PAF-Acether–Degrading Acetylhydrolase in Plasma LDL Is Inactivated by Copper- and Cell-Mediated Oxidation

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Abstract In peripheral blood, native low-density lipoprotein (LDL) is a major carrier of acetylhydrolase, the enzyme that hydrolyzes the sn-2 acetate of PAF-acether, converting it to lyso PAF-acether. By controlling the level of PAF-acether, the acetylhydrolase may regulate the biologic effects of this potent inflammatory and thrombotic mediator. The biologic oxidation of LDL appears to underlie its atherogenicity. We report here that oxidative modification of LDL led to progressive loss of associated acetylhydrolase activity. Reductions of approximately 90% and 40% of acetylhydrolase activity occurred respectively in LDL oxidized for 24 hours by copper ions (2.5 μmol/L) in phosphate-buffered saline and in LDL incubated with human monocyte-like THP-1 cells in Ham’s F-10 medium. Acetylhydrolase activity decreased as a function of the degree of LDL oxidation and was correlated with an increase in net negative charge and in the content of thiobarbituric acid–reactive substances (r = −.94 and r = −.88, respectively; P < .001). The acetylhydrolase of mildly oxidized LDL displayed a similar $K_m$ for PAF-acether compared with native LDL, whereas its $V_{	ext{max}}$ was lower. Thus, acetylhydrolase conserved its affinity for PAF-acether, whereas a nondefined and noncompetitive inhibitor, apparently produced during oxidation, might account for the observed loss in enzymatic activity. Acetylhydrolase activity was totally recovered in LDL modified by both acetylation and malondialdehyde. In contrast, a reduction of 75% or more in acetylhydrolase activity was observed in LDL modified by 4-hydroxynonenal; this effect may be due to either a direct modification of amino acid side chains of acetylhydrolase by 4-hydroxynonenal or a modification of the phospholipid environment of the enzyme at the surface of the LDL particle. Because PAF-acether is probably synthesized and released by cells present in the atheromatous plaque, it may play a potentially proatherogenic role. Thus, the loss of PAF-acether–degrading acetylhydrolase activity in oxidized LDL confers this lipoprotein with a new atherogenic role. (Arterioscler Thromb. 1994;14:353-360.)

Key Words • monocyte-like cells • 4-hydroxynonenal • apolipoprotein B • atherogenesis

Oxidatively modified low-density lipoproteins (Ox-LDLs) play a major role in the pathogenesis of atherosclerosis (reviewed in Reference 1) and have been identified in atherosclerotic plaques from Watanabe rabbits2-3 and human subjects.3 Ox-LDL may exert several proatherogenic effects, which include uptake by scavenger receptors of macrophages with subsequent transformation into foam cells,4 at a typical component of fatty streaks and advanced plaques, and the ability to enhance monocyte recruitment and macrophage retention in atherosclerotic lesions.5 Moreover, Ox-LDLs are markedly cytotoxic for both endothelial cells and macrophages.6

LDL can be oxidatively modified in vitro by exposure to redox-active metal ions such as copper7 or by incubation with various cells in culture, including aortic smooth muscle cells,8,9 endothelial cells,9 monocytes,10 and macrophages.4 The oxidation of LDL involves a complex series of reactions that are incompletely established; one of the most important involves peroxidation of esterified polyunsaturated fatty acids.11 Apolipoprotein B-100 (apoB-100) of LDL may be modified by interaction with several products of lipid peroxidation,11,12 including malondialdehyde (MDA)13,14 and 4-hydroxynonenal (4-HNE).14-16 Moreover, oxidized phospholipids, in which cleavage of the polyunsaturated sn-2 fatty acid results in formation of a short hydrocarbon chain or hydroxyl group, are generated.11,17,18 In addition, some 40% of phosphatidylcholine, the major phospholipid of LDL, is hydrolyzed to lysophosphatidylcholine on oxidation; this effect is mediated by a phospholipase A$_2$ intrinsic to LDL.19 Such enzymatic activity may play a key role in the oxidation of LDL, as its inhibition blocks the enhanced macrophage uptake of LDL.19

