Increased Phospholipase A Activities in Sera of Intensive-Care Patients Show sn-2 Specificity but No Acyl-Chain Selectivity

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Phospholipase A (PLA) activities were measured by high-performance liquid chromatography in two enzyme preparations purified from human duodenal juice and a serum pool as well as in 52 sera from 31 intensive-care patients with various diseases. On the basis of a position-specific fatty acid analysis of the natural substrate ("soybean lecithin") from a commercial PLA kit, serum activities of PLA, could be clearly distinguished from those of PLA2, which is not possible in the usual measurements made with single-label radioactive substrates. Independent of the type of disease, all sera with highly increased PLA activities (40–200 U/L) showed nearly pure PLA2 characteristics without any preference among oleic, linoleic, and linolenic acid in the sn-2 position of the glycerophospholipid substrate. Nevertheless, very low PLA1 activities (<5 U/L, most likely due to heparin perfusion therapy) could also be detected by palmitic and stearic acid release from the sn-1 position, leading to small changes in fatty acid release patterns of sera with low PLA activities. Measurements with sera from heparin-treated volunteers demonstrated that heparin therapy may initially contribute as much as 22 U/L to increased PLA1 activities but is not important under prolonged therapy. The absence of selectivity with respect to acyl-chain desaturation supports the concept of serum PLA2 as an acute-phase protein rather than a regulator of the arachidonic acid cascade.

Indexing Terms: enzyme activity · isoenzymes · lecithin · glycerophosphate substrate · heparin perfusion therapy · acute-phase proteins · variation, source of · fatty acids · chromatography, thin-layer · chromatography, reversed-phase

Recently, increased phospholipase A (PLA) activities have been detected in sera of patients with various diseases such as myocardial infarction, infections, severe diabetes, pancreatitis, leukemia, cancer, and schizophrenia (1, 2).6 Serum PLA concentrations have also been shown to correlate with the mortality of intensive-care patients (2, 3) with pancreatitis (4), multiple injuries (5), or lung failure (6). The source and function of this serum PLA remain unclear. Increased concentrations of serum PLA, originally thought to be exclusively of pancreatic origin (7), were found even in pancreatectomized patients (8). Immunoassays with antibodies against human pancreatic PLA2 (EC 3.1.1.4) (9) also suggested the existence of a serum PLA2 of nonpancreatic origin. Speculations about its source included inflammatory cells such as granulocytes and monocytes or thrombocytes (3, 10). Recent results favor serum PLA being a secretion product of hepatocytes during the acute-phase reaction (11).

The acyl-chain selectivity of some human cellular PLA2s, e.g., for arachidonic acid, is controversial (see 12 and references cited therein), and no data on such properties of serum PLA2 have been available until now. A clear distinction between PLA2, phospholipase B (PLB; EC 3.1.1.5), and a PLA1/lyso phospholipase (EC 3.1.1.3/3.1.1.5) combination was also not possible because most measurements of serum PLA activities were performed with radioactively monolabeled substrates (see 2 and references cited therein). However, clinical investigations of serum PLA activities have been facilitated since the introduction of a commercial test involving a micellar soybean lecithin as substrate and photometric detection of the liberated fatty acids in total (13).

In this study, we investigated the positional and acyl-chain specificities of two purified human PLAs and of PLA in sera of intensive-care patients with various diseases through the use of fatty acid and phospholipid analysis of the above-cited soybean lecithin substrate. Furthermore, we wanted to estimate the influence of a prolonged heparin therapy on the increased concentrations of serum PLA because a PLA2 from the plasma membrane of the liver is known to be released into the blood by heparin (14, 15).

Materials and Methods

Materials

Lipid standards, fatty-acid-free bovine serum albumin (BSA), PLA2 from bee venom (Sigma, Deisenhofen, Germany), PLA2 from porcine pancreas, test kit for the photometric determination of phosphorus (Boehringer Mannheim, Mannheim, Germany), phosphomonoxylic acid (Fluka, Buchs, Switzerland), solvents in "pro analysis" quality, 20 × 20 cm glass plates coated with Silica 60 for thin-layer chromatographic (TLC) analyses (without fluorescent indicator; Merck, Darmstadt, Germany) and a test kit for the enzymatic determination of nonesterified fatty acids ("NEFA C": Wako Chemicals, Neuss, Germany) were purchased as indicated.

