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# Minimal oxidation and storage of low density lipoproteins result in an increased susceptibility to phospholipid hydrolysis by phospholipase A<sub>2</sub>

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## Abstract

In vitro-studies have shown that phospholipid hydrolysis of low density lipoproteins (LDL) by bee venom or porcine pancreatic phospholipase A<sub>2</sub> (PLA<sub>2</sub>) leads to an increased uptake of these lipoproteins by macrophages transforming them into foam cells. Recently, a secretory phospholipase A<sub>2</sub>, group II, was detected in human atherosclerotic plaques. In order to investigate the role of this enzyme in the pathogenesis of atherosclerosis, a structurally identical human secretory PLA<sub>2</sub> was purified from the medium of HepG2 cells stimulated with interleukin-6 and tumor necrosis factor- $\alpha$ . The activity of the purified enzyme towards the phospholipids of native and modified low density lipoproteins was compared with the activity towards *Escherichia coli*-membranes and other phospholipid substrates. Compared to *E. coli*-membranes, native LDL proved to be a poor substrate for group II PLA<sub>2</sub>. After mild oxidation induced by copper ions or by 2,2-azobis(2-amidinopropane) (AAPH), the susceptibility of LDL to phospholipid hydrolysis was found to be increased by 25 and 23%, respectively, whereas extensive copper-mediated oxidation caused a decreased hydrolysis. Aging of LDL at 6°C for weeks or at 37°C for hours resulted in an increase in PLA<sub>2</sub>-catalyzed phospholipid hydrolysis of up to 26-fold. LDL protected from oxidation by probucol during aging showed a lesser increase in susceptibility to phospholipid hydrolysis. Our results suggest that PLA<sub>2</sub>, group II, can increase the atherogenicity of LDL by its ability to hydrolyze the phospholipids of these lipoproteins, especially after modifications that are likely to occur in vivo. © 1997 Elsevier Science Ireland Ltd.

**Keywords:** Atherosclerosis; Lipid oxidation; Low density lipoprotein; Phospholipase A<sub>2</sub>

## 1. Introduction

It is well established that the formation of foam cells in arterial intima, a characteristic histologic finding in early atherosclerotic lesions, is mainly based on the accumulation of LDL lipids in monocyte-derived macrophages. In vitro studies have shown, however, that native LDL are poorly degraded by macrophages. By contrast, certain modifications of lipoproteins, which are likely to occur in vivo, have been shown to lead to an increased uptake of LDL by macrophages in vitro. These modifications include peroxidation of polyunsaturated fatty acids [1–3], aggregation [4–6],

hydrolysis of triglycerides by lipoprotein lipase or hepatic lipase [7,8] as well as phospholipid hydrolysis by the actions of phospholipases A<sub>2</sub> [9,10], C [5] and D [11]. Phospholipid hydrolysis of LDL mediated by phospholipase A<sub>2</sub> activities has attracted considerable attention in recent years. Steinbrecher et al. [12] demonstrated that an oxidative modification of LDL in the presence of endothelial cells is accompanied by an extensive release of fatty acids from the *sn*-2 position of LDL phospholipids. This phospholipid hydrolysis was ascribed to an intrinsic PLA<sub>2</sub> activity of apolipoprotein B-100 towards the phospholipids of oxidized LDL [13–15]. Results of other studies, however, suggest that platelet-activating factor acetylhydrolase (PAF-AH), which is associated with LDL particles and exhibits hydrolytic activity toward oxidatively fragmented fatty

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acid residues at the *sn*-2 position of phospholipids, is more likely to be responsible for the intrinsic PLA<sub>2</sub> activity of oxidized LDL [16–19]. Phospholipid hydrolysis of LDL leads to an accelerated biological degradation of the lipoproteins which may either prevent or promote atherosclerosis depending on where the biological degradation of LDL occurs. It was demonstrated in hypercholesterolemic rabbits that low density lipoproteins treated with immobilized snake venom PLA<sub>2</sub> in an extracorporeal circuit are rapidly cleared from plasma by the liver. Based on these results the implantation of a device containing immobilized PLA<sub>2</sub> was suggested as a useful way to lower high plasma levels of cholesterol [20–22]. On the other hand, phospholipid hydrolysis may accelerate the formation of foam cells if it occurs in low density lipoproteins that are trapped in the subendothelial space. LDL incubated with bee venom phospholipase A<sub>2</sub> were shown to be incorporated at increased rate by murine macrophage-like cells in vitro [9]. Similar results were obtained with LDL treated with porcine pancreatic PLA<sub>2</sub> [10]. In contrast, Sparrow et al. [23] observed an increased LDL uptake by macrophages only when PLA<sub>2</sub>-mediated phospholipid hydrolysis was followed by the action of soybean lipoxygenase. No effect was detected after incubation of the lipoproteins with either lipoxygenase or PLA<sub>2</sub> alone. These conflicting results can possibly be explained by different degrees of phospholipid hydrolysis (the details of which were not reported by Sparrow et al.) and differing methods for the quantitation of LDL degradation by macrophages.

Recently a secretory PLA<sub>2</sub>, group II, was detected in atherosclerotic plaques [24]. The enzyme was predominantly located at the surface of macrophages and to a lesser extent in necrotic areas and in the extracellular matrix of the tissues. No PLA<sub>2</sub> expression was observed in media and in non-affected parts of the arterial walls. Secretory phospholipase A<sub>2</sub>, group II, is similar in structure and catalytic properties to group I phospholipase A<sub>2</sub>, which is predominantly found in the pancreas. Both enzymes have a molecular weight of approximately 14 kDa and require calcium ions in millimolar concentrations for their catalytic action. PLA<sub>2</sub>, group II, is constitutively synthesized in prostatic, intestinal and placental tissue and in chondrocytes. Upon stimulation with inflammatory cytokines many other cells including hepatocytes, vascular smooth muscle cells and renal mesangial cells are able to express and secrete the enzyme (reviewed in Kudo et al. [25]). High levels of group II PLA<sub>2</sub> activity can be found in human plasma in inflammatory disorders and septic shock. Therefore, it is generally assumed that this enzyme plays an essential role in local and systemic inflammation [26–28]. Since atherosclerosis is associated with several features of inflammation, the presence of group II PLA<sub>2</sub> in atherosclerotic plaques may be part of an inflammatory

response to a pre-existing lesion of the arterial wall. On the other hand, this phospholipase might play an important role in the formation of foam cells if the enzyme is able to hydrolyze the phospholipids of LDL in a similar way to bee venom and pancreatic PLA<sub>2</sub>. The aim of the present study was to purify a PLA<sub>2</sub> structurally identical to the enzyme detected in atherosclerotic plaques, and to determine its hydrolytic activity towards the phospholipids of native and modified low density lipoproteins.