The phospholipase A$_2$ activity associated with LDL might in part be due to the acetylhydrolase,20 previously described as a platelet-activating factor or a PAF-acether (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, PAF)–degrading enzyme. However, it was recently established that the substrate specificity of the purified acetylhydrolase is restricted to oxidatively fragmented phosphatidylcholine bearing a short-chain fatty acid (up to five carbon atoms) at the sn-2 position.20 Initially, the acetylhydrolase was described as a calcium-independent enzyme that hydrolyzes the sn-2 acetate of PAF, converting it to lyso PAF.21-23 This enzyme is synthesized and released by several types of human cells such as macrophages,24 platelets,25 and hepatocytes.26 Acetylhydrolase is principally transported in blood in association with LDL.27,28 Furthermore, acetylhydrolase appears to control the biologic activity of PAF.29 PAF is a potent inflammatory mediator that is implicated in the immune response. The role of PAF in
several pathologies such as anaphylactic shock and ischemia has been suggested (reviewed in Reference 30), and this molecule may play an important role in atherogenesis (reviewed in Reference 31). PAF is synthesized by various cells on activation, including monocytes, macrophages, endothelial cells, and platelets; such cells contribute to the development of atheromatous plaque. A fraction of the synthesized PAF is released from cells and transported in association with albumin28 and plasma lipoproteins, including very-low-density lipoprotein, LDL, and high-density lipoprotein.28,32 PAF may be implicated in atherogenesis in different ways. First, as shown in vitro, membrane-bound PAF may act as an adhesion molecule between polymorphonuclear neutrophils and endothelial cells33 or platelets,34 and it may enhance vascular permeability in vivo.35 Second, PAF may act in vivo as a chemoattractant activator with the capacity to attract monocytes, which interact with endothelial cells, and thus may activate their infiltration into the intimal space. In addition, PAF may induce the synthesis and release of several inflammatory cytokines, such as tumor necrosis factor and growth factors in various cells of the arterial wall, which may contribute to the development of atherosclerotic lesions.31 Furthermore, PAF induces release of proteases, such as elastase, from macrophages;36 such enzymes may degrade components of the extracellular matrix of the intima. PAF also induces release of active oxygen species from monocytes,37 macrophages,37 endothelial cells,38 and lymphocytes;39 in the subintimal space, active oxygen species may induce tissue damage and equally contribute to LDL oxidation (reviewed in Reference 1).

In the present study, we investigated the activity of the LDL-associated acetylhydrolase during copper- and cell-induced oxidation to establish whether Ox-LDL retains the capacity to modulate PAF-mediated effects. Our results show that progressive loss of LDL-associated acetylhydrolase activity occurred during both copper- and cell-mediated oxidation. The Ox-LDL-associated acetylhydrolase displayed the same affinity for PAF (similar Km) as native LDL; by contrast, its maximal velocity (Vmax) decreased progressively during oxidation, suggesting that the loss of enzymatic activity is probably due to a noncompetitive product of oxidation.

**Methods**

**Materials**

PAF (hexadecyl), obtained as a powder from Novabiochem, was dissolved at a final concentration of 10−2 mol/L in ethanol (80% vol/vol) and mixed with [3H-acetyl]PAF (1-O-hexadecyl-2-[3H]acetyl-sn-glycerol-3-phosphocholine, 10 Ci/mmol; Du Pont–New England Nuclear). The mixture was brought to dryness under a nitrogen stream. l-O-Stearoyl-2-arachidonyl-sn-glycero-3-phosphocholine (Sigma Chemical Co) was dissolved in chloroform and dried under nitrogen. Dry samples were redissolved in an NaCl (150 mmol/L) solution to which 7-Ketocholesterol and cholesterol-5α-6α-epoxide (Sigma) were added. Before oxidation, LDL was dialyzed against a 0.22-μm filter (Costar) and stored at 4°C. The purity of LDL preparations was evaluated as described earlier.40 The protein content of LDL was determined by the procedure of Lowry et al.41

