Modified Phosphatidylethanolamine as the Active Component of Oxidized Low Density Lipoprotein Promoting Platelet Prothrombinase Activity*

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We analyzed the influence of the atherogenic oxidized low density lipoproteins (LDL) on the activity of the platelet prothrombinase complex, a major contributor to overall thrombin formation in vivo. Platelet dependent thrombin generation was found to be strongly stimulated by in vitro oxidized LDL. The enhancement was additive to that observed with the platelet agonist thrombin. Oxidized LDL increased the platelet binding of annexin-V, suggesting that the augmented surface exposure of aminophospholipids promoted the prothrombinase activity. All of the stimulatory activity of the oxidized LDL could be recovered in the microemulsions prepared from the lipid portion of the modified particles. Phospholipid vesicles were prepared containing the total lipids of the oxidized LDL but lacking specifically in one lipid component. Following the selective removal of the ethanolamine phospholipids (PE) from the LDL lipids, the platelet-dependent thrombin formation was markedly reduced. Vesicles enriched with the isolated PE fraction alone enhanced the thrombin generation. Analyses with autoxidized phospholipids indicated that oxidation products of unsaturated diacyl-PE were mainly responsible for the increased prothrombinase activity. Oxidized LDL and its PE fraction lost their stimulatory activity after treatment with NaCNBH₃, a chemical reductant of Schiff base adducts. Phospholipid vesicles supplemented with synthetic aldehyde-PE adducts largely reproduced the stimulation of the thrombin generation. We conclude that the oxidized LDL particles elicit a pronounced prothrombotic response by increasing the activity of the platelet prothrombinase complex. Specific oxidative modifications of the LDL-associated ethanolamine phospholipids are mainly responsible for this stimulation.

The protease thrombin is known to play a central role in physiologic hemostasis. Thrombin catalyzes the formation of fibrin from fibrinogen and amplifies the coagulation process through the proteolytic activation of factors V, VIII, and XI. By means of the proteolysis of specific cell membrane receptors, thrombin activates platelets and regulates multiple cellular processes such as proliferation and chemotaxis (1, 2). The rapid formation of thrombin from prothrombin is thought to be mediated mainly by the prothrombinase complex assembled on the outer leaflet of the plasma membrane of the activated platelets. There is general agreement that under in vivo conditions this complex mediates the large scale synthesis of thrombin. The assembly of factors Va and Xa on the platelet surface is most probably triggered initially by the translocation of aminophospholipids such as phosphatidylserine (PS) from the inner to the outer leaflet of the cell membrane (3). The appearance of specific phospholipids on the platelet surface facilitates the interaction of factor Va with the platelet cell membrane. This, in turn, enables the binding of factors Xa and II (prothrombin), thereby allowing the proteolysis of prothrombin to thrombin as catalyzed by factor Xa.

High local concentrations of thrombin within and around the atherosclerotic plaque (4) suggest that the protease may also be of importance for the development of thrombosis. Because arterial thrombosis is the major single cause of mortality in industrialized countries, the elucidation of the mechanisms causing the increased formation of thrombin is of particular interest. According to recent evidence, coronary thrombosis leading to myocardial infarction is in most cases induced by a thrombus that develops following the rupture of a lipid-rich, unstable atherosclerotic plaque (5). The nature of the lipidic material present in the unstable plaque is apparently of great relevance for the extent of pathological activation of coagulation. Among other components, the plaques are enriched with oxidized low density lipoproteins (LDL). Substantial evidence obtained over the last decade suggests a causal role for oxidized LDL in the development of atherosclerosis (6). Recent data, moreover, support the view that oxidized LDL may also directly promote thrombogenesis. This hypothesis is based on results demonstrating that oxidized LDL are able to promote the initiation of coagulation by enhancing the expression of tissue factor (7, 8) and to mitigate anticoagulant mechanisms such as thrombomodulin-dependent protein C activation (9) and the expression of tissue factor pathway inhibitor (10). In the present study, we investigated the influence of oxidized LDL on the activity of the platelet prothrombinase complex. The modified lipoproteins were observed to increase thrombin formation strongly, suggesting a relevant role for oxidized LDL in the development of thrombosis. A series of separation procedures led to the identification of oxidized phosphatidylethanolamine (PE) as the lipoprotein component mediating the strongest stimulation of the platelet thrombin generation.