## 2. Materials and methods

### 2.1. Materials

HepG2 cells were obtained from the American Tissue Culture Collection. Culture medium (Dulbecco's MEM) was purchased from Seromed, Berlin, Germany; fetal bovine serum, L-glutamine, penicillin–streptomycin, and phosphate-buffered saline (PBS) were from Gibco Life Technologies, Eggenstein, Germany. Interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and murine monoclonal antibodies to human phospholipase A<sub>2</sub>, group II, were purchased from biomol, Hamburg, Germany. Polyclonal peroxidase-labeled goat antibodies to murine IgG from Dakko, Hamburg were used for the enzyme-linked immunosorbent assay (ELISA). CM-Sephadex C25, Heparin–Sephadex, Sephadex G25, probucol, phosphatidylcholine, phosphatidylethanolamine, dithioerythritol and bovine serum albumin were products of Sigma, St. Louis, MO. <sup>14</sup>C-labeled oleic acid and phosphatidylcholine labeled with [<sup>14</sup>C]arachidonic acid at the *sn*-2 position were purchased from DuPont de Nemours, Bad Homburg, Germany. Commercially available test kits from Wako, Neuss, Germany and Boehringer Mannheim were used for the determination of free fatty acids and phosphatidylcholine, respectively. Purified porcine pancreatic phospholipase A<sub>2</sub> was obtained from Boehringer Mannheim. All other chemicals were analytical grade and purchased from Merck, Darmstadt, Germany.

### 2.2. Methods

#### 2.2.1. Induction of PLA<sub>2</sub> secretion by HepG2 cells

HepG2 cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 I.U./ml penicillin–streptomycin. PLA<sub>2</sub> secretion was stimulated by adding 25 ng/ml TNF- $\alpha$  and 25 ng/ml IL-6 to the medium of confluent cell cultures. Three days after addition of the cytokines the medium was removed and centrifuged for 10 min at 2000  $\times$  g. The supernatant was either used immediately for the purification of PLA<sub>2</sub> or stored at –20°C until use.

### 2.2.2. Purification of group II phospholipase A<sub>2</sub>

Phospholipase A<sub>2</sub>, group II, was purified from HepG2 medium by conventional liquid chromatography using a 'Gradifrac System 1' from Pharmacia Biotech, Freiburg, Germany. The optical densities of eluents were continuously monitored at 280 nm and recorded; fractions containing PLA<sub>2</sub> were identified by ELISA as described below. In a typical experiment 100–120 ml medium of cytokine-stimulated HepG2 cells was applied to a 2.5 × 17 cm column of CM-Sephadex C25, equilibrated with a 2.5 mM Tris–HCl buffer, pH 7.4 containing 150 mM NaCl (buffer A). The column was washed with the same buffer, until no further decrease in optical density of the eluent was observed. Subsequently, the bound proteins were eluted with a 25 mM Tris–HCl buffer, pH 7.4 containing 1.5 M NaCl (buffer B) and collected in 6 ml fractions. Fractions containing PLA<sub>2</sub> were pooled and dialysed overnight against a 15-fold volume of buffer A. The enzyme was further purified by chromatography on a 1.5 × 5 cm column of heparin–sepharose. The conditions for binding, washing, elution and detection of the protein were generally the same as for CM-Sephadex chromatography. Fractions containing PLA<sub>2</sub> were pooled and dialysed overnight against a 10-fold volume of saline. Subsequently the purified enzyme was concentrated to a final volume of 1–2 ml in 'Centriprep 10' microconcentrators (Amicon) and stored in fractions of 20–30 µl at –20°C.

### 2.2.3. Characterization of the purified enzyme

The homogeneity of the enzyme preparations was examined by SDS-electrophoresis on commercially available (Pharmacia Fine Chemicals) polyacrylamide gradient gels (8–25%) using the 'Phast' power supply and cooling system from the same manufacturer. PLA<sub>2</sub> samples were applied either without pretreatment or after reduction of disulfide bonds with 1% (w/v) dithioerythritol. Six standard proteins (14.3–66 kDa) were used as molecular weight markers. The gels were run with 50 V/cm at 16°C for 45 min and subsequently stained with Coomassie Brilliant Blue R. In order to determine the thermostability of the catalytic activity, samples of the enzyme were heated to 60°C for 80 min, centrifuged (1 min at 27 000 × g), and the activities of the supernatants were measured using *Escherichia coli*-membranes and LDL as substrates. Extraction with 0.18 N H<sub>2</sub>SO<sub>4</sub> [29] was employed to examine the acid-stability of purified PLA<sub>2</sub>.

### 2.2.4. Preparation of substrates

*E. coli*-membranes were labeled with [<sup>14</sup>C]oleic acid as previously described [30,31]. For the preparation of monomeric phospholipid substrates, 0.44 nmol 2-(arachidonyl-1-<sup>14</sup>C), 1-palmitoyl-*sn*-glycerophosphorylcholine or 0.47 nmol 2-(arachidonyl-1-<sup>14</sup>C),

1-palmitoyl-*sn*-glycerophosphorylethanolamine were dispersed in 100 µl phosphate-buffered saline, pH 7.4, containing 2 mM CaCl<sub>2</sub> and 2% (w/v) bovine serum albumin. A method described by Schädlich et al. [32] was used to obtain micellar phosphatidylcholine. Six µmol 1-palmitoyl, 2-arachidonyl-*sn*-glycerophosphorylcholine and 10 nmol 2-(arachidonyl-1-<sup>14</sup>C), 1-palmitoyl-*sn*-glycerophosphorylcholine were dissolved in 1 ml of a 20 mM glycine buffer, pH 8.0, containing 6 mM sodium desoxycholate and 0.1% Triton X-100. The mixture was heated to 60°C for 1 min and after cooling to room temperature 10 µl 200 mM CaCl<sub>2</sub> solution was added to obtain a clear solution. Low density lipoproteins were isolated from pooled EDTA plasma by sequential ultracentrifugation [33]. The lipoproteins were either used immediately after isolation or stored with 10% sucrose at –20°C [34] until use. Before cryopreservation and before use, LDL were separated from density solution or sucrose, respectively, on 1.5 cm × 2.5 cm columns of Sephadex G25 equilibrated with saline.

### 2.2.5. Modification of LDL

Freshly prepared LDL were modified by 'aging' and by oxidation using copper ions or 2,2-azobis(2-amidinopropane)hydrochloride (AAPH). Aging was achieved by storing the lipoproteins in saline at 6°C for up to 8 weeks or at 37°C for 1–28 h. Before storage the LDL preparations were passed through a 0.2 µm filter to remove bacteria. Oxidation was induced by incubating LDL for 16 h at 37°C with 2 and 55 µmol CuSO<sub>4</sub> or with 176 µmol AAPH per µmol apolipoprotein B (apo B). Subsequently the oxidants were separated from the lipoproteins by chromatography on 1.5 cm × 2.5 cm columns of Sephadex G25 equilibrated with saline.

### 2.2.6. Enzymatic hydrolysis of phospholipid substrates

Phosphatidylethanolamine of *E. coli*-membranes labeled with [<sup>14</sup>C]oleic acid was used as substrate to determine the activity of phospholipase A<sub>2</sub>. One unit (U) of PLA<sub>2</sub> was defined as the amount of enzyme catalyzing the release of 1 µmol fatty acids from phospholipids of the bacterial membranes per min. In general, 100 µl of substrate was mixed with 25 µl enzyme solution and 25 µl of a 500 mM Tris–HCl buffer, pH 8.0, containing 10 mM CaCl<sub>2</sub> and 10% bovine serum albumin as described by Aufenanger et al. [31]. After incubation at 37°C for 15 min the reaction was stopped by adding 100 µl 30 mM EDTA solution. The lipids were extracted with 1250 µl modified Dole reagent (2-propanol-*n*-heptane–1 M H<sub>2</sub>SO<sub>4</sub> (40:10:1, v/v/v)), 500 µl *n*-heptane and 750 µl distilled water. After phase separation, 500 µl of the organic phase was passed through a column containing 100 mg aminopropyl silica gel. Free fatty acids were eluted with 3 × 500 µl of diethylether–acetic acid (98:2) into a scintillation vial.