**Chemical Modification of LDL**

Acetylated LDL (Ac-LDL) was prepared according to the procedure of Basu et al.42 MDA-LDL was prepared by incubating LDL (0.5 mg protein/mL in PBS containing 3 mmol/L EDTA) for 3 hours at 37°C with MDA (final concentration, 25 mmol/L) according to the protocol of Palinski et al.43 4-Hydroxynonenal (4-HNE)-LDL was prepared as described by Palinski et al.14 LDL (2 mg protein/mL in PBS containing 3 mmol/L EDTA) was incubated for 24 hours at 37°C with 4-HNE (final concentration, 5 mmol/L) in the presence or absence of the reducing agent NaCNBH3 (final concentration, 20 mmol/L). In defined experiments, concentrations of 4-HNE, ranging from 0.3 to 10 mmol/L final concentration, were incubated with LDL for 5 hours at 37°C. In time-course studies, 4-HNE (final concentration, 10 mmol/L) was incubated with LDL for 1 to 5 hours at 37°C.

**Preparation of Cells**

The human monocyte-like cells THP, (TIB 202) (American Type Culture Collection) were grown in RPMI 1640 containing 20 μmol/L L-mercaptoethanol, 10% heat-inactivated fetal calf serum, and 40 μg/mL gentamicin. Cell viability was assessed by trypan blue exclusion at different periods of incubation in the presence or absence of LDL.

**Cell-Induced Modification of LDL**

THP, cells were washed three times with Ham's F-10 medium to remove serum. Cells were then plated at 3×104/35×10-mm dishes (Costar) in 1 mL Ham's F-10 supplemented with gentamicin (40 μg/mL). LDL was added to the cells to give a final concentration of 0.5 mg LDL protein/mL in the absence or presence of CuCl2 (2.5 μmol/L). Incubations were then carried out for 0 to 24 hours (unless otherwise indicated) at 37°C in a humidified atmosphere containing 5% CO2. Control dishes, in which either cells or LDL was omitted, were incubated under similar conditions. At the end of the incubation period, EDTA (3 mmol/L) was added to prevent further oxidation. Oxidized and chemically modified LDLs were extensively dialyzed at 4°C against PBS containing 3 mmol/L EDTA to remove unreacted chemical reagents.

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were measured. The second part was adjusted to a density of approximately 1.050 g/mL with NaCl-KBr solution containing EDTA (3 mmol/L) and ultracentrifuged in a Ti rotor of the Beckman TL100 ultracentrifuge (Beckman Instruments) at 100 000 rpm for 3.5 hours at 10°C. LDL was recovered (as a 1-mL fraction) at the meniscus of the tube and extensively dialyzed against PBS containing 3 mmol/L EDTA. The electrophoretic mobility, protein content, and [3H]acetate hydrolase activity of this reisolated LDL were measured.

Cells were disrupted by sonication at 4°C for 15 seconds (Sonic and Materials Inc), and cell-associated acetylhydrolase activity was determined.

Assessment of LDL Modification

The time course of copper-induced oxidation of LDL (62 μg protein/mL) in PBS containing 2.5 μmol/L CuCl2 was monitored continuously for periods up to 6 hours, as conjugated diene formation, by measuring the increase in absorbance at 236 nm.3 The content of lipid aldehydes in copper- and cell-oxidized LDL was estimated before dialysis as the fluorescent products of the reaction with thiobarbituric acid as described by Buege and Aust43; the results are expressed as nanomoles of equivalent MDA per mg of LDL protein. The net electrical charge on both native and modified LDL at pH 8.6 was estimated by electrophoresis in agarose gel (Cornig).44 The electrophoretic mobility of the modified LDL was compared with the native LDL from which it was derived and expressed as the relative electrophoretic mobility (REM). Acetylhydrolase Assay

