Development and Use of ELISA to Quantify Type II Phospholipase A2 in Normal and Uremic Serum

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Previously we reported that uremic plasma contained eight times more phospholipase A2 (PLA2) activity than control plasma (Costello et al., Clin Chem 1990;36:198–200). That study, however, did not distinguish between various PLA2s that could contribute to the observed increase. Therefore, we developed a sandwich ELISA to specifically quantify serum type II PLA2. By ELISA, uremic sera contained significantly more type II PLA2 than control sera (median = 1025 μg/L, range = 52–3320 μg/L vs median = 9.2 μg/L, range = 4.6–17.5 μg/L; P = 0.002). When serum samples were incubated with 1-[^14C]oleate-labeled autoclaved Escherichia coli, activity was increased 14.6-fold in uremic vs normal serum, with a median of 6.5 μmol/min per liter (range 1.1–16.3) vs a control median of 0.49 μmol/min per liter (range 0.32–0.60; P = 0.002). Thus, ELISA detects about eightfold more immunoreactive type II PLA2 in uremic serum than does enzymatic analysis. Evidently, the increase in the PLA2 activity previously observed in uremic plasma is primarily due to increased concentrations of type II PLA2.

Indexing Terms: uremia/immunoassay/enzyme activity

Phospholipase A2 (PLA2; EC 3.1.1.4) catalyzes the hydrolysis of the acyl ester bond at the sn-2 position of phospholipids. In mammalian cells this activity releases predominately cis-unsatuated fatty acids and the corresponding lysophospholipid, products that perturb the membrane bilayer. Activation of these enzymes contributes to membrane phospholipid turnover and alterations in fluidity of membranes, and is considered to be the rate-limiting step for the mobilization of arachidonic acid to initiate the eicosanoid cascade (1). Two major classes of PLA2s are thought to contribute to arachidonic acid mobilization in human cells and tissues (2). A hormonally sensitive, 85-kDa PLA2 found in the cytosol is a prime candidate in the regulation of arachidonate mobilization, since it associates with cell membrane upon agonist stimulation and may be regulated by phosphorylation (3). The other major enzyme, a 14-kDa type II secretory PLA2 (sPLA2), is typically granule-associated (4), and the newly synthesized protein contains a signal peptide that may be responsible for its subcellular localization, secretion from cells, and its role as a potential extracellular effector enzyme (4). Thus, purified human 14-kDa PLA2 not only is proinflammatory and induces edema and (or) synovitis when injected into animal joints (5, 6), but also reportedly functions in synergy with other cytokines to stimulate arachidonic acid mobilization when added extracellularly (7). For these reasons, then, increased concentrations of type II PLA2 in biological fluids may contribute to various pathologies.

Indeed, numerous studies have reported increased concentrations of PLA2 in various human disease states (8–12). For example, PLA2 activity is increased 2–40-fold vs controls in various forms of arthritis (8), renal failure (9), malaria (10), and septic shock (11; for review see ref. 12). However, these reports measured enzymatic activity and generally did not distinguish between the various types of PLA2s that could account for the observed increase. More recently, immunochemical methods such as RIA and ELISA have been used to demonstrate an increase in immunoreactive PLA2 in serum/plasma from patients with pancreatitis (13), arthritis (14), shock, and hematologic malignancies (15).

Previously we reported that the concentrations of PLA2 activity in uremic plasma (9) were increased up to eightfold compared with normal. In these studies, we noted that dilution of either control or uremic plasma increased total enzymatic activity three- to fourfold. These results suggested that the amounts of enzymatic activity in undiluted plasma, and perhaps in other crude biological fluids, are consistently underestimated because of the presence of components that interfere with enzymatic analysis. In this report, we describe a specific sandwich ELISA to quantify type II PLA2 in human serum and demonstrate with this immunochemical method that the increase of PLA2 activity in uremic serum is due to increased amounts of the proinflammatory, secretory enzyme.

Materials and Methods

Antibody Preparation

Monoclonal antibodies were prepared by our institutional hybridoma facility by established methods involving purified human sperm PLA2 (16). Ascites fluid was precipitated with 400 g/L ammonium sulfate and further purified by anion-exchange chromatography. The monoclonal antibody was subtyped as IgG1.