The activity was calculated by multiplying the counted radioactivity (DPM) with a factor that had to be determined for each lot of substrate [31].

Hydrolysis of monomeric phosphatidylcholine, monomeric phosphatidylethanolamine and micellar phosphatidylcholine was started by adding 50  $\mu$ l enzyme solution to 100  $\mu$ l of freshly prepared substrate. After incubation at 37°C for 1 h, the reaction was stopped by the addition of 150  $\mu$ l 20 mM EDTA solution. The lipids were extracted and the released fatty acids were determined in the same way as described for the *E. coli*-based PLA<sub>2</sub> assay.

LDL phospholipids were hydrolyzed by incubating the lipoproteins (apo B concentrations: 1.1–2.9 g/l) with purified human group II PLA<sub>2</sub> or porcine pancreatic PLA<sub>2</sub> in a 100 mM Tris–HCl buffer, pH 7.4, containing 2 mM CaCl<sub>2</sub> and 2% (w/v) bovine serum albumin. After incubation the concentrations of free fatty acids were determined using the ‘NEFA C’ test kit from Wako as described below. Each experiment was run in triplicate with three blanks containing an equal volume of saline instead of the enzyme solution. PLA<sub>2</sub>-catalyzed phospholipid hydrolysis was calculated from the increase in concentration of free acids during incubation. All values were corrected for intrinsic PLA<sub>2</sub> activity by subtracting the corresponding blank values.

#### 2.2.7. Quantitative determination of free fatty acids

The concentration of free fatty acids was measured by a discontinuous enzymatic method using the ‘NEFA C’ test kit from Wako [35,36]. The method relies on the acylation of coenzyme A by the fatty acids in the presence of acyl-coenzyme A synthetase, ATP and magnesium cations. Subsequently, Acyl-CoA is oxidized by acyl-coenzyme A oxidase leading to the production of 2,3-trans-enoyl-CoA and hydrogen peroxide. The presence of peroxidase and hydrogen permits the oxidative condensation of 4-aminoantipyrine with 3-methyl-*N*-ethyl-*N*-( $\beta$ -hydroxyethyl)-aniline to form a colored adduct which can be measured at 550 nm. The assay, which determines non-oxidized and oxidized free fatty acids with equal sensitivity, is performed with two different color reagents (A and B) containing the following ingredients. Color reagent A: acyl-coenzyme A synthetase, ascorbate oxidase, coenzyme A, ATP, 4-aminoantipyrine, and magnesium chloride dissolved in a 50 mM phosphate buffer, pH 6.9. Color reagent B: acyl-coenzyme A oxidase, peroxidase, and 3-methyl-*N*-ethyl-*N*-( $\beta$ -hydroxyethyl)-aniline dissolved in distilled water. The sensitivity of the assay was enhanced by dissolving the lyophilized ingredients in 25% of the diluent volumes recommended by the manufacturer and changing the volume ratio of sample:reagent A:reagent B from 1:20:40 to 1:5:10. The samples were incubated for 10 min at room temperature with reagent A in wells of a microtiter plate. Subsequently reagent B was added

and after another 5 min the optical densities were measured in a microplate reader at 550 nm. Oleic acid in concentrations of 125, 250, and 500  $\mu$ M was used as standard.

#### 2.2.8. Other methods

The concentration of total protein during PLA<sub>2</sub> purification was estimated by the method of Macart and Gerbaut [37]. The concentration of apo B-100 in LDL preparations was determined turbidimetrically using polyclonal rabbit antibodies to the apolipoprotein and purified apo B as standard. Phosphatidylcholine content of LDL was determined using the ‘PL MPR 2’ enzymatic test kit from Boehringer Mannheim.

Thiobarbituric acid reactive substances (TBARS) in LDL were determined by a modification of the method described by Stocks and Dormandy [38]. LDL samples, trichloroacetic acid (0.22%), and thiobarbituric acid (1% in 50 mM NaOH) were mixed in a volume ratio of 1:1:0.5 and boiled in a water bath for 15 min. After cooling to room temperature the mixtures were centrifuged at 22 000  $\times g$  for 2 min to pellet denatured proteins. The TBARS content of the samples, expressed as ‘malonaldehyde equivalents’, was calculated from the optical densities of the supernatants at 532 nm using 1,1,3,3-tetramethoxypropane (malonaldehyde bis[ $\alpha$ -dimethyl acetal]) as standard.

Purified group II PLA<sub>2</sub> was examined for an oxidizing effect on fatty acids by incubating 1590 U/l of the enzyme with 333  $\mu$ M linoleic acid at 37°C for 3 h in a 200 mM borate buffer, pH 9.0. Pure linoleic acid and the pure enzyme treated in the same way were used as blanks. The formation of conjugated dienes was estimated by measuring the optical densities at 234 nm before and after incubation.

An enzyme-linked immunosorbent assay (ELISA) was employed to detect and quantitate group II PLA<sub>2</sub> protein. The assay was performed on 96-well microtiter plates using a monoclonal mouse antibody to human group II PLA<sub>2</sub> and peroxidase-labeled polyclonal antibodies to murine IgG.

Thin-layer chromatography of LDL lipids was carried out on 20 cm  $\times$  20 cm  $\times$  0.2 cm silica plates purchased from Merck, Darmstadt, Germany. Lipids were extracted three times with equal volumes of chloroform–methanol (2:1). The pooled extracts were dried under nitrogen and redissolved in chloroform–methanol (one third of the original volume). 10 ml of the concentrated extracts were applied to the plates which were developed with the solvent systems chloroform–methanol–water–acetic acid (78:30:4.8:9.6) for the separation of phospholipids and heptane–isopropyl ether–acetic acid (75:50:5) for the separation of neutral lipids. The plates were stained by spraying molybdotophosphoric acid (10 g dissolved in 100 ml ethanol plus 4 ml 37% HCl) onto the surface of the plates and heating to 120°C for 3 min.

### 3. Results

Human group II PLA<sub>2</sub> was purified more than 800-fold from the medium of cytokine-stimulated HepG2 cells. On the average, 200  $\mu$ g enzyme protein with a specific activity of 12 U/mg was obtained from 100 ml medium. During purification PLA<sub>2</sub> displayed a distinct hydrophobicity leading to binding of the protein to surfaces of tubes and vials at low salt concentrations (< 150 mM NaCl). On SDS electrophoresis under non-reducing conditions, the purified enzyme migrated as two bands with apparent molecular weights of approximately 19 and 14 kDa, respectively (Fig. 1, lane 1). These two bands were also observed after additional purification procedures like 'acid extraction' as described by Kramer et al. [29], gel filtration on Sephadex G80, and high performance liquid chromatography (HPLC) using a reversed phase column of octadecyl silica gel (results not shown). After reduction of disulfide bonds by treatment with 1% (w/v) dithioerythritol, however, a single band with an apparent molecular weight of approximately 16 kDa was obtained (Fig. 1, lane 3). Heating to 60°C for 80 min resulted in a complete loss of enzyme activity toward *E. coli*-mem-

