Extracellular phospholipase A₂: causative agent in circulatory collapse of septic shock?

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Introduction

The syndrome of septic shock is still a common cause of death in spite of recent advances in antimicrobial therapy. This is, paradoxically, a reflection of medical progress in that the factors predisposing to septic shock are those of old age, debilitating underlying disease, prior antibiotic therapy, use of immunosuppressive agents and increasing reliance on invasive diagnostic and therapeutic interventions [1]. There are an estimated 100,000 to 300,000 cases of gram-negative sepsis in the United States per year [2] with mortality rates ranging from 30–80%. These high mortality rates have remained virtually unchanged for decades [3].

In attempting to understand the pathogenesis of the septic shock syndrome, various circulating mediators of cardiovascular collapse have been sought. Among these putative mediators are included tumour necrosis factor [4], histamine, kinins, endorphins, myocardial depressant factors and various eicosanoids [5]. So far, none of these candidates has fulfilled the requisite criteria for mediators in the setting of septic shock [6], although products of the arachidonic acid cascade are certainly amongst the frontrunners. In spite of the lack of success in the search for such a mediator, the concept of a circulating factor, released into the systemic circulation in response to bacte-remia or endotoxemia remains an attractive notion.

On the basis of early studies in our laboratory describing the mediation of regional blood flow in inflamed sites by extracellular phospholipase A₂ (PLA₂) [7–9], we postulated that the intravascular release of a soluble PLA₂ initiates a sequence of events culminating in cardiovascular collapse in the setting of septicemia. The experimental and clinical studies described below suggest that circulating PLA₂ may well play a central role in the pathogenesis of circulatory collapse in septic shock.

Extracellular phospholipase A₂ as a local vasoactive and inflammatory mediator

Phospholipase A₂ has been found in association with localized sites of inflammation in both experimental animals and in man. High levels of PLA₂ activity have been identified in glycogen-induced ascitic fluid in rabbits [10] and in lymph plasma in sheep subsequent to intradermal challenge with vaccinia virus or purified protein derivative of Bacille Calmette-Guerin [8]. A soluble PLA₂ has been described [9, 11], characterized [12] and purified [13] from synovial fluids of patients with rheumatoid arthritis. The levels of PLA₂ were found to correlate significantly (p < 0.0001) with disease activity in rheumatoid arthritis [14].

Soluble PLA₂ is secreted extracellularly in lymph by activated macrophages [8, 15], whereas the
cellular sources of the synovial fluid enzyme include osteoblasts [16] and chondrocytes [17]. Extracellular PLA\textsubscript{2} is both pro-inflammatory and vasoactive. In view of the association of PLA\textsubscript{2} to inflamed sites, the development of inflammatory changes in rabbit skin were studied subsequent to intradermal injection of both active and inactivated venom and pancreatic PLA\textsubscript{2}. PLA\textsubscript{2}, at concentrations encountered in human disease, caused acute inflammatory changes characterized grossly by erythema and induration, and histologically by inflammatory cell infiltration, vascular and tissue damage, and microabscess formation [18]. The severity of the resultant lesions was both time- and dose-dependent.

The development of inflammatory lesions subsequent to intradermal injection was also associated with increased regional blood flow or hyperemia [19]. Using the microsphere method for quantitation of regional blood flow [20], PLA\textsubscript{2} was found to induce a profound and sustained increase in dermal blood flow after intradermal injection [7]. This hyperemia was abrogated by pretreatment of local injection sites with indomethacin, suggesting that at least part of the vasoactive effect of PLA\textsubscript{2} was mediated by cyclooxygenase products. Thus, a soluble PLA\textsubscript{2} is released extracellularly in response to local inflammatory stimuli and exogenous PLA\textsubscript{2} is locally vasoactive causing increased regional blood flow.

**Extracellular phospholipase A\textsubscript{2} in experimental endotoxin shock**

From these earlier data we postulated that vasoactive changes in a systemic inflammatory response may well involve fundamentally similar pathways as for the local inflammatory response, but differing in magnitude. Endotoxemia, the experimental counterpart to clinical gram-negative septic shock, has been regarded as a systemic inflammatory response [21, 22], and was therefore chosen as our experimental model. Endotoxin shock was induced in rabbits by i.v. challenge with E. coli endotoxin 0127:B8. Changes in mean arterial blood pressure (MABP) and serum PLA\textsubscript{2} activity were followed over a 5-hour period. Animals challenged with an LD\textsubscript{50} dose of E. coli endotoxin became hypotensive over the period of observation with a progressive fall in MABP. Serum PLA\textsubscript{2} activity rose concomitantly by a mean of 11.2-fold over baseline levels. The correlation of the rise in serum PLA\textsubscript{2} activity with the fall in MABP yielded a statistically significant monotonic relationship [23]. In contrast, there was no change in either MABP or serum PLA\textsubscript{2} activity in control animals injected with non-pyrogenic saline.