Acetylhydrolase activity was measured in preparations of both native and modified LDL, in cell lysates, and in cell supernatants (both before and after reisolation of LDL) according to Palmantier et al45 with some modifications. Samples were diluted in 490 μL of a buffer (pH 8) containing (mmol/L) HEPES 4.2, NaCl 137, KCl 2.6, and EDTA 2. After 5 minutes of preincubation at 37°C, the reaction was initiated and performed for 10 minutes at 37°C by addition of 10 μL [3H-acetyl]PAF representing 0.05 μCi and at a final concentration of 10 μmol/L (or 0.15 μCi; 100 μmol/L when mentioned). The reaction was stopped in an ice bath. Unreacted [3H-acetyl]PAF was bound to an excess of bovine serum albumin (16.7 mg/mL; final concentration for 10 minutes) and precipitated by addition of trichloroacetic acid (8% vol/vol, final concentration) as described by Miwa et al.46 The samples were centrifuged (3000 g for 20 minutes), and the [3H]acetate released into the aqueous phase was measured by liquid scintillation counting. Control assays (blanks), reflecting any nonenzymatic degradation of [3H-acetyl]PAF, were performed using the heat-denatured enzyme of human serum (100°C for 10 minutes); in the presence of 0.05 or 0.15 μCi of [3H-acetyl]PAF, less than 5% and approximately 10%, respectively, of the radiolabel was released with that liberated by native LDL. The results after subtraction of controls (blanks) are expressed as nanomoles of free [3H]acetate per 10 minutes per milligram of LDL protein unless otherwise indicated.

The specificity of acetylhydrolase toward PAF was verified in various samples, suggesting that the activity we measured was not classical Ca2+-dependent phospholipase A2. On the one hand, an excess of cold 1-O-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (40 μmol/L), the substrate for phospholipase A2, was added to the assay mixture together with [3H-acetyl]PAF (10 μmol/L), and acetylhydrolase activity was totally recovered (data not shown). On the other hand, EDTA (2 mmol/L) was replaced by CaCl2 (10 μmol/L) in the acetylhydrolase reaction buffer. Under such conditions, [3H-acetyl]PAF degradation was not modified by the presence of Ca2+ (data not shown). In addition, the presence of the serine-protease inhibitor phenylmethylsulfonyl fluoride (1 mmol/L) during LDL isolation did not diminish the activity of acetylhydrolase as opposed to the much more potent serine-protease inhibitor 4-[2-aminoethyl]benzenesulfonyl fluoride (1 mmol/L), which totally inhibited the enzymatic activity.

Kinetic Studies of Acetylhydrolase

The kinetic properties of acetylhydrolase associated with native and copper-oxidized LDL were evaluated at 37°C for 10 minutes using 1 to 100 μmol/L PAF and 0.05 to 0.15 μCi/500 μL [3H-acetyl]PAF. The Km and Vmax of acetylhydrolase were calculated using the Lineweaver-Burk representation of the data.

Statistical Analysis

Results are expressed as mean±SD. Mean values were compared by Student's t test, with significance defined at a value of P<0.05.

Results

We initially evaluated the activity of acetylhydrolase as a function of the degree of oxidation of LDL. The time course of LDL oxidation achieved at 37°C in PBS in the presence of 2.5 μmol/L CuCl2 is shown in Fig 1. In LDL preparations subjected to oxidation, the absorbance of conjugated dienes at 236 nm increased approximately fourfold between the first and third hour of incubation. The generation of aldehydes, estimated as the content of TBARS, increased rapidly, to reach a maximum between 8 and 16 hours (Fig 1A); the TBARS level decreased thereafter. The REM on agarose gel, and thus net negative charge, of oxidized LDL was compared with that of native LDL (Fig 1B) and found to be substantially elevated, attaining a plateau at 16 to 24 hours. Finally, the activity of a PAF-degrading enzyme, a specific acetylhydrolase, showed a progressive and dramatic decrease to less than 20% of control levels within 4 to 18 hours of the initiation of oxidation (Fig 1C); the rate of loss of acetylhydrolase activity as well as the time course and kinetics of the oxidation of LDL varied markedly between LDL preparations (Fig 1).

We performed several additional experiments to establish whether the oxidative mechanism was indeed responsible for the observed decrease in LDL-associated acetylhydrolase activity. Incubation of LDL for 24 hours under conditions similar to those previously described (PBS containing 2.5 μmol/L CuCl2 at 37°C) but in the presence of butylated hydroxytoluene (60 μmol/L) protected LDL from oxidation (REM=1.20±0.10) and resulted in 95±4.1% (n=3) recovery of acetylhydrolase activity compared with the initial activity. These results indirectly argue for a non-temperature- and non-time-dependent loss of LDL-associated acetylhydrolase activity during our experiments performed at 37°C for up to 48 hours. Furthermore, the addition of CuCl2 (2.5 μmol/L) directly to the acetylhydrolase assay did not modify the enzymatic activity (data not shown). From the present results, it is apparent that a strong correlation exists between the degree of LDL oxidation and the diminution in acetylhydrolase activity. Indeed, significant correlations between the reduction in acetylhydrolase activity and increase in TBARS content (r=−.98±.01, P<.001) and equally between acetylhydrolase activity and the increase in REM (r=−.94±.03, P<.001) were found. Such correlations were strengthened by the observation that one of the LDL preparations was oxidized more rapidly, and in this case alteration in the parameters of oxidation closely paralleled the decrease in acetylhydrolase activity (Fig 1).