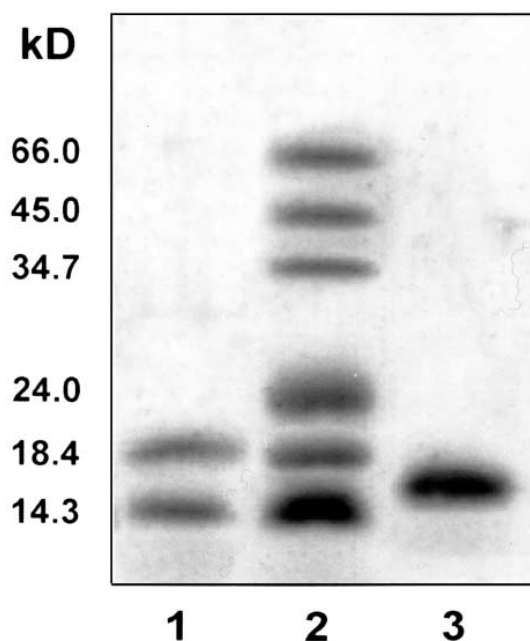


Fig. 1. SDS electrophoresis of purified secretory phospholipase A<sub>2</sub>, group II, and standard proteins. Electrophoresis was carried out on a polyacrylamide gradient gel (8–25%) at 16°C and 50 V/cm using the PHAST System from Pharmacia. Lane 1, non-reduced group II PLA<sub>2</sub>; lane 2, molecular weight standard containing bovine serum albumin (66 kDa), ovalbumin (45 kDa), pepsin from porcine stomach mucosa (34.7 kDa), trypsinogen from bovine pancreas (24 kDa), subunit of  $\beta$ -lactoglobulin from bovine milk (18.4 kDa), and lysozyme from egg white (14.3 kDa); lane 3, group II PLA<sub>2</sub> reduced with 1% (w/v) dithioerythritol. Protein bands were stained with Coomassie Brilliant Blue R.

Table 1

Activities of secretory phospholipases A<sub>2</sub> group I and group II toward different phospholipid substrates

Substrate	PLA <sub>2</sub> activity (nmol/l min)	
	Group 1	Group 11
<i>E. coli</i> membranes	15 000.0	14 890.0
Micellar phosphatidylcholine	305.0	280.6
Monomeric phosphatidyl-ethanolamine	82.6	10.8
Monomeric phosphatidylcholine	7.5	0.8
Native LDL	0.5–7.8	0.1–1.3

branes and other substrates (not shown). By contrast, after acid extraction a loss in activity of less than 10% was observed. The hydrolytic activity of the purified enzyme was compared with the activity of commercially available porcine pancreatic PLA<sub>2</sub> using *E. coli*-membranes, micellar and monomeric phosphatidylcholine, monomeric phosphatidylethanolamine and native LDL as substrates. Both enzymes showed the same order of substrate preference with the highest activities toward *E. coli*-membranes and the lowest towards LDL phospholipids (Table 1). The enzyme activities toward native LDL varied widely from preparation to preparation of the substrate. When enzyme concentrations were used that showed comparable activities with *E. coli*-membranes as substrate, LDL phospholipids were hydrolyzed approximately 6 times more rapidly by porcine pancreatic PLA<sub>2</sub> than by human group II PLA<sub>2</sub>. On the average, 0.7 nmol/l fatty acids per min were released from 1.3 mmol/l phospholipids of native LDL during incubation with 15 U/l of purified group II PLA<sub>2</sub>.

After storage of LDL at 6°C for several weeks, the lipoproteins showed a striking increase in the release of fatty acids during incubation with PLA<sub>2</sub> (Fig. 2A). Similar results were obtained with native LDL stored for several h at 37°C prior to incubation with the enzyme (Fig. 2B). Under these conditions, however, the PLA<sub>2</sub>-mediated release of fatty acids from stored LDL usually exhibited a characteristic time-course with an initial increase up to a maximum followed by a decrease after prolonged storage. The storage times leading to a maximum of hydrolysis varied from preparation to preparation of the lipoproteins.

The origin of the fatty acids released from native and stored LDL upon treatment with group II PLA<sub>2</sub> was investigated by thin-layer chromatography of organic extracts from the reaction mixtures. As shown in Fig. 3, treatment of both native and stored LDL with group II PLA<sub>2</sub> resulted in a decrease in phosphatidylcholine content which was accompanied by increased concentrations of lysophosphatidylcholine and free fatty acids. No effect of the enzyme on other LDL components like

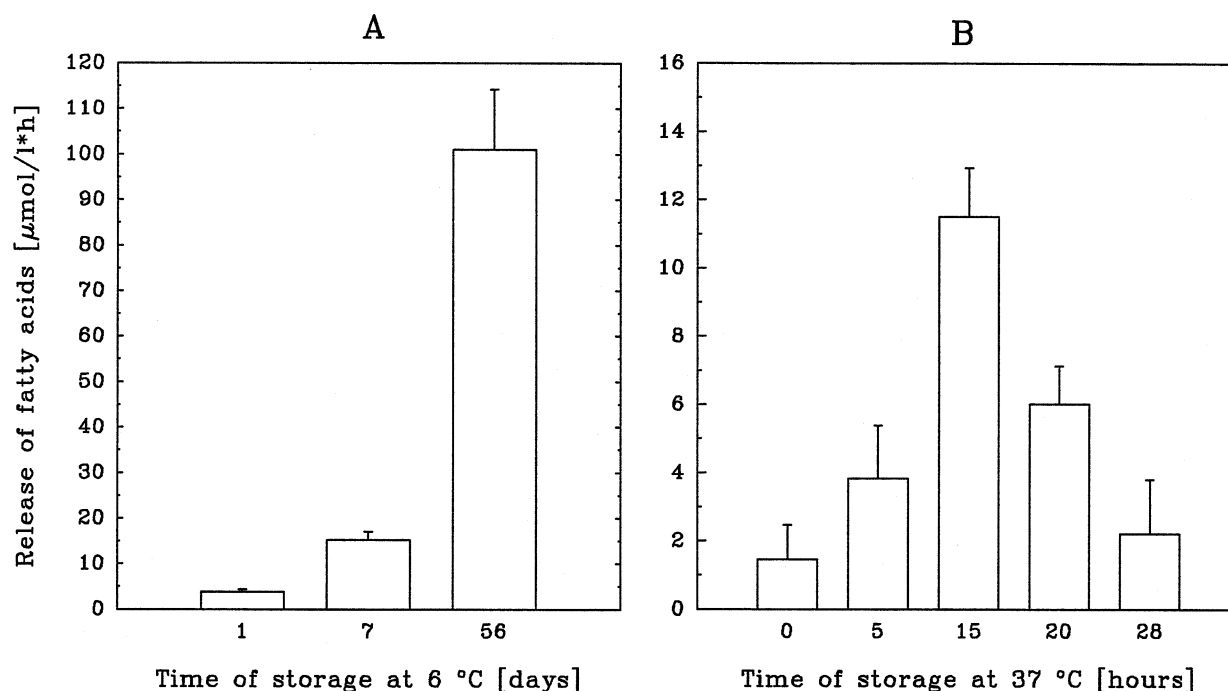


Fig. 2. Effect of LDL aging at 6°C and 37°C on the release of fatty acids mediated by phospholipase A<sub>2</sub>, group II. LDL samples (apo B concentration 1.1 g/l) stored at 6°C (A) or 37°C (B) in saline for different times were incubated with 660 U/l (A) or 520 U/l (B) of purified PLA<sub>2</sub> group II, in a 100 mM Tris–HCl buffer, pH 7.4, containing 2 mM CaCl<sub>2</sub> and 2% (w/v) bovine serum albumin. The values were corrected for intrinsic PLA<sub>2</sub> activities of the lipoproteins by subtracting the release of fatty acids in the absence of enzyme. Bars represent means of three experiments  $\pm$  S.D.

triglycerides or cholesteryl esters was observed. The fatty acids extracted from group II PLA<sub>2</sub>-treated LDL migrated in the same way as 'native' linoleic or arachidonic acid (not shown) indicating that the vast majority, if not all, of the released fatty acids were not oxidized.

