methods

A simple assay for a human serum phospholipase A_2 that is associated with high-density lipoproteins

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Abstract Phospholipase A_2 (PLA_2) activity is usually assayed with expensive radioactive or chromogenic substrates unsuitable for performing large numbers of assays. We have designed a simple microplate assay for human serum PLA_2 using the chromogenic substrate 4-nitro-3-octanoyloxy-benzoic acid. Using this substrate, serum PLA_2 activity was similar to that measured with the previously characterized chromogenic phospholipid substrate 1,2-bis-heptanoylthio-glycerophosphocholine. However, the assay described here appears to be more sensitive. The mean PLA_2 activity in serum from healthy volunteers (n = 30) measured by this assay was 10.4 ± 1.6 μmol · h^{-1} · ml^{-1}. The assay is reproducible and is suitable for the analysis of large numbers of samples in a clinical setting. We have also demonstrated that 94% of the PLA_2 activity in normal human serum is associated with high-density lipoproteins and that serum PLA_2 activity is positively correlated with the lipoprotein parameters total triglyceride ($P < 0.0001$), total cholesterol ($P < 0.0001$), and atherogenic index ($P = 0.008$). The serum PLA_2 activity was calcium dependent and was inhibited by the serine protease inhibitor 3,4-dichloroisocoumarin (EC_{50} = 0.4 mM). The PLA_2 activity characterized here is unlikely to be due to plasma platelet-activating factor acetylhydrolase or low molecular weight His-Asp $sPLA_2$, and may represent a new $sPLA_2$ type.—Petrovic, N., C. Grove, P. E. Langton, N. L. A. Misso, and P. J. Thompson. **A simple assay for a human serum phospholipase A_2 that is associated with high-density lipoproteins. *J. Lipid Res.* 2001.** 42: **1706–1713.**

Supplementary key words enzyme • substrate • cholesterol • atherogenic

Phospholipase A_2 (PLA_2) plays a crucial role in a number of diverse cellular responses, including phospholipid digestion and metabolism, host defense, and signal transduction, and they also provide precursors for eicosanoid generation (1). PLA_2 comprises a large group of enzymes that share the capacity to hydrolyze fatty acids from the *sn*-2 position of glycerophospholipids. PLA_2 provides precursors for the generation of eicosanoids when the cleaved fatty acid is arachidonic acid and precursors for platelet-activating factor when the *sn*-1 position of the phosphatidylcholine contains an alkyl ether linkage and some bioactive lysophospholipids, such as lysophosphatidic acid or lysophospha-tidylcholine. Mammalian cells generally contain more than one PLA_2, each of which is regulated independently and exerts a distinct effect (2). PLA_2 enzymes are found in all mammalian tissues including plasma and serum.

PLA_2 enzymes are generally classified into two large groups on the basis of their localization, namely, extracellular or secreted ($sPLA_2$) (3) and intracellular or cytosolic ($cPLA_2$) (1). In mammalian extracellular fluids, several types of small (~14 kDa), calcium-dependent $sPLA_2$ enzymes have been characterized (4). Although plasma $sPLA_2$ are physiologically important, they have not been well characterized. It has not been determined how many different PLA_2 enzymes exist in plasma and what are their respective roles in different physiological states. The studies performed to date on plasma PLA_2 vary in their conclusions partly because plasma was taken from a variety of organisms (including humans) and in different physiological states. These include samples from patients with severe inflammatory diseases (5), sepsis, rheumatoid arthritis (6), and acute pancreatitis (7) or from rats treated intravenously with heparin (8). In these reports, the presence of several different PLA_2 enzymes in plasma has been suggested. Furthermore, it was also observed that these enzymes exist in plasma as high molecular weight complexes with unidentified molecular species. Gijon et al. (9) have suggested that in patients with septic shock, plasma $sPLA_2$ could be physically associated with HDL.

Other recent studies also suggest that plasma lipoproteins could be the major source of PLA_2 in plasma or serum. The best-characterized lipoprotein-associated PLA_2 is the type VII enzyme found in plasma under normal

Abbreviations: DCIC, 3,4-dichloroisocoumarin; 1,2bHGPC, 1,2-bis-heptanoylthio-glycerophosphocholine; PAF-AH, platelet-activating factor acetylhydrolase; Pefabloc, 4-[2-aminoethyl]-benzenesulfonyl fluoride; PLA_2, phospholipase A_2; 4N3OBA, 4-nitro-3-octanoyloxy-benzoic acid.

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physiological conditions. This PLA_2 was initially characterized as platelet-activating factor (PAF) acetylhydrolase (PAF-AH) and is also known as human plasma LDL-PLA_2 because it is associated with the LDL class of lipoproteins (10, 11). This enzyme is a single polypeptide containing a lipase consensus motif and is expressed in inflammatory cells, in particular macrophages, or in tissues infiltrated with large numbers of inflammatory cells. In addition to its PAF-AH activity, this enzyme also hydrolyzes phosphatidylcholines with either oxidized or short chain fatty acids at the *sn*-2 position (11). An important characteristic of this enzyme is that it has no requirement for calcium.