Male New Zealand White rabbits (2.3 kg) were used to generate polyclonal antibodies by using purified human vertebral disc sPLA2. After the initial subcutaneous injection of 127 μg of purified human disc sPLA2 in complete Freund’s adjuvant, the rabbits were
boosted at 3 and 6 weeks with an additional 127 μg of the enzyme in incomplete Freund’s adjuvant. After 13 weeks the rabbits were bled, and serum was processed and stored at −20°C (17). Serum was subjected to Protein A column chromatography, and the immunoglobulin fraction was eluted with 0.1 mol/L glycine buffer, pH 3.0. Purified polyclonal antibody (2.0–2.5 g/L) was stored at −20°C.

Western Blot Analysis

Proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were electroblotted to polyvinylidene difluoride membranes (Millipore, Bedford, MA) for immunologic detection. The amounts of PLA2 applied to each lane were: human sperm, vertebral disc, and synovial fluid sPLA2 (1 μg); and porcine pancreatic, Naja naja, and Crotalus adamanteus (7 μg). A 1:50 (by vol) dilution of primary monoclonal antibody and then a 1:2000 (by vol) dilution of horseradish peroxidase (HRP)-conjugated secondary antibody were used. Diaminobenzidine was used as the substrate for the peroxidase reaction.

Sandwich ELISA

Serum concentrations of type II PLA2 were measured by a sandwich ELISA essentially as described by Voller et al. (18). Mouse monoclonal antibody was first used as the capture antibody, rabbit polyclonal antisera the secondary antibody, and goat anti-rabbit IgG conjugated with HRP (IgG-HRP) as the tertiary, reporter antibody. Costar (Cambridge, MA) microtiter plates were coated overnight at 4°C with primary antibody (4 mg/L) in 100 μL of isotonic saline (9 g/L NaCl) and 100 mmol/L sodium bicarbonate buffer, pH 9.6. Plates were then decanted and blocked with 100 μL of 50 g/L Carnation Instant dry milk (milk) in isotonic saline for 90 min at 25°C. Plates were washed five times with 180 μL of 0.5 mL Tween 20 in isotonic saline, and then incubated with 100 μL of serum sample or PLA2 calibrator. After a 90-min incubation at 25°C, plates were washed five times (as above) and then incubated for 90 min with 4 μg of secondary antibody in 100 μL of 50 g/L milk in saline. Plates were then washed five times (as above) and incubated with IgG-HRP at 1:1000 (by vol) (Cappel, West Chester, PA) in 100 μL of 50 g/L milk in saline for 90 min. Plates were washed five times (as above), and HRP activity was measured by using 2,2’-‐azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid) as substrate (18). Reaction mixtures were incubated for 15 min at 25°C and the absorbance was measured at 405 nm. Purified human sperm type II PLA2 was used as a calibrator; a linear relation between absorbance and enzyme quantity was obtained between 0.2 and 5.4 ng/well.

Measurement of PLA2 Activity

PLA2 activity was measured by an established method involving autoclaved 1-14C]oleate-labeled Escherichia coli (1). Briefly, reaction mixtures contained: 10 nmoL of labeled E. coli phospholipid (10 000 dpm), 5 mmol/L CaCl2, 50 mmol/L sodium acetate, pH 6.0, and 10–80 μL of normal or uremic serum. Concentrations of protein and time of incubation were adjusted to ensure linear kinetics. Samples were extracted and processed as previously described (9). Activity was expressed as μmol/min per liter. Human seminal fluid type II PLA2 calibrator was purified by cation-exchange and reversed-phase HPLC, and its purity and protein content were assessed by amino acid composition, N-group, and sequence analysis. The purified type II PLA2 had a single asparagine N-group, and the sequence for the N-terminal 26 amino acid residues was identical to that reported for type II PLA2 purified from various human sources by similar methods (19, 20).

Protein Determination

Antibody concentration was determined by absorbance at 280 nm (an absorbance reading of 1 = 1.6 g/L). Protein concentration for Western blot analysis was determined by the method of Bradford (21).

Blood

Blood was obtained immediately before dialysis from consenting patients by the hemodialysis unit at the Medical College of Virginia, Richmond, VA. Serum from 11 patients currently undergoing dialysis treatments was used for this study. Healthy subjects (n = 10) on no known medication were used as controls.

Blood was collected and serum was obtained and stored in sterile microfuge tubes at −20°C. Snake venom and porcine pancreatic PLA2s were purchased from Sigma Chemical Co. (St. Louis, MO).

Statistics

SAS Institute (version 6) statistical procedures were performed by the Department of Biostatistics at Virginia Commonwealth University for the analysis of variance and linear regression. Wilcoxon nonparametric analysis was performed to determine P values for nongaussian data populations.