A slow but measurable release of fatty acids was also observed upon storage of LDL. The concentrations of free fatty acids ranging from 30 to 55  $\mu\text{mol}/\text{g}$  apo B in freshly isolated LDL was found to be increased by 21–86  $\mu\text{mol}/\text{g}$  apo B after storage for 4 weeks at 6°C.

The release of fatty acids reflecting the intrinsic PLA<sub>2</sub> activity of the lipoproteins varied among different preparations and was considerably accelerated during incubation with reaction buffer at 37°C. For an accurate determination of group II PLA<sub>2</sub>-catalyzed phospholipid hydrolysis in low density lipoproteins, blank values had to be subtracted from the total release of fatty acids. The blank values were obtained by incubating the lipoproteins under the same conditions in the absence of PLA<sub>2</sub>. As demonstrated in Table 2, the intrinsic PLA<sub>2</sub> activities of an LDL preparation increased with the age of the lipoproteins. Since LDL used in our experiments were isolated from pooled plasma which can contain high levels of secretory phospholipase A<sub>2</sub>, the intrinsic PLA<sub>2</sub> activity might be partly due to this enzyme bound to the lipoproteins. On the other hand, calcium-independent PAF-AH co-

purified from HepG2 medium might be responsible for a part of the catalytic activity of the enzyme preparations. To exclude these possibilities, we determined the intrinsic and the group II PLA<sub>2</sub> activity toward LDL phospholipids both in the absence and presence of calcium. As shown in Fig. 4, the intrinsic PLA<sub>2</sub> activity of LDL proved to be calcium-independent in contrast to group II PLA<sub>2</sub> purified from HepG2 medium, which was found to be completely inactive in the absence of calcium.

Besides a release of fatty acids, other changes in the physicochemical properties of stored LDL were observed including a shift in absorbency spectrum to greater wavelengths associated with a tendency to aggregate, increased concentrations of thiobarbituric acid reactive substances (TBARS), and an accelerated electrophoretic migration (not shown). Storage at 37°C for up to 24 h had no detectable effect on the mobility of the apo B in SDS electrophoresis or on its reactivity with polyclonal antibodies to the protein (results not shown).

The effect of LDL oxidation on PLA<sub>2</sub>-mediated phospholipid hydrolysis was examined by treatment of lipoproteins with 176  $\mu\text{mol}$  AAPH/ $\mu\text{mol}$  apo B and with 2 or 55  $\mu\text{mol}$  CuSO<sub>4</sub>/ $\mu\text{mol}$  apo B prior to incubation with the enzyme. After moderate oxidation with AAPH or 2  $\mu\text{mol}$  CuSO<sub>4</sub>/ $\mu\text{mol}$  apo B, the PLA<sub>2</sub>-catalyzed release of fatty acids was increased by approxi-

mately 23 and 25%, respectively. After extensive oxidation with 55  $\mu\text{mol CuSO}_4/\mu\text{mol apo B}$ , a 17% decrease in hydrolysis rate was observed (Fig. 5). During copper-induced oxidation, a considerable amount of fatty acids was released from the lipoproteins. Only a minor part of these fatty acids could be removed by dialysis or gel filtration, indicating that they were tightly bound to the LDL particles. In order to investigate whether the reduced activity of PLA<sub>2</sub> towards extensively oxidized LDL might be due to inhibition by free fatty acids, the effect of different concentrations of oleic acid on PLA<sub>2</sub> activity was determined using *E. coli*-membranes as substrate. Both group I and group II phospholipase A<sub>2</sub> were inhibited by increasing concentrations of the free fatty acids with a complete loss of enzyme activity at

Table 2

Intrinsic PLA<sub>2</sub> activities of an LDL preparation after different times of storage

LDL	Release of fatty acids ( $\mu\text{mol/l h}$ )
Native	$3.47 \pm 0.77$
Stored at 6°C for 4 weeks	$5.26 \pm 1.08$
Stored at 6°C for 8 weeks	$9.66 \pm 0.40$

The activities were obtained by measuring the release of fatty acids from 1.1 g/l LDL during incubation at 37°C in a 100 mM Tris–HCl buffer, pH 7.4, containing 2 mM CaCl<sub>2</sub> and 2% bovine serum albumin.

concentrations of 700 and 500  $\mu\text{M}$ , respectively (results not shown).

Probucol, a potent antioxidant, was used to estimate the role of oxidation in the enhancement of phospholipid hydrolysis by aging. Aliquots of freshly prepared LDL were preincubated for 17 h at 37°C with equal volumes of probucol (310  $\mu\text{M}$  final concentration) and saline, respectively. An additional aliquot representing native LDL was kept at 6°C for the same time. In contrast to the unprotected lipoproteins, probucol-protected LDL showed no increase in TBARS after incubation (not shown). Samples of the three aliquots were subsequently incubated with purified group II PLA<sub>2</sub> to determine their susceptibility to phospholipid hydroly-

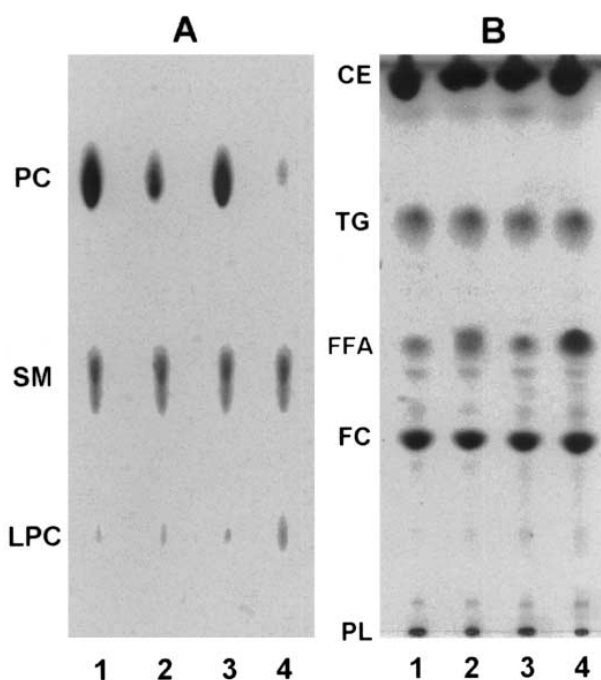


Fig. 3. Thin-layer chromatographic analysis of lipids from freshly isolated and stored LDL after incubation with saline or purified group II PLA<sub>2</sub>. 30  $\mu\text{l}$  LDL containing 12.15 mM phosphatidylcholine and 8.6 g/l apo B were mixed with equal volumes of purified group II PLA<sub>2</sub> (4237 U/l) or saline (controls), and a 300 mM Tris–HCl buffer, pH 7.4, containing 6 mM calcium chloride and 6% bovine serum albumin. The mixtures were incubated at 37°C for 27 h and subsequently extracted 3 times with 90  $\mu\text{l}$  chloroform–methanol (2:1). The 3 extracts were pooled, dried under nitrogen and redissolved in 30  $\mu\text{l}$  chloroform–methanol (2:1). 10  $\mu\text{l}$  of the extracts were applied to silica plates which were developed with the solvent systems chloroform–methanol–water–acetic acid (78:30:4.8:9.6) for the separation of phospholipids (A) and heptane–isopropyl ether–acetic acid (75:50:5) for the separation of neutral lipids (B). Lane 1, native LDL incubated with saline; lane 2, native LDL incubated with group II PLA<sub>2</sub>; lane 3, stored LDL (4 weeks at 6°C) incubated with saline; lane 4, stored LDL treated with group II PLA<sub>2</sub>. The migration distances of the different lipids are indicated on the left. Abbreviations: PC, phosphatidylcholines; SM, sphingomyelins; LPC, lysophosphatidylcholines; CE, cholesteryl esters; TG, triglycerides; FFA, free fatty acids; FC, free cholesterol; PL, phospholipids.