There may be other sPLA_2 enzymes, besides LDL-PLA_2, that are associated with lipoprotein particles. Thus, apolipoprotein B-100, an integral component of some lipoproteins (VLDL, IDL, and LDL) may itself possess PLA_2 activity (12, 13). An intrinsic PLA_2-like activity in LDL, which might be LDL-PLA_2 or a completely separate enzyme, has also been proposed (14, 15). Interestingly, human acute phase HDL and LDL are the first reported biological substrates for human group IIA PLA_2, with this activity resulting in the formation of numerous biologically active lipid products (16).

PLA_2 activity is usually assayed with expensive radioactive or chromogenic substrates that are unsuitable for assaying large numbers of patient samples in a clinical setting. The aim of this work was to design and validate a simple microplate spectrophotometric assay for serum PLA_2 using the chromogenic lipid substrate 4-nitro-3-octanoyloxy-benzoic acid (4N3OBA) and examine its relationship to serum lipoprotein parameters. The use of this substrate was originally reported by Cho and Kezdy (17), and it has been subsequently used to measure PLA_2 activity in snake venoms (18–20) and purified immobilized PLA_2 activity (21). 4N3OBA was originally used in a kinetic assay protocol to measure PLA_2 enzyme activity purified from snake venom and porcine pancreas (17). However, the assay as originally described may not be directly applicable to high-throughput screening of enzyme activity in a complex medium such as serum. Therefore, we have optimized the assay with 4N3OBA as substrate to measure PLA_2 activity in serum from healthy volunteers and have demonstrated that this PLA_2 activity is exclusively associated with HDL and is strongly correlated with serum lipoprotein parameters.

METHODS

Human serum collection

Blood samples were collected by forearm venepuncture from healthy volunteers who had fasted overnight. Serum was separated and stored at −70°C until assayed for PLA_2 activity. The study was approved by the Human Research Ethics Committee of the University of Western Australia, and all subjects gave written informed consent.

PLA_2 assays

4N3OBA as substrate. 4N3OBA (Sigma, Castle Hill, New South Wales, Australia) was dissolved in chloroform at a concentration of 50 mg/ml. Aliquots (80 μl, 4 mg) were distributed into Eppendorf tubes and evaporated under vacuum. The dry 4N3OBA residue (4 mg per tube) was stored at −20°C. Immediately before the assay, substrate (4 mg) was resuspended in 1 ml of PLA_2 assay buffer (150 mM KCl, 10 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.5). The suspension was vortexed vigorously for 1 min and centrifuged (2,000 *g*, 2 min) at room temperature, and the supernatant was used as a substrate solution (190 μl) in enzyme assays. There was no obvious micelle formation during substrate solubilization, indicating that 4N3OBA was completely soluble at the final concentration of 2 mM used in the standard assay. Dilutions of serum in a final volume of 10 μl were added to 190 μl substrate solution, and samples were incubated in microtiter plate wells at room temperature for 1 h. The absorbances at 425 and 600 nm (to correct for any turbidity in the sample) were determined on a microplate reader (Molecular Devices, Sunnyvale, CA). In experiments in which PLA_2 activity was expressed as units, one unit was defined by the following equation:

$$PLA_2 \text{ activity } [\mu mol \cdot h^{-1} \cdot ml^{-1}] = (A_{425nm} - A_{600nm})\ [OD_{425nm}/h] \times 0.07862\ [\mu mol/OD_{425nm}] \times (1/\text{serum volume } [1/ml])$$

The correction factor (0.07862) represents the concentration of product (μmoles in 200 μl) producing an absorbance of 1.0 at 425 nm.

1,2-Bis-heptanoylthio-glycerophosphocholine as substrate. Aliquots (250 μg) of 1,2-bis-heptanoylthio-glycerophosphocholine (1,2bHGPC; Cayman Chemical, Ann Arbor, MI) supplied as a solution in methyl acetate were distributed into Eppendorf tubes and evaporated under vacuum, and the dry substrate was stored at −20°C. Immediately before the assay, substrate (250 μg) was resuspended in 190 μl of PLA_2 assay buffer. 5,5′-Dithiobis-2-nitro-benzoic acid (DTNB, Sigma) in PLA_2 assay buffer (1 mM, 40 μl) was added, and the resulting mixture was vortexed vigorously for 1 min and used as a substrate solution (190 μl) in enzyme assays (final substrate concentration 2 mM). In separate experiments we determined that DTNB did not inhibit human serum PLA_2 activity, and it was therefore included in the substrate solution for simplicity. After addition of serum dilutions, samples were incubated in microtiter plate wells at room temperature for 1 h. The absorbances at 415 and 600 nm were measured.

Effects of inhibitors on PLA_2 activity. The protease inhibitor 3,4-dichloroisocoumarin (DCIC; Sigma) was dissolved in DMSO (Sigma) and stored at −20°C. In PLA_2 assays, the effects of DCIC were corrected for the effects of DMSO. A second protease inhibitor, 4-[2-aminoethyl]-benzenesulfonyl fluoride (Pefabloc; Roche Diagnostics, Castle Hill, New South Wales, Australia) was dissolved in PLA_2 assay buffer and stored at −20°C. The effects of these inhibitors on PLA_2 activity were assessed by adding 5 μl of inhibitor solution to the serum/substrate mixture (5 μl/190 μl) followed by incubation at room temperature for 1 h before measurement of the absorbances at 425 and 600 nm.