Results

Antibody Specificity

Figure 1 illustrates the specificity of the mouse anti-human monoclonal antibody toward various PLA2s by Western blot analysis. In reducing SDS-PAGE gels, the 14-kDa type II PLA2 migrates as a single band with an apparent molecular mass of 16–17 kDa. The mouse monoclonal antibody at 1:1000 (by vol) dilution readily detects 1 μg of sPLA2 purified from human sperm, vertebral disc, and synovial fluid; the lower limit of detectability was 0.25–0.5 μg. Porcine pancreatic (type I), N. naja (type I), and C. adamanteus (type II) were not detected at concentrations ≤7 μg. A similar profile was obtained with the rabbit anti-human type II PLA2 polyclonal antibody (data not shown). These results demonstrate that both antibody
preparations are highly specific for human 14-kDa type II PLA₂.

Calibration Curve, Precision, Accuracy and Analytical Recovery

Mouse monoclonal antibody was used to initially bind the type II PLA₂ in a sandwich ELISA in which the rabbit polyclonal served as the secondary antibody. The detection of increasing concentrations of purified human seminal type II PLA₂ by sandwich ELISA is shown in Fig. 2. With goat anti-rabbit IgG-HRP as a tertiary antibody, the absorbance is linear with protein in the concentration range of 0.2–5.4 ng/well, and is nonlinear when the absorbance exceeds 1.3.

Accuracy and precision of the sandwich ELISA was determined by performing duplicate experiments with four replicates/dose per day for 20 days (40 individual experiments). For 5, 20, and 80 μg of type II PLA₂ per liter, the within-run CVs were 17.5%, 10.9%, and 8.9%, whereas the between-run CVs were 13.7%, 14.4%, and 10.4%, respectively. The limit of detection, defined as the concentration corresponding to the mean absorbance of the conjugate + 3 SD, was 2.1 μg/L sPLA₂.

Analytical recovery was determined by adding 10, 25, and 50 μg/L purified sPLA₂ to plasma containing 11–16 μg/L type II PLA₂. The analytical recoveries of enzyme, defined as micrograms of recovered sPLA₂ divided by micrograms of added sPLA₂ times 100, were 116.9%, 96.9%, and 87.6%, respectively (mean 100.4%).

Linear regression analysis of the calibration curve (not shown) yielded a y-intercept of 0.16 A₄₀₅nm and a slope of 0.14 A₄₀₅nm/pg sPLA₂; SEE was 0.092 and r² was 0.95.

sPLA₂ Concentrations in Uremic Serum

Uremic and control sera were analyzed by the sandwich ELISA to measure the content of type II PLA₂ (Figs. 3 and 4). Type II PLA₂ was readily detected in undiluted control serum (median = 9.2 μg/L, range 4.6–17.5 μg/L). By contrast, undiluted uremic serum yielded absorbance values ≥2.0; therefore, dilutions of 50- to 400-fold were necessary for quantification (Fig. 3). Immunochemical concentrations of type II PLA₂ in uremic serum were increased ~111-fold relative to control (Fig. 4); uremic serum contained a median of 1025 μg/L (range 52–3320 μg/L, n = 11) vs a control median of 9.2 μg/L (range 4.6–17.5 μg/L, n = 13). These data demonstrate high concentrations of immunoreactive type II PLA₂ in uremic serum and an extraordinarily wide range (52–3320 μg/L) of serum concentrations in patients with renal failure. Interestingly, the type II PLA₂ content of uremic serum was comparable before and 20 min after heparinization during the early stages of the dialysis procedure, and was increased 1.5- to 2-fold at the termination of dialysis in three of four patients tested (data not shown).

We previously reported that PLA₂ in uremic plasma was increased eightfold relative to normal plasma when measured by its enzymatic activity (9). Table 1 compares the content of type II PLA₂ in uremic and
normal serum by enzymatic activity and ELISA. Enzymatic analysis demonstrates a 14.6-fold increase in uremic vs control serum: median = 6.5 μmol/min per liter, range 1.1–16.3 vs median = 0.49 μmol/min per liter, range 0.32–0.60, respectively. In contrast, the ELISA detects 111 times as much type II PLA₂ in uremic serum vs control (median = 1025 vs 9.2 μg/L). Thus, the increase in type II PLA₂ in uremic vs control serum is nearly eight times greater when measured by immunochemical analysis than by enzymatic activity.

