

Original Article

Haemodialysis activates phospholipase A₂ enzyme

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Abstract

Background. Clinical and experimental evidence suggest that haemodialysis (HD) procedure is an inflammatory process. For the production of pro-inflammatory lipid mediators in many inflammatory reactions, the release of arachidonic acid by phospholipase A₂ (PLA₂) enzyme is a prerequisite. Therefore, the purpose of the present investigation was to establish whether the activity of PLA₂ increases during HD and whether the increase depends on the type of dialyser used.

Methods. We performed dialysis in eight chronic HD patients. Blood samples entering and leaving the dialyser were obtained before and at 15, 60, 120 and 180 min after the dialysis was started, on one occasion using a cuprophane and on another occasion a cellulose triacetate dialyser. PLA₂ activity was assessed in crude plasma and in plasma extract.

Results. PLA₂ activity in plasma extract exhibited similar biochemical properties to that of inflammatory human synovial fluid PLA₂ enzyme which is of group II PLA₂. PLA₂ activity in crude plasma represents a type of PLA₂ other than the synovial type. In HD patients, baseline PLA₂ activities in crude plasma and plasma extract were significantly increased when compared to normal subjects. An increase in PLA₂ activity was observed in crude plasma with a peak appearing at 15 min when the patients were dialysed with cuprophane and cellulose triacetate membranes. This increase was observed in both arterial and venous blood samples and was more pronounced when the patients were dialysed with cuprophane than with cellulose triacetate membranes. When PLA₂ was assessed in plasma extract, the activity increased only with cuprophane but not with cellulose triacetate membranes.

Conclusion. PLA₂ activity in plasma is increased in HD patients and increases during the dialysis procedure to a greater extent with a less biocompatible membrane. Continuous activation of PLA₂ might be relevant for long-term deleterious consequences of HD.

Key words: arachidonic acid; biocompatible materials; inflammation; membranes artificial

Introduction

Haemodialysis in humans and in experimental animals may result in adverse anaphylactic symptoms such as hypotension, respiratory distress, oedema of the skin, muscle cramps, nausea, headache, diaphoresis, feeling of fatigue, and lack of energy [1–3]. Some of these symptoms are used as clinical endpoints to assess acute side-effects due to the methods and materials used during dialysis [1]. One of the major cause for anaphylactic symptoms has been ascribed to the bioincompatibility of the blood/membrane contact [4–6]. For bioincompatibility, the cellulosic nature of the membranes has been implicated. In different membranes the cellulose has been treated differently or different cellulose material has been used to form porous membranes. These membranes are treated with copper and ammonia in cuprophane membranes, whereas acetylated cellulose (CT 110, CT 190, Baxter, IL, USA) or saponified cellulose ester (135 sce, C-D Medical) are used in other membranes. Cellulose is regenerated by removing acetate moieties and other chemicals. In cellulose acetate membranes the acetate moieties are retained [7]. The use of these membranes is associated with a variable incidence of side-effects. Compared to other membranes, cuprophane membranes are considered to be less biocompatible [4–11].

As a result of blood/membrane interaction several studies reported leukopenia due to complement activation [2,8], increased production of β_2 -microglobulin [12], interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor (TNF) [9–11,13,14], prostacyclin, thromboxane, platelet activating factor (PAF), hydroxyeicosatetraenoic acids (HETS) [9,15–17] during haemodialysis. Many inflammatory cells release prostaglandins and thromboxanes *in situ* with cuprophane membranes [9]. Proinflammatory autacoids like prostaglandin, prostacyclin, thromboxanes, PAF, HETS are the metabolites of free arachidonic acid. Free arachidonic acid is primarily released by phospholipase

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A₂ (PLA₂) enzyme from phospholipids by hydrolysing a fatty acyl ester bond at sn-2 position [18]. PLA₂ enzyme is activated by endotoxins and cytokines like interleukins and TNF [19–21]. Thus activation of PLA₂ during haemodialysis might account for some of the clinical signs and symptoms during dialysis. Therefore we investigated the impact of dialysis on the activation of PLA₂ *in vivo* by comparing the effect of a membrane considered to be rather bioincompatible with that of a biocompatible one.