Isolation of lipoproteins

Lipoprotein classes (HDL, LDL, and VLDL) were isolated by ultracentrifugation of serum through NaBr/NaCl gradients, as previously described (22, 23). Briefly, the serum density was adjusted to 1.26 g/ml by addition of NaBr. The serum was then overlayed on several layers of NaCl and NaBr solutions of decreasing density to produce a discontinuous density gradient that by diffusion becomes a continuous gradient. Lipoprotein fractions isolated from the gradients were desalted on gel permeation PD-10 columns (Sigma) before assay for PLA_2 activity. Lipoproteins were collected at the following gradient densities: VLDL (<1.006 g/ml), LDL (1.006–1.063 g/ml), HDL (1.063–1.210 g/ml).

Fractionation of human serum by size-exclusion Superose 6 chromatography

Serum samples (0.5 ml) were applied to a column (10 ml, 7 × 1.5 cm) packed with Superose 6 gel (Amersham Pharmacia Biotech, Castle Hill, New South Wales, Australia) according to the manufacturer's instructions. The column was equilibrated and run in PLA_2 assay buffer, and chromatography was performed at a flow rate of 0.2 ml/min at room temperature with collection of 0.5-ml fractions.

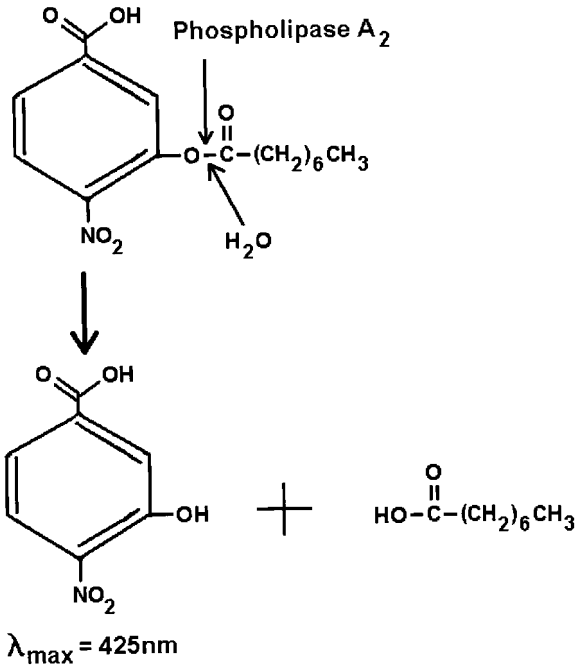
Measurement of lipoprotein parameters

Total cholesterol, HDL cholesterol, and total triglyceride concentrations in human serum samples were determined at the Department of Clinical Biochemistry (PathCentre, Perth, Western Australia). The concentration of LDL cholesterol was calculated according to the following equation:

LDL cholesterol =
total cholesterol − HDL cholesterol − total triglyceride/2.2

Statistical analyses

Results are expressed as means of three independent experiments, each done in triplicate. In independent measurements of PLA_2 activity using the same serum sample, the coefficient of variation did not exceed 10%. In enzyme activity measurements using different serum samples, results were expressed as mean ± standard deviation. Correlation between parameters was evaluated by linear regression. Statistical significance was set at $P < 0.05$.



Scheme 1. Structures of the PLA_2 substrate, 4-nitro-3-octanoyloxybenzoic acid (4N3OBA), and the chromophore, 4-nitro-3-hydroxybenzoic acid, detected at 425 nm.

RESULTS

A simple assay for the determination of serum PLA_2 activity

To determine the correlation between absorbance at 425 nm and chromophore concentration, known amounts of 4N3OBA were subjected to NaOH hydrolysis of the ester bond (**Scheme 1**), causing complete conversion to the chromophore, 4-nitro-3-hydroxy-benzoic acid. The resulting absorbance at 425 nm was measured in a total volume of 0.2 ml using a microplate reader. The relationship between chromophore concentration and absorbance at 425 nm was linear (**Fig. 1A**). A chromophore concentration of 1.0 mM produced an absorbance at 425 nm of 2.54 ± 0.04 OD. This correlation factor was used to calculate the concentration of chromophore produced as a result of PLA_2 activity and thereby the unit of PLA_2 activity.

The solubility of 4N3OBA in aqueous solutions is limited. To determine its maximum solubility, known amounts of 4N3OBA were resuspended in PLA_2 reaction buffer. The suspension was centrifuged (2000 *g*, 2 min) to remove undissolved substrate, and the concentration of dissolved 4N3OBA was determined by quantitative hydrolysis with NaOH. The maximum solubility of 4N3OBA in PLA_2 assay buffer was 3.14 ± 0.15 mM. In subsequent assays for PLA_2 activity, 2 mM 4N3OBA was chosen as the standard substrate concentration. In a separate experiment, the reproducibility of substrate solubilization was determined by repeated resuspension of 4 mg of dry 4N3OBA in 1 ml of assay buffer, centrifugation to remove insoluble material, and measurement of the soluble substrate concentration by quantitative hydrolysis with NaOH. The mean 4N3OBA concentration was 1.93 mM (SD 0.097, coefficient of variation 5%, n = 9), indicating that interassay variation in the final substrate concentration obtained by this method was not significant. Furthermore, as shown in Fig. 1B, a substrate concentration of 2 mM was sufficient to saturate PLA_2 enzyme activity under the assay conditions used.