Because acetylhydrolase activity decreased as a function of LDL oxidation in vitro, we performed similar
times up to 48 hours with 2.5 \( \mu \)mol/L CuCl\(_2\) in 0.01 mol/L phosphate-buffered saline (PBS) at pH 7.4. At the end of the incubation period, oxidation was stopped by addition of EDTA (3 mmol/L). Oxidatively modified LDL was extensively dialyzed in Ham's F-10 medium with or without added 2.5 \( \mu \)mol/L CuCl\(_2\) and in the presence or absence of THP-1. As shown in Fig 2A, the TBARS content of LDL in cell supernatants increased rapidly in the presence of cells and CuCl\(_2\), with a maximum being attained by 24 hours. In the absence of CuCl\(_2\), the TBARS content of LDL reached a plateau at 48 hours and was twofold less than that when CuCl\(_2\) was added to cells. The electrophoretic mobility of LDL was also increased and in the presence of cells and CuCl\(_2\) was maximal (threefold elevation) at 48 hours (Fig 2B). The electrophoretic mobility of LDL oxidized by cells alone was almost doubled after incubation for 48 hours and was comparable to that in the presence of cells and CuCl\(_2\) after incubation for 96 hours. The oxidation of LDL in Ham's F-10 medium in the presence of added CuCl\(_2\) showed a lag phase of 24 hours compared with our previous experiments in PBS. The acetylhydrolase activity associated with LDL that had been incubated with cells and CuCl\(_2\) decreased by approximately 80% after 24 hours (Fig 2C) and paralleled the evolution of the oxidation parameters (Fig 2A and 2B). A reduction in acetylhydrolase activity was also observed, although to a lesser extent (approximately 50% decrease after 48 hours), when LDL was incubated either with cells alone or without cells in the presence of CuCl\(_2\) (Fig 2C). The evolution of the oxidation parameters with time under these conditions always paralleled the diminution of acetylhydrolase activity (Fig 2A and 2B). Finally, the cellular content of acetylhydrolase was not affected by prolonged incubation with LDL in the presence or absence of CuCl\(_2\) (data not shown). No cellular release of acetylhydrolase was observed under any of our experimental conditions up to 96 hours of incubation.

These results suggested that the oxidation of LDL might be responsible for the observed decrease in acetylhydrolase activity. However, the generation of oxidized phospholipids (mainly oxidized phosphatidylcholine), bearing short chains at the sn-2 position of glycerol and which are hydrolyzed to form lyso-compounds,17,18,20 may interfere with the assay for acetylhydrolase, which is based on the cleavage of \([^{3}H]\)acetate from the labeled PAF. Therefore, we performed several sets of experiments using 10-fold higher concentrations of \([^{3}H]\)acetate[PAF in the assay (100 instead of 10 \( \mu \)mol/L), and under all conditions, we observed similar decreases (±10%) in acetylhydrolase activity as a function of the oxidation of LDL (data not shown). These results support the suggestion that oxidized phospholipids did not dilute the substrate. Moreover, the enzymatic activity was also measured in each sample before resolation and dialysis of LDL; the activity associated with control native LDL and the kinetics of loss of acetylhydrolase activity during oxidation were comparable in such LDL samples (data not shown). These findings show that enzyme activity is recovered after resolation of LDL and that a potential inhibitor generated during oxidation is not readily lost into aqueous solution.
TABLE 1. Kinetic Constants of Low-Density Lipoprotein-Associated Acetylhydrolase Activity

