Immunoochemical Detection of Group I and Group II Phospholipases A₂ in Human Serum

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Time-resolved fluoroimmunoassay was developed for the detection of synovial-type phospholipase A₂ (s-PLA₂) in human serum. This solid-phase, sandwich assay uses a polyclonal rabbit antibody raised against synovial-type group II PLA₂ produced in Escherichia coli. No cross-reactions were detected between s-PLA₂ and PLA₂ from human or porcine pancreas, human ascitic fluid, or bee or cobra venom. In healthy individuals, the average concentration of s-PLA₂ is 3.7 µg/L, with a 95% reference interval from 1.3 to 10.8 µg/L. We investigated pancreatic PLA₂, which is a group I PLA₂, and synovial-type group II PLA₂ in sera of patients with hematological malignancies and septic fever. The concentration of s-PLA₂ was increased in patient sera and correlated significantly with the catalytic activity of PLA₂ and the concentration of C-reactive protein. No correlation with the concentration of pancreatic PLA₂ was found. The results suggest that the increased catalytic activity of PLA₂ in sera of patients with septic fever results from synovial-type group II PLA₂.

Additional Keyphrases: fluoroimmunoassay · C-reactive protein · carcinoma

Phospholipase A₂ (PLA₂) catalyzes the hydrolysis of the acyl ester bond on the sn-2 position of phosphoglycerides. The enzyme is widespread in bacteria, plants, snake and bee venoms, and mammalian cells and secretions (1, 2). Rich sources of PLA₂ include pancreatic tissue and juice (3, 4) and inflammatory exudate (5). PLA₂ is an enzyme of low molecular mass, ~14 kDa, and requires the presence of calcium ions for its enzymatic activity. Pancreatic and venom PLA₂s are divided into two groups on the basis of the amino acid sequence of the enzyme proteins (6). Group I PLA₂s contain a cysteine at position 11 that forms a disulfide bridge with a cysteine at position 77, whereas group II enzymes lack these cysteines and the corresponding disulfide bridge. Group I PLA₂s are those found in the pancreas, Elapidae (cobra) and Hydrophiidae (sea snake) venoms, and group II enzymes are found in Crotalidae (rattlesnake) and Viperidae (old-world viper) venoms (7).

PLA₂ was postulated to be associated with the pathologies of several diseases involving infection, tissue damage, and inflammation, such as acute pancreatitis (8, 9), septic shock (10), peritonitis (11), and multiple injuries (12). The cellular source of the nonpancreatic PLA₂ found in serum is unknown.

The genes of both group I (13) and group II PLA₂ (14, 15) were cloned and localized in chromosomes 12 and 1, respectively (16, 17). Oligonucleotide probes deduced from the N-terminal amino acid sequence were used in cloning the gene of human synovial fluid (14) and human platelet PLA₂ (15). The amino acid sequences of these enzyme proteins deduced from the cDNAs are closely related to each other and to pancreatic PLA₂. The synovial fluid and platelet PLA₂s lack cysteines at positions 11 and 77 and therefore are classified as group II PLA₂.

We recently raised an antibody to human synovial fluid-type PLA₂ (s-PLA₂) produced by a recombinant technique in Escherichia coli (Nevalainen et al., J Rheumatol, in press). This antibody inhibited PLA₂ in synovial fluid of patients suffering from rheumatoid arthritis, and PLA₂s immunoreactivity was found in articular and laryngeal cartilage by immunohistochemistry. In the present study, we developed a time-resolved fluoroimmunoassay (TR-FIA) for the detection of s-PLA₂ in human body fluids and investigated the presence of group I and group II PLA₂s in sera of patients with hematological malignancies and septic fever. We found considerably increased catalytic PLA₂ activities in many patients sera and a highly significant correlation between the catalytic PLA₂ activity and the concentration of the immunoreactive synovial-type group II PLA₂ but not the pancreatic group I PLA₂.

Materials and Methods

Instrumentation

Time-resolved fluorescence was measured with an Arcus fluorometer (Wallac, Turku, Finland). Radioactivity was counted with a liquid scintillation counter (Rackbeta, Wallac). The plate washer (Wellwash) and plate shaker (Wellmixin 3) used in TR-FIA were from Denley (Billinghurst, UK).