Subjects and methods

Subjects and study design

Eight haemodialysis (HD) patients (4 males and 4 females) gave their written consent to participate in the following study which was approved by the ethical committee of the Inselspital, University of Bern. Their age ranged from 24 to 67 years (mean age 44.5 years). Eight healthy volunteers matched for age and sex with the HD patients (4 males and 4 females; range of age 26–64 years, mean age 45 years) were used for comparison of baseline PLA₂ activity before the dialysis was started. All HD patients had a history of at least 6 months on haemodialysis treatment and were undergoing haemodialysis regularly twice or three times a week using bicarbonate dialysate. These HD patients had the following underlying diseases causing endstage renal failure: pyelonephritis (4 patients), glomerulonephritis (3 patients) and autosomal dominant polycystic kidney disease (1 patient). No medication known to interfere with PLA₂ activity were taken either by the patients or by the volunteers.

The patients were routinely dialysed with a cellulose triacetate hollow-fibre dialyser (CT 110, Baxter, IL, USA). For the study day, in addition a less biocompatible filter with a similar surface area was used for comparison (Cuprophane capillary dialyser, Hemoflow E4S, Fresenius). Dialysis was performed by puncturing a-v fistulas at the forearm with a 15-G needle. The first blood sample was collected before dialysis treatment. Additional blood samples were obtained either from the blood line coming directly from the body ('arterial') or from the blood line coming from the dialyser ('venous') at the following time points: 15 min after bolus injection of heparin (3000 U), 60 min and 120 min thereafter, and at the end of dialysis (~180 min).

To study the effect of heparin, two patients were dialysed using cellulose triacetate hollow-fibre dialyser and with cuprophane capillary dialyser without administering any heparin. Arterial blood samples were collected at the start of dialysis and at 15 min on dialysis. The dialysis was later on continued by administering heparin.

Analytical procedures

Blood collection and preparation of crude plasma. Four millilitres of whole blood was collected using 4-ml Monovette KE tubes containing 1.6 mg EDTA/ml blood (Sarstedt, Neumbrecht Germany). Within 30 min the blood samples were centrifuged at 3000 g for 10 min. The upper cell free plasma was carefully collected and stored at –20°C until further use.

Extraction of plasma for PLA₂ activity measurements. Crude plasma (750 µl) was extracted with equal volumes of 0.36 N sulphuric acid and was kept on ice water for 60 min. The

sulphuric acid was removed by dialysing (membrane with a molecular weight cut-off of 6000–8000 daltons) against 10 mM sodium acetate buffer pH 4.5. The dialysed plasma sample was incubated for 30 min at 80°C, which resulted in the formation of a white precipitate. This precipitate was resuspended in 10 mM sodium acetate pH 4.5 to a final volume of 5 ml. This sample was centrifuged at 20 000 g for 30 min. The supernatant was separated and stored at –20°C until further use. This supernatant is designated below as 'plasma extract'.

In-vitro effect of heparin on plasma PLA₂ activity. Crude plasma was mixed with indicated concentrations of heparin and PLA₂ activity was measured in crude plasma directly or in the plasma extract.

Determination of protein concentrations. Protein concentrations in crude plasma and in the plasma extracts were determined using the bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, IL, USA).

Assay of PLA₂ activity. [³H]-oleic-acid (specific activity 10 Ci/mmol, Amersham International plc, Buckinghamshire, UK)-labelled *Escherichia coli* was prepared according to the procedure of Patriarca *et al.* [22]. PLA₂ activity in the crude plasma and in the plasma extract was assayed using [³H]-oleate-labelled, autoclaved *E. coli* as the substrate [23]. The reaction mixture 350 µl contained 100 mM Tris-HCl pH 7.3/8.0 or sodium acetate buffer pH 6.0; 5 mM Ca²⁺, 2.85 × 10⁸ cells of autoclaved *E. coli* cells (corresponding to 10 000 c.p.m. and 5.5 nmol lipid phosphorus). The amount of protein was chosen such that 6–15% hydrolysis of substrate was obtained when incubated at 37°C for 120 min. The reaction was terminated by adding 100 µl of 2 N hydrochloric acid. 100 µl of fatty acid-free BSA (100 mg/ml) was added, and the tubes were vortexed and centrifuged at 13 000 g for 5 min. An aliquot (140 µl) of the supernatant containing released [³H]-oleic acid was mixed with scintillation cocktail (Dynagel, J. T. Baker, B. V. Deventer, Holland) and counted in a liquid scintillation counter. This method was further characterized for the hydrolysis products in the supernatant by extracting the fatty acids by the method of Bligh and Dyer [24]. The extracted products were separated on silicagel TLC plates (Merck, Basel, Switzerland) using the solvent system petroleum ether:diethyl ether:acetic acid (80:20:3 by v/v). Arachidonic acid (Sigma Chemie, Buchs, Switzerland) was used as a standard. The specificity of this assay method using *E. coli* as substrate was also characterized using phospholipase C (PLC) and phospholipase D (PLD) enzymes (Sigma Chemie, Buchs, Switzerland) using the same condition as described for PLA₂ assay. The released fatty acids were analysed by fatty acid entrapment with fatty acid free BSA as well as by separation on silica gel TLC plates as described above after extraction by the method of Bligh and Dyer [24].

