

In separate experiments, LDL were preoxidized and subsequently incubated with HDL in the presence of EDTA. After 2-hour incubation, PCOOH levels in LDL+HDL3 mixtures were significantly decreased (−42%, P<0.01; Figure 1A) as compared to oxLDL alone. Even greater effects on PCOOH levels were noted after prolonged (6-hour) incubation (−62%, P<0.01; Figure 1A). Consistent with these data, HDL3 potently inactivated PCOOH in oxLDL reisolated after 0.5-hour incubation (−64%, P<0.01; Figure 1B), an effect which was even...
more pronounced at 2 hours (−87%, P<0.001) and at 6 hours (−95%, P<0.001; Figure 1B). In reisolated HDL3, slow accumulation of PCOOH was observed (Figure 1C) consistent with the transfer of PLOOH from oxLDL.

HDL2 equally attenuated PLOOH content in preoxidized LDL, albeit less markedly than HDL3 when compared on a total mass basis. Indeed, PCOOH levels were significantly decreased by HDL2 both in LDL+HDL2 mixtures (−25% and −53%, P<0.05, after 2-hour and 6-hour oxidation, respectively; Figure 1A) and in reisolated oxLDL (−72%, P<0.01, −80%, P<0.001, and −90%, P<0.001, after 0.5-hour, 2-hour, and 6-hour oxidation respectively; Figure 1B). In reisolated HDL, PCOOH levels were preferentially elevated (15-fold) in HDL2 as compared to HDL3 after 0.5-hour incubation, and were 5-fold and 4-fold elevated after 2 hours and 6 hours, respectively (Figure 1C).

Table. Influence of Reconstituted HDL on LDL Oxidation by AAPH

<table>
<thead>
<tr>
<th>ApoA-I/POPC</th>
<th>Oxidation Rate in the Propagation Phase (% LDL Alone)</th>
<th>Duration of the Propagation Phase (% LDL Alone)</th>
<th>Maximal Diene Concentration (% LDL Alone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA-I/POPC</td>
<td>36±12***</td>
<td>204±64**</td>
<td>73±16**</td>
</tr>
<tr>
<td>ApoA-I/POPC+chloramine T</td>
<td>97±20 §§§</td>
<td>80±10 §§</td>
<td>73±11</td>
</tr>
<tr>
<td>ApoA-I/POPC/SM</td>
<td>82±15 §§</td>
<td>98±20 §§§</td>
<td>81±15†</td>
</tr>
<tr>
<td>ApoA-I/POPC/FC</td>
<td>89±10 §§§</td>
<td>91±10 §§§</td>
<td>80±17</td>
</tr>
</tbody>
</table>

LDL (10 mg TC/dL) were incubated with AAPH (1 mmol/L) in the absence or presence of rHDL (10 mg total protein/dl) in PBS at 37°C. rHDL prepared by cholate dialysis contained either apoA-I/POPC (molar ratio, 1.0/77.1), apoA-I/POPC/FC (molar ratio, 1.0/66.4/5.1), or apoA-I/POPC/SM; molar ratio, 1.0/65.8. In some experiments rHDL was preincubated with chloramine T (see Figure 2 for details). Oxidation of LDL+ rHDL mixtures was compared to LDL oxidized alone and measured as the increment in absorbance at 234 nm. Data are means±SD of 3 independent experiments with 2 independent LDL samples. *P<0.05 vs LDL alone.

**CEOOh**

In striking contrast, no change in CEOOH concentrations was observed in oxLDL+HDL3 mixtures incubated under the same conditions (supplemental Figure IIIA). Likewise, no effect of HDL3 on CEOOH content in oxLDL reisolated after coincubation was observed (supplemental Figure IIIB). Reisolated HDL3 slowly accumulated low CEOOH levels (supplemental Figure IIIC), thereby resembling the pattern of PCOOH accumulation (Figure 1C). Similarly, no effect of HDL2 on CEOOH content of preoxidized LDL was observed (data not shown). CEOOH accumulation was considerably more pronounced in reisolated HDL2 than in reisolated HDL3 (approximately 30-fold after 0.5-hour and 2-hour oxidation and 23-fold after 6 hours; n=4, P<0.05).

**HDL-Mediated Inactivation of PCOOH: Role of ApoA1**

To define the role of apoA1 in the HDL-mediated inactivation of PCOOH, rHDL containing only purified apoA1 and palmitoyloleoyl phosphatidylcholine (POPC) at a molar ratio of 1.0/77.1 were prepared. Importantly, the size and density of such rHDL closely resemble those of small dense HDL3.21 When such enzyme-deficient, small, dense rHDL were incubated with oxLDL for 2 hours at 37°C, PCOOH levels were significantly decreased (−43%) as compared to those in oxLDL incubated alone (2.37±0.40 versus 3.96±0.95 μmol/L respectively, n=3; P<0.01). Furthermore, such rHDL displayed potent activity in the LDL oxidation assay; indeed, significant reduction (−64%, P<0.05; Table) in the propagation rate of lipid peroxidation in LDL incubated with apoA1/POPC complexes as compared to LDL oxidized alone was observed. ApoA1/POPC rHDL equally induced a pronounced prolongation of the propagation phase (+104%) and a moderate decrease in the maximal diene concentration (−27%; Table). The capacity of such rHDL to protect LDL from oxidative damage was comparable to that of small dense HDL3b +3c isolated from control human plasma (−79% decrement in the propagation rate, +57% increment of the propagation phase duration and −27% decrement in the
maximal diene concentration when recalculated to equivalent concentration of protein of 10 mg/dL; n = 11).

**Implication of Met Residues of ApoAI in HDL-Mediated Inactivation of PCOOH**

Met sulfoxide-containing forms of apoAI represented 9.5 ± 2.3% (n = 9) of total apoAI in freshly isolated HDL3, suggesting that some Met oxidation occurs in the circulation in vivo or during HDL isolation in vitro. After incubation of HDL3 in the presence of oxLDL, both redox-active Met residues of apoAI (Met112 and 148) were significantly transformed (by 58% and 79% after 0.5 hours and 2 hours, respectively) into the corresponding sulfoxides (Figure 2A), thereby demonstrating that inactivation of LDL-derived PCOOH by HDL3 was associated with the conversion of apoAI Met into Met(O). Furthermore, concomitant formation of PCOH occurred, which was accelerated by the presence of HDL3. Indeed, levels of PCOH were elevated 2.1-fold in the presence of HDL3 (P < 0.05) and were accompanied by significant reduction in PCOOH levels (−59%; Figure 2B; P < 0.05), resulting in the 5.6-fold elevated PCOH/PCOOH ratio (0.67 versus 0.12).