Reagents

L-α-Dipalmitoylphosphatidylcholine and PLA₂ were obtained from Sigma Chemical (St. Louis, MO). L-α-Dipalmitoyl-phosphatidylcholine (50 kCi/mol) was from DuPont NEN (Boston, MA). Human pancreatic PLA₂ and ascitic fluid PLA₂ were purified as described earlier (13, 18, respectively). Microtitration plates were from Eflab (Helsinki, Finland), and assay buffer for TR-FIA was from Wallac.
Serum samples. Sera for the determination of the reference interval of immunoreactive s-PLA₂ were obtained from 59 healthy volunteers (26 women and 33 men), average age 41 years (range 20-64 years). Serum samples were obtained from 11 patients (4 women and 7 men, 66 samples), average age 54 years (range 25-76 years) with malignant hematological diseases. Six patients had multiple myeloma, two had acute myeloid leukemia, two had acute lymphatic leukemia, and one had non-Hodgkin lymphoma. The patients were neutropenic because of cytotoxic drug therapy and had fever and increased concentrations of C-reactive protein (CRP) in their sera. Bacterial blood cultures were negative. All sera were stored frozen at −70 °C until assayed.

PLA₂ standards. PLA₂ standards were prepared from recombinant s-PLA₂ in a solution containing 0.15 mol of NaCl, 0.5 mg of NaN₃, and 10 mg of bovine serum albumin per liter of 50 mmol/L Tris-HCl buffer, pH 7.75.

Procedures

Preparation of platelet and leukocyte homogenates. Leukocytes were separated from 40 mL of buffy coat representing 450 mL of blood from a healthy donor. Erythrocytes were sedimented with 13 mg/L dextran at room temperature. The leukocyte-rich supernate was centrifuged at 300 × g for 10 min.

Platelets were separated from platelet-rich plasma representing 450 mL of blood from a healthy donor. Leukocytes were first sedimented by centrifugation at 300 × g for 10 min, and platelets were separated from the supernate at 2500 × g for 15 min. Isolated leukocytes and platelets were homogenized in 10 mmol/L sodium phosphate buffer, pH 7.6 (200 g of cell suspension per liter of buffer), and centrifuged at 10 000 × g for 1 h at 4 °C. The supernate was stored at −20 °C until assayed.

Labeling of anti-recombinant PLA₂ antibody. A polyclonal antibody to recombinant s-PLA₂ was raised in a rabbit as described (Nevalainen et al., J Rheumatol, in press). Protein A-purified anti-recombinant PLA₂ antibody was labeled with an isothiocyanate derivative of a europium chelate [Eu³⁺-N-(p-isothiocyanatobenzyl)-diethylene-triamine-N⁷,N²,N⁹,N⁹-tetraacetate] by using a Eu-labeling kit (Wallac) according to the manufacturer's instructions.

Time-resolved fluoroimmunoassays. For the TR-FIA, microtitration plates were coated overnight with Protein A-purified anti-recombinant PLA₂ antibody (25 µg/mL in 50 mmol/L Tris-HCl, pH 7.75 containing 0.15 mmol/L NaCl and 0.5 g/L NaN₃, 200 µL/well) treated with three volumes of HCl/water (125 µL of 11.6 mol/L HCl in 50 mL of water) for 5 min. The coated plates were washed two times, and 25 µL of recombinant PLA₂ standard (0, 1.5, 9, 54, and 324 µg/mL) or sample was pipetted into the wells containing 175 µL of assay buffer. After 1-h incubation with shaking at room temperature, followed by washing six times, the Eu-labeled anti-recombinant PLA₂ antibody (2.5 µg/mL in assay buffer, 200 µL/well) was added. The washing step was repeated after 1 h, and 200 µL of enhancement solution was added. Fluorescence was measured after an additional 5 min of shaking and 10 min of standing.

The concentration of pancreatic PLA₂ was measured by TR-FIA by use of a monoclonal anti-pancreatic PLA₂ antibody as the catching antibody and a polyclonal sheep antibody as the detecting antibody (19).

Measurement of PLA₂ catalytic activity. The catalytic activity of PLA₂ was measured by use of a mixture of unlabeled dipalmitoyl phosphatidylincholine (6 mmol/L) and 2-[^3H]palmitoylphosphatidylincholine as a substrate (20). Serum samples (10 µL) were assayed in duplicate. The radioactivity of free fatty acids released from the substrate during the incubation was measured in a liquid scintillation counter.

Measurement of C-reactive protein. The concentration of CRP in the serum samples was determined by immunoturbidimetry. Commercial antisera and calibrators (Orion Diagnostica, Helsinki, Finland) were used in the assay. The upper limit of the reference interval is 10 mg/L.