To determine the optimum incubation time, different amounts of serum were assayed for PLA_2 activity with incubation times ranging from 5 to 120 min (Fig. 1C). The PLA_2 enzyme reaction showed biphasic kinetics for all serum concentrations. An initial fast phase during the first 40 min was followed by a slower linear reaction rate for incubation times >40 min. For subsequent assays of PLA_2 activity, 60 min was chosen as the standard incubation time. One unit of serum PLA_2 activity was defined as the formation of 1 μmol of product by 1 ml of serum during 1 h of incubation at room temperature in the standard microplate assay.

To determine the optimum amount of serum in the assay, different amounts of serum were incubated for 60 min in the standard PLA_2 assay (Fig. 1D). A linear relationship between serum concentration and product formation was observed when the volume of serum did not exceed 2.5 μl per 200 μl of reaction mixture and the final absorbance did not exceed 0.325 OD (Fig. 1D). Therefore, in subsequent PLA_2 assays, values were accepted only if the absorbance at 425 nm did not exceed 0.325 OD. If a higher absorbance was observed, then the serum was diluted and the assay repeated.

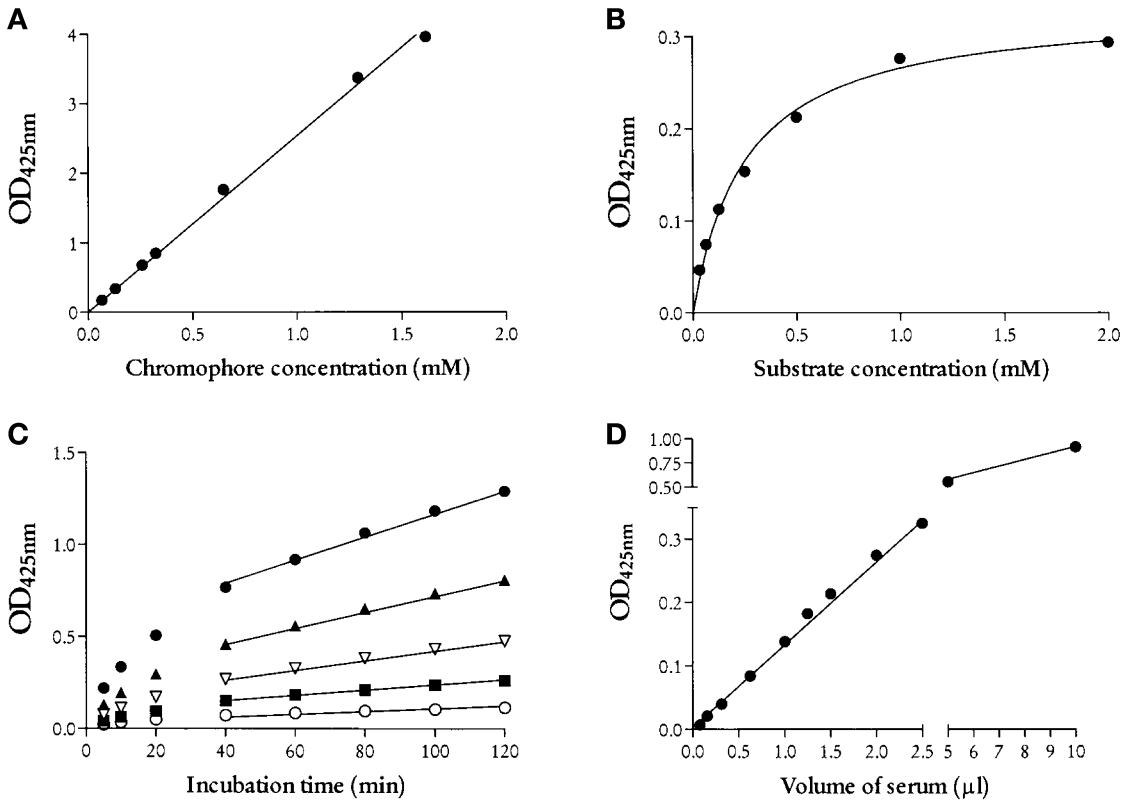


Fig. 1. Optimization of the human serum PLA_2 assay. A: Relationship between absorbance at 425 nm and chromophore concentration. Known concentrations of 4N3OBA were subjected to quantitative NaOH hydrolysis. B: Substrate concentration dependence of the serum PLA_2 activity assay. A range of 4N3OBA concentrations were incubated with 2 μl serum for 60 min. C: Time dependence of the serum PLA_2 activity assay. Different amounts of serum [0.625 μl (open circle), 1.5 μl (solid square), 2.5 μl (open triangle), 5 μl (solid triangle) and 10 μl (solid circle) were incubated with 2 mM 4N3OBA for a range of times (5–120 min). D: Serum concentration dependence of the PLA_2 activity assay. A range of serum volumes (0.1–10 μl) were incubated with 2 mM 4N3OBA for 60 min. All experiments were performed in a final volume of 0.2 ml in microtiter plates, and the absorbance at 425 nm was measured on a microplate reader. Each point is the mean of three independent experiments, each performed in triplicate.