To further evaluate the role of Met residues in the capacity of HDL to inactivate PCOOH, chloramine T was used to selectively oxidize apoAI Met112 and Met148.11 Both apoAI Met residues were fully oxidized by chloramine T (data not shown); such HDL3 revealed diminished capacity to inactivate PCOOH, resulting in increased PCOOH levels (+33%; supplemental Figure IVA). In parallel, a minor increase (+10%) in PCOOH was observed in oxLDL reisolated from the mixture of oxLDL and HDL3 pretreated with chloramine T as compared to oxLDL reisolated from the mixture with native HDL3 (supplemental Figure IVB). Marked increment in PCOOH occurred in reisolated HDL3 pretreated with chloramine T as compared to native HDL3 (+350%, P < 0.05; supplemental Figure IVC). As a result, levels of PCOH in oxLDL + HDL3 mixtures were significantly decreased (−70%; supplemental Figure IVD) in the presence of HDL3 pretreated with chloramine T as compared to native HDL3, resulting in a 4.2-fold diminished PCOH/PCOOH ratio.

Finally, apoAI Met residues were selectively oxidized with chloramine T in rHDL. The antioxidative activity of such chloramine T-pretreated rHDL was markedly decreased, both in terms of its capacity to decrease LDL oxidation rate and to prolong LDL oxidation (Table). Furthermore, treatment of apoAI/POPC rHDL with chloramine T diminished (−45%) its capacity to inactivate oxLDL-derived PCOOH as compared to nontreated rHDL (PCOOH levels at the end of the incubation, 3.08 ± 0.70 μmol/L versus 2.37 ± 0.40 respectively; n = 3).

**Implication of HDL-Associated Enzymes in HDL3-Mediated Inactivation of PCOOH**

To evaluate the potential role of HDL-associated enzymes, including LCAT, PAF-AH, and PON1, in the inactivation of LDL-derived LOOH, HDL3 was pretreated with a cocktail of enzymatic inhibitors (DFP which inhibits all 3 enzymes, Pefabloc which selectively inhibits PAF-AH, and EDTA which selectively inhibits PON1; supplemental Table III) and then incubated with oxLDL. Pretreatment significantly decreased the activities of HDL-associated LCAT (~50%), PAF-AH (~90%), and PON1 (~99%; supplemental Figure VA). However, no decrease in the capacity of such HDL3 to inactivate LOOH in oxLDL (supplemental Figure VB) or to delay LDL oxidation (data not shown) was observed. Similarly, no significant loss of the capacity of HDL3 to delay the accumulation of conjugated dienes in LDL was noted as a result of the pretreatment of HDL3 with any given single inhibitor (supplemental Table III).

In a separate experiment, HDL3 was preincubated with DTNB to derivatize free SH-groups involved in LCAT and PON1 activities,11,23 Again, such treatment exerted no influence on HDL3 capacity to inactivate preformed LOOH (data not shown). As expected, treatment of HDL3 with DTNB did not cause oxidation of Met residues of apoAI (data not shown). Finally, 10-minute incubation of HDL3 at 56°C significantly decreased PON1 and LCAT activities but did not influence its capacity to delay LDL oxidation (supplemental Table III).

To evaluate the role of hydrolytic enzymes, products of hydrolytic degradation of PC were equally determined. Levels of lysoPC in reisolated LDL oxidized alone and in oxLDL reisolated from oxLDL + HDL3 mixtures after 6-hour oxidation were similar (2.11 ± 1.02 versus 1.75 ± 1.25 μmol/L, respectively; n = 3). Furthermore, no difference was observed between lysoPC levels in HDL3 reisolated from LDL + HDL3 mixtures after 6-hour oxidation and nonoxidized HDL3 (1.00 ± 0.44 versus 0.97 ± 0.31 μmol/L, respectively; n = 3). Finally, after 6-hour incubation, no accelerated accumulation of free fatty acids was noted in LDL + HDL3 mixtures versus LDL alone (40.0 ± 4.1 versus 43.5 ± 4.9 μmol/L, respectively; n = 3).

**Implication of Surface PL in the HDL-Mediated Inactivation of PCOOH: Role of Acceptor Monolayer Rigidity**

To elucidate whether PCOOH could be transferred to acceptor particles from oxLDL, oxLDL was incubated with LOOH-free PC liposomes prepared from nonoxidizable POPC.
Indeed, PCOOH accumulation in the liposomes was observed with concomitant decrease in PCOOH content of oxLDL (−50%, *P<0.001; Figure 3A). To investigate whether such PCOOH transfer depended on physical characteristics of the acceptor PL monolayer, liposomal membrane rigidity was varied by adding sphingomyelin (SM) or free cholesterol (FC). On addition of 30 mol% SM to POPC, transfer of PCOOH to acceptor liposomes was diminished by −10%; addition of 10 and 30 mol% FC decreased PCOOH transfer from oxLDL by −20% and −25%, respectively (Figure 3A).

As a result, the rigidity of the liposomal PL monolayer, assessed as generalized polarization (GP) of Laurdan fluorescence, was strongly and negatively correlated with PCOOH transfer (r = −0.98, *P<0.02; Figure 3B).

LDL was then oxidized in the presence of small dense rHDL containing either apoAI/POPC, apoAl/POPC/FC, or apoAl/(POPC+SM). The propagation rate of LDL oxidation was significantly higher in the presence of apoAI/POPC/FC and apoAl/POPC/SM rHDL (+53%, *P<0.05, and +46%, *P<0.01, respectively; Table) as compared to LDL oxidized in the presence of apoAl/POPC rHDL. In parallel, the propagation phase was markedly shortened in the presence of apoAl/POPC/FC and apoAl/POPC/SM rHDL (−113%, *P<0.07, and −106%, *P<0.06, respectively; Table). By contrast, no difference in the maximal diene concentration was observed between different rHDL preparations, consistent with data for plasma HDL known to influence the rate, rather than the total amount, of LOOH accumulation under the oxidative conditions employed.7

Kinetic Analysis of LDL and HDL Oxidation

Kinetic analysis of LDL and HDL oxidation performed according to Bowry and Stocker24 revealed that the lipid peroxidation rate (Rs) in LDL was decreased by 55% as a result of the addition of HDL3 (supplemental Table IV). The lipid peroxidation rate of HDL3 was markedly (up to 21-fold) lower as compared to those for LDL. Importantly, the sum of Rs values obtained for LDL and HDL3 oxidized the same mixture was lower as compared to the value of Rs obtained for LDL oxidized alone, indicative of the protective effect of HDL toward LDL oxidation.