Results

Time-Resolved Fluoroimmunoassay for Synovial-Type PLA₂

Standard curve, precision profile, and assay range. The standard curve for the TR-FIA for s-PLA₂ and the precision profile calculated from 12 replicates for each PLA₂ concentration are shown in Figure 1. The coefficient of intra-assay variation ranged from 3.7% to 19.4%. The sensitivity of the assay was determined to be 0.65 µg/L. This concentration corresponds to the mean fluorescence of zero standard (12 replicates) plus 3 SD.

Analytical recovery. Two different concentrations (10 and 100 µg/L) of recombinant s-PLA₂ were added to three serum samples with different concentrations of s-PLA₂. The percentage recovery of s-PLA₂ was calculated as (amount of added PLA₂ found/amount of PLA₂ added) × 100. The analytical recoveries were 107.2% and 112.5%, respectively, with an overall mean of 109.9% (Table 1).

![Fig. 1. Standard curve (○) and precision profile (■) for human s-PLA₂.](https://example.com/figure1.png)
Table 1. Analytical Recovery of Recombinant S-PLA$_2$

<table>
<thead>
<tr>
<th>Concentration of s-PLA$_2$, µg/L</th>
<th>Added PLA$_{a}$</th>
<th>Added PLA$_{b}$</th>
</tr>
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<tbody>
<tr>
<td>49.0</td>
<td>128.5</td>
<td>117.6</td>
</tr>
<tr>
<td>27.9</td>
<td>96.0</td>
<td>112.4</td>
</tr>
<tr>
<td>5.9</td>
<td>97.1</td>
<td>107.6</td>
</tr>
</tbody>
</table>

*The recovery of recombinant PLA$_a$ added to three serum samples with different endogenous synovial-type PLA$_2$ concentrations was determined by time-resolved fluoroimmunoassay. The overall mean analytical recovery was 109.9%.

**Linearity.** Statistical evaluation of linearity was performed with the BMDP software library following the guidelines of National Committee for Clinical Laboratory Standards' Document EP6-P. Sera from two pools with s-PLA$_2$ concentrations of 850.3 and 3.9 µg/L, respectively, were mixed in proportions that resulted in calculated s-PLA$_2$ concentrations (n = 7) from 8.2 to 427.1 µg/L. Measurements performed in quadruplicate indicated linearity of the test to a calculated concentration of 215.5 µg/L.

**Specificity.** Cross-reactions of anti-recombinant PLA$_2$ antiserum with PLA$_{a,b}$ from various sources were studied. The antiserum did not react with PLA$_{a,b}$s from human or porcine pancreas, human ascitic fluid, or bee or cobra venom; at 50% saturation, the cross-reactivities were 0.10%, 0.20%, 0.11%, 0.25%, and 0.04%, respectively (Figure 2). The mean concentration of s-PLA$_2$ in leukocyte and platelet homogenates (200 g of cells per liter; n = 5) was 2.5 (SD 0.4) and 8.3 µg/L (SD 3.4), respectively.

Concentrations of s-PLA$_2$ in Sera of Healthy Individuals

The concentration of s-PLA$_2$ was determined in sera of 59 apparently healthy individuals (26 women and 33 men). Because of the skewed distribution of the s-PLA$_2$ values (Figure 3), the data were log-transformed. The obtained geometric mean of the s-PLA$_2$ values was 3.7 µg/L with a 95% reference interval from 1.3 to 10.8 µg/L. We saw no sex-related difference in s-PLA$_2$ values of healthy individuals.

Concentrations of s-PLA$_2$ in Sera of Patients with Septic Fever

Figure 4 shows the catalytic activity and concentrations of s-PLA$_2$ in sera of patients with hematological malignancies and septic fever. Increased concentrations of s-PLA$_2$ were observed in 77% (51 of 66) of the samples. The PLA$_2$ catalytic activity was above normal (>2 U/L, 21) in 94% (62 of 66). As shown in Figure 5,
there was a highly significant positive correlation between the concentration of immunoreactive s-PLA\(_2\) and the catalytic activity of PLA\(_2\) \((r = 0.98, P < 0.001)\).

The concentrations of CRP and pancreatic phospholipase A\(_2\) (p-PLA\(_2\)) in sera of the patients were also measured (Figure 4). The concentration of CRP was above normal (\(>10\) mg/L) in 94% (62 of 66) of the samples. CRP correlated well with the catalytic activity of PLA\(_2\) \((r = 0.77, P < 0.001)\) and with the concentration of s-PLA\(_2\) \((r = 0.78, P < 0.001)\) (Figure 6). The concentration of p-PLA\(_2\) was increased \((>9\) \(\mu\)g/L; 19) in one patient only. There were no correlations between p-PLA\(_2\) and s-PLA\(_2\) \((r = -0.1, P = 0.423)\) or p-PLA\(_2\) and the PLA\(_2\) catalytic activity \((r = -0.37, P = 0.77)\).