Calculations and statistical analysis

The area under the activity of PLA₂ versus time curve (AUC) from plasma samples was calculated by the linear trapezoidal rule [25]. The values are expressed as mean (±SD). The significance of differences was determined using the Student *t* test for the paired observations or the Wilcoxon test.

Results

PLA₂ activity measured in the crude plasma and in the plasma extract by the method using oleate labelled

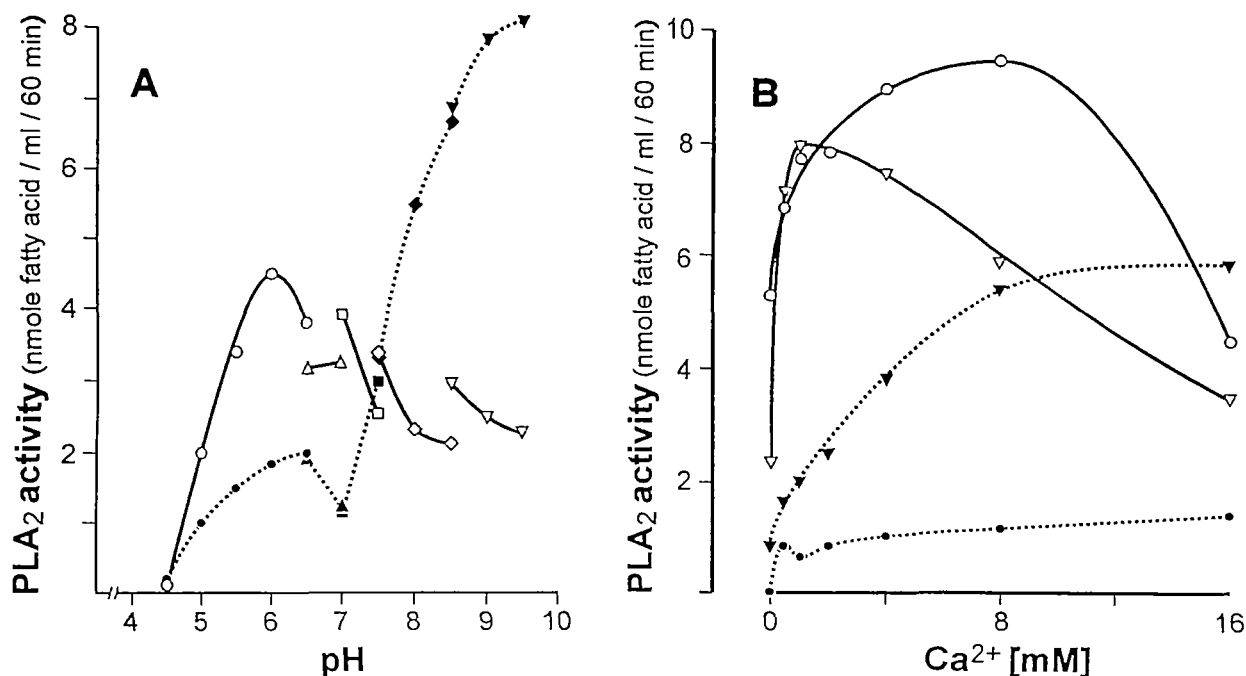


Fig. 1. Effect of pH (A) and calcium (B) on PLA₂ enzyme activity in crude plasma (—, solid line and open symbols) and in plasma extract (....., open line and closed symbols). Each value represents the average of three determinations. The buffers used were 100 mM sodium acetate for pH 4.5–6.5 (●); 100 mM bis-tris for pH 6.5–7.0 (▲); 100 mM HEPES for pH 7.0–7.5 (■); 100 mM tris-HCl for pH 7.5–8.5 (◆) and 100 mM glycyl-glycine for pH 8.5–9.5 (▼) (optimum pH was measured in the presence of 5 mM calcium). The effect of calcium concentrations on PLA₂ activity in crude plasma (—, solid lines) and in plasma extract (....., open lines) was measured at pH 6.0 (●) at pH 8.5 (▼).