Discussion

The present study illustrates that immunochemical concentrations of type II PLA₂ are dramatically increased in patients with renal failure and confirm our previous report that the eightfold increase in PLA₂ activity in uremic serum is due to an increase in type II PLA₂ (9). Thus, renal failure joins a host of pathologies (i.e., endotoxic shock, inflammatory bowel diseases, malaria) and stressful circumstances (i.e., schizophrenia, surgery) in which the amounts of PLA₂ activity in serum are increased either persistently or transiently (for review see refs. 22 and 23). Several other laboratories have recently used ELISA (14, 15, 24) and RIA (13) methods to establish the identity of the enzyme responsible for the increased PLA₂ activity observed in biological fluids.

We were surprised to find that the apparent increase of type II PLA₂ in uremic serum estimated by ELISA was more than seven times greater than that by direct

<table>
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<th>Table 1. sPLA₂ in normal and uremic serum.</th>
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<td>Immunoreactivity, μg/L</td>
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<td>Normal</td>
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Values were determined by duplicate determinations and converted to μg/L PLA₂ protein or μmol/min per liter of hydrolyzed phospholipid as described in Materials and Methods.

measurement of enzymatic activity. One possible explanation for this discrepancy is that proteins with epitopes common to the type II PLA₂ are expressed and accumulate in renal failure. This notion has potential credence since the human gene for the type II PLA₂ contains five exons, with exons 3, 4, and 5 encoding the mature enzyme (4). It is possible that exon shuffling could result in the generation of proteins immunochemically related to the type II PLA₂ (25). This is thought to be the basis for antibody diversity (26) and may explain the occurrence of a Ca²⁺-dependent translocation domain in the mammalian 85-kDa cytosolic PLA₂ with homology to protein kinase C and GTPase-activating protein (27).

Several lines of evidence suggest that the increased amounts of enzyme detected by our ELISA may be due to accumulation of enzymatically inactive or "suppressed" type II PLA₂ in uremia. First, both our mouse monoclonal and rabbit polyclonal antibodies are highly specific to human type II PLA₂. Neither antibody reacted with type I or type II 14-kDa PLA₂s from venom or pancreas, proteins sharing invariant domains but only 25% amino acid sequence homology (28). Among the various PLA₂s, there is significantly greater homology in the Ca²⁺ binding domain (29) and the amino-terminal alpha helix (30). The lack of cross-reactivity by our antibodies suggests that they are not directed toward epitopes common to these enzymes. This selectivity of monoclonal and polyclonal antibodies generated to type II PLA₂ has also been noted by others (13–15, 24, 31). For these reasons, we believe that the specificity of our antibodies also diminishes the likelihood of nonspecific recognition. Because the kidney is the major site of catabolism and excretion of proteins and peptides ≤25 kDa (32), the accumulation of 14-kDa enzyme and catalytically inactive metabolites in serum would be an expected consequence of renal failure.

It is also possible that the well-established presence of endogenous suppressor and activator molecules (33, 34) in crude biological fluids such as serum and plasma contributes to an inaccurate measurement of protein content when estimated by enzymatic analysis. Indeed, in our previous report, we noted that dilution of both normal and uremic plasma increased the total enzymatic activity in both plasma samples by as much as 400% but, curiously, did not significantly alter the extent of the increase when compared at the same dilution. Thus, enzymatic analysis in crude samples is generally unsatisfactory for quantification but may be useful for comparative purposes. Immunochemical methods such as the ELISA, on the other hand, may be the method of choice for quantification but, as we have noted for uremic serum, may not reflect catalytic potential.

Markedly increased amounts of type II PLA₂ may have pathologic consequences for the uremic patient. Numerous studies have shown that the isolated human type II PLA₂ is proinflammatory or toxic. Thus, when exogenously administered, the enzyme induces edema...
(5, 6), compromises phagocyte bactericidal and degranulating activity (35), and causes convulsions or death (36). Of particular interest in renal failure is the apparent ability of the type II PLA₂ to lower blood pressure in endotoxic shock (37), in which instance serum type II PLA₂ is increased 11-fold and its concentration is directly related to the decrease in mean arterial blood pressure. Since type II PLA₂ is not diminished by hemodialysis (or may be increased), it is tempting to speculate that the inability to normalize serum concentrations of this enzyme may contribute to the patient’s complex pathology, blood pressure changes, and possibly ill-defined malaise, which frequently persists in chronic renal failure despite adequate dialysis.

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References