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1 The abbreviations used are: PS, phosphatidylserine; LDL, low density lipoproteins; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; HNE, 4-hydroxynonenal; AAPH, 2,2′-azobis-(2-amidinopropane hydrochloride); BHT, butylated hydroxytoluene; NaCNBH₃, sodium cyanoborohydride.
Experimental Procedures

Materials—Egg phosphatidylethanolamine (PC), 1-palmitoyl,2-palmitoyl-PC (dipalmitoyl-PC), 1-palmitoyl,2-linoleoyl-PC, egg PE, 1-palmitoyl,2-linoleoyl-PE, 1-palmitoyl,2-lyso-PC (lyso-PE), 1-palmitoyl,2-linoleoyl-phosphatidyl ethanolositol (PS), sphingomyelin (from bovine brain), 1-palmitoyl,2-lyso-lyso-PC (lyso-PC), sphingomyelin (bovine brain), copper acetate, a-thrombin, collagen (type VIII), myeloperoxidase, diethylenetriaminepentaacetic acid, NaCNBH3, and factor Xa were obtained from Sigma. Fluorescein isothiocyanate-labeled annexin V was purchased from Alexis, and diethylpyridyl-labeled anti-P-selectin antibody was obtained from ImmunoTech. 2,2'-Azobis(2-aminopropane) hydrochloride (AAPH) was provided by Polysciences Inc. Factor Va and factor II were from American Diagnostica, and factor S-2238 was obtained from Chromogenix. Plasmalogen-PE was isolated from total bovine brain PE by alkaline methanolysis as described (11).

Lipoprotein Isolation and Oxidation—LDL was prepared from healthy donors by ultracentrifugation (12), dialyzed against argon-bubbled phosphate-buffered saline containing 0.3 mM EDTA and stored under argon at 4 °C. The amount of LDL-associated protein was determined using the Bradford procedure (13). Before the start of oxidation, LDL was freshly dialyzed against argon-bubbled phosphate-buffered saline. To oxidize the lipoproteins with copper and peroxyl radicals, the particles (0.2 mg protein/ml) were treated for different time intervals at 37 °C with 10 μM copper acetate and for 4 h with 4 mM AAPH, respectively. Oxidation was terminated by the addition of butylated hydroxytoluene (BHT, 20 μM). Myeloperoxidase-mediated LDL oxidation was performed according to a previously published protocol (14). LDL (0.5 mg protein/ml) was treated for 1 h at 37 °C with 100 nm myeloperoxidase in the presence of 100 μM l-tyrosine, 100 μM diethylenetriaminepentaacetic acid, 1 mM H2O2 suspended in 100 mM NaCl, and 20 mM Na2HPO4. After the addition of BHT, the suspensions were passed through centrifugal filters, and the lipoproteins were recovered.

Preparation of Microemulsions and Lipid Phases—Following the oxidation of the lipoproteins, the particles (50 μg of protein) were divided into aqueous and lipid phases according to the Bligh and Dyer procedure (15). The upper two-thirds of aqueous fractions (containing less than 10% of the total protein contents of the whole LDL particles) was tested directly for its effects on platelet procoagulant activity. The organic phase was evaporated under N2 and dispersed as a microemulsion by three sonication steps (3 min each, with 1-min breaks in between) at 4 °C under a constant stream of N2. Thereafter, the microemulsions were incubated with the platelets for analysis of the thrombin formation. To prepare the lipid mixtures from oxidized LDL deficient in specific lipid components, the lipid phases (from 1 mg LDL) were first resuspended in 500 μl of CHCl3/CH2OH (2:1). Then, 10 identical portions of the suspensions were applied in parallel onto Silica plates using CHCl3/CH3OH (1:4). Thereafter, the lipids were further separated by two-dimensional tlc using the solvents CHCl3/CH3OH/(NH3/H2O (90:54:5.5:5.5; vol:vol). Therefore, only a single spot became visible that could be stained with the amino group reagent ninhydrine. Fraction IV of the oxidized LDL therefore predominantly contained modified ethanolamine phospholipids.