Discussion

Our present investigations have identified 2 key determinants of the potent capacity of physicochemically-defined HDL3 particles to protect LDL from oxidative damage by free radicals. Firstly, the HDL PL monolayer, which ensures the transfer of PLOOH from LDL to HDL3 in a process modulated by HDL surface lipid rigidity, and secondly apoAI, which reduces PLOOH to the corresponding redox-inactive PLOH by virtue of Met residues. By contrast, our data does not support the contention that HDL-associated enzymes (ie, PON1, PAF-AH, and LCAT) contribute significantly to the inactivation of LDL-derived LOOH.

We have established that the rigidity of the PL monolayer of HDL particles is a key modulator of the transfer efficiency of PCOOH from LDL to HDL. Indeed, the strong negative correlation of the PLOOH transfer rate to liposomes with membrane rigidity emphasizes the importance of both physical and chemical properties in this process. Furthermore, both the PLOOH transfer rate from oxLDL and the capacity of HDL to delay LDL oxidation decreased in parallel with surface lipid rigidity in rHDL.

The spontaneous translocation of PL-derived LOOH between erythrocytes and LDL is established.24 LOOH transfer equally occurs between plasma lipoproteins in systems consisting of donor/acceptor vesicles and rHDL.19,20 Lipid transfer proteins, including cholesteryl ester transfer protein (CETP), can accelerate the transfer of both PLOOH and CEOOH between plasma lipoproteins.19,20 As HDL and LDL are the major vehicles for transport of PL and CE in plasma, exchange of LOOH with other lipoproteins (eg, VLDL) should be of secondary importance. Clearly then, the transfer of LDL-associated PLOOH to HDL constitutes a key step in the HDL-mediated attenuation of LDL lipid peroxidation.

Consistent with the earlier data of Stocker et al,11,12 HDL3-associated apoAI inactivated LDL-derived PLOOH with reduction to the corresponding PLOH; apoAI methionine residues 112 and 148 presumably constitute the site of such reduction.11,12 Indeed, concentrations of redox-active Met residues in apoAI and of PLOOH in our assay ranged from 1.9 to 4.5 μmol/L and from 1.2 to 4.7 μmol/L, respectively, consistent with a 1:1 reaction stoichiometry. Kinetic analysis revealed that lipid peroxidation rate of HDL3 was markedly lower as compared to that of LDL despite similar molar content of oxidizable fatty acids in HDL3 and LDL.6,25 Furthermore, the sum of lipid peroxidation rates in LDL and in HDL3 oxidized in the mixture was considerably lower than that of LDL oxidized alone under identical conditions. As simple inhibition of oxidation by the addition of less oxidizable substrate can be excluded, these data provide direct evidence for HDL3 as a site of the inactivation of LDL-derived LOOH on exposure to oxidative stress.

Oxidation of specific Met residues in apoAI and apoAll to Met(O) plays a major role in the two-electron reduction of PCOOH and CEOOH in HDL particles.11,12 In addition, lipid-free apoAI can prevent formation of LDL-derived oxidized PL by the removal of LOOH from LDL or arterial wall.
cells\(^1\); LOOH reduction by apoAI Met residues may contribute significantly to such an antioxidative effect. Importantly, rHDL containing only purified apoAI and POPC, but devoid of enzymatic components, and authentic small, dense HDL\(^{3b +3c}\), were comparable in their capacities to delay lipid peroxidation of LDL. ApoAI therefore constitutes the central element in the HDL\(^3\)-mediated protection of LDL from free radical–induced oxidation. This conclusion is consistent with the potently capacity of HDL\(^3\) to inactivate LOOH as HDL\(^3\) is enriched in apoAI as compared to HDL\(^2\).\(^6\)

The dissociation of PON1 activity from the capacity of HDL to inactivate LOOH indicates that HDL-associated PON1 does not contribute significantly to the inactivation of LDL-derived LOOH. We and others\(^{11,26}\) demonstrated the existence of PON1-independent antioxidative activity of HDL. The major activity of PON1 has been recently demonstrated as that of a lactonase rather than a peroxidase\(^{17}\); the affinity of PON1 for LOOH is several orders of magnitude lower than its affinity for lactones.\(^{17,27}\) Together with our present data, these findings demonstrate that LOOH do not constitute a significant substrate of PON1 and that the previously proposed mechanism for the PON1-mediated attenuation of LDL lipid peroxidation via hydrolysis of LOOH is not tenable. We hypothesize therefore that the established antatherosclerotic properties of PON1\(^{28}\) are unrelated to its capacity to inactivate LOOH, but rather involve its major activity as a lactonase through a still unknown pathway upstream of the regulation of systemic oxidative stress.

In a similar manner, plasma PAF-AH readily hydrolyzes PAF-like oxPL\(^{29}\); furthermore, PAF-AH can hydrolyze PLOOH,\(^{18}\) leading to the formation of lysoPC and a fatty acid hydroperoxide. In our studies, inhibition of PAF-AH by Pefabloc was without effect on the HDL-mediated inactivation of LDL-derived LOOH, thereby indicating that PAF-AH cannot account for this activity. LCAT may equally hydrolyze short-chain oxidized PC generated during lipoprotein oxidation,\(^{15}\) but is inactive toward oxidized CE, the most abundant oxidized lipid species in LDL.\(^1\) Data herein demonstrate that LCAT is at most a minor factor in LOOH inactivation.

We propose a 2-step mechanism for the HDL\(^3\)-mediated protection of LDL against oxidative damage induced by free radicals. Initially, PLOOH is transferred from LDL to HDL; such transfer is governed by the rigidity of the surface monolayer of HDL, decelerates with increasing rigidity, and can be facilitated by lipid transfer proteins, such as CETP.\(^{19}\) Subsequently, reduction of PLOOH by redox-active Met residues of apoAI results in the formation of PLOOH and methionine sulfoxides. Preferential degradation of PCOOH by HDL as compared to CEEOH is consistent with the PCOOH location in the surface monolayer of LDL, resulting in their easier accessibility for transfer to HDL.\(^{24}\) Nonetheless, accumulation of CEEOH in LDL can be inhibited by HDL on LDL+HDL cooxidation as a consequence of decreased accumulation of PLOOH and shorter chain length of lipid peroxidation. The minor role of HDL-associated enzymes in this mechanism is consistent with their inability to supply reductive equivalents for LOOH reduction.