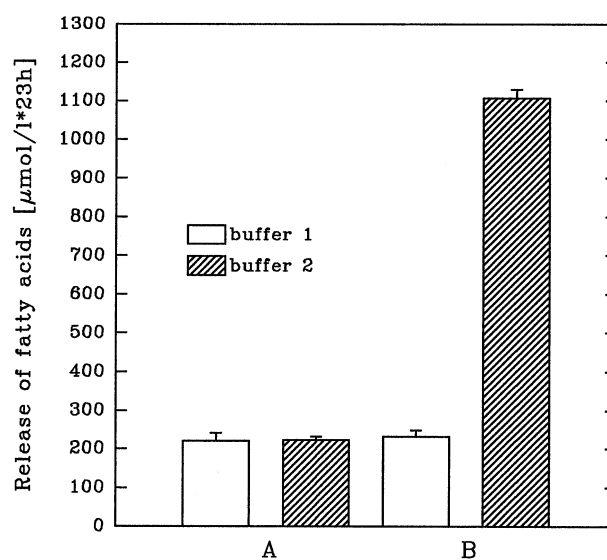


Fig. 4. Effect of calcium on the release of fatty acids from LDL catalyzed by the intrinsic PLA<sub>2</sub> activity and by added group II PLA<sub>2</sub>. After storage at 6°C for 8 weeks, LDL samples containing 1.1 g/l apo B, 1.3 mM phosphatidylcholine, and 197  $\mu\text{M}$  free fatty acids were incubated at 37°C for 23 h with saline and 4237 U/l of group II PLA<sub>2</sub>, respectively. Two different reaction buffers were used. Buffer 1: 100 mM Tris–HCl, pH 7.4, containing 2% (w/v) bovine serum albumin; buffer 2: 100 mM Tris–HCl, pH 7.4, containing 2% (w/v) bovine serum albumin and 2 mM CaCl<sub>2</sub>. A, release of fatty acids caused by intrinsic PLA<sub>2</sub> activity of LDL; B, release of fatty acids caused by intrinsic + group II PLA<sub>2</sub> activity. Bars represent means of three experiments  $\pm$  S.D.



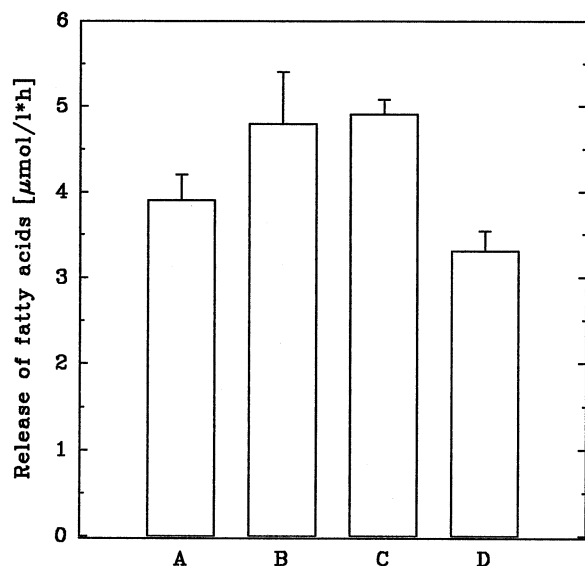


Fig. 5. Activity of  $\text{PLA}_2$ , group II, towards phospholipids of native and oxidized LDL. LDL oxidation was performed by incubation (16 h at  $37^\circ\text{C}$ ) with AAPH (176  $\mu\text{mol}/\mu\text{mol}$  apo B) or with  $\text{CuSO}_4$  (2 or 55  $\mu\text{mol}/\mu\text{mol}$  apo B). Native and oxidized LDL (apo B concentration: 1 g/l) were treated with 660 U/l of purified  $\text{PLA}_2$  in a 100 mM Tris-HCl buffer, pH 7.4 containing 2 mM  $\text{CaCl}_2$  and 2% (w/v) bovine serum albumin. Substrates: A, native LDL; B, LDL oxidized with 176  $\mu\text{mol}$  AAPH/ $\mu\text{mol}$  apo B; C, LDL oxidized with 2  $\mu\text{mol}$   $\text{CuSO}_4$ / $\mu\text{mol}$  apo B; D, LDL oxidized with 55  $\mu\text{mol}$   $\text{CuSO}_4$ / $\mu\text{mol}$  apo B. The values were corrected for intrinsic  $\text{PLA}_2$  activities of the lipoproteins by subtracting the release of fatty acids in the absence of the enzyme. Bars represent means of three experiments  $\pm$  S.D.

sis. LDL aged at  $37^\circ\text{C}$  without probucol were hydrolyzed approximately 177% more rapidly than the native lipoprotein, whereas probucol-protected LDL showed an increase in hydrolysis of only 110% (Fig. 6).

After incubation of LDL with purified  $\text{PLA}_2$ , substantially higher concentrations of TBARS were measured in the reaction mixtures. In order to investigate if phospholipid hydrolysis leads to an accelerated lipid peroxidation we have determined the TBARS content in LDL samples after treatment with porcine pancreatic  $\text{PLA}_2$  and with both active and heat-inactivated group II  $\text{PLA}_2$ . Native LDL treated with active group II  $\text{PLA}_2$  showed approximately 44-fold higher TBARS concentrations than LDL incubated under the same conditions with an equal volume of saline. Incubation with heat-inactivated group II  $\text{PLA}_2$  resulted in a 26-fold increase in the reaction products of lipid oxidation. In contrast, no increase in TBARS was observed after phospholipid hydrolysis by porcine pancreatic  $\text{PLA}_2$  (Fig. 7). The same negative result was obtained with ultrafiltrates of purified group II  $\text{PLA}_2$  (not shown) indicating that the enzyme preparations did not contain redox-active heavy metals or other low-molecular contaminants that might account for the observed increase in TBARS.