Enrichment of LDL with Phospholipids—Fresh venous blood obtained from healthy donors was drawn into tubes containing EDTA (1 mg/ml) and plasma was prepared by centrifugation. For the enrichment of LDL with ethanolamine phospholipids, 1 μmol of either 1-palmitoyl,2-linoleoyl-PS or plasmalogen-PE (from bovine brain) was dissolved in 1 µl of ethylene glycol and added to the control samples. For the preparation of the control samples, 4 µmol of egg PC was dissolved in the same volume of ethanol. The solutions were added very slowly under stirring to 10 ml of plasma, and the suspensions were incubated at 37 °C under argon for 6 h. The LDL particles were isolated by ultracentrifugation and dialyzed against phosphate-buffered saline under argon at 4°C. Synthesis of Aldehyde-PE Adducts—Synthetic HNE-PE adducts were prepared by adding 1 mol of 1-palmitoyl,2-linoleoyl-PE (dissolved in 1 ml of diethylether) to 2 mol of HNE suspended in 1 ml of 0.75 M NaCl and 1 mM Hepes (pH 8.5) (17). The mixture was incubated under vigorous shaking for 2 h in the dark in the presence of argon (30 °C). Thereafter, the lipids of the ether phase were separated by one-dimensional tlc using the solvents CHCl3/CH2OH/ CH2COOH (65:25:10) (first direction) and CHCl3/CH2OH/HCOOH/H2O (65:25.8:9.1:1) (second direction). Therefore, only a single spot became visible that could be stained with the amino group reagent ninhydrine. Fraction IV of the oxidized LDL therefore predominantly contained modified ethanolamine phospholipids.
Platelet prothrombinase activity is stimulated by oxidized LDL. a, platelets (2 × 10^7/ml) were incubated for 5 min at 37 °C with native LDL (nLDL) and oxidized LDL (oxLDL) (both at 50 μg protein/ml). Oxidation of the particles was performed by a 4-h incubation with copper (10 μM). Platelets were also stimulated with thrombin (T, 0.5 units/ml) and collagen (C, 10 μg/ml). The results are expressed as fold increase versus the prothrombinase activity of untreated platelets. b, LDL was oxidized for the indicated time intervals with 10 μM copper and the prothrombinase activity analyzed in the absence (open symbols) and presence (filled symbols) of thrombin. The mean of 4–12 independent experiments is shown.

RESULTS

Enhancement of Platelet Prothrombinase Activity by Oxidized LDL—In a first series of experiments, native LDL as well as lipoprotein particles previously oxidized for 4 h with copper were added to the platelet suspensions. Although native LDL did not affect the platelet dependent thrombin formation, the copper oxidized LDL enhanced the synthesis of the protease by 5.5-fold (Fig. 1a). When platelets had been incubated for 5 min with copper (10 μM) in the absence of the lipoproteins, their prothrombinase activity remained unchanged (1.2 ± 0.4-fold versus control). Moreover, when the 4-h incubation of LDL with copper was performed in the presence of the hydrophilic radical scavenger BHT (50 μg/ml), again no stimulation of the platelet-dependent thrombin formation was observed (1.0 ± 0.2-fold versus control; means ± S.D. from three independent experiments). Taken together, these results make it unlikely that soluble or lipoprotein bound copper per se contributed to the enhancement of the prothrombinase activity by copper oxidized LDL. The platelet agonists thrombin and collagen, known stimulators of platelet prothrombinase activity, augmented the thrombin formation by 3.6 (thrombin)- and 6.0-fold (thrombin plus collagen) (Fig. 1a). In separate experiments, the particles were oxidized for 4 h with the peroxyl radical generator AAPH (4 mM). Therefore, thrombin generation was stimulated by 4.6 ± 1.2-fold (means ± S.D. from four independent experiments). LDL oxidized for 2, 4, and 12 h with copper elevated platelet prothrombinase activity by 3.1-, 5.7-, and 4.6-fold, respectively, as compared with the untreated platelets (Fig. 1b). In the presence of thrombin, the LDL particles previously treated for different time periods with copper accelerated the prothrombinase activity by 7.4 (oxidation, 2 h)-, 10.5 (4 h)-, and 10.9-fold (12 h), respectively. Because the 4-h oxidation of LDL appeared to be the shortest time period inducing the maximal stimulation of the thrombin formation, this time interval was adopted in the subsequent experiments.