E. coli as substrate showed specificity only for PLA₂ enzyme. The measured radioactivity in the *E. coli* supernatant was due to the release of free fatty acid and not due to a mixture of hydrolysed fatty acids and unhydrolysed *E. coli* phospholipids, as determined by analysing the extracted lipids of the *E. coli* supernatant on silica gel TLC plates (data not given). No free fatty acids were measurable when PLC or PLD were used instead of PLA₂.

In order to define the optimum conditions for the assessment of the PLA₂ activity, the effect of variable pH and calcium concentrations was determined. As shown in Figure 1, PLA₂ activity measured in crude plasma and in plasma extract showed different biochemical properties. In the presence of 5 mM Ca²⁺ PLA₂ enzyme activity was optimal at pH 6.0 for crude plasma, whereas in plasma extract the pH optimum was shifted from the acidic to basic pH with a maximum activity at pH 8.0–9.0 (Figure 1A). PLA₂ enzyme in the plasma extract was resistant to heat (80°C for 30 min). Based on this analysis the following conditions were chosen for all subsequent measurements: In the presence of 5 mM Ca²⁺ crude plasma was measured at pH 6.0 and plasma extract at pH 8.0. PLA₂ activity in crude plasma and in plasma extract was also measured at a physiological pH of 7.3.

In all eight HD-patients the basal PLA₂ activity was higher than the corresponding activity from their matched controls, when PLA₂ was assessed in crude plasma ($P < 0.001$) (Figure 2). The mean (\pm SD) values for all patients and control subjects were

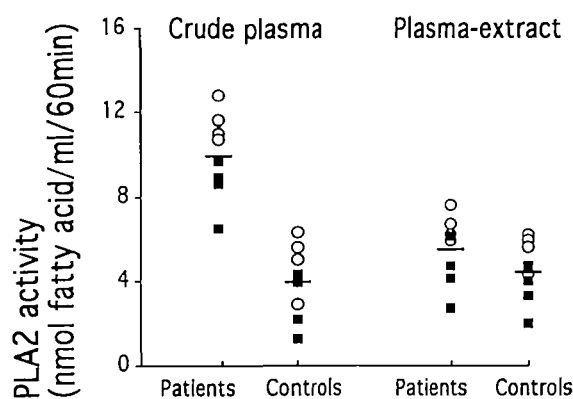


Fig. 2. PLA₂ activity in plasma samples from HD-patients before dialysis and in control subjects. Open circles represent females and closed squares represent males. The individual values of all subjects and the mean values (bars) are shown. Matched controls had lower PLA₂ activity than HD-patients when measured in crude plasma ($P < 0.001$) and in plasma extract ($P < 0.05$).

9.95 ± 1.97 nmol fatty acid/ml/60 min and 3.93 ± 1.7 nmol fatty acid/ml/60 min respectively. The corresponding values for PLA₂ activity in plasma extract were 5.5 ± 1.6 nmol fatty acid/ml/60 min and 4.5 ± 1.4 nmol fatty acid/ml/60 min respectively (Figure 2). Six of eight HD-patients had higher PLA₂ activity in plasma extract than their matched control subjects ($P < 0.05$). PLA₂ activity was lower in plasma extract than in crude plasma ($P < 0.001$) from patients, but not from controls. Female HD patients and female

controls had slightly higher PLA₂ activities than males (Figure 2).

The activation of PLA₂ during dialysis was assessed in eight HD patients with a cuprophane filter and with a cellulose triacetate filter (Figure 3). Measurements were performed both in arterial and in venous blood. PLA₂ activities in crude plasma (Figure 3, A and B) and in plasma extract (Figure 3, C and D) were significantly increased in HD patients dialysed with cuprophane filter compared to cellulose triacetate filter. The increase in PLA₂ activity is not due to haemoconcentration because, all the patients were under steady state and their protein levels in the samples did not increase during dialysis (data not shown). In order to get a quantitative estimate of the activation over time the AUC was determined for each patient (Figure 4).