Identification of HDL as a major source of serum PLA_2 activity

To identify the molecular characteristics of the human serum enzyme(s) exhibiting PLA_2 activity, initial gel permeation chromatography experiments were performed using Sephadex G-100. In these experiments, PLA_2 activity eluted in the column void volume without any PLA_2 activity being detected in the lower molecular weight fractions (data not shown). To further characterize the higher molecular weight species with PLA_2 activity in serum, we performed gel permeation chromatography on Superose-6 (5–5000 kDa separation range). To eliminate potential differences between individual serum samples, we used a pooled sample from 30 healthy volunteers. In these experiments, a single peak of PLA_2 activity was detected using both the artificial substrate 4N3OBA and the structurally more appropriate phospholipid substrate 1,2bHGPC (**Fig. 2A**). However, 4N3OBA was a more sensitive substrate for the measurement of serum PLA_2 activity than was 1,2bHGPC (Fig. 2A). In separate experiments, activity assays optimized for each substrate showed that 4N3OBA was five times more sensitive for assaying PLA_2 activity in human serum samples compared with 1,2bHGPC (data not shown).

To test the hypothesis that serum lipoproteins might be the carriers of PLA_2 activity, HDL, LDL, and VLDL were separated from normal human serum, and Superose 6 chromatography was performed on each of these fractions to determine the elution profiles of the three lipoprotein types. Only HDL eluted in a similar molecular weight range to serum PLA_2 activity, whereas LDL and VLDL eluted in the higher molecular weight range (Fig. 2A).

To further substantiate the findings that PLA_2 activity might be linked with serum HDL, we determined PLA_2 activity in purified HDL, LDL, and VLDL samples from healthy volunteers (n = 3) (Fig. 2B). Most of the total lipoprotein PLA_2 activity (HDL, LDL, and VLDL combined) was detected within the HDL fraction (9.848 ± 1.497 units or 94%) (Fig. 2B). LDL and VLDL contained 4.9% (0.516 ± 0.128 units) and 1.1% (0.114 ± 0.042 units) of the total PLA_2 activity, respectively. The total PLA_2 activity in the HDL fractions was similar to the total serum PLA_2 activity measured in a pooled sample from 30 healthy volunteers (10.40 ± 1.56 units). These findings, taken together, suggest that most of the PLA_2 activity in normal human serum is associated with HDL.

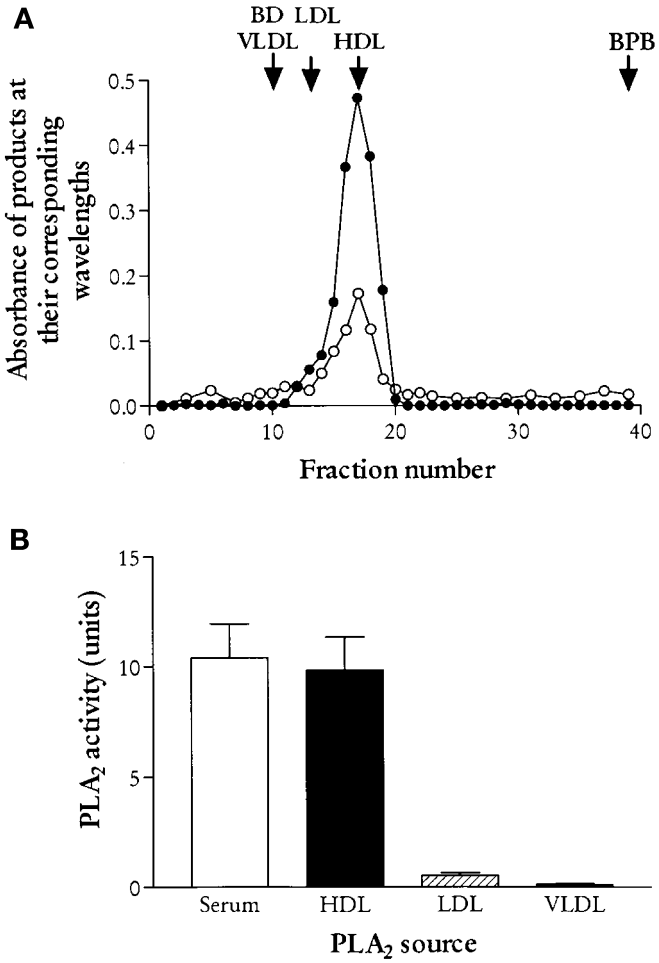


Fig. 2. Identification of HDL as the major source of human serum PLA_2 activity. A: Superose-6 gel permeation chromatography of human serum was performed using pooled serum from 30 healthy volunteers. PLA_2 activity in the eluted fractions was determined using 4N3OBA (solid circle) and 1,2-bis-heptanoylthioglycerophosphocholine (1,2bHGPC) (open circle) as substrates (both at a concentration of 2 mM). Absorbance was measured at 425 nm for 4N3OBA and at 415 nm for 1,2bHGPC. The elution positions of the three lipoprotein fractions (HDL, LDL, and VLDL) are indicated (arrows). The elution positions for Blue Dextran (BD), indicating column void volume, and for bromphenol blue (BPB), indicating the low-molecular-weight end of the separation range, are also shown (arrows). Each point is the mean of three independent experiments. B: PLA_2 activity in purified lipoprotein fractions. PLA_2 activity was determined in purified HDL, LDL, and VLDL fractions from healthy volunteers (n = 3) and compared with total serum PLA_2 activity from healthy volunteers (n = 30). The unit of PLA_2 activity in lipoprotein fractions is corrected for the initial volume of serum from which these lipoproteins were isolated. Bars represent the mean (± standard deviation) of three independent experiments, each performed in triplicate.