<table>
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<tr>
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<th>( K_m ), ( \mu \text{mol/L} )</th>
<th>( V_{\text{max}} ), ( (\text{nmol/min})/\text{mg LDL Protein} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native LDL ( (n=3) )</td>
<td>14.2±0.8</td>
<td>1.26±0.11</td>
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<tr>
<td>Native LDL+Ox-LDL ( (n=3) )*</td>
<td>16.2±0.9</td>
<td>0.65±0.09</td>
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<tr>
<td>Ox-LDL†</td>
<td></td>
<td></td>
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<tr>
<td>3 Hours</td>
<td></td>
<td>0.66</td>
</tr>
<tr>
<td>6 Hours</td>
<td></td>
<td>0.61</td>
</tr>
<tr>
<td>8 Hours</td>
<td></td>
<td>11.5±2.2†</td>
</tr>
<tr>
<td>12 Hours</td>
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<td>0.45</td>
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LDL indicates low-density lipoprotein; Ox-LDL, oxidatively modified LDL. LDL preparations were incubated at 37°C for 10 minutes with defined concentrations of \( ^3H \)-acetylPAF (1 to 100 \( \mu \text{mol/L} \), 0.05 to 0.15 \( \mu \text{Ci} \)). \( K_m \) and \( V_{\text{max}} \) of acetylhydrolase were calculated from the Lineweaver-Burk representation of the data and are the means±SD of three experiments (unless otherwise indicated), each with LDL from a different donor.

*Fully oxidized LDL (undetectable acetylhydrolase activity) added as twofold excess. \( V_{\text{max}} \) was calculated with respect to the native LDL protein content.

†Values from one representative experiment of two, each with LDL from a different donor.

‡Mean±SD of the four \( K_m \) values of the experiment.

Experiments were then designed to provide more insight into the mechanisms of the reduction of acetylhydrolase activity induced by oxidation. Fully oxidized LDL, totally lacking acetylhydrolase activity, was mixed with native LDL, and kinetic studies were performed. The Michaelis-Menten kinetics were determined, and \( K_m \) and \( V_{\text{max}} \) values were calculated using the Lineweaver-Burk representation. We observed that the acetylhydrolase activity associated with native LDL possessed the same \( K_m \) in the presence or absence of Ox-LDL; in contrast, the \( V_{\text{max}} \) of the enzymatic activity decreased twofold in the presence of Ox-LDL (Table 1).

In the latter case, some exchange of enzyme and/or inhibitor probably occurs in both directions between native LDL and Ox-LDL, and such exchange may be responsible for the decrease in the \( V_{\text{max}} \) of acetylhydrolase.

Furthermore, we performed kinetic experiments using preparations of LDL that had undergone mild oxidation at 37°C in PBS containing CuCl2 (2.5 \( \mu \text{mol/L} \)) under the same conditions as previously described. The \( K_m \) of acetylhydrolase in Ox-LDL remained essentially unchanged compared with that of acetylhydrolase associated with native LDL, whereas the \( V_{\text{max}} \) decreased as a function of oxidation (decreasing by approximately 60% after 8 hours of oxidation). These results are in favor of the generation of a noncompetitive inhibitor of acetylhydrolase during the oxidative process and argue against substrate dilution by oxidized phospholipids.

We subsequently evaluated the nature of the non-competitive inhibition of LDL-associated acetylhydrolase activity. First, we examined the effects of two oxysterols, 7-ketocholesterol and cholesterol-5α,6α-epoxide, which are quantitatively significant components of oxidized LDL.46 Native LDL was incubated at 37°C for 20 minutes with each of the two oxysterols and acetylhydrolase activity measured. The total enzymatic activity of the control native LDL (150 \( \mu \text{g protein} \) was recovered for each concentration of oxysterol studied from 1 to 100 \( \mu \text{mol/L} \) (data not shown); the oxysterol concentrations were chosen in relation to their content in oxidized LDL.46

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As was already described, 15\(^\pm\)16 However, in LDL incubated with MDA under controlled conditions and subsequent chemical modifications of LDL, low-density lipoprotein (LDL) modified with 4-hydroxynonenal (4-HNE) led to a dramatic loss of acetyhydrolase activity associated with Ox-LDL; the progressive loss of enzymatic activity always paralleled the evolution of the oxidation parameters, which involved an increase in both the net negative electrical charge of LDL and TBARS content.