For each patient the AUC of the PLA₂ activity was calculated for venous and arterial blood while dialysed with a cuprophane or with a cellulose triacetate membrane. When the values derived from PLA₂ measurements in crude plasma were considered, each patient had a higher AUC value in arterial and in venous blood during dialysis with cuprophane than with cellulose triacetate membranes (arterial, cuprophane

vs cellulose triacetate membrane 2243 ± 404 vs 1756 ± 320 nmol fatty acid/ml/60 min, $P < 0.001$; venous, cuprophane vs cellulose triacetate membrane 3129 ± 890 vs 2499 ± 611 nmol fatty acid/ml/60 min, $P < 0.001$) (Figure 3 A and 3 B). Similarly the values of PLA₂ activity obtained from measurements in plasma extracts from venous blood were higher in eight of eight patients when cuprophane than when cellulose triacetate membranes were used (1559 ± 397 vs 997 ± 340 nmol fatty acid/ml/60 min, $P < 0.001$) (Figure 3D). Seven of eight patients had higher AUCs in plasma extracts from arterial blood when dialysed with cuprophane than with cellulose triacetate membranes (1713 ± 436 vs 1035 ± 472 nmol fatty acid/ml/60 min $P < 0.02$) (Figure 3C). PLA₂ activity measured at physiological pH in crude plasma and in plasma extract also increased more, when the samples were obtained during cuprophane than cellulose triacetate membrane dialysis. Both plasma extract and crude plasma showed a similar profile when determined at pH 7.3 as measured at the optimal conditions (data not shown).

For each individual the values of the AUC of PLA₂ activity derived from measurements in crude plasma