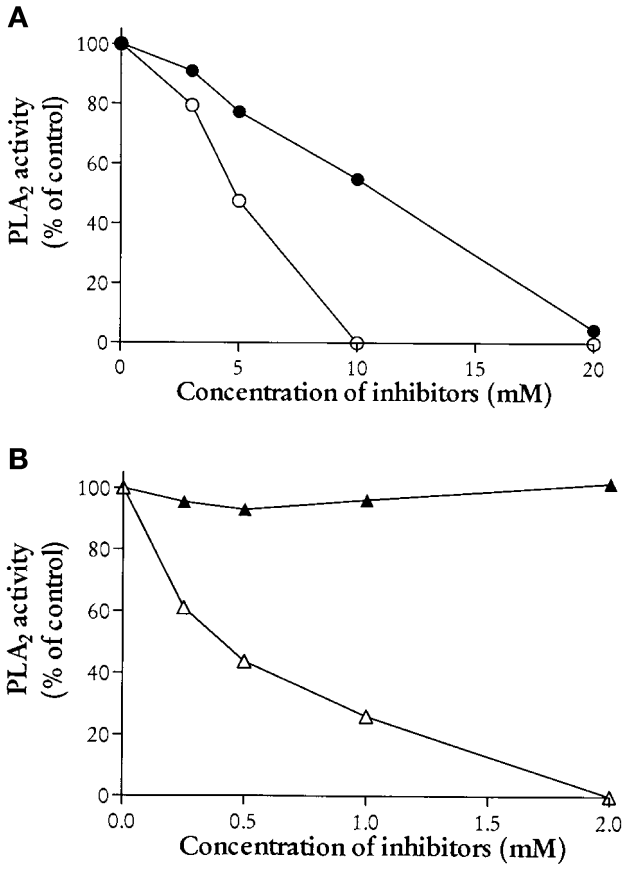


Fig. 3. Inhibition of serum PLA_2 activity. A: The effects of increasing concentrations of EDTA (solid circle) and EGTA (open circle) in assay buffer (150 mM KCl, 10 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.5) on PLA_2 activity in pooled serum from healthy volunteers (n = 30). PLA_2 activity is expressed as a percentage of the activity in a control sample without EDTA or EGTA, and each point is the mean of three independent experiments, each performed in triplicate. B: The effects of increasing concentrations of 3,4-dichloroisocoumarin (DCIC) (open triangle) and Pefabloc (solid triangle) on PLA_2 activity in pooled serum from healthy volunteers (n = 30). PLA_2 activity is expressed as a percentage of the activity in a control sample without inhibitor, and each point is the mean of three independent experiments, each performed in triplicate.

Inhibitors of serum PLA_2 activity

To further characterize human serum PLA_2, we determined the effects of several known PLA_2 inhibitors (**Fig. 3**). Serum PLA_2 activity was not inhibited by manoalide or 4-bromo-phenacyl bromide, which have previously been reported to inhibit the known calcium-dependent small molecular weight PLA_2 (24, 25) (data not shown). However, the PLA_2 activity detected in our assay appears to be calcium dependent, because EDTA and EGTA inhibited this activity (Fig. 3A). EGTA and EDTA inhibited PLA_2 activity in the presence of 10 mM calcium with EC_{50} values of 5 and 11 mM, respectively.

The effect of DCIC, which has been reported to inhibit lipoprotein-linked serine-dependent PLA_2 activity (11), was also determined. DCIC inhibited serum PLA_2 activity with an EC_{50} of 0.4 mM (Fig. 3B). The irreversible serine protease inhibitor Pefabloc has been reported to efficiently inhibit both human and rat PAF-AH and to totally inactivate human plasma-, VLDL-, IDL-, LDL-, and HDL-associated PAF-AH (LDL-PLA_2) (26, 27). In contrast, Pefabloc, at concentrations up to 2 mM, did not inhibit the serum PLA_2 activity measured in our assay (Fig. 3B).

The possibility that the PLA_2 enzyme activity assayed in our experiments is due to a known secretory PLA_2

(type II or type V) was investigated by determining the effects of blocking polyclonal antibodies to PLA_2 IIA and PLA_2 V on serum PLA_2 activity. Neither of these antibodies had any inhibitory effect on the PLA_2 activity (data not shown).

It is unlikely that the PLA_2 activity observed in our study is due to lipoprotein lipase because 1M NaCl did not inhibit serum PLA_2 activity, whereas it has been shown to inhibit lipoprotein lipase activity (28). Furthermore, serum PLA_2 activity was not inhibited by tetrahydrolipstatin, a potent inhibitor of serine lipases (29).