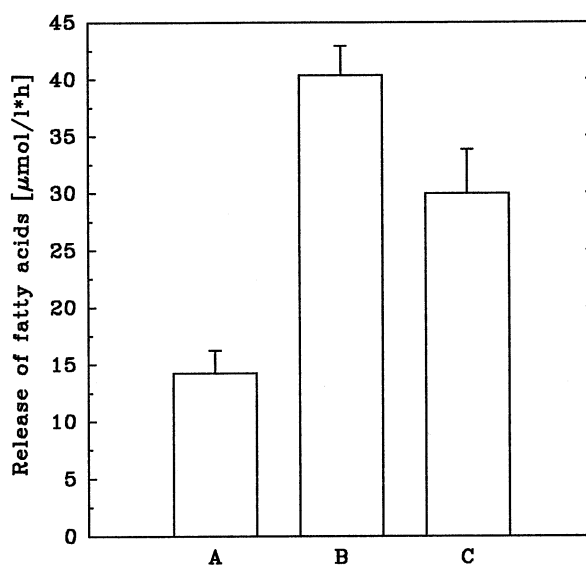


Fig. 6. Effect of probucol during storage of LDL on the susceptibility of the lipoproteins to phospholipid hydrolysis catalyzed by group II  $\text{PLA}_2$ . LDL samples containing 1.6 g/l apo B and 1.9 mM phosphatidylcholine were stored for 17 h at  $6^\circ\text{C}$  or at  $37^\circ\text{C}$  in the absence or presence of 0.31 mM probucol and subsequently incubated with 1300 U/l group II  $\text{PLA}_2$ . A, release of fatty acids from LDL stored at  $6^\circ\text{C}$  in the absence of probucol; B, release of fatty acids from LDL stored at  $37^\circ\text{C}$  in the absence of probucol; C, release of fatty acids from LDL stored at  $37^\circ\text{C}$  in the presence of 0.31 mM probucol. The values were corrected for intrinsic  $\text{PLA}_2$  activities of LDL by subtracting the release of fatty acids in the absence of the enzyme. Bars represent means of three experiments  $\pm$  S.D.

To examine if the enzyme or contaminants of the preparations had an oxidative effect on polyunsaturated fatty acids, purified group II  $\text{PLA}_2$  was also

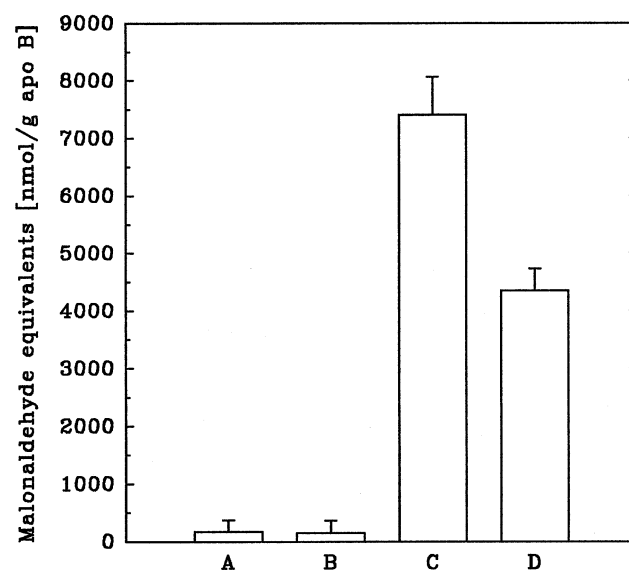


Fig. 7. Concentration of thiobarbituric acid reactive substances in 1.3 g/l LDL after incubation with saline (A), group I  $\text{PLA}_2$  (B), active group II  $\text{PLA}_2$  (C), and heat-inactivated group II  $\text{PLA}_2$  (D). Bars represent means of three experiments  $\pm$  S.D.

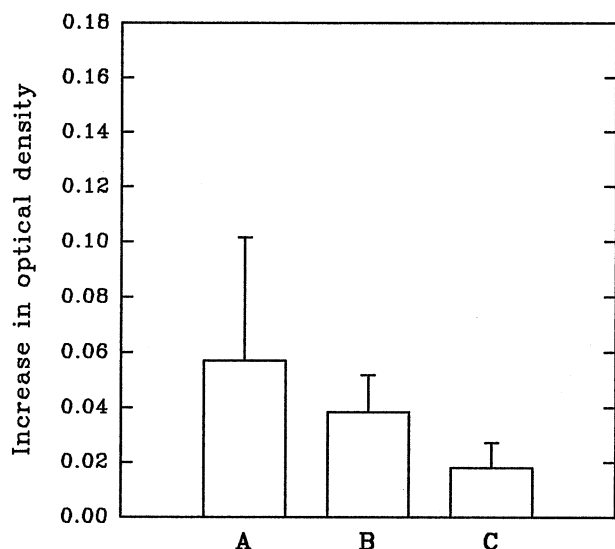


Fig. 8. Increases in optical densities of 333  $\mu$ M linoleic acid + 1590 U/l of purified group II PLA<sub>2</sub> (A), pure linoleic acid (B), and pure group II PLA<sub>2</sub> (C) during incubation (3 h at 37°C) in a 200 mM borate buffer, pH 9.0. Each bar represents the mean of three experiments  $\pm$  S.D.

incubated with linoleic acid. The formation of conjugated dienes, early products of lipid oxidation, was estimated by comparing the optical densities at 234 nm before and after a 3 h incubation at 37°C. The results of these experiments are shown in Fig. 8. Although the absorbancies of the reaction mixtures containing both linoleic acid and purified group II PLA<sub>2</sub> were slightly increased after incubation, this increase did not exceed the combined increases in absorbancy of the two controls containing either linoleic acid or group II PLA<sub>2</sub> alone.

#### 4. Discussion

In vitro-studies have shown that the hydrolysis of LDL phospholipids leads to an enhanced degradation of the lipoproteins by macrophages which may accelerate the formation of foam cells in vivo. The aim of this study was to examine whether the secretory phospholipase A<sub>2</sub> detected in human atherosclerotic plaques [24] is capable of hydrolyzing the phospholipids of LDL, similar to bee venom and porcine pancreatic phospholipase A<sub>2</sub>. For this purpose, a phospholipase A<sub>2</sub> was isolated and purified from culture medium conditioned by cytokine-stimulated HepG2 cells. Previously, we observed that this enzyme immunologically cross-reacted with four different monoclonal antibodies which were raised against human placental, synovial and sperm PLA<sub>2</sub> and also recognized the PLA<sub>2</sub> enzyme identified in atherosclerotic plaques. This observation suggested that the HepG2-specific and the plaque-associated en-

zyme are at least immunologically related. We have shown that the PLA<sub>2</sub>-specific mRNA isolated from lysates of cytokine-stimulated HepG2 cells and the mRNA extracted from human atherosclerotic plaques have the same nucleotide sequences indicating that the two enzymes are products of the same gene (Menschikowski M, Eckey R, Aufenanger J, Jaross W, unpublished results).

The enzyme isolated from medium of cytokine-stimulated HepG2 cells proved to be heat-labile, acid-stable, calcium-dependent and had obviously no oxidative effect on linoleic acid. These properties indicate that the enzyme preparations were not contaminated with group I PLA<sub>2</sub>, PAF-AH, lipoxygenases or redox-active metals. The two bands observed on SDS electrophoresis under non-reducing conditions are more likely to be caused by two different conformations of the native enzyme than by two different proteins.

The results of this study suggest that PLA<sub>2</sub>, group II, is capable of hydrolyzing the phospholipids of low density lipoproteins in vitro. However, consistent with previously reported results [39], native LDL proved to be a poor substrate for the enzyme. The susceptibility of the lipoproteins to phospholipid hydrolysis was shown to be increased after storage at 6 or 37°C and after mild oxidation by AAPH or copper ions. By contrast, prolonged aging at 37°C as well as extensive copper-catalyzed oxidation resulted in a decreased PLA<sub>2</sub>-mediated phospholipid hydrolysis. Thus, the physicochemical state of the lipoproteins seems to be a decisive factor for the susceptibility of LDL to phospholipid hydrolysis by phospholipases A<sub>2</sub>.