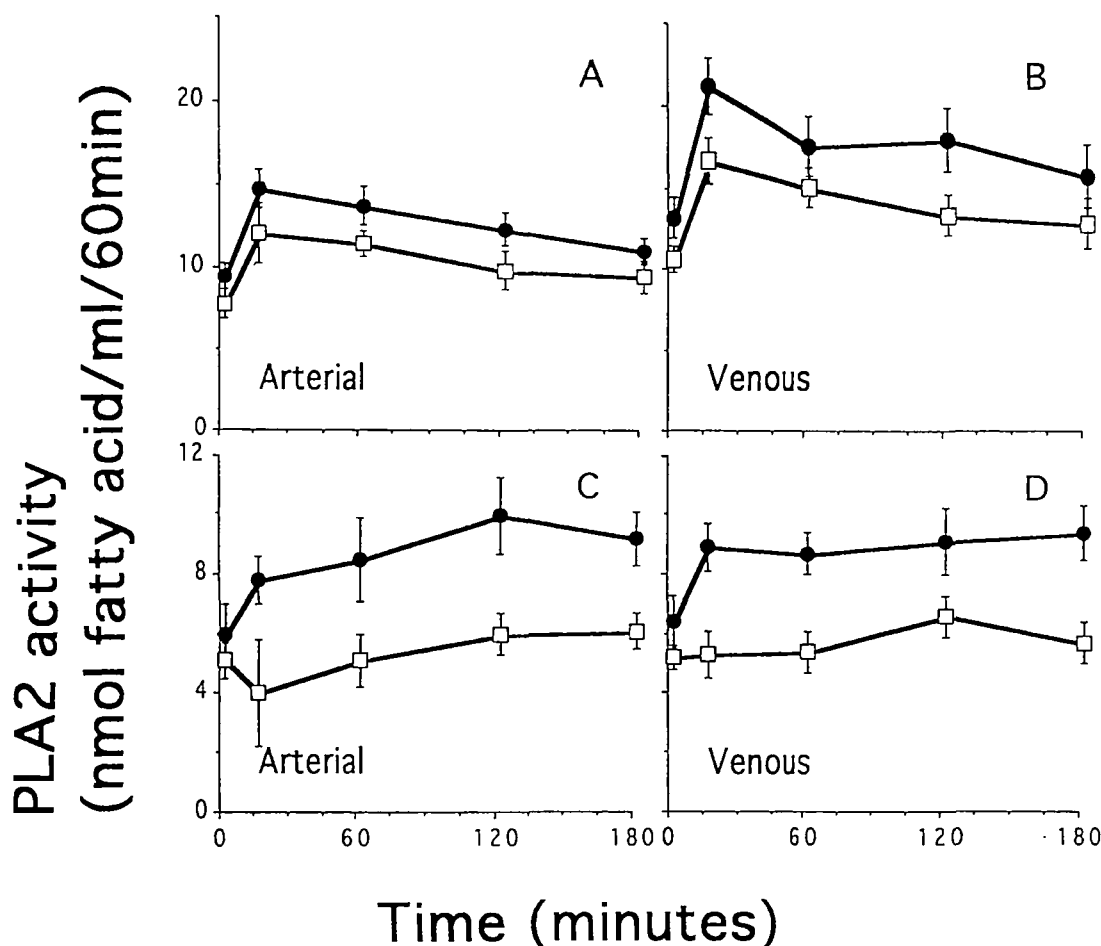


Fig. 3. PLA₂ activity measurements in crude plasma (A and B) and in plasma extract (C and D) before (0 min) and at several time points during dialysis. The patients were dialysed with cuprophane (●) and cellulose triacetate (□) membranes. Each value represents the mean value (\pm SD) from eight patients.

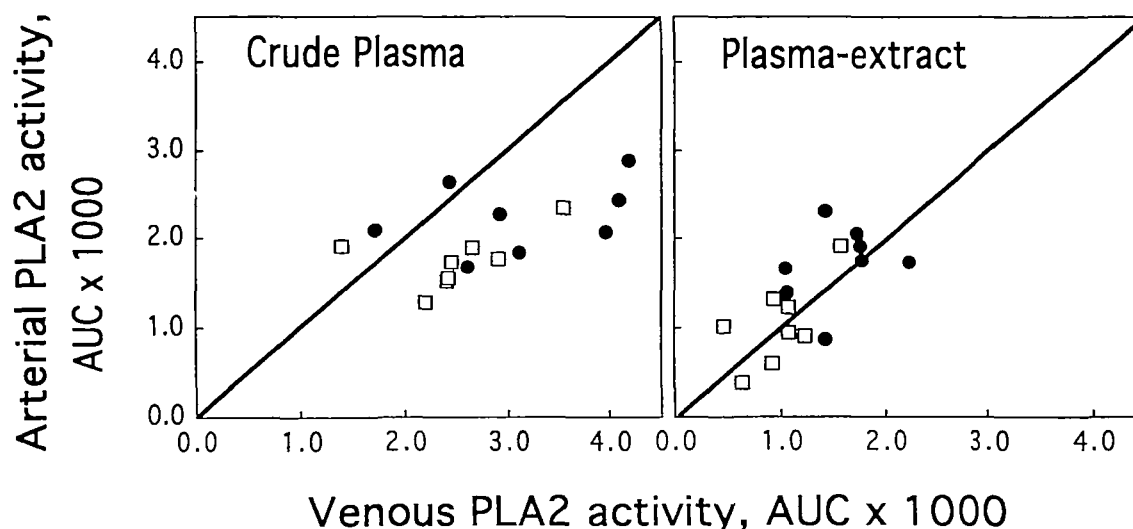


Fig. 4. The AUC of PLA₂ activity was calculated for crude plasma and plasma extract in venous blood and plotted against the corresponding AUC of PLA₂ activity in arterial blood for each patient. The patients were dialysed using cuprophane (●) and cellulose triacetate membranes (□). The oblique line is the line of identity. The activity measured in crude plasma was higher in venous blood than in arterial blood ($P < 0.02$), whereas no such difference was found for measurements of plasma extract.

and plasma extract were calculated. The individual AUC value of the PLA₂ activity of arterial blood was plotted against the corresponding AUC value of the PLA₂ activity of venous blood (Figure 4). In crude plasma 13 of 16 values were below the line of identity indicating higher PLA₂ activity in venous than in arterial blood ($P < 0.02$). In plasma extract the values were randomly distributed on both sides of the line of identity, indicating similar PLA₂ activity in both arterial and venous plasma extract.