The prothrombinase activity in the absence of platelets was barely promoted by the copper oxidized LDL (0.012 ± 0.004 (oxidized LDL) versus 0.006 ± 0.003 milliunits/ml (control)), the value being below the one determined in the presence of the untreated platelets (0.028 ± 0.007 milliunits/ml, means ± S.D. from three independent experiments). Thus, the presence of the platelets was necessary to elicit substantial stimulation of the thrombin formation by oxidized LDL. Activated platelets are known to enhance the prothrombinase activity by exposing aminophospholipids on their surface. To register the appearance of the aminophospholipids, we analyzed the platelet binding of annexin-V (Fig. 2a). Native LDL did not alter the aminophospholipid exposure. In contrast, oxidized LDL caused a
The modified LDL, we further separated the modified lipids into five different fractions by a one-dimensional TLC procedure. The lipids of the lanes were reisolated in a way that only one respectively different fraction was left on the plate. Thereafter, the fractions were mixed with dipalmitoyl-PC and tested for their influence on the prothrombinase activity. Oxidized LDL lipids deficient in fraction I and those lacking fraction II increased the prothrombinase activity by 13 and 30%, respectively, as compared with the thrombin formation in the presence of all modified lipids (Fig. 3b). The removal of fraction III resulted in a 24% decrease of the thrombin generation. An even stronger reduction (by 44%) was observed in the presence of the modified lipid mixture specifically deficient in fraction IV. When fraction V was selectively removed, the thrombin formation was comparable with the one determined in the presence of the total oxidized LDL lipids (Fig. 3b). We concluded from these results that the active components of the oxidized LDL mediating the stimulation of the thrombin generation were present mainly in fractions IV and III.

Next, we quantified the changes in the contents of the single lipid fractions as induced by oxidation of LDL. Because control experiments indicated that fractions I–IV contained native and modified phospholipids (see “Experimental Procedures”), their phosphate contents were determined. In Table I, the phosphate contents of the single fractions are expressed as percentages of the total phosphate content of the LDL lipids. Although the percentages of fraction I were elevated by 3.4-fold, those of fraction II were unchanged at the end of the oxidation period (Table I). The proportion of fraction III was reduced by 23%. In addition, the oxidation procedure elicited a 45% decrease in the percentage of fraction IV (Table I). Because the modified lipids of fractions III and IV emerged as likely candidates for causing the enhancement of the thrombin formation by oxidized LDL, we isolated these fractions from the modified lipoproteins and incorporated them into dipalmitoyl-PC vesicles. As a control, vesicles containing all oxidized LDL lipids were analyzed, which accelerated the prothrombinase activity by 7.1-fold (Fig. 4a). Vesicles supplemented with fraction III of the oxidized LDL led to a 2.1-fold elevation of the prothrombinase activity. In the presence of vesicles enriched with fraction IV of the oxidized LDL, the thrombin formation was increased by 5.2-fold (Fig. 4a). Together, the results demonstrated that fraction IV and, to a considerably lesser extent, fraction III were the active components of the oxidized LDL enhancing the formation of thrombin.