Correlation between serum lipoprotein parameters and PLA_2 activity

To further assess the relationship between serum lipoproteins and PLA_2 activity, we collected fasting serum samples from 30 healthy volunteers. PLA_2 activity as well as serum lipoprotein-related parameters (total cholesterol, LDL cholesterol, HDL cholesterol, total triglyceride concentration, and atherogenic index) were determined in these samples. Atherogenic index is defined as the ratio of total cholesterol concentration to HDL concentration. Linear regression analysis showed that serum PLA_2 activity was positively correlated with total triglyceride (r^2 = 0.6244, $P < 0.0001$), total cholesterol (r^2 = 0.4651, $P < 0.0001$), and LDL (r^2 = 0.3213, P = 0.0011) concentrations (**Fig. 4A**) and with atherogenic index (r^2 = 0.225, P = 0.0081) (Fig. 4B). Interestingly, serum PLA_2 activity did not correlate significantly with HDL cholesterol concentration (P = 0.4236).

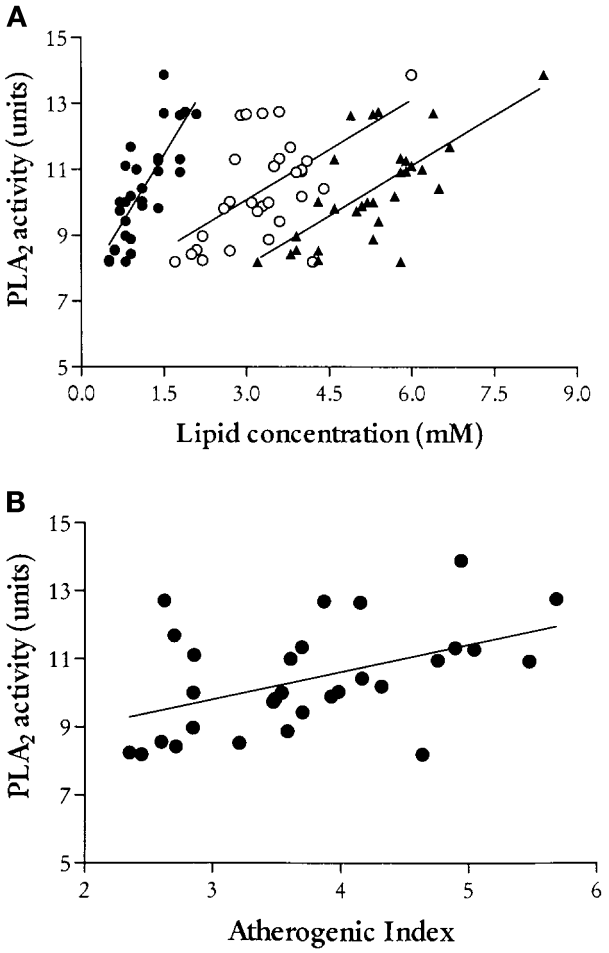


Fig. 4. Correlation between serum PLA_2 activity and serum lipoprotein-related parameters. PLA_2 activity, total cholesterol (solid triangle), LDL cholesterol (open circle), total triglyceride concentrations (solid circle) (A) and atherogenic index (total cholesterol/HDL) (B) were determined in serum samples from healthy volunteers (n = 30). PLA_2 activity was correlated with triglyceride (r^2 = 0.6244, $P < 0.0001$), total cholesterol (r^2 = 0.4651, $P < 0.0001$), and LDL (r^2 = 0.3213, P = 0.0011) concentrations and with atherogenic index (r^2 = 0.225, P = 0.0081). Each point is the mean of three independent experiments, each performed in triplicate.

DISCUSSION

Our findings suggest that the chromogenic substrate 4N3OBA can be successfully used to measure PLA_2 activity in human serum. Cleavage by PLA_2 of the ester bond between the octanoyl group and the chromophore 4-nitro-3-hydroxy-benzoic acid results in absorbance at 425 nm (17).

Here we describe a simple PLA_2 assay that employs 4N3OBA and microplate spectrophotometric measurement of PLA_2 activity. The assay does not require radioactive or expensive substrates and is suitable for the analysis of large numbers of patient samples in a clinical setting. PLA_2 assays performed on human serum fractions separated by Superose-6 chromatography gave similar results using 4N3OBA compared with the previously characterized chromogenic phospholipid PLA_2 substrate 1,2bHGPC (30), although 4N3OBA appears to be a more sensitive substrate.

Biochemical characterization of the molecular species exhibiting PLA_2 activity in normal human serum indicates that the majority of serum PLA_2 appears to be physically associated with HDL. Several previous reports support the conclusion that serum HDL could be the major source of $sPLA_2$ in normal serum. The $sPLA_2$ activity has been previously shown to be associated with HDL in patients with septic shock (9). Furthermore, acute phase HDL has been shown to be the primary natural substrate for type IIA $sPLA_2$ (16), and transgene overexpression of $sPLA_2$ IIA specifically increases HDL catabolism in acute and chronic inflammatory conditions (31, 32).