Figure 5 depicts the effect of heparin on PLA₂ activity *in vitro*. Heparin inhibited PLA₂ activity in crude plasma as well as in plasma extract when high concentrations of heparin were used. During dialysis the concentrations of heparin following an i.v. bolus dose of 3000 U are probably much lower than one unit

per millilitre. To exclude the effect of heparin, *in vivo* PLA₂ activity was measured in two patients when dialysed without heparin administration for 15 min. Compared to zero min sample PLA₂ activity measured in crude plasma (at pH 6.0 or 7.3) and plasma extract increased similarly after 15 min in both with and without heparin administration (data not shown).

Discussion

PLA₂ enzymes characterized so far are classified into group I, II, III and IV based on their primary and secondary structure [26,27]. PLA₂ enzymes purified from human platelets and from human synovial fluid are classified under group II and are considered to account for inflammatory reactions, whereas the group I from pancreas appears not to be involved in inflammation [28–30]. Group III enzyme has not been detected in mammals and the function of cytosolic PLA₂ (Group IV) is not very well defined yet [27]. Inflammatory human synovial fluid PLA₂ (group II) is resistant to low pH, optimally active in the presence of mM concentrations of calcium and at basic pH [31,32]. PLA₂ activity measured in plasma extract in the present investigation exhibited similar biochemical/biophysical properties as those known for PLA₂ derived from inflammatory human synovial fluid PLA₂. The PLA₂ assessed in crude plasma showed an optimum activity at acidic pH. PLA₂ activity measured at pH 6.0 was abrogated when plasma was treated with acid and heat suggesting that the activity measured in crude plasma represents a different type of PLA₂ than the synovial type (group II PLA₂). A similar pH optimum was observed by others in crude plasma of uremic patients [33].

Before dialysis was started all patients received a

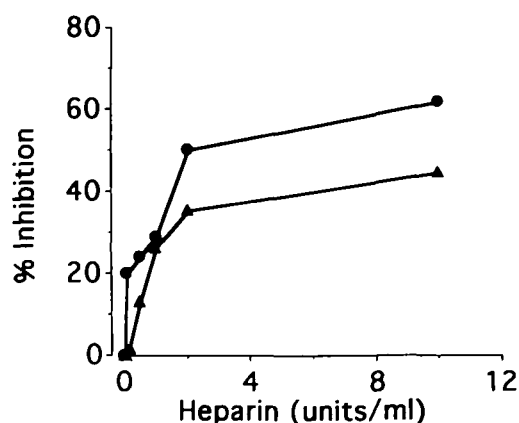


Fig. 5. *In-vitro* effect of heparin on PLA₂ activity. Following the addition of heparin (0.1, 0.5, 1.0, 2.0, and 10.0 units/ml) to plasma, PLA₂ activity was measured directly in crude plasma (●) and in plasma extract (▲). PLA₂ activity was expressed as nmol fatty acid/ml/60 min incubation. Each value represents the average of three determinations.

bolus dose of heparin (~ 3000 U). When added *in vitro* heparin inhibited PLA₂ activity in crude plasma and in plasma extract. This observation is in line with a previous publication [33], but is at variance with an observation made by Nakamura *et al.*, who showed that heparin enhanced plasma PLA₂ activity in patients undergoing cardiac surgery [34]. Dialysis with and without heparin administration resulted in a similar increase in PLA₂ activity. Thus, the increase in PLA₂ activity during dialysis cannot be attributed to the administered dose of 3000 U of heparin. Furthermore, the increase in PLA₂ activity in plasma cannot be related to haemoconcentration because in all patients ultrafiltration during dialysis was negligible as shown by the absence of an increase in the concentrations of plasma proteins.

When PLA₂ activity was measured in crude plasma the peak PLA₂ activity was observed in all patients at 15 min after dialysis was started (Figure 3 A and B). Previous investigations revealed similar peaks of biological events during the initial 15 min on dialysis. These changes comprise among others, a decline in the leukocyte count in peripheral blood, evidence for activation of the complement system, release of TNF and interleukins, including IL-1 β and arachidonic acid metabolites, such as PGE₂, TXB₂, PAF and HETS [2,8–17]. The release of many arachidonic acid derivatives suggests activation of PLA₂, as shown by the present investigations. The question arises what mediator(s) activate(s) PLA₂ activity during dialysis? Potential candidates are TNF, possibly complement and IL-1 β [21,35]. IL-1 β is a well established activator of group II PLA₂ [25]. Thus the link between IL-1 β and PLA₂ activity during dialysis is an attractive hypothesis. However, *in vivo* this has to be considered carefully for the following reasons.