Second, we conducted three distinct chemical modifications of LDL under controlled conditions and subsequently measured LDL-associated acetyhydrolase activity. Modification of LDL was monitored by the increased electrophoretic mobility and the enhanced TBARS content when modification was performed with MDA. In Table 2, we show that neither acetylation nor modification by MDA affected LDL-associated acetyhydrolase activity, which was totally recovered. In contrast, modification by 4-HNE led to a dramatic loss of acetyhydrolase activity, which was more marked in the absence of the reducing agent NaCNBH\(_3\). The loss of acetyhydrolase activity was always proportional to the extent of 4-HNE modification of LDL, as shown in Fig 3. Indeed, when LDL was incubated for 5 hours with increasing concentrations of 4-HNE, the increment in REM was correlated (r=−.93, P≤.0001) with the diminution of acetyhydrolase activity. In the presence of 10 mmol/L 4-HNE, a decrease in enzymatic activity was detected at 1 hour of incubation; after 5 hours, only approximately 40\% of the initial activity remained. The aggregation of 4-HNE-LDL particles was visually observed when the REM of 4-HNE-LDL attained a value of approximately 2, as was already described.15\(^\pm\)16 However, in LDL incubated with 5 mmol/L 4-HNE for 5 hours, acetyhydrolase activity had already decreased by 35\% in the absence of lipoprotein aggregates. Several control experiments were performed to ensure the specificity of the loss of LDL-associated acetyhydrolase activity by 4-HNE. We verified that the addition of 4-HNE and/or NaCNBH\(_3\) for 20 minutes at 37°C to native LDL did not affect the enzymatic activity. NaCNBH\(_3\) added alone to the assay protected the activity of acetyhydrolase to a minor degree (approximately 15\%).

**Discussion**

LDL-associated acetyhydrolase is the major enzyme that regulates the level of circulating PAF.29 Because the oxidative modification of LDL renders this particle atherogenic (reviewed in Reference 1), we explored the fate of LDL-associated acetyhydrolase under oxidative conditions—either in a cell-free system containing copper ions or using a cellular model of human monocyte-like cells, THP\(_1\). Using both systems, we observed a dramatic decrease in the activity of the acetyhydrolase associated with Ox-LDL; the progressive loss of enzymatic activity always paralleled the evolution of the oxidation parameters, which involved an increase in both the net negative electrical charge of LDL and TBARS content.

The \(K_a\) of LDL-associated acetyhydrolase for PAF remained constant during oxidation and corresponded to that found earlier for both native LDL28 and the enzyme purified from LDL.23 From our kinetic studies, it is apparent that the acetyhydrolase conserves its affinity for PAF during oxidation of LDL; in contrast, its \(V_{\text{max}}\) decreased progressively. Because the acetyhydrolase in our preparations was not purified, the decrease in the \(V_{\text{max}}\) reflects the decrease in the amount of active enzyme present in oxidized LDL. These results argue against the possibility of a competitive inhibition of acetyhydrolase by an unidentified oxidized phospho-
lipid in the assay for acetylhydrolase. This question is of some importance, because it has been shown that the acetylhydrolase may hydrolyze several oxidized phospholipids that might dilute the radioactive substrate in the assay. It has been shown that acetylhydrolase may hydrolyze a purified, oxidized derivative of phosphatidylcholine (with a short chain in the sn-2 position) with the same affinity as that for PAF but with a $V_{\text{max}}$ that is approximately 60% lower.20 Steinbrecher and Pritchard17 found that a small fraction of the numerous oxidation products of LDL inhibits the hydrolysis of PAF by acetylhydrolase associated with this lipoprotein. Others18 have suggested that the phospholipase A$_2$ activity that hydrolyzes the oxidized phosphatidylcholine in LDL is distinct from acetylhydrolase and may be associated with apoB-100 of LDL. The respective contributions of phospholipase A$_2$ and acetylhydrolase activity to the hydrolysis of oxidized phospholipids to lysophospholipids during LDL oxidation are not clearly defined yet.