Dense HDL\(^3\) was distinct in accumulating lower levels of PCOOH and CEEOH and inactivating lipid hydroperoxides more potently than HDL\(^2\). According to our proposed mechanism, the elevated capacity of HDL\(^3\) to incorporate or to inactivate (P)LOOH may derive from (1) depletion of sphingomyelin, (2) enrichment in apoAI, or (3) altered conformation of apoAI as compared to HDL\(^2\).\(^6\)

The pathophysiological relevance of (P)LOOH inactivation by HDL is highlighted by prooxidative and proinflammatory properties of LOOH.\(^1\) In the arterial intima, free radical–induced peroxidation of LDL lipids results in the formation of LOOH as primary products.\(^1\) We used AAPH, a well-characterized azo-initiator of oxidation, to model free radical–induced LDL oxidation which involves LOOH formation as a major step.\(^{21}\) Furthermore, the HDL to LDL ratio used in our experiments was close to that in the interstitial fluid.\(^{30}\) Together with glutathione peroxidase (GPx), apoA-I represents a key LOOH-reducing protein in human plasma.\(^{31}\) LOOH reduction mediated by apoAI might therefore be especially relevant in microenvironments depleted of low-molecular-weight compounds, including glutathione and GPx, such as the arterial intima.

The capacity of HDL\(^3\) to protect LDL from free radical–induced oxidative damage is deficient in the atherogenic dyslipidemias of type 2 diabetes and metabolic syndrome.\(^{14}\) Elevation in the rigidity of the PL monolayer or deficiency of apoAI may underlie this observation. We therefore propose that induction of selective increase in the concentration of functional HDL\(^3\) particles displaying decreased surface rigidity and rich in apoAI may constitute an efficacious therapeutic approach to attenuate oxidative damage, inflammation, and atherosclerosis in dyslipidemic subjects at high cardiovascular risk.

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Disclosures

None.

References

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Supplement Material

HDL3-mediated inactivation of LDL-associated phospholipid hydroperoxides is determined by the redox status of apolipoprotein A-I and HDL particle surface lipid fluidity: Relevance to inflammation and atherogenesis

Amal Zerrad-Saadi a,b,c, Patrice Therond c, Sandrine Chantepie a,b,c, Martine Couturier a,b, Kerry-Anne Rye e, M. John Chapman a,b,c, and Anatol Kontush a,b,c *

Short title: Molecular determinants of antioxidative activity of HDL3

a Dyslipidemia and Atherosclerosis Research Unit (UMR 939), National Institute for Health and Medical Research (INSERM), Hôpital de la Pitié, Paris, France; bUPMC Paris 6, Paris, France; c Department of Biochemistry, University Paris Descartes, EA 3617, Paris, France; d University Paris 6, Paris, France and e Heart Research Institute, Sydney, Australia.

* Corresponding author: Dr. Anatol Kontush, INSERM Unité 551, Pavillon Benjamin Delessert, Hôpital de la Pitié, 83 boulevard de l'Hôpital, 75651 Paris Cedex 13, France. Tel. (33) (1) 4217 7976. Fax (33) (1) 4582 8198. E-mail kontush@chups.jussieu.fr
EXPERIMENTAL PROCEDURES

Isolation of plasma lipoproteins. Lipoproteins were preparatively fractionated by isopycnic density gradient ultracentrifugation from normolipidemic human serum or EDTA plasma as previously described. Subjects recruited for the study were healthy males, aged 8 to 70 years, non-smokers and either abstainers or only moderate alcohol consumers (<25g/d).

Five major subfractions of HDL were isolated, large light HDL2b (d 1.063-1.087 g/mL) and HDL2a (d 1.088-1.110 g/mL), and small, dense HDL3a (d 1.110-1.129 g/mL), HDL3b (d 1.129-1.154 g/mL), and HDL3c (d 1.154-1.170 g/mL). The validity and reproducibility of this density gradient fractionation of HDL particle subspecies has been extensively documented.

Small, dense HDL3 was a mixture of two HDL3 subclasses, notably HDL3b (d 1.129-1.154 g/mL) and HDL3c (d 1.154-1.170 g/mL); large, light HDL2 was a mixture of HDL2b and HDL2a. The HDL3a subclass was excluded in order to avoid overlap between the physicochemical and functional properties of small vs. large HDL.

The chemical compositions of isolated HDL subfractions were within the range for control subjects (Supplementary Table V).

A reference preparation of LDL was isolated from a single healthy normolipidemic donor in the density interval 1.018-1.065 g/ml by ultracentrifugation for 72h at 31,000g and 15°C. Following ultracentrifugation, KBr and EDTA salts were removed by exhaustive dialysis against phosphate-buffered saline (PBS) at 4°C.

Preparation of reconstituted (rHDL). Discoidal rHDL was prepared as previously described by cholate dialysis and contained either apoAI/ palmitoyloleoyl phosphatidylcholine (POPC) at a molar ratio of 1.0/77.1, apoAI/POPC/FC at a molar ratio of 1.0/66.4/5.1, or apoAI/ (POPC+ sphingomyelin (SM)) at a molar ratio of 1.0/65.8. The molar ratio of POCP to SM was 10/1. Recombinant HDL were dialyzed against 0.01 mol/l Tris-buffered saline (pH 7.4) containing 0.15 mol/l NaCl, 0.005% EDTA-Na2 and 0.006% NaN3.
Preparation of liposomes. Individual purified lipids (6mg) or mixtures of lipids were diluted in chloroform and lyophilized to remove traces of organic solvents. Dried lipids were suspended by vortexing in 4ml PBS and sonicated using a W-500 sonicator under nitrogen for 15-20 min at a temperature above the phase transition temperature of each lipid. Following sonication, the dispersion was centrifuged in the TL-100 tabletop ultracentrifuge using a fixed angle rotor for 20min at 30,000 rpm and subsequently for 1h at 40,000 rpm to remove non-liposomal lipid and large multilamellar liposomes respectively.

Characterisation of chemical composition of lipoproteins. Total protein, total cholesterol (TC), free cholesterol (FC), phospholipid (PL) and triglyceride (TG) contents of isolated lipoprotein subfractions were determined using commercially available assays. Cholesteryl ester (CE) was calculated by multiplying the difference between total and free cholesterol concentrations by 1.67. ApoAI and apoAII were quantitated by immunonephelometry. To estimate molar concentrations of HDL particles, molecular weights of 199 and 163 kDa calculated as described elsewhere were used for HDL3b and 3c subfractions, respectively.

Measurement of enzymatic activities of HDL. Paraoxonase (PON) 1 activity of HDL subfractions (100 µg protein/ml) was assessed as arylesterase activity determined photometrically in the presence of CaCl₂ (1 mM) using phenyl acetate as a substrate. Activity of PAF-AH was assessed using 1-palmitoyl 2-(6-[7-nitrobenzoxadiazolyl]amino) caproyl phosphatidylcholine (C₆NBD PC) as a fluorescent substrate. Activity of LCAT was measured using a commercially available kit (Roar Biomedical, New York, USA).