In vitro studies clearly demonstrate that first, cytokine releasing agents cross the membranes from the dialysate to the blood side, second, complement factors induced during blood contact with dialysis membranes *in vitro* enhance transcription of IL-1 and third, cellulose directly stimulates cytokine production [9,36,37]. Thus *in-vitro* data clearly support the hypothesis of IL-1 to account for PLA₂ activation. However, the *in-vivo* observations are more difficult to reconcile with that hypothesis, since only some [38–40] but not all [41–43] studies reveal increased concentrations of IL-1 β in blood. In line with our observation of a more pronounced PLA₂ activation possibly mediated through IL-1 release with a more complement-activating membrane is the recent report from Schindler *et al.* [14] demonstrating an increased gene expression of IL-1 β in peripheral blood mononuclear cells leaving the dialyser 5 min after start of haemodialysis. These authors found a significant correlation between the increase in IL-1 β mRNA and increase in activated complement C5a. It is likely that IL-1 β contributes significantly to the increase in PLA₂ activity. However, it is open to speculation whether differential activation of IL-1 β by cuprophane and cellulose triacetate membranes accounts for the differences in

PLA₂ activity observed during dialysis with these two membranes. Note that no difference between complement- and non-complement-activating membranes has recently been found with respect to IL-1 release [38]. Besides differential activation of IL-1, a different binding capacity of cuprophane and triacetate membranes for PLA₂ could also account for the differences in PLA₂ increase.

The origin of the PLA₂ in plasma is open to speculation. We have recently shown that lung tissue exhibits much higher activities of group II PLA₂ than liver, spleen, and kidney [28]. The lung is considered to account for leukocyte sequestration during dialysis [44]. It is conceivable that part of the activity of PLA₂ in peripheral blood originates from lung tissue. Alternatively activated and/or sequestered leukocytes might account directly for the increase in PLA₂ activity [45,46]. The presence of PLA₂ activity in granulocytes and blood mononuclear cells was previously seen by several groups [32,47] including our own (unpublished data).

A higher PLA₂ activity was measured in venous than in arterial blood, when measurements were performed in crude plasma (Figure 4), suggesting an activation process or removal of an inhibitor by membrane material. This venoarterial difference is in line with the increased transcription of IL-1 β in peripheral blood mononuclear cells obtained from venous, but not from arterial blood, as previously described by Schindler *et al.* [14] and the increased production of TXB₂ in venous when compared with arterial blood by Gawaz and Ward [16]. Interestingly the PLA₂ activity in the plasma extract was the same in arterial and venous blood (Figure 3C and D). PLA₂ from plasma extract (presumably group II) has different biochemical properties than that measured in crude plasma. Furthermore the regulation and tissue distribution of the PLA₂ enzymes assessed in crude plasma and plasma extract might be different and by that explain the different behaviour with respect to the activation during dialysis. With respect to the regulation we and others have recently shown a differential effect of glucocorticoids on group I and II PLA₂ in rats [28,29].

Recently PLA₂ has been considered as a relevant factor for another type of arthropathy, i.e. rheumatoid arthritis [48]. High levels of PLA₂ (presumably group II) had been found in blood and synovial fluid from these patients [49,50]. Injection of PLA₂ (group II) into animal joints resulted in a massive inflammatory response with synovial cell swelling and hyperplasia [51,52]. These changes eventually cause periarticular subchondral bone erosions [53]. Periarticular erosions, albeit with a distinct distribution from that in patients with rheumatoid arthritis, is a feature of dialysis arthropathy [54–56]. It has been suggested that dialysis arthropathy has an inflammatory basis, since increased C-reactive protein concentrations had been found in patients with dialysis arthropathy [57]. Furthermore amyloid deposits consisting of β_2 -microglobulin in joints and periarticular

tissue were described in patients with dialysis arthropathy [58]. The synthesis of β_2 -microglobulin might locally be increased by activation of mononuclear cells [59] and by that mechanism favour β_2 -microglobulin amyloid deposition in the presence of a decreased renal clearance of that protein. Thus it is conceivable that repeated PLA₂ activation due to inflammatory effects caused by extracorporeal circulation are relevant for the understanding of some of these signs and symptoms, such as the pathogenesis of dialysis arthropathy.

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