Since we had previously shown that mitogen-stimulated macrophages secreted PLA\textsubscript{2} extracellularly whereas those coincubated with dexamethasone sodium phosphate did not [15], we investigated the effect of dexamethasone pretreatment in the setting of endotoxin shock. Animals pretreated with dexamethasone remained normotensive and serum PLA\textsubscript{2} levels remained unchanged suggesting that steroids administered prior to systemic endotoxemia may prevent the liberation of active PLA\textsubscript{2} into the systemic circulation [23].

Finally, the PLA\textsubscript{2}-containing fraction of septic shock serum (MW 10000–13000) obtained by gel filtration chromatography, was re-infused into normal rabbits. Infusion of the exogenous PLA\textsubscript{2}-containing fraction to levels encountered in endotoxemia, reproduced the hypotensive effect of endotoxin itself. Pretreatment of the PLA\textsubscript{2} fraction with the PLA\textsubscript{2} inhibitor p-bromophenacyl bromide (pBPB) prior to re-infusion resulted in inhibition of PLA\textsubscript{2} activity, and as well, abrogated the hypotensive effect of exogenous PLA\textsubscript{2}.

Linear regression analyses of MABP responses of those groups of rabbits treated with (a) saline, (b) endotoxin, (c) PLA\textsubscript{2}-active fraction of shock serum, and (d) pBPB-treated PLA\textsubscript{2}-active fraction of shock serum revealed that the rate of fall in MABP in the PLA\textsubscript{2}-active fraction-treated group exactly paralleled that of the endotoxin-treated group consistent with the postulated common pathway of action.

These data demonstrated that PLA\textsubscript{2} is 1) released in massive amounts into the systemic circulation following endotoxin challenge in a model of experimental endotoxin shock, 2) the rise in serum PLA\textsubscript{2} activity is directly proportional to the fall in MABP, 3) dexamethasone pretreatment prior to endotoxin challenge protects against hypotension and prevents release of active PLA\textsubscript{2} into the systemic circulation, and 4) exogenous PLA\textsubscript{2} isolated from septic shock plasma causes systemic hypotension.
Extracellular phospholipase A<sub>2</sub> in clinical gram-negative septic shock: a retrospective study

Studies of the role of circulating PLA<sub>2</sub> in the pathogenesis of hypotension were extended to the clinical counterpart of experimental endotoxin shock, i.e. gram-negative septic shock (GNSS). Sera taken from patients during the acute hypotensive phase of GNSS were assayed for PLA<sub>2</sub> activity and correlated to the presence (or absence) of adult respiratory distress syndrome (ARDS).

Thirty-four patients with septic shock were subdivided into two groups: those with GNSS with ARDS vs those with GNSS without ARDS, as diagnosed by Hallman's criteria [24].

During the acute, hypotensive phase of septic shock, serum PLA<sub>2</sub> was elevated in 34 of 34 patients (100%) by a mean of 16-fold over that of serum PLA<sub>2</sub> activity of 21 in-hospital controls matched for underlying disease ($p < 0.0001$) [25]. Patients with GNSS and ARDS had a mean serum PLA<sub>2</sub> activity of 20.2-fold greater than controls ($p < 0.0001$) whereas those with GNSS without ARDS had a mean 14-fold increase in PLA<sub>2</sub> activity over controls ($p < 0.0001$). The difference in serum PLA<sub>2</sub> activity in patients with confirmed GNSS with and without ARDS was statistically significant ($p = 0.01$) [25].

Thus in this cross-sectional study of 34 patients with GNSS, 1) serum PLA<sub>2</sub> activity was increased in all patients during the acute hypotensive phase of GNSS and 2) septic patients with concurrent ARDS had a mean serum PLA<sub>2</sub> activity 70% greater than septic patients without respiratory insufficiency.