Measurement of antioxidative activity of HDL. The antioxidative activity of small, dense HDL3 was evaluated as the capacity to attenuate the oxidation of reference LDL by 2,2'-azobis-(2-amidinopropane) hydrochloride (AAPH), an azo-initiator of lipid peroxidation and a well-characterised model of free-radical-induced LDL oxidation. Small, dense HDL3 consisted of a mixture of HDL3b and 3c particles.
Accumulation of lipid hydroperoxide (LOOH). LDL were oxidised at 20mg TC/dl in the presence or absence of individual HDL subfractions (40mg/dl total mass/dl) with 2mM AAPH at 37°C for 2h and 6h; these time points correspond approximately to the end of the lag and the propagation phases of LDL oxidation respectively. Oxidation was terminated by addition of EDTA (10µM) and BHT (10µM). AAPH was employed to oxidise LDL at it provides a highly constant production of free radicals of defined properties. Levels of oxidised and non-oxidised lipids were determined by HPLC (see below).

Accumulation of conjugated dienes. The time course of lipoprotein oxidation was equally characterized by accumulation of conjugated dienes, which were measured as the increment in absorbance at 234 nm. LDL (10 mg TC/dl) was oxidised with 1mM AAPH at 37°C for 16h in the presence or absence of HDL (20mg total mass /dl). Absorbance kinetics at 234 nm revealed three consecutive phases: the lag, propagation and plateau phases; the durations of the lag and propagation phases, average oxidation rates within these phases and amount of dienes formed at the end of the propagation phase (maximal amount of dienes) were calculated for each absorbance curve. Corrections for the absorbance increase due to AAPH itself were made.

Determination of the capacity of HDL to inactivate LOOH. Available data indicate that HDL3 from interstitial fluid is similar to plasma HDL3. The HDL to LDL ratio is slightly elevated in the interstitial fluid as compared to plasma, an observation which was reflected by our experimental conditions (see below). LDL isolated from atherosclerotic lesions contains oxidised lipids, including lipid hydroperoxides. Levels of lipid peroxidation products in LDL from atherosclerotic tissue are similar to those present in oxLDL at the end of the oxidation lag-phase (10-100 mol LOOH/mol LDL; see refs above and). As compared to those levels, artefactual lipid peroxidation lipids during lipoprotein purification was negligible.
in our studies (<1 mol/mol LDL). It is important to mention in this regard that we used EDTA to protect LDL during isolation.

LDL (40mg TC/dl) were preoxidised with 4mM AAPH for 16h at 37°C. Oxidative modification was terminated by addition of EDTA (10µM) and BHT (10µM). Oxidised LDL (oxLDL) was dialysed against PBS at 4°C to remove EDTA and excess BHT, and incubated at a concentration of 20 mg TC/dl for 0.5, 2 and 6h in the presence or absence of native HDL3 (40 mg total mass/dl) in PBS at 37°C. EDTA (100 µM) was present to inhibit lipid peroxidation during the incubation. Subsequently, oxLDL and HDL3 were reisolated by density gradient ultracentrifugation as described above. Lipid hydroperoxides and non-oxidised lipids were identified and quantified in oxLDL and HDL3 before and after incubation by HPLC (see below).

Measurement of LOOH and non-oxidised lipids. LOOH were evaluated by HPLC (with chemiluminescent and UV detection) as molecular species of PL and CE hydroperoxides (PLOOH and CEOOH respectively) in mixtures of LDL+HDL3 incubated in the presence of the oxidant (AAPH). Molecular species of PLOOH other than PCOOH were not detected using this approach (data not shown). Concomitantly, consumption of molecular species of non-oxidised PL and CE was measured by HPLC with UV detection as described elsewhere. In parallel, the time course of PL and CE hydroperoxide accumulation and of concomitant PL and CE consumption was assessed in individual LDL and HDL3 fractions after reisolation from the same reaction mixture using density gradient ultracentrifugation.

In some experiments, lipoperoxides were measured on the basis of their ability to oxidise N-benzoyl leucomethylene blue to methylene blue in the presence of haemoglobin as described by Auerbach et al. In brief, oxLDL (20mg TC /dl) incubated in the presence or absence of HDL3 (40mg total mass /dl) at 37°C was mixed with a reagent containing 5mg of N-benzoyl leucomethylene blue dissolved in 8ml of dimethylformamide, which was then added to a 0.05
M potassium phosphate buffer (pH 5) containing 1.4 % Triton X-100 and 0.0055 % hemoglobin. The sample (50µl) was then mixed with 100µl of the reagent and read at 660 nm.

**Measurement of lipid hydroxides (LOH).** Total levels of PCOOH and PCOH present in lipoprotein samples were identified and quantified by HPLC using a C18-Kromasil column and UV detection at 234 nm. Levels of PCOH were calculated as the difference between total PCOOH+PCOH and PCOOH measured by HPLC with chemiluminescent detection (see above).

**Measurement of lysophosphatidylcholine (lysoPC) and free fatty acids.** LysoPC content was evaluated by HPLC with external standardisation using UV detection on a Kromasil Silica (Si-100; 2.1 x 25 cm) column. The column was eluted at 0.4ml/min and 50°C with an isopropanol/hexane/potassium acetate (25mM) gradient at pH 7, with continuous monitoring at 205 nm. Following initial equilibration with a 57.5/36/6.5 % solvent mixture for 5 min, the gradient was changed to 43.5/50/6.5% and eluted for 30 min.

Free fatty acids were measured using a commercially available kit (Diasys, Bouffemont, France) which does not distinguish between non-oxidised and oxidised species.

**Determination of the fluidity of PL surface monolayer.** Liposomes (5mg total lipid/dl) were incubated with 33µM of Laurdan fluorescent probe at 37°C for 30 min in the dark. The fluorescence spectrum of Laurdan was obtained using an excitation wavelength of 340nm. The value of generalized polarization (GP) of the Laurdan fluorescence was employed as a measure of PL monolayer rigidity and calculated as GP = (F440-F490)/ (F440+F490), where F440 and F490 are the emission intensities at the blue (440 nm) and red (490 nm) edges of the emission spectrum that correspond to the fluorescence emission maxima in the gel, and the liquid-crystalline phases respectively, of a PL bilayer.\textsuperscript{19,20}

**Transfer of phosphatidylcholine hydroperoxide (PCOOH) and cholesteryl ester hydroperoxide (CEOOH) from oxLDL to liposomes.** Transfer of PL and CE
hydroperoxides was evaluated using LDL (40 mg TC/dl) preoxidised for 16h by AAPH (4mM). The oxLDL was dialysed and incubated at a concentration of 20 mg TC/dl for 2h in the presence or absence of liposomes (20mg lipid/dl) in PBS at 37 °C. Subsequently, oxLDL and liposomes were reisolated by density gradient ultracentrifugation as described above; liposomes were collected as the top fraction (1.6ml). LOOH and non-oxidised lipids were measured in oxLDL and liposomes before and after incubation using HPLC as described above.