To learn more about the identity of the modified lipids present in fraction IV, vesicles supplemented with specific phospholipids were oxidized and the oxidation products separated by TLC (see “Experimental Procedures”). Part of the products generated by oxidation of egg PE migrated at the same height as fraction IV. Moreover, fraction IV of the oxidized LDL stained...
Modified PE of Oxidized LDL Stimulates Thrombin Formation

To analyze in more detail the role of the phospholipids for the stimulation of the thrombin generation, we oxidized lipoproteins containing higher contents of plasmalogen-PE and mixed with dipalmitoyl-PC (100 nmol). Subsequently, they were dispersed in the resuspension buffer and tested for their effects on platelet prothrombinase activity.

Lipoproteins previously enriched with egg PC alone (4 nmol, PC). Subsequently, the modified lipoproteins were oxidized for 4 h with copper (10 μM). Thereby the LDL concentrations of diacyl-PE were reduced by 33% (control), 31% (DPE-enriched LDL), and 15% (PPE-enriched LDL). The same pro-oxidant lowered the contents of plasmalogen-PE by 73% (control), 66% (DPE-enriched LDL), and 15% (PPE-enriched LDL). The oxidized LDL particles were then added to the platelets for the measurement of prothrombinase activity. The mean of 3–5 independent experiments is shown.

Aldehydes generated by the oxidation of unsaturated fatty acids are known to react with the free amino group of the PE head group yielding adducts such as Schiff bases (17). To analyze whether Schiff bases contributed to the stimulation of the prothrombinase activity, we oxidized lipid vesicles consisting of defined species of the phospholipids. Copper oxidation of vesicles consisting of the 1-palmitoyl,2-linoleoyl-(diacyl)-PE species of PC led to a 1.7-fold increase of the thrombin generation (Fig. 5b).

Lipoproteins made from dipalmitoyl-PC did not alter the thrombin formation. Following the oxidation of PC vesicles supplemented with 5 mol % of plasmalogen-PE (from bovine brain) and with 5 mol % of the 1-palmitoyl,2-linoleoyl-PE enhanced the prothrombinase activity by 3.1- and 5.9-fold, respectively. Native PC/diacyl-PE vesicles that had been incubated in the absence of copper did not stimulate the thrombin formation. As expected, oxidized vesicles made from dipalmitoyl-PC did not alter the thrombin formation by the platelets (Fig. 5b). However, oxidation of dipalmitoyl-PC vesicles (95 mol %) supplemented with 5 mol % of the 1-palmitoyl,2-linoleoyl species of PI and PE resulted in a 2.6- and 5.6-fold-enhancement of the prothrombinase activity. Concomitant activation of the platelets with thrombin additionally enhanced the effect of the oxidized diacyl-PE and -PI (Fig. 5b).

The results indicated that oxidized unsaturated diacyl-PE elicits the most effective stimulation of the platelet prothrombinase activity. Both the ethanolamine head group and the unsaturated fatty acid at the C-2 atom were required for the stimulation induced by the ethanolamine phospholipid.

Fig. 5. Oxidation of unsaturated diacyl-PE promotes thrombin formation. a, 95 mol % of 1-palmitoyl,2-linoleoyl-(diacyl)-PC was mixed with 5 mol % of 1-palmitoyl,2-linoleoyl species of PC, PI, and PE (DPE), as well as with plasmalogen-PE (PPE, from bovine brain) and 1-palmitoyl,2-lyso-PE (LPE). The vesicles prepared from the mixtures were oxidized for 10–12 h at 37 °C with 10 μM copper and the oxidized vesicles were added to the platelet suspensions for the analysis of the prothrombinase activity. Oxidation of vesicles; a, untreated vesicles, b, vesicles were prepared from mixtures of 95 mol % dipalmitoyl-PC and 5 mol % 1-palmitoyl,2-linoleoyl species of PI and PE (DPE). After oxidation with copper, the vesicles were tested for their influence on platelet-dependent thrombin formation. Light grey columns, absence of thrombin; dark grey columns, presence of thrombin. 