Correlation of extracellular phospholipase A<sub>2</sub> with the severity of circulatory collapse in septic shock: a prospective study

Twelve consecutive patients with the diagnosis of GNSS were entered into a prospective study of serum PLA<sub>2</sub> profiles and concomitant hemodynamic changes from diagnosis to death or discharge. The magnitude of circulatory instability or collapse was quantified by the hemodynamic instability score (HIS) [26]. In all 12 patients, serum PLA<sub>2</sub> activity correlated with the hemodynamic status of each patient as reflected by HIS scores. Peak serum PLA<sub>2</sub> activity consistently corresponded to periods of maximal circulatory collapse with a return towards normal levels in the convalescent phase. Peak serum PLA<sub>2</sub> activities as high as 291-fold over controls were observed. The correlation of PLA<sub>2</sub> and HIS achieved nominal levels of significance of $p < 0.001$ using a logistic regression model for ordinal data. Correlations were positive in 12 of 12 patients, consistent with a highly statistically significant correlation of PLA<sub>2</sub> activities and HIS scores ($p < 0.02$) [26]. This was the first prospective study of serum PLA<sub>2</sub> profiles in patients with septic shock. This study demonstrated that endogenous serum PLA<sub>2</sub> activity correlated with the magnitude of circulatory collapse in clinical septic shock, as it did in rabbits with experimental endotoxic shock [23].

Concordance of phospholipase A<sub>2</sub> and endogenous cortisol levels in gram-negative septic shock

Since we had previously shown that dexamethasone prevents the intravascular release of active PLA<sub>2</sub> following endotoxin challenge [23], and since others have shown that steroids induce the synthesis of specific phospholipase inhibitory proteins known as lipocortins (reviewed in [27, 28]), it was of interest to examine the serum profiles of endogenous cortisol, ACTH and PLA<sub>2</sub> in patients during the course of septic shock.

Eight patients with septic shock were studied prospectively. Serum cortisol and ACTH levels were quantitated by radioimmunoassay simultaneously with PLA<sub>2</sub> determination. In 4 survivors, peak cortisol levels ranged from 662–2480 nmol/l (reference range 50–600 nmol/l). Cortisol levels in 4 non-survivors ranged from 450–2320 nmol/l. In contrast, there was little fluctuation in serum ACTH levels in all patients studied, with ACTH levels consistently less than 5 pmol/l. The correlation coefficient of simultaneous serum PLA<sub>2</sub> and cortisol levels in survivors of septic shock showed a consistent statistical correlation of $r > 0.833$ ($p < 0.01$) whereas the coefficient of correlation of PLA<sub>2</sub> and cortisol in non-survivors was consistently less than $r = 0.505$. There was no correlation in any patient of serum ACTH with cortisol or PLA<sub>2</sub> [29]. Using McCullagh's regression model for ordinal data [30], a statistically significant association between PLA<sub>2</sub> and cortisol was detected amongst survivors ($p < 0.0001$) as well as for the total sample ($p < 0.001$). In pa-
tients who died, the association between PLA₂ and cortisol achieved only borderline significance ($p = 0.07$).

Thus, in the first prospective study of cortisol levels in man during septic shock we noted a strong statistical correlation of cortisol and PLA₂ in survivors of septic shock, while such a correlation was absent in all non-survivors. The absence of a correlation of cortisol and ACTH in patients with septic shock suggested that the stimulus for cortisol release arises from outside the hypothalamic-pituitary axis.

Characterization of human septic shock serum phospholipase A₂

Septic shock serum was fractionated by gel filtration column chromatography as described [7]. Fractions were assayed for PLA₂ activity using radiolabeled E. coli substrate. Human shock serum PLA₂ was eluted as a single peak of molecular weight 14000 [7].

Human septic shock serum PLA₂ activity was assayed [31] using the substrates 2-[14C]-palmitoyl-l-palmitoyl-3-phosphatidylcholine or 1-[14C]-palmitoyl-sn-glycero-3-phosphocholine in micellar dispersion in aqueous buffer. Assays were performed in substrate excess, in the linear range of the assay with respect to incubation times and enzyme concentration.

Incubation of human septic shock serum phospholipase with synthetic 2-[14C]-palmitoyl-l-palmitoyl-3-phosphatidylcholine resulted in hydrolysis of 23.2% of substrate. Of the total products formed, 96.6% of radiolabel was associated with free fatty acid, and 3.4% was associated with lysophosphatidate. In comparison, incubation of radiolabeled substrate with authentic PLA₂ from Naja Naja venom resulted in 97.1% of radiolabeled product comigrating with free fatty acid and 2.9% with lysophosphatidate. These data were consistent with a 2-acyl positional specificity of septic shock serum phospholipase [32].

The pH optimum of HSS-PLA₂ activity was determined in the presence of either 7 mM CaCl₂ or 5 mM EDTA, since PLA₂ may be absolutely calcium-dependent or optimally active in the absence of exogenous calcium [27]. In the presence of calcium, HSS-PLA₂ displayed a monophasic pH optimum of 7.5. Chelation of calcium by EDTA significantly reduced PLA₂ activity at pH 7.5 (by 97%) and did not reveal a calcium-independent species of PLA₂, particularly in the acidic range [32].