The nature of modifications that occur in LDL during aging and lead to an increase in susceptibility to phospholipid hydrolysis is not known. The increase in TBARS concentration in aged LDL, the accelerated phospholipid hydrolysis in moderately oxidized LDL and the inhibitory effect of the antioxidant probucol, suggest that oxidation of unsaturated fatty acyl residues is responsible, at least in part, for this phenomenon. There are several reports documenting the accelerated hydrolysis of oxidized phospholipids by PLA<sub>2</sub> [40–43]. A hypothesis explaining this substrate preference was proposed by van den Berg et al. [40]. Experiments with monolayers of oxidized and non-oxidized 1-palmitoyl-2-linoleylphosphatidylcholine indicated that oxidized phospholipids exhibit an altered conformation, which may facilitate the access of phospholipases A<sub>2</sub> to the *sn*-2 ester bonds. In the case of aged LDL, we found no evidence for a preferential release of oxidized fatty acids by group II PLA<sub>2</sub>. There seem to be other mechanisms by which oxidation can lead to an activation of the enzyme towards LDL phospholipids. Previous studies have shown that the activity of phospholipases A<sub>2</sub> toward phospholipid mono- and bilayers is determined not only by the acyl chains and polar head groups of

the phospholipids, but also by certain properties of the whole layers, like packing order, curvature, and lateral surface pressure. Phospholipases prefer substrate surfaces with an inhomogeneous structure, a low lateral pressure, and a strong curvature [44–53]. Perturbations and membrane packing defects lead to an activation of PLA<sub>2</sub>. LDL oxidation is likely to cause perturbations of the phospholipid monolayer via two different mechanisms: firstly by inducing a conformational change of individual phospholipids which may affect the packing order of the whole layer, and secondly by increasing the intrinsic activity of the lipoproteins resulting in an accumulation of reaction products. Free fatty acids and lysophospholipids when present in bilayers above a threshold concentration were shown to have a PLA<sub>2</sub>-activating effect [47,50,52,54], which might be due to perturbations of the phospholipid layer induced by small amounts of these reaction products.

The intrinsic PLA<sub>2</sub> activity may also account for the decreased rate of group II PLA<sub>2</sub>-catalyzed phospholipid hydrolysis after extensive oxidation and prolonged storage of LDL at 37°C. An increased or prolonged phospholipid hydrolysis mediated by the intrinsic PLA<sub>2</sub> activity can lead to a substrate depletion as well as to concentrations of free fatty acids that are inhibitory to secretory phospholipases A<sub>2</sub>. An enzyme-inhibiting effect can also be caused by end products of lipid peroxidation, like malondialdehyde, as demonstrated by Balevska et al. [55]. Although oxidized LDL used in our study were subjected to gel filtration on Sephadex G25 before incubation with the enzyme, the free fatty acids were only partially removed by this procedure. Though not measured, it can be assumed that aldehydic compounds formed during oxidation remained bound to apo B, because most aldehydes tend to form adducts with proteins.

Compared to aging at 6 or 37°C, minimal *in vitro* oxidation of LDL by copper ions or AAPH resulted in a rather moderate increase in susceptibility to phospholipid hydrolysis. Furthermore, even the phospholipids of LDL protected from oxidation during storage by probucol were hydrolyzed approximately 110% more rapidly than the phospholipids of the native lipoproteins. From these findings it can be concluded that not only oxidation but also other modifications associated with aging contribute to the preferred hydrolysis of LDL phospholipids by PLA<sub>2</sub>, group II. Aggregation, for example, which can be observed after storage, or interaction with surfaces of the vials used for storage can cause perturbations of the phospholipid monolayer and facilitate the hydrolytic action of phospholipases.

There is evidence that LDL can be oxidized *in vivo*, particularly when the lipoproteins have entered the subendothelial space. Oxidized LDL-like particles have been isolated from human atherosclerotic plaques [56,57]. Endothelial and smooth muscle cells [12,58,59]

as well as monocytes and neutrophils [60] were shown to be capable of oxidizing LDL *in vitro*. The oxidative action of these cells in atherosclerotic plaques is facilitated by an increased retention of the lipoproteins in arterial walls susceptible to atherosclerosis [61].

Additional modifications of LDL are likely to occur in atherosclerotic plaques and may contribute to an increased susceptibility of LDL phospholipids to hydrolysis by PLA<sub>2</sub>. Interactions with cellular proteoglycans, for example, or the action of proteases and non-secretory phospholipases released from necrotic cells can lead to alterations in shape of the whole lipoprotein particle as well as to local packing defects of the phospholipid monolayer. Thus, it can be assumed that LDL retained in atherosclerotic plaques are a preferred substrate for the secretory phospholipase A<sub>2</sub> detected at the same sites. Hydrolysis of phospholipids by PLA<sub>2</sub> results in substantially altered physicochemical properties of LDL like an increase in negative net charge, in the concentrations of free fatty acids, lysophospholipids and cholesterol at the surface and a decreased fluidity of the phospholipid monolayer [62,63]. These alterations obviously lead to an enhanced degradation of modified LDL by macrophages transforming them into foam cells.

Recent *in vitro*-studies suggest that the increased uptake of PLA<sub>2</sub>-modified LDL by macrophages may not be the only mechanism by which PLA<sub>2</sub> can contribute to the development of atherosclerotic lesions. Several of the biological effects of oxidized LDL that are believed to play a role in the pathogenesis of atherosclerosis, were found to be mimicked by lysophosphatidylcholine, a reaction product of LDL phospholipid hydrolysis. Lysophosphatidylcholine was shown to induce the production of heparin-binding epidermal growth factor by macrophages [64], to enhance expression of the vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells [65] and to be chemotactic for human monocytes [66] and T-lymphocytes [67]. The generation of lysophospholipids in oxidized LDL is mainly attributed to the intrinsic PLA<sub>2</sub> activity of the lipoprotein. The detection of a secretory phospholipase in atherosclerotic plaques suggests that the release of lysolecithin *in vivo* and the resulting biological effects can be substantially increased by the action of this enzyme.

A secondary effect of PLA<sub>2</sub>-induced phospholipid hydrolysis in LDL may be an enhanced oxidation of the lipoproteins as suggested by Parthasarathy et al. [13]. The increased TBARS concentration in LDL after phospholipid hydrolysis by group II PLA<sub>2</sub> observed in our study seemed to support the hypothesis that oxidation and PLA<sub>2</sub> activity are reciprocally reinforcing. Our control experiments with porcine pancreatic phospholipase A<sub>2</sub>, however, have shown that phospholipid hydrolysis does not necessarily promote lipid peroxidation

in LDL. Furthermore, an increase in thiobarbituric acid reactive substances was also observed after incubation of LDL with heat-inactivated group II PLA<sub>2</sub> whereas the active enzyme showed no oxidative effect on linoleic acid. Therefore, the increase in oxidation products in LDL treated with group II PLA<sub>2</sub> is rather due to a non-enzymatic interaction of the enzyme protein with components of the reaction mixtures or with thiobarbituric acid. Further studies are required to elucidate the chemical mechanisms responsible for this phenomenon.

In conclusion, group II phospholipase A<sub>2</sub> detected in atherosclerotic plaques is likely to promote the formation of foam cells by its ability to hydrolyze the phospholipids of LDL, especially when these lipoproteins have been modified by oxidation or other alterations that are likely to occur in the subendothelial space.

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