**Oxidation of HDL methionine (Met) residues.** HDL3 (1.5 mg protein/ml) was incubated for 1 h at 4°C in PBS containing 1 mM EDTA in the presence of chloramine T (500 µM), a reagent that selectively oxidises protein Met residues into corresponding sulfoxides but is also able to modify free SH groups. Consistent with its preferential reactivity towards the protein moiety, chloramine T did not modify the fluidity of the lipid phase in HDL3 (data not shown). Unreacted chloramine T was removed by gel filtration on a Sephadex PD-10 column (Amersham Biosciences, Uppsala, Sweden).

**Measurement of oxidised Met residues in HDL.** HDL3 were subjected to HPLC on a 5 µm, 25 x 0.46 cm, 300Å, C18 column (ACE, AIT, Houilles, France). The column was eluted at 1ml/min and 50°C with an acetonitrile/water gradient containing 0.1% (v/v) trifluoroacetic acid (Pierce, Rockford, USA) monitored at 214 nm. Initial equilibration was performed with 40% acetonitrile buffer; acetonitrile concentration was subsequently increased linearly to 65% over 50 min (modified from (Pankhurst G and coll, JLR, 2003)).

**Inhibition of lecithin:cholesterol-acyltransferase (LCAT), platelet-activating factor-acetyl hydrolase (PAF-AH) and paraoxonase 1 (PON1).** HDL3 were incubated overnight at 4°C with a cocktail of inhibitors containing DFP (5mM), Pefabloc (0.1mM) and EDTA (5mM) to inhibit LCAT, PAF-AH and PON1. Subsequently, the inhibitors were removed by dialysis against PBS at 4°C.
HDL3 were also pre-treated individually with each of the inhibitors. To pre-treat HDL with Pefabloc or EDTA, serum was incubated with Pefabloc (0.1 mM) or EDTA (4.8 mM) for 16h at 4°C and HDL3 isolated by density gradient ultracentrifugation. Control HDL3 was isolated from serum incubated in parallel in the absence of the inhibitors. In a separate experiment, HDL3 was directly pre-treated with DFP (5mM) in PBS at 37°C. Control HDL3 was incubated in parallel in the absence of the inhibitor. In another experiment, HDL3 was incubated at 56°C for 10min to heat-inactivate the enzymes. Control HDL3 was incubated in parallel at 37°C.

To derivatise HDL-associated thiols, HDL3 (1.5 mg total protein/ml) was incubated overnight at 4°C in PBS containing 1mM EDTA and 2mM DTNB following by dialysis against PBS at 4°C.

**Calculation of kinetic parameters of LDL and HDL oxidation.** The kinetics of LDL and HDL3 oxidation were analysed according to Bowry and Stocker. The free radical generation rate at 37°C was calculated as $R_g = 1.4 \times 10^{-6} [\text{AAPH}] \text{ s}^{-1}$, where $[\text{AAPH}]_0$ is the initial concentration of the azo-initiator of oxidation, AAPH. The initiation rate of lipid peroxidation was calculated as $R_i = 2 [\alpha-\text{TocH}]_0/t_{\text{inh}}$, where $[\alpha-\text{TocH}]_0$ is the initial concentration of $\alpha$-tocopherol and $t_{\text{inh}}$ is the duration of the lag-phase of lipoprotein oxidation. The contribution of other antioxidants (ubiquinol-10, carotenoids) to the total radical-scavenging capacity of lipoproteins was considered to be negligible. Concentrations of $\alpha$-tocopherol of 9.0 mol/mol LDL and 0.3 mol/mol HDL3 were used, according to our earlier studies. The duration of the lag-phase of lipoprotein oxidation was determined in a separate experiment; values of 106, 48 and 113 min (means of at least 3 independent experiments) were used for LDL alone, HDL3 alone and the mixture of LDL+HDL3 respectively. To convert between mass and molar concentrations, molecular weights of 2500 and 180 kDa were used for LDL and HDL3. The phase-transfer efficiency was defined as...
\[ \varepsilon = \frac{R}{R_i} \] and expressed as a percentage.\(^8\) The **rate of lipid peroxidation** \( R_p \) was calculated as a sum of concentrations of PLOOH and CEOOH at a given time-point divided by the time of oxidation.\(^8\) The **fractional rate of lipid peroxidation** was calculated as \( \Phi = 2 \frac{R_p}{[LH]} \), where [LH] is the concentration of non-oxidised lipid (PL or CE).\(^8\) The order of magnitude of all calculated parameters was in excellent agreement with that reported by Bowry and Stocker.\(^8\)

**Statistical analysis.** All data are shown as means ± SD unless otherwise indicated. Between-group differences were analyzed by Student’s t-test or Wilcoxon test for dependent samples when applicable. Pearson’s moment-product correlation coefficients were calculated to evaluate relationships between variables.
RESULTS

Kinetic analysis of LDL and HDL oxidation

Kinetic analysis of LDL and HDL oxidation performed according to Bowry and Stocker\textsuperscript{8} revealed that the initiation rate of lipid peroxidation by AAPH ($R_i$) was similar in LDL oxidised alone and in LDL reisolated from mixtures with HDL3 (Supplementary Table IV). The value of $R_i$ calculated for reisolated HDL3 was lower than those for LDL. The values of the phase-transfer efficiency ($\varepsilon$) of free-radical induced oxidation paralleled those of the initiation rate.\textsuperscript{8}

The rate of lipid peroxidation ($R_p$) in reisolated LDL was decreased by 55\% as a result of the addition of HDL3 (Supplementary Table IV). The rate of lipid peroxidation of reisolated HDL3 was markedly (up to 21-fold) lower as compared to those for LDL. Importantly, the sum of $R_p$ values obtained for LDL and HDL3 reisolated from the same mixture was lower as compared to the value of $R_p$ obtained for LDL alone, indicative of the protective effect of HDL towards LDL oxidation. Consistent with this result, the fractional rate of lipid peroxidation ($\Phi$) in reisolated LDL was reduced by 56\% as a result of the addition of HDL3; moreover, the sum of $\Phi$ values obtained for LDL and HDL3 reisolated from their mixture was 31\% lower than the value of $\Phi$ obtained for LDL alone.
REFERENCES


7. Gowri MS, Van der Westhuizen DR, Bridges SR, Anderson JW. Decreased protection by HDL from poorly controlled type 2 diabetic subjects against LDL oxidation may be due to the abnormal composition of HDL. *Arterioscler Thromb Vasc Biol.* 1999;19:2226-2233.