Aldehydes generated by the oxidation of unsaturated fatty acids are known to react with the free amino group of the PE head group yielding adducts such as Schiff bases (17). To analyze whether Schiff bases contributed to the stimulation of the prothrombinase activity, fraction IV containing the oxidized PE was isolated from the modified LDL and enriched in dipalmitoyl-PC vesicles containing the ethanolamine phospholipids. Thereby the LDL concentrations of diacyl-PE were reduced by 33% (control), 31% (DPE-enriched LDL), and 15% (PPE-enriched LDL). The same pro-oxidant lowered the contents of plasmalogen-PE by 73% (control), 66% (DPE-enriched LDL), and 15% (PPE-enriched LDL). The oxidized LDL particles were then added to the platelets for the measurement of prothrombinase activity. The mean of 3–5 independent experiments is shown.
activity elicited by the vesicles was reversed by 82% following altered by NaCNBH3. In further experiments, whole oxidized
vented by NaCNBH3. The mean of 4–6 independent experiments is
diagonally striped column additional presence of NaCNBH3.

FIG. 6. Prothrombinase activation by oxidized LDL is pre-
vented by NaCNBH3. a, LDL particles (0.2 mg protein/ml) were oxi-
dized for 4 h with copper (10 μM), and the suspensions were treated
subsequently for 2 h with NaCNBH3 (18 mM) or vehicle. Fraction IV
containing the modified ethanolamine phospholipids was isolated from
the lipoproteins, enriched in dipalmitoyl-PC vesicles, and analyzed for
its influence on platelet prothrombinase activity. In further experi-
ments, platelets (2 × 10^7/ml) were stimulated with thrombin (0.5 units/
ml) in the absence or presence of NaCNBH3 (18 mM). Gray columns,
absence of NaCNBH3; diagonally striped columns, presence of NaC-
BNH3. b, copper-oxidized LDL was treated for 2 h with NaCNBH3 (18
mM) or vehicle and thereafter added to the platelets for the analysis of
the thrombin generation. Gray columns, presence of oxidized LDL;
diagonally striped column, additional presence of NaCNBH3. c, the
enhancement of platelet-annexin-V binding by oxidized LDL is reversed
by NaCNBH3. Gray columns, untreated platelets (co) and platelets
treated with oxidized LDL (as indicated; diagonally striped column,
presence of NaCNBH3. The mean of 4–6 independent experiments is
shown.

oxidized LDL treated with NaCNBH3. The reducing agent sup-
presed by 88% the stimulation of the aminophospholipid ex-
posure by the modified LDL. To further substantiate the role of
the PE adducts for the stimulation of the prothrombinase activi-
thy, synthetic adducts were prepared from egg PE and HNE, a
major lipid peroxidation product of unsaturated fatty acids
accumulating in the oxidized particles (21). The synthetic PE
adducts, which had been enriched in dipalmitoyl-PC vesicles,
enhanced thrombin formation by 3.3-fold (Table II). Treatment
of the synthetic adducts with NaCNBH3 prevented stimulation
of the prothrombinase activity by 87% suggesting that Schiff
bases were responsible for the increased generation of throm-
bin. To investigate whether the presence of the fatty acid at sn2
of the PE-HNE adduct was essential for the enhancement of the
thrombin generation, we synthesized adducts between
1-palmitoyl,2-lyso-PE and HNE. The lyso-PE-HNE adducts in-
creased the prothrombinase activity by 3.0-fold, the activation
being lowered by 90% subsequent to the treatment with
NaCNBH3. The results suggested that the imino group be-
tween the ethanolamine head group and the aldehyde was the
major determinant for the stimulation of the prothrombinase
activity (see the Discussion).