The chemical modification of amino acid residues, mainly lysine, in LDL—apoB-100 by acetylation and MDA did not significantly alter acetylhydrolase activity associated with LDL. By contrast, modification of LDL by 4-HNE led to a marked and progressive decrease in acetylhydrolase activity. The 4-HNE modification of LDL primarily modifies the protein moiety of LDL and to a lesser extent its lipid moiety.15 Furthermore, aggregation of 4-HNE—LDL was also observed.15,16 In our study, however, reduction in acetylhydrolase activity was probably independent of such aggregation. Modification of LDL with 4-HNE is particularly severe and cytotoxic compared with that by MDA.14,15,16 However, at this stage we cannot distinguish between the respective role of 4-HNE modification of protein, phospholipid, or both in the loss of acetylhydrolase activity. We assume that in our experiments the number of 4-HNE amino acid adducts in 4-HNE—LDL and in fully oxidized LDL in the presence of copper ions (incubation for approximately 24 hours) is similar, according to Chen et al.47 Indeed, antibodies raised against the 4-HNE amino acid adducts react to the same extent with Ox-LDL as with 4-HNE—LDL.14 Thus, it appears probable that the time-dependent formation of 4-HNE amino acid adducts may be involved in the progressive decrease in acetylhydrolase activity in Ox-LDL. As the acetylhydrolase has not yet been cloned, the question remains as to whether the modifications of lysine, tyrosine, or histidine residues at the active site of the enzyme may be implicated in its progressive loss of activity.

The activity of several enzymes, such as those of the phospholipase A$_2$ family, is sensitive to the nature of the phospholipid environment,18 and acetylhydrolase may display such a property. Variations in the $K_m$ and $V_{\text{max}}$ values of acetylhydrolase found for LDL, very low-density and high-density lipoproteins, and lipoprotein[a] may be explained by the existence of distinct environments of the enzyme in the surface of these particles25,27,28; our results are consistent with this hypothesis.

Parthasarathy and Barnett18 showed that the activity of LDL-associated phospholipase A$_2$, which hydrolyzes oxidized phospholipids, diminishes dramatically during copper- and endothelial cell—mediated oxidation and during photooxidation (total inactivation after 90 minutes). In contrast, under the same conditions of photooxidation, LDL-associated acetylhydrolase activity decreased by only 25%. The authors suggest that whereas modification and fragmentation of apoB-100, and particularly alteration of histidine, are involved in phospholipase A$_2$ inactivation, they are not responsible for the onset of the loss of acetylhydrolase activity. The active site of acetylhydrolase is therefore either protected or composed of amino acids that are less sensitive to such modifications. In contrast to phospholipase A$_2$, which is insensitive to serine-protease inhibitors,29 we have totally inhibited acetylhydrolase with 4-[2-aminoethyl]-benzenesulfonyl fluoride (see "Methods"). The latter compound did not interfere with the oxidation of LDL (results not shown).

The loss of acetylhydrolase activity in Ox-LDL may be relevant to the development of the atheromatous lesion. Indeed, PAF is probably synthesized and released by cells present in the plaque and may exert proatherogenic effects in the microenvironment of the arterial intima. Thus, the loss of acetylhydrolase activity, the enzyme that may regulate the level of PAF,29 may enhance the biologic effects of this potent inflammatory mediator. In blood plasma, besides the phospholipase A$_2$ activity intrinsic to apoB-100, intact LDL transports several proteins, such as acetylhydrolase and tissue factor pathway inhibitor, which confer it with both anti-inflammatory and antithrombotic properties. Thus, the decrease in acetylhydrolase activity, as shown in this study, is a potentially important feature of LDL oxidation, which may contribute to the diminution of the local protective role of LDL in microenvironments of the arterial intima.

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References


38. Stewart AG, Dubbin PN, Harris T, Dusting GJ. Platelet-activating factor may act as a second messenger in the release of iocoasodan and superoxide anions from leukocytes and endothelial cells. *Proc Natl Acad Sci USA.* 1990;87:3215-3219.


PAF-acether-degrading acetylhydrolase in plasma LDL is inactivated by copper- and cell-mediated oxidation.
C Dentan, P Lesnik, M J Chapman and E Ninio

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