PLA2 of human origin was purified from duodenal juice (16) and pooled serum (17) as described. The PLA
test kit, containing the lyophilized phospholipid substrate, a buffer solution, a stop reagent, and a lyophilized control serum, was a kind gift from Boehringer Mannheim.

Sera

Serum samples were taken from 22 men and 9 women patients (median age: 40 years, range: 21 to 72 years) at an anesthesiological intensive-care unit (Klinikum Mannheim, Mannheim, Germany). The subjects had multiple injuries (n = 9), pancreatitis (n = 10), septicemia (n = 13), peritonitis (n = 3), and (or) lung failure (n = 14) as the main diagnoses. PLA activities were monitored several times a week until convalescence or death. Sera from a group of 16 students (7 men, 9 women; median age: 24 years, range: 21 to 36 years) served to estimate the normal range of PLA activities.

Four apparently healthy volunteers received an intravenous bolus of 5000 IU of heparin. Blood was drawn from these subjects before and 10, 30, 60, 120, 240, and 360 min after injection.

TLC Analysis of Substrate Phospholipids

The lipid composition of the PLA kit substrate was investigated by TLC from a total lipid extract (18) of 100 μL of the micellar substrate solution to which had been added 100 nmol of heptadecanoic acid, 50 nmol of diheptadecanoyl phosphatidylethanolamine, and 50 nmol of diheptadecanoyl phosphatidylcholine in 20 μL of methanol. Plates were developed twice with CHCl₃/CH₃OH/NH₄OH (16 mol/L), 75/25/5, by vol, then split in two in the middle. On one half of the plate, lipid spots were made visible by staining with phosphomolybdate (19); the corresponding areas on the symmetrically loaded other half were scratched off for fatty acid analysis by HPLC and phosphorus determination (20). An acid eluent, CHCl₃/CH₃OH/CH₃COOH/H₂O (78/30/9.6/4.8, by vol), was also used to develop the TLC plates, to confirm the identity of the spots.

Fatty acids in the sn-1 and sn-2 positions of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were determined by incubating 500 μL of the micellar substrate solution at 37 °C with 3.5 U of PLA₂ from bee venom or porcine pancreas in 100 μL of isotonic saline containing BSA, 40 g/L. After 5, 20, 60, and 240 min, we pipetted 100-μL aliquots of the incubation solution into 50 μL of the stop reagent (15 mmol/L EDTA in 50 mmol/L phosphate buffer, pH 5.5), added 100 nmol of heptadecanoic acid, extracted the total lipids (18), and separated the phospholipids by TLC as above. Free (nonesterified) fatty acids (FFA) and lysophospholipid spots were analyzed for fatty acid composition by HPLC (see below).

PLA Activities and Fatty Acid Release Patterns

We incubated at 37 °C 100 μL of serum or enzyme preparations in isotonic saline containing 40 g/L BSA with 500 μL of the buffered (Tris · HCl, 125 mmol/L, pH 8.0) micellar substrate solution from the PLA test kit (13). After 5 min (twice), 20 min, and 65 min, we pipetted 100-μL aliquots into 50 μL of the stop reagent, then measured FFA concentrations enzymatically (5- and 20-min aliquots) and by the HPLC method (5- and 65-min aliquots). We added 20 μL of a 5 mmol/L solution of heptadecanoic acid in methanol as internal standard to the samples processed further for HPLC analysis. Samples with activities >60 U/L were diluted two- or fourfold with BSA/isotonic saline. Blank incubations were done in the same manner with 100 μL of the BSA/isotonic saline instead of serum (reagent blank) or with 100 μL of serum and 500 μL of a detergent/buffer solution containing deoxycholate 1 mmol/L, Triton X-100 2.5 g/L, CaCl₂ 4 mmol/L, and Tris · HCl 125 mmol/L at pH 8.0 (serum blank). PLA activities (U/L) were calculated by:

\[
PLA = D \cdot \frac{[FFA(t₂) - FFA(t₁)]}{(t₂ - t₁)}
\]

where D = serum dilution factor (=9) and FFA(t) = FFA concentration, μmol/L, in stopped aliquots after incubation times of t₁ and t₂ (5 and 20 or 5 and 65 min, respectively). Total PLA activity measured by HPLC was the sum of the activities calculated for each of the five fatty acids in the substrate.