SUPPLEMENTARY FIGURE LEGENDS

**Supplementary Fig. I. Accumulation of PLOOH in LDL and HDL3 oxidised in a mixture by AAPH for 2 and 6h.**

LDL (20 mg TC/dl) were oxidised by AAPH (2 mM) in the presence or absence of HDL3 (filled bars; 40 mg total mass/dl) in PBS at 37°C. LDL oxidised alone was used as a control (open bars). LDL and HDL3 were reisolated by density gradient ultracentrifugation. PCOOH and PL were evaluated by HPLC with chemiluminescent and UV detection in LDL+HDL3 mixtures (A), reisolated LDL (B) and reisolated HDL3 (C). Data are means ± SD of four independent experiments with four independent HDL samples; *p<0.05 vs incubation without added HDL3. Concentrations of PCOOH in LDL oxidised alone for 2h and 6h were 1.10±0.85 and 4.20±0.61 µM respectively.

**Supplementary Fig. II. Accumulation of CEOOH in LDL and HDL3 oxidised in a mixture by AAPH for 2 and 6h.**

LDL (20 mg TC/dl) were oxidized by AAPH (2 mM) in the presence or absence of HDL3 (filled bars; 40 mg total mass/dl) in PBS at 37°C. LDL oxidized alone was used as a control (open bars). LDL and HDL3 were reisolated by density gradient ultracentrifugation. CEOOH and CE evaluated by HPLC with chemiluminescent and UV detection in LDL+HDL3 mixtures (A), reisolated LDL (B), and reisolated HDL3 (C). Data are means +/- SD of four independent experiments with four independent HDL3 samples. Concentrations of CEOOH in LDL oxidised alone for 2h and 6h were 3.5±1.3 and 11.8±3.0 µM respectively.

**Supplementary Fig. III. Inactivation of CEOOH in LDL preoxidized by AAPH and incubated with native HDL3.**

LDL (40 mg TC/dl) were preoxidized by AAPH (4 mM), dialysed and incubated at 20 mg TC/dl for 0.5, 2 and 6h in the presence or absence of HDL3 (filled bars; 40 mg total mass/dl) in PBS at 37 °C. LDL oxidized alone was used as a control (open bars). LDL and HDL3 were
Supplement Material

reisolated by density gradient ultracentrifugation. CEOOH and CE were evaluated by HPLC with chemiluminescent and UV detection in the LDL and HDL3 mixture (A), reisolated LDL (B), and reisolated HDL3 (C). Data are means +/- SD of four independent experiments with four independent HDL samples. Concentration of CEOOH in preoxidised LDL was 5.0±0.5 µM.

**Supplementary Figure IV. Influence of Met oxidation on PCOOH accumulation in preoxidised LDL+HDL3 (A), reisolated LDL (B) and reisolated HDL3 (C), and on PCOH accumulation in preoxidised LDL+HDL3 (D).**

To selectively oxidize Met residues, HDL (1.5 mg protein/ml) were incubated for 1 h at 4°C in PBS containing 1 mM EDTA in the presence of chloramine T (chlor T; 500 µM). LDL preoxidised by AAPH (see Fig. 1 and 2 for details) were incubated for 2h in the presence of native HDL3 (open bars) or HDL3 pretreated with chloramine T (filled bars); *p<0.05 vs native HDL3.

**Supplementary Fig. V. Influence of the inhibition of HDL-associated enzymes on HDL3-mediated inactivation of LOOH in preoxidised LDL.**

HDL3 were incubated overnight with DFP (5mM), Pefabloc (0.1mM) and EDTA (5mM). Enzymatic activities (A) and the capacity to inactivate LOOH in oxLDL (B) were measured in native HDL3 (grey bars) and in HDL3 pretreated with the inhibitors (filled bars). OxLDL incubated alone was the control (open bars); **p<0.01, *p<0.05 vs oxLDL.
**Supplementary Table I. Molecular species of LOOH in LDL and LDL+HDL3**

<table>
<thead>
<tr>
<th>Lipoprotein (incubation time)</th>
<th>Molecular species of LOOH</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCOOH, % of initial PC</td>
<td>CEOOH, % of initial CE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18:2/16:0</td>
<td>20:4+22:6/16:0</td>
<td>20:4+18:2</td>
</tr>
<tr>
<td>LDL (6h)</td>
<td>12.0±2.0</td>
<td>12.6±1.0</td>
<td>4.5±1.0</td>
</tr>
<tr>
<td>LDL+HDL3 (6h)</td>
<td>7.5±1.7</td>
<td>6.8±1.9**</td>
<td>3.3±0.6</td>
</tr>
</tbody>
</table>

LDL (20 mg TC/dl) was oxidised by AAPH (2 mM) in the presence or absence of HDL3 (40 mg total mass/dl) in PBS at 37°C. LDL oxidised alone was used as a control. PCOOH and CEOOH were evaluated by HPLC with chemiluminescent detection. Data are means +/- SD of four independent experiments with four independent HDL samples; **p<0.01 vs LDL alone.
Supplementary Table II. PCOOH and CEOOH in LDL oxidised alone, HDL3 oxidised alone and LDL+HDL3 oxidised together.

<table>
<thead>
<tr>
<th></th>
<th>PCOOH (2h)</th>
<th>PCOOH (6h)</th>
<th>CEOOH (2h)</th>
<th>CEOOH (6h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of initial PC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL alone</td>
<td>2.84±1.75</td>
<td>12.27±1.72</td>
<td>1.89±0.98</td>
<td>4.47±0.97</td>
</tr>
<tr>
<td>HDL3 alone</td>
<td>2.10±0.14</td>
<td>5.75±0.34</td>
<td>0.96±0.15</td>
<td>1.62±0.06</td>
</tr>
<tr>
<td>LDL+HDL3 mixture</td>
<td>1.18±0.90</td>
<td>5.54±2.91*</td>
<td>1.16±0.25</td>
<td>3.32±0.63</td>
</tr>
<tr>
<td>% of initial CE</td>
<td></td>
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</tbody>
</table>

LDL (20 mg TC/dl) or HDL3 (40 mg total mass/dl) were oxidized separately or together by AAPH (2 mM) for 2h and 6h. PCOOH and CEOOH were evaluated by HPLC with chemiluminescent detection. Data are means +/- SD of four independent experiments with four independent HDL samples; *p<0.05 vs LDL alone.
## Supplementary Table III. Influence of the inhibition of HDL enzymes on the capacity of HDL3 to inhibit LDL oxidation by AAPH