The calcium dependence of the enzyme was examined over the range of 0–10 mM CaCl₂. PLA₂ was optimally active in the presence of 2 mM calcium with a progressive loss of enzyme activity at higher concentrations. The effect of temperature on the rate of hydrolysis of radiolabeled E. coli phospholipids was examined. Enzyme activity increased linearly with temperature over the range of 30–40°C [32].

Septic shock serum PLA₂ was tested for immunoreactivity against anti-human pancreatic PLA₂ by radioimmunoassay [26]. Sera with PLA₂ activities as high as 260-fold normal did not cross-react with anti-human pancreatic PLA₂.

Discussion

We had postulated that the presence of bacteria or bacterial products in the systemic circulation causes the intravascular release of large amounts of a soluble PLA₂ and by generation of vasoactive products of PLA₂ hydrolysis, contributes to circulatory collapse in septic shock.

Exogenous PLA₂ is vasoactive both locally and systemically. Intravenous infusion of snake venom PLA₂ caused a rapid and profound drop in MABP in dogs [33], cats [34] and rats [35]. Similarly, infusion of septic shock serum PLA₂ produced hypotension in rabbits at a rate parallel to that induced by endotoxin [23].

Serum levels of endogenous PLA₂ correlated significantly with the fall in MABP in both experimental endotoxin shock as well as in patients with septic shock. Hyperphospholipasemia has been confirmed in endotoxin shock in rats [36] as well as in patients with severe septicemia [37].

The source of circulating PLA₂ is, as yet, undefined. The septic shock PLA₂ appears to be of extra-pancreatic origin based on the absence of cross-reactivity with anti-human pancreatic PLA₂ antiserum. Nor does the enzyme appear to originate from spleen or circulating leukocytes in that comparable elevations of serum PLA₂ have been noted in septic patients post-splenectomy or with profound leukopenia post-cytotoxic chemotherapy (unpublished observations).
While the mechanism of PLA₂-induced hypotension is not known, the release of PLA₂ into the systemic circulation may lead to the generation of vasoactive eicosanoids and lysosphosphatides. Perfusion of rat liver ex vivo with exogenous porcine pancreatic PLA₂ resulted in the release of 6-keto-prostaglandin F₁α and thromboxane B₂ from intact cells and tissues [38]. Intravenous infusion of snake venom PLA₂ in rats caused a significant fall in MAP which correlated with the rise in the ratio of 6-keto-PGF₁α to TXB₂ [39, 40]. PLA₂ was also shown to cause an increase in lung perfusion pressure [41], which correlated linearly with the ratios of TXB₂ to 6-keto-PGF₁α. These data suggest that circulating PLA₂ attacks phospholipid substrate of intact cells and tissues leading to the generation of a number of vasoactive lipids.

Glucocorticoids appear to exert at least part of their anti-inflammatory effect through the synthesis and release of endogenous phospholipase inhibitory proteins known as lipocortins (reviewed in [27, 28]). Several lines of evidence have been advanced to support this concept. Exposure of a number of cell types to steroids in vitro resulted in the extracellular release of lipocortin [42, 43]. As well, lipocortin was recovered in body fluids after parenteral administration of steroids [34]. Significantly, the anti-inflammatory effects of steroids were abrogated in vivo by coadministration of a monoclonal anti-lipocortin antibody [28]. Experimentally, we have shown that pretreatment of rabbits with steroids prior to endotoxin challenge protected against endotoxin-induced hypotension and prevented PLA₂ release into the systemic circulation. These observations suggest an antiphospholipase effect of exogenous or endogenous corticosteroids. In patients with septic shock, cortisol levels were discordant in all survivors and concordant in all non-survivors. Interestingly, there was evidence of complete dissociation of the pituitary-adrenal axis in these patients, with presumptive evidence of extrapituitary regulation of adrenocortical function. Thus, postulated mechanisms of pro-inflammatory and anti-inflammatory pathways involving phospholipase A₂, phospholipase inhibitory proteins (PLIP) and corticosteroids, will require further investigation. These data demonstrate that PLA₂ fulfills several requisite criteria for mediators of circulatory shock as proposed by Lefer [6]. First, there is a demonstrable increase in circulating PLA₂ in response to bacteria or bacterial products. Second, exogenous PLA₂ was shown to cause hypotension in experimental animals and endogenous PLA₂ levels correlated with the severity of hypotension in both animals and man. The third and final criterion, amelioration of circulatory function by inhibiting the formation or antagonizing the action of PLA₂ awaits the development of a selective and non-toxic inhibitor of circulating PLA₂ in man.

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