HPLC Determination of Single Fatty Acid Concentrations

FFAs were extracted from 100 μL of stopped PLA-incubation aliquots by our modified Dole extraction (21) or from FFA-containing TLC spots by methanol and determined as p-bromophenacyl derivatives by isocratic reversed-phase HPLC with acetonitrile/water (77/23, by vol) on a C₁₈ 3-μm-particle column exactly as described recently for serum FFA (21). The HPLC injection volume was 20 μL. Overall recoveries of the internal standard C₁₇:0 were usually 90-95%.

Esterified fatty acids extracted from 100 μL of the micellar substrate solution or from the scratched off TLC spots were first hydrolyzed in 1 mL of KOH/ethanol at 70 °C and extracted with n-hexane according to a standard procedure (22) then derivatized with p-bromophenacyl bromide and determined by HPLC as described for the FFAs above (21).

Enzymatic Determination of Total FFA Concentrations

We reacted 70 μL of the stopped PLA reaction solutions with 175 μL of solution A and 350 μL of solution B of the NEFA C test kit according to the manufacturer's instructions. The test is based on an acyl-CoA synthetase and acyl-CoA oxidase reaction, with photometric detection of the H₂O₂ formed by a condensation reaction (23).

Results

Analysis of the substrate. The commercial substrate used for the PLA determinations was first analyzed by TLC for lipid composition (see reagent blanks in Figure 1). PE and PC are the major phospholipid components, with concentrations of ~3.5 mmol/L each according to their fatty acid and phosphorus determinations in the lipid spots. There were only traces (<2%) of FFA or
lyso phospholipids in the TLC assay, which clearly separates neutral lipids, FFA, PE, PC, LPE, and LPC. There were two spots very close together, more polar than LPC in the alkaline run, and three spots, less polar than FFA, that did not change remarkably during incubations with various PLAs, even when incubated much longer than 1 h (e.g., serum PLA in Figure 1).

Fatty acid analysis (Figure 2, Table 1) of the alkaline hydrolysat e of the total substrate by HPLC showed that five C₁₈–C₁₉ fatty acids make up >98% of the total fatty acid content. This fatty acid pattern is very similar to that of the components PE and PC. Gas-chromatographic analysis performed by the method of Lepage and Roy (24) yielded essentially the same results (not shown). The enzymatic hydrolyses of the substrate with PLA₂ from bee venom and porcine pancreas liberated mostly linoleic acid, as well as oleic and linolenic acids, but no saturated fatty acids. We obtained identical results for both enzymes with no time dependency of the fatty acid patterns released. The alkaline hydrolysates of the lyso phospholipids formed during the enzymatic reaction contained the saturated fatty acids. A significantly greater percentage of palmitic acid was found in LPE than in LPC.

**Table 1. Fatty Acid Analysis of Phospholipids in Soybean Lecithin Substrate**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Total substrate</th>
<th>PE</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:0</td>
<td>17.1 &lt;1</td>
<td>16.8 33.5</td>
<td>14.4 24.4</td>
</tr>
<tr>
<td>18:1</td>
<td>3.4 &lt;1</td>
<td>2.3  5.0</td>
<td>3.7  7.0</td>
</tr>
<tr>
<td>18:1ω9</td>
<td>8.0  8.0</td>
<td>7.2  6.2</td>
<td>8.8  8.4</td>
</tr>
<tr>
<td>18:2ω6</td>
<td>63.0 81.9</td>
<td>68.9 48.9</td>
<td>65.2 53.9</td>
</tr>
<tr>
<td>18:3ω3</td>
<td>7.4  8.4</td>
<td>6.8  6.3</td>
<td>8.0  8.3</td>
</tr>
</tbody>
</table>

* Each value is the mean of 8 determinations. All standard deviations were <1%.