Oxidation of LDL with myeloperoxidase in the presence
of tyrosine and H2O2 yields specific aldehydes that react with PE
to form Schiff bases similar to those occurring in vivo
in the atherosclerotic vessel wall (14). LDL was oxidized with myel-
operoxidase (see “Experimental Procedures”), and the spot
migrating at the same height as fraction IV (of the copper-
oxidized LDL) was isolated. Subsequently, the modified lipid
fraction was enriched in dipalmitoyl-PC vesicles and treated
for 2 h with NaCNBH3 or vehicle. The vesicles incubated with
the buffer alone enhanced the prothrombinase activity by 5.9 ±
2.4-fold. Following treatment of the vesicles containing the
lipids modified with NaCNBH3 (18 mM, 2 h incubation), the
stimulatory effect was completely abolished (0.8 ± 0.4-fold
versus untreated platelets, means ± S.D. of triplicate deter-
nations from two independent experiments). Accordingly, also
the enhancement of prothrombinase activity by myeloperoxid-
ase-treated LDL is likely to be caused by the PE-aldehyde
adduct.

DISCUSSION

The importance of thrombin in the physiologic and patho-
physiologic activation of coagulation led us to analyze whether
oxidized LDL, a proatherogenic agent, affected the synthesis of
the protease. We observed a pronounced stimulation of platelet-
dependent thrombin formation by oxidized LDL, which was
nearly equally as potent as the one elicited by the strong
platelet agonists thrombin plus collagen. Oxidized LDL in-
creased the prothrombinase activity by enhancing the exposure
of aminophospholipids on the platelet surface. To characterize
the active components within the oxidized LDL, the particles
were separated into aqueous and lipid phases. Although the lipid phases increased the prothrombinase activity to an extent similar to that in total oxidized LDL, no stimulatory activity was found when the platelets were exposed to the aqueous phases. From these results the possibility cannot be excluded that the modified protein components of the oxidized LDL might have contributed to the promotion of the thrombin formation. The strong effect elicited by the lipid phases led us to separate them into different oxidized lipid fractions. Although several fractions were found to increase platelet-prothrombinase activity, the most active component was detected in the portion of the oxidized lipoproteins containing modified ethanolamine phospholipids. We therefore decided to analyze in more detail the oxidative modification of the ethanolamine phospholipids mediating the procoagulant response of the platelets.

Under in vivo conditions, the platelets are continuously exposed to PE present in the plasma lipoproteins. The LDL-associated PE is known to consist mainly of the plasmalogen and diacyl subgroups, both types of ethanolamine phospholipids being enriched particularly with unsaturated fatty acids (22). To evaluate the contributions of oxidation products of the different PE subtypes for enhanced thrombin formation, we oxidized phospholipid vesicles and LDL enriched with unsaturated species of diacyl- and plasmalogen-PE. Oxidation of the diacyl-PE-supplemented lipid carriers resulted in a more pronounced acceleration of thrombin formation as compared with the oxidative modification of the particles enriched with plasmalogen-PE. The presence of the plasmalogen-specific enol ether thus prevented to some extent the generation of the oxidation product promoting thrombin formation. The enol ether has been shown previously to attenuate the oxidative degradation of unsaturated fatty acids as induced by different oxidants (11, 23–25). Oxidation of vesicles containing unsaturated PC and PI resulted in a weaker stimulation of the prothrombinase activity as compared with oxidation of vesicles with unsaturated diacyl-PE. Moreover, oxidized lyso-PE barely enhanced the prothrombinase activity. Together, the results demonstrate that only in the presence of both the ethanolamine head group and unsaturated fatty acids are oxidation products generated that are capable of activating the thrombin formation.

Because the ethanolamine head group per se is insensitive toward oxidation, whereas, in contrast, unsaturated fatty acids are easily degraded, the latter components will be decomposed first by the oxidative attack. Oxidation of the unsaturated fatty acids results in the formation of aldehydes, which are known to react with the free amino groups of lysine residues and of phospholipids. Thereby, several types of adducts are generated including Schiff bases. When we treated the modified ethanolamine phospholipids isolated from the oxidized LDL with NaCNBH₃, a specific reductant for Schiff bases, the stimulatory effect of the oxidized phospholipids on prothrombinase activity was lost. Furthermore, NaCNBH₃ inhibited the exposure of the aminophospholipids on the platelet surface as promoted by the oxidized LDL. The reducing agent also prevented the enhancement of the prothrombinase activity elicited by the synthetic aldehyde-PE adducts. Taken together, the results suggest that Schiff base PE-aldehyde adducts are the most active components of the oxidized LDL provoking the stimulation of the prothrombinase activity.