<table>
<thead>
<tr>
<th></th>
<th>Inhibition of LCAT activity, %</th>
<th>Inhibition of PAF-AH activity, %</th>
<th>Inhibition of PON1 activity, %</th>
<th>Propagation rate, % LDL alone</th>
<th>Propagation phase duration, % LDL alone</th>
<th>Maximal diene concentration, % LDL alone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DFP treatment</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control HDL3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>52±13</td>
<td>201±29</td>
<td>120±13</td>
</tr>
<tr>
<td>Pretreated HDL3</td>
<td>71±13</td>
<td>80±20</td>
<td>95±4</td>
<td>74±54</td>
<td>197±123</td>
<td>119±15</td>
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<tr>
<td><strong>Pefabloc treatment</strong></td>
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<tr>
<td>Control HDL3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>56±10</td>
<td>174±37</td>
<td>101±22</td>
</tr>
<tr>
<td>Pretreated HDL3</td>
<td>7±9</td>
<td>96±4</td>
<td>2±4</td>
<td>52±6</td>
<td>163±21</td>
<td>87±14</td>
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<td><strong>EDTA treatment</strong></td>
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<tr>
<td>Control HDL3</td>
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<td>0</td>
<td>42±4</td>
<td>220±34</td>
<td>103±4</td>
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<tr>
<td>Pretreated HDL3</td>
<td>3±6</td>
<td>0±0</td>
<td>83±11</td>
<td>37±6</td>
<td>249±45*</td>
<td>100±2</td>
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<tr>
<td><strong>Heat inactivation</strong></td>
<td></td>
<td></td>
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<tr>
<td>Control HDL3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>49±13</td>
<td>228±31</td>
<td>138±5</td>
</tr>
<tr>
<td>Pretreated HDL3</td>
<td>30±37</td>
<td>7±7</td>
<td>100±0</td>
<td>46±5</td>
<td>229±12</td>
<td>132±8</td>
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</tbody>
</table>
Enzymatic activities are expressed relative to control HDL3. Data are means ± SD of three to five independent experiments with three independent HDL samples from three different donors. LDL (10 mg TC/dL) was incubated with AAPH (1 mM) in the presence of either control HDL3, HDL3 pre-treated with DFP (5mM) in PBS at 37°C, HDL3 isolated by density gradient ultracentrifugation from serum pre-incubated with Pefabloc (0.1 mM) for 16h at 4°C, HDL3 isolated by density gradient ultracentrifugation from serum pre-incubated with EDTA (4.8 mM) for 16h at 4°C, or HDL3 pre-incubated at 56°C for 10min. Control HDL3 was incubated in parallel in the absence of the inhibitor or isolated from serum incubated in the absence of the inhibitor. HDL3 concentration in the assay was 10 mg total mass/dl in each case. Inhibition of enzymatic activities in HDL3 is shown as % of initial levels; *p<0.05 vs corresponding control HDL3.
## Supplementary Table IV. Kinetic parameters of LDL and HDL3 oxidation

<table>
<thead>
<tr>
<th></th>
<th>Ri, nM/s</th>
<th>ε, %</th>
<th>Rp, nM/s</th>
<th>Φ, ppm/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL alone</td>
<td>0.35</td>
<td>25</td>
<td>0.42</td>
<td>1.6</td>
</tr>
<tr>
<td>LDL reisolated from</td>
<td>0.42</td>
<td>30</td>
<td>0.19</td>
<td>0.7</td>
</tr>
<tr>
<td>mixtures of LDL and HDL3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL3 reisolated from</td>
<td>0.23</td>
<td>17</td>
<td>0.02</td>
<td>0.4</td>
</tr>
<tr>
<td>mixtures of LDL and HDL3</td>
<td></td>
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</table>

Initial concentrations: AAPH, 2 mM; LDL, 20 mg total cholesterol/dl (equivalent to 0.24 µM); HDL3, 40 mg total mass/dl (equivalent to 1.12 µM); α-tocopherol, 2.20 µM in LDL and 0.34 µM in HDL3. Calculations were performed using values obtained at 2h oxidation.
Supplementary Table V. Chemical composition of HDL3 subfractions

<table>
<thead>
<tr>
<th></th>
<th>TP</th>
<th>FC</th>
<th>PL</th>
<th>CE</th>
<th>TG</th>
<th>CE/TG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Individual normolipidemic donor</strong> (means ± SD of eight independent experiments)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3b</td>
<td>47.9±1.6</td>
<td>2.8±0.3</td>
<td>27.8±1.7</td>
<td>18.6±1.8</td>
<td>2.7±0.8</td>
<td>7.4±2.5</td>
</tr>
<tr>
<td>3c</td>
<td>55.4±1.4</td>
<td>2.5±0.3</td>
<td>23.3±1.2</td>
<td>15.0±1.4</td>
<td>2.9±0.8</td>
<td>5.2±2.2</td>
</tr>
<tr>
<td><strong>Normolipidemic plasma pool</strong> (means ± SD of four independent experiments)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3b</td>
<td>48.9±1.3</td>
<td>2.6±0.1</td>
<td>25.8±0.6</td>
<td>20.1±0.5</td>
<td>2.5±0.2</td>
<td>7.9±0.5</td>
</tr>
<tr>
<td>3c</td>
<td>54.1±2.0</td>
<td>2.5±0.2</td>
<td>22.1±0.7</td>
<td>18.1±1.6</td>
<td>3.3±0.9</td>
<td>5.8±1.8</td>
</tr>
</tbody>
</table>

HDL3 subfractions were isolated by density gradient ultracentrifugation and their content of total protein (TP), free cholesterol (FC), phospholipids (PL), cholesteryl esters (CE), triglycerides (TG) and the ratio of CE/TG were determined.
Supplementary Fig. I. Accumulation of PLOOH in LDL and HDL3 oxidised in a mixture by AAPH for 2 and 6h.
Supplementary Fig. II. Accumulation of CEOOH in LDL and HDL3 oxidised in a mixture by AAPH for 2 and 6h.
Supplementary Fig. III. Inactivation of CEOOH in LDL preoxidized by AAPH and incubated with native HDL3.
Supplementary Figure IV. Influence of HDL3 pretreatment with Chloramine T (chlor T) on the accumulation of PCOOH in LDL+HDL3 (A), reisolated LDL (B) and reisolated HDL3 (C) and on the accumulation of PCOH in LDL+HDL3 (D).
Supplementary Figure V. Influence of the inhibition of HDL-associated enzymes on HDL3-mediated inactivation of LOOH in preoxidised LDL.