**Characteristics of the analytical methods.** The enzymatic and the chromatographic method for determining total serum PLA activities correlated well (Figures 3 and 4). The lower values (about 14%) gained with the HPLC method probably reflect the four times longer incubation period of 1 h [as well as the underestimation of ~5% caused by incomplete extraction of linoleic and linolenic acid (21)]. The longer incubation was chosen to enhance the sensitivity of the HPLC method so that a FFA release of 6.7 ×mol/L (after addition of the stop reagent) corresponds to a PLA activity of 1 U/L. The use of reagent blanks led to detection limits (mean + 3 SD) of 2 U/L for the enzymatic method (n = 10) and about 0.5–1 U/L per fatty acid species in the HPLC method (n = 6). The intraassay CVs for the enzymatic method (n = 8) were 3.9% at 55 U/L and 12.8% at 13 U/L for the HPLC method (n = 10) these were 4.0% and 7.7%, respectively. CV ranges for single fatty acid activities (HPLC, n = 10) were 25–10% for activities between 1 and 5 U/L and 10–20% at >5 U/L. Day-to-day imprecisions were 11.3% (HPLC, n = 6) and 12.5% (enzymatic, n = 10) for assays of fresh thawed portions of a frozen serum with an activity of 55 U/L.

**Reference values.** All sera from a group of 16 apparently healthy students led to PLA activities below or close to the detection limit of 2 U/L (as measured with the enzymatic method). Only two sera liberated linoleic acid in concentrations above the detection limit of 1 U/L (1.5 and 2.5 U/L) if the HPLC method was applied, but the values for the corresponding palmitic acid releases (0.6 and 0.4 U/L, respectively) were too low to differentiate between PLA₁ and PLA₂. Therefore, we estimated a “normal range” for serum PLA of 0–3 U/L, which is
Incubations with sera of patients. The sera of patients with highly increased PLA activities (>40 U/L) all showed patterns of fatty acid release essentially identical with that of the purified PLa₁₂₂; i.e., no dependence on the type of disease was noticeable. Nevertheless, in many cases very low releases of palmitic acid, <1.7 U/L, were measurable; these did not correlate with the total PLA activity but did influence the percentage pattern of released fatty acids in sera with lower PLA activities (Figure 5, d and e). Serum blanks, even from sera with PLA activities >100 U/L, were all below the detection limit of 1 U/L. Serum PLA from an intensive-care patient with multiple injuries apparently preferred PE over PC as substrate, as shown by the TLC analysis in Figure 1.

The influence of heparin on PLA activities was investigated in healthy volunteers who received an intravenous bolus injection of 5000 IU of heparin. Total PLA 10 min after injection was as great as 22 U/L but dropped to <5 U/L after several hours, with a half-life of about 50 min (Figure 6). In contrast to the sera of intensive-care patients, the postheparin sera liberated palmitic (32.4%) and stearic acid (6.1%) from the substrate (Figure 5f). There was no significant difference between fatty acid patterns from samples drawn 10 and 60 min after heparin injection.

Discussion

The prerequisite for interpretation of the fatty acid patterns released by PLA was the knowledge of the phospholipid and fatty acid composition of the commercial substrate. It might be an advantage of this substrate to contain not only “soybean lecithin” (as declared by the manufacturer), but also an approximately equimolar mixture of PE and PC, because mammalian PLAs very often prefer PE over PC (25). The other fatty-acid-containing components were apparently not degraded by PLAs from bee venom, porcine pancreas, or serum; therefore, we focused our interest on the analysis of PE and PC.

Bee venom and porcine pancreas PLAs liberate all fatty acids in the sn-2 position of PE and PC in the substrate because of their absolute sn-2 specificity, regardless of the acyl compositions at positions 1 and 2 (26). Because PE is the initially preferred substrate (TLC results not shown), but the pattern of released fatty acids is unchanged until both components are completely degraded, the acyl composition of the sn-2 position should be essentially identical for PE and PC. The fatty acid patterns found in the alkaline hydrolysates of the enzymatically produced lysophospholipids reflect the sn-1 substitution of PE and PC. Only here were palmitic and stearic acid found. Release of these fatty acids may therefore serve as an indicator for sn-1 activity (PLa₁, PLB). The alkaline hydrolysates of PE and PC confirm the positional analyses, the calculated fatty acid percentages (sn-1 + sn-2) being approximately the means of both positions.

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activities (>40 U/L) in sera of intensive-care patients released fatty acid patterns identical to that of the sn-2 position of the substrate and should therefore be classified as PLA₂. This is the first clear evidence for such a classification of serum PLA activities: measurements with monolabeled substrates do not allow one to exclude PLB or PLA₁/lysophospholipase A₂ combinations (27). To our knowledge, experiments with double-labeled substrates have been done only with postheparin plasma (28, 29) but not with sera from intensive-care patients.

Theoretically, PLA₁ or PLB activities with exclusive specificity for unsaturated acyl chains and a simultaneous slight preference for linoleic acid also could "mimic" the sn-2 pattern from the sn-1 pattern. This would require a shift in the molar ratio of unsaturated fatty acids (18:1/18:2/18:3) from about 1/8/1 to 1/10/1 during the release. This theoretical possibility seems very unlikely, given that there is no evident physiological necessity and also no example for such PLA₁ or PLB activities, which usually have broad substrate specificities (14). The PLA character is demonstrated by the simultaneous appearance of FFA and lysophospholipids during the incubation with serum in Figure 1. The combined action of a phospholipase C and (e.g.) pancreatic lipase (which reacts well with diglycerides) would produce only monoglycerides.

The low values for serum blanks (<1 U/L), which we obtained even for sera with very high PLA activities (up to 218 U/L), suggest that endogenous phospholipids do not seem to be good substrates for serum PLA₂ under the conditions chosen (detergents, serum dilution 1:6, pH 8.0), perhaps because of their binding to lipoproteins.

The low release of palmitic acid (<1.7 U/L) by sera of many of the intensive-care patients is explained by the low PLA₁ activities in these patients. A PLA₁ produced
by hepatocytes and located at the outer side of the plasma membrane of liver and endothelial cells is known to be released into the blood by heparin (14). Most intensive-care patients had received heparin from a perfusor as prophylaxis against disseminated intravascular coagulation. Because palmitic acid accounts for about one-third of the fatty acids in the sn-1 position of PE (the preferred substrate) and because the enzyme does not possess acyl-chain selectivity (14, 28, and our results below), the PLA\(_1\) activity in a serum might be roughly estimated by:

\[
\text{PLA} \approx 3 \times \text{palmitic acid release, U/L}
\]

Therefore, the PLA\(_1\) concentrations in the plasma of the intensive-care patients in this study would be in a range of 0–5 U/L.

Our findings in the heparin-treated volunteers support the above interpretation. The heparin-induced serum PLA activities are comparable with values reported earlier by Becker et al. (30) and release a fatty acid pattern that matches very well that of the sn-1 position of PE, the preferred substrate (14; and own results, TLC runs not shown), confirming the PLA\(_1\) classification already done by Vogel and Bierman (29). The elimination kinetics, with a mean half-life of ~50 min, probably reflect a redistribution of the enzyme to the endothelial membranes of the vessel walls caused by the elimination of heparin (\(t_{1/2} \approx 50–60\) min). A displacement of other enzymes, e.g., lipoprotein lipase, has been described that was clearly related to the kinetics of heparin (31). Nevertheless, a complete elimination from the circulation by other mechanisms is also possible.

We conclude, therefore, that a contribution of heparin infusion therapy to increases in PLA\(_2\) activities is initially possible but not important with prolonged therapy, as indicated by the low PLA\(_1\) activities in the intensive-care patients. The constant fatty acid patterns—released from either position—show the absence of acyl-chain selectivity of the involved PLAs, at least among the five C\(_{16}-\)C\(_{18}\) fatty acids occurring in the soybean substrate. This behavior parallels results by Schalkwijk et al. (12), who showed that cytosolic PLAs\(_2\) from human platelets and polymorphonuclear leukocytes, two candidates discussed as sources for serum PLA, possess no or low acyl-chain selectivity. Our preliminary results (unpublished) from experiments with Escherichia coli membranes enriched with polyunsaturated fatty acids as substrate suggest that serum PLA\(_2\) from intensive-care patients also does not prefer arachidonic or eicosapentaenoic acid over the other physiologically occurring fatty acids. The two- to threefold higher activities gained with bacterial membranes as substrates in comparison with the micellar substrate resemble again findings with PLAs\(_2\) from platelets and synovial fluid (10, 32, and references cited therein). We speculate that the task of the PLAs in sera of intensive-care patients is to destroy bacterial membranes rather than to regulate the arachidonic acid cascade, an idea that supports also the acute-phase concept by Crowl et al. (11).

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References