Oxidation of vesicles made from saturated PC and unsaturated diacyl-PE stimulated prothrombinase activity to an extent comparable to that in the oxidized vesicles composed of unsaturated PC plus unsaturated diacyl-PE. This finding indicated that the extent of non-saturation of PC molecules does not play a major role for the promotion of the activity of the platelet prothrombinase complex. Most likely, therefore, the aldehyde partner for the adduct formation originates mainly from the PE-associated unsaturated fatty acid itself. Thus, the condensation reaction most likely occurs intramolecularly. The generation of the aldehyde-PE adduct results in the loss of the positive charge of the ethanolamine moiety. Consequently, PE acquires a net negative charge. One might assume that this modification could enable the phospholipid to interact with CD36, a platelet cell membrane protein with high affinity for anionic phospholipids and for oxidized LDL (26, 27). However, because the negative charge of the modified PE is retained after treatment with NaCNBH₃, whereas the stimulatory influence on prothrombin cleavage is lost, the enhancement of the prothrombinase activity must be caused by other structural components of the adduct. As the treatment with NaCNBH₃ results in the selective chemical reduction of the Schiff base-specific imino group, this moiety appears to be crucial for the stimulation of the prothrombinase activity. Together with the α-, β-unsaturated double bond of the aldehydes, the imino group forms a system of conjugated double bonds. The high reactivity of the conjugated double bonds could enable the modified PE to interact with platelet cell membrane receptors involved in the activation of the platelets. Earlier data already indicate that extracellular N-substituted phosphatidylethanolamine is able to stimulate platelet aggregation and secretion (28). Our results suggest that the platelet activation induced by the modified PE of oxidized LDL results in the enhanced exposure of aminophospholipids on the platelet surface, a mechanism facilitating the assembly of the prothrombinase complex.

It is well known that aldehydes originating from the oxidation of unsaturated fatty acids can react with lysine residues of apoprotein B100 yielding adducts that are recognized by the classic scavenger receptors of the A type (29). Such aldehyde-protein adducts were recently shown to be responsible for the stimulation of macrophage growth induced by oxidized LDL (30) and for the decreased binding of the modified particles to aortic proteoglycans (31). However, the protein adducts are unlikely to have contributed to a major extent to the stimulation of platelet prothrombinase activity by oxidized LDL. Indeed, the lipid microemulsions prepared from the oxidized LDL enhanced the prothrombinase activity in a manner comparable to the whole particles. Furthermore, the inhibitory effect elicited by chemical reduction with NaCNBH₃ was rather similar when the activation of the platelets had been induced by the oxidized LDL as compared with the one triggered by the modified PE fractions of the lipoproteins.

Our results might contribute to a more profound understanding of the mechanisms leading to the formation of thrombi at the site of the unstable atherosclerotic plaques. Upon rupture of the plaque, the oxidized LDL of the vascular wall is exposed immediately to the blood platelets. According to the results of our study, this will strongly augment platelet prothrombinase activity. Therefore, the local formation of thrombin is expected to be markedly increased; this will result in the enhanced generation of fibrin with subsequent intravascular thrombus formation. In summary, we find that among several modified lipid fractions of oxidized LDL, that increase platelet prothrombinase activity, oxidation products of ethanolamine phospholipids elicit the strongest stimulation. Schiff bases formed by the condensation of the head group of ethanolamine phospholipids with aldehydes generated by the oxidation of unsaturated fatty acids contribute to a major extent to the enhancement of the prothrombinase activity. By promoting the large scale production of thrombin through the prothrombinase com...
plex, oxidized LDL is expected to implement a strong prothrombotic stimulus under in vivo conditions.

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Modified Phosphatidylethanolamine as the Active Component of Oxidized Low Density Lipoprotein Promoting Platelet Prothrombinase Activity
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