In spite of our findings that serum PLA_2 is found almost exclusively on HDL particles, serum PLA_2 activity did not show a significant correlation with HDL cholesterol, whereas significant correlations were observed with total cholesterol, total triglyceride, and LDL cholesterol concentrations. The observed correlation with LDL cholesterol could be a consequence of the correlation with total cholesterol. The absence of a correlation with HDL cholesterol suggests that the PLA_2 activity of HDL is not related to total HDL concentration but rather to an as yet undetermined functional or structural property of HDL particles, possibly associated with total cholesterol or triglyceride levels. Structurally and functionally, HDL are a heterogeneous population of lipoproteins, particularly during inflammation and acute phase responses. Thus,

the acute phase protein serum amyloid A is also expressed during inflammation and associates with HDL, altering its physicochemical composition. Acute phase serum amyloid A, but not the constitutive form, markedly enhances $sPLA_2$ activity (33).

Considering the established cardioprotective role of HDL, an associated proinflammatory enzyme such as $sPLA_2$ could be involved in catabolism of HDL and the consequent loss of its anti-inflammatory properties. In the acute phase response and in diseases with prolonged inflammation, normal HDL is converted into acute phase HDL, which is proinflammatory and unable to protect LDL against oxidative modification (34). These changes in HDL metabolism could be responsible for the depressed HDL levels in chronic inflammatory diseases. In support of such a hypothesis, it has been reported that HDL from transgenic mice and human HDL treated with recombinant $sPLA_2$ failed to protect against the formation of biologically active phospholipids in LDL (35).

In addition, biologically active lipids formed as a result of PLA_2 action on serum lipoproteins may also affect cellular function. Thus, lysophosphatidylcholine, which is generated as a consequence of PLA_2 activity during LDL oxidation, is a potent chemotactic factor for monocytes (36). Enzymatic generation of lysophosphatidylcholine by $sPLA_2$ using plasma lipoprotein phospholipids as a substrate is essential for the $sPLA_2$-mediated inhibition of platelet activation (37). Incubation of LDL with purified soybean lipoxygenase, in the presence of pure PLA_2, can mimic endothelial cell-induced oxidative modification leading to enhanced recognition of LDL by macrophages (38). Our finding that serum PLA_2 activity correlated positively with atherogenic index supports the hypothesis that the HDL-associated PLA_2 characterized in this work has a proatherogenic role.

Although there is some evidence to suggest that $sPLA_2$ IIA may be the PLA_2 associated with HDL, the PLA_2 activity characterized in this work does not appear to belong to this type. Thus, serum PLA_2 activity was not inhibited by manoalide or 4-bromo-phenacyl bromide, which have previously been reported to inhibit the known calcium-dependent small molecular weight PLA_2 (24, 25). However, the PLA_2 detected in our assay appears to be calcium dependent, and this is one of the main biochemical characteristics of PLA_2 IIA. The previously characterized calcium-dependent $sPLA_2$ enzymes that have histidine- and asparagine-dependent active sites have not been reported to be inhibited by DCIC, an inhibitor of serine-dependent PLA_2 activity. However, DCIC did inhibit the serum PLA_2 activity identified in our experiments. Furthermore, blocking polyclonal antibodies to PLA_2 IIA and PLA_2 V did not inhibit the PLA_2 enzyme activity that we measured in serum. Experiments using antibodies to block the activity of an enzyme bound to a lipoprotein particle should be interpreted with caution. Lack of specificity of the antibody for the active site, or steric hindrance and inaccessibility of the active site to the antibody, may limit the ability of the antibody to block enzyme activity. Nevertheless, taken together with the data obtained with low molecular weight PLA_2 inhibitors, our findings strongly suggest that this enzyme is not a type IIA or type V PLA_2. The possibility that HDL-associated PLA_2 activity is the result of several distinct PLA_2 types within the lipoprotein particle cannot be excluded. Further studies involving purification of HDL-associated PLA_2 will be needed to specifically address that issue.

The PLA_2 activity characterized in this work is unlikely to be due to the previously characterized plasma PAF-AH or LDL-PLA_2 that were found to be calcium-independent enzymes, a characteristic not observed in our PLA_2 assays. Furthermore, the new irreversible serine protease inhibitor, Pefabloc, which efficiently inhibits both human and rat PAF-AH and totally inactivates human plasma-, VLDL-, LDL-, and HDL-associated PAF-AH (LDL-PLA_2) (26, 27) did not inhibit serum PLA_2 activity.

We also tested the possibility that the observed PLA_2 activity might be due to the action of lipoprotein lipase, although in our experiments we did not use post-heparin plasma, which usually contains high levels of this enzyme. Serum PLA_2 activity was not inhibited by 1 M NaCl, which has been reported to inhibit lipoprotein lipase (28). Furthermore, PLA_2 activity was not inhibited by tetrahydrolipstatin, a potent inhibitor of serine lipases (29).

Enzymes within lipoproteins are just beginning to be characterized, and their physiological roles are still unclear. Circulating lipoproteins contain a number of associated enzymes other than PLA_2, including lecithin cholesterol acyl transferase, phospholipid transfer protein, cholesterol ester transfer protein, and paraoxonase 1. Together with lipoprotein lipase and hepatic lipase, these enzymes are involved in lipoprotein remodelling and modulation of lipoprotein structure and function in normal physiological conditions as well as in inflammation and the acute phase response (39).

Lipoproteins and PLA_2 play key roles in a number of important processes in mammalian organisms, and further studies are therefore necessary to characterize the structural and functional interactions between these molecules in a variety of physiological states.

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