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 anti-microbial 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 100000 to 300000 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_2 is both pro-inflammatory and vasoactive. In view of the association of PLA_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_2. PLA_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_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_2 was mediated by cyclooxygenase products. Thus, a soluble PLA_2 is released extracellularly in response to local inflammatory stimuli and exogenous PLA_2 is locally vasoactive causing increased regional blood flow.

*Extracellular phospholipase A_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_2 activity were followed over a 5-hour period. Animals challenged with an LD_{50} dose of E. coli endotoxin became hypotensive over the period of observation with a progressive

fall in MABP. Serum PLA_2 activity rose concomitantly by a mean of 11.2-fold over baseline levels. The correlation of the rise in serum PLA_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_2 activity in control animals injected with non-pyrogenic saline.

Since we had previously shown that mitogen-stimulated macrophages secreted PLA_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_2 levels remained unchanged suggesting that steroids administered *prior* to systemic endotoxemia may prevent the liberation of active PLA_2 into the systemic circulation [23].

Finally, the PLA_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_2-containing fraction to levels encountered in endotoxemia, reproduced the hypotensive effect of endotoxin itself. Pretreatment of the PLA_2 fraction with the PLA_2 inhibitor p-bromophenacyl bromide (pBPB) prior to re-infusion resulted in inhibition of PLA_2 activity, and as well, abrogated the hypotensive effect of exogenous PLA_2.

Linear regression analyses of MABP responses of those groups of rabbits treated with (a) saline, (b) endotoxin, (c) PLA_2-active fraction of shock serum, and (d) pBPB-treated PLA_2-active fraction of shock serum revealed that the rate of fall in MABP in the PLA_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_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_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_2 into the systemic circulation, and 4) exogenous PLA_2 isolated from septic shock plasma causes systemic hypotension.

Extracellular phospholipase A₂ in clinical
gram-negative septic shock: a retrospective study

Studies of the role of circulating PLA₂ 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 PLA₂ 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 PLA₂ was elevated in 34 of 34 patients (100%) by a mean of 16-fold over that of serum PLA₂ activity of 21 in-hospital controls matched for underlying disease ($p < 0.0001$) [25]. Patients with GNSS and ARDS had a mean serum PLA₂ activity of 20.2-fold greater than controls ($p < 0.0001$) whereas those with GNSS without ARDS had a mean 14-fold increase in PLA₂ activity over controls ($p < 0.0001$). The difference in serum PLA₂ 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 PLA₂ activity was increased in all patients during the acute hypotensive phase of GNSS and 2) septic patients with concurrent ARDS had a mean serum PLA₂ activity 70% greater than septic patients without respiratory insufficiency.

Correlation of extracellular phospholipase A₂
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 PLA₂ profiles and concomitant hemodynamic changes from diagnosis to death or discharge. The magnitude of circulatory instability or collapse was quantitated by the hemodynamic instability score (HIS) [26]. In all 12 patients, serum PLA₂ activity correlated with the hemodynamic status of each patient as reflected by HIS scores. Peak serum PLA₂ activity consistently cor-

responded to periods of maximal circulatory collapse with a return towards normal levels in the convalescent phase. Peak serum PLA₂ activities as high as 291-fold over controls were observed. The correlation of PLA₂ 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 PLA₂ activities and HIS scores ($p < 0.02$) [26].

This was the first prospective study of serum PLA₂ profiles in patients with septic shock. This study demonstrated that endogenous serum PLA₂ 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 A₂ and endogenous
cortisol levels in gram-negative septic shock

Since we had previously shown that dexamethasone prevents the intravascular release of active PLA₂ 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 PLA₂ 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 PLA₂ 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 PLA₂ 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 PLA₂ 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 PLA₂ [29]. Using McCullagh's regression model for ordinal data [30], a statistically significant association between PLA₂ 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 assocciation between PLA$_2$ 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 PLA$_2$ 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 A$_2$

Septic shock serum was fractionated by gel filtration column chromatography as described [7]. Fractions were assayed for PLA$_2$ activity using radiolabeled E. coli substrate. Human shock serum PLA$_2$ was eluted as a single peak of molecular weight 14000 [7].

Human septic shock serum PLA$_2$ activity was assayed [31] using the substrates 2-[^{14}C]-palmitoyl-l-palmitoyl-3-phosphatidylcholine or 1-[^{14}C]-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-[1-^{14}C]-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 lysophosphatide. In comparison, incubation of radiolabeled substrate with authentic PLA$_2$ from Naja Naja venom resulted in 97.1% of radiolabeled product comigrating with free fatty acid and 2.9% with lysophosphatide. These data were consistent with a 2-acyl positional specificity of septic shock serum phospholipase [32].

The pH optimum of HSS-PLA$_2$ activity was determined in the presence of either 7 mM CaCl$_2$ or 5 mM EDTA, since PLA$_2$ may be absolutely calcium-dependent or optimally active in the absence of exogenous calcium [27]. In the presence of calcium, HSS-PLA$_2$ displayed a monophasic pH optimum of 7.5. Chelation of calcium by EDTA significantly reduced PLA$_2$ activity at pH 7.5 (by 97%) and did not reveal a calcium-independent species of PLA$_2$, particularly in the acidic range [32].

The calcium dependence of the enzyme was examined over the range of 0–10 mM CaCl$_2$. PLA$_2$ 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 PLA$_2$ was tested for immunoreactivity against anti-human pancreatic PLA$_2$ by radioimmunoassay [26]. Sera with PLA$_2$ activities as high as 260-fold normal did not cross-react with anti-human pancreatic PLA$_2$.

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 PLA$_2$ and by generation of vasoactive products of PLA$_2$ hydrolysis, contributes to circulatory collapse in septic shock.

Exogenous PLA$_2$ is vasoactive both locally and systemically. Intravenous infusion of snake venom PLA$_2$ caused a rapid and profound drop in MABP in dogs [33], cats [34] and rats [35]. Similarly, infusion of septic shock serum PLA$_2$ produced hypotension in rabbits at a rate parallel to that induced by endotoxin [23].

Serum levels of endogenous PLA$_2$ 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 PLA$_2$ is, as yet, undefined. The septic shock PLA$_2$ appears to be of extra-pancreatic origin based on the absence of cross-reactivity with anti-human pancreatic PLA$_2$ antiserum. Nor does the enzyme appear to originate from spleen or circulating leukocytes in that comparable elevations of serum PLA$_2$ have been noted in septic patients post-splenectomy or with profound leukopenia post-cytotoxic chemotherapy (unpublished observations).

While the mechanism of PLA_2-induced hypotension is not known, the release of PLA_2 into the systemic circulation may lead to the generation of vasoactive eicosanoids and lysophosphatides. Perfusion of rat liver ex vivo with exogenous porcine pancreatic PLA_2 resulted in the release of 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2 from intact cells and tissues [38]. Intravenous infusion of snake venom PLA_2 in rats caused a significant fall in MABP which correlated with the rise in the ratio of 6-keto-$PGF_{1\alpha}$ to TXB_2 [39, 40]. PLA_2 was also shown to cause an increase in lung perfusion pressure [41], which correlated linearly with the ratios of TXB_2 to 6-keto-PGF_α. These data suggest that circulating PLA_2 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 PLA_2 release into the systemic circulation. These observations suggest an antiphospholipase effect of exogenous or endogenous corticosteroids. In patients with septic shock, cortisol levels were concordant with serum PLA_2 levels in all survivors of septic shock but discordant 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 A_2, phospholipase inhibitory proteins (PLIP) and corticosteroids