Discussion

Group I and group II PLA\(_2\)s are not only structurally unique proteins but also functionally distinct enzymes. p-PLA\(_2\) (group I) functions as a digestive enzyme within the intestinal lumen (22, 23) but is nontoxic (24, 25). Mammalian group II PLA\(_2\) occurs intra-articularly in synovial fluid, platelets, placenta and inflammatory cells, and exudate (15). The molecular masses of extracellular (secretory) group I and group II PLA\(_2\)s are \(\sim 14\) kDa. PLA\(_2\)s of greater molecular mass have been purified, for example, from monocyte cell lines (26). The latter are intracellular enzymes that possibly are responsible for arachidonic acid release from membrane phospholipids rather than the secretory group II PLA\(_2\)s (27).

The TR-FIA described here is specific for synovial-type group II PLA\(_2\). The antibody used in this assay was raised in a rabbit against human s-PLA\(_2\) produced by a recombinant technique in E. coli (Nevalainen et al., J Rheumatol, in press). The anti-s-PLA\(_2\) antibody did not react with PLA\(_2\) from human pancreas or ascitic fluid, and only tiny amounts of immunoreactivity were found by this assay in leukocyte and platelet homogenates.

Increased catalytic activity of PLA\(_2\) in serum is associated with many diseases involving infection, tissue destruction, and inflammation, such as septic shock, acute pancreatitis, rheumatoid arthritis, and multiple injuries (8–12). PLA\(_2\) is effectively eliminated from the circulation: the half-life of intravenously injected pancreatic PLA\(_2\) in rat plasma is \(\sim 3\) min, and the administered enzyme protein can be found in the proximal tubule cells of the kidney by immunohistochemistry (28). Nephrectomies in experimental animals (28) and renal insufficiency in humans (29) slow down the elimination and increase the enzyme concentrations in plasma. However, in inflammatory diseases, PLA\(_2\) activity in serum increases without concomitant renal failure.

Exocrine pancreas is a well-known source of increased concentration of PLA\(_2\) in sera of patients with acute pancreatitis (8, 30) and pancreatic cancer (31). In our study, the concentration of pancreatic PLA\(_2\) was not increased in sera of patients with hematological malignancies and septic fever. On the other hand, increased concentrations of s-PLA\(_2\) were observed in 55 of 66 serum samples from these patients. The catalytic activity of PLA\(_2\) was increased in 62 of 66 samples and showed significant positive correlations with the concentrations of s-PLA\(_2\) and CRP \((r = 0.98\) and \(r = 0.78\), respectively). Our results support the view that the increase of the catalytic activity and the concentration of s-PLA\(_2\) in serum could represent an acute-phase response (32). We also found slightly increased s-PLA\(_2\) concentrations in 2 of 59 serum samples from healthy individuals (11 and 25 \(\mu\)g/L; the upper limit of the reference interval is 10.8 \(\mu\)g/L).

Nonpancreatic group II PLA\(_2\) may be released into the extracellular compartment from a variety of sources. mRNA of group II PLA\(_2\) was demonstrated in several human tissues (15). Polymorphonuclear leukocytes (33), platelets (34), and chondrocytes (35) are believed to secrete group II PLA\(_2\). We found s-PLA\(_2\) by immunohistochemistry in chondrocytes of articular and
laryngeal cartilage but not in rheumatic synovial tissue, which contains various inflammatory cells including polymorphonuclear leukocytes (Nevalainen et al., unpublished observations, 1992). This observation supports the view that polymorphonuclear leukocytes are not the source of circulating PLA₂ (36, 37). Our results show that the amount of s-PLA₂ released by homogenization from polymorphonuclear leukocytes is negligible. In addition, the patients had malignant hematological diseases and were leukopenic as a result of treatment with cytotoxic drugs. The concentration of s-PLA₂ in homogenates of platelets from healthy individuals was low. These and earlier results suggest that platelets in humans are not a quantitatively important source of PLA₂ found in serum (38).

In conclusion, our results show that the concentration of synovial-type group II PLA₂ is greatly increased in sera of patients with septic fever and correlates remarkably well with the catalytic activity of PLA₂ and with the concentration of CRP in these samples. The source of the circulating group II PLA₂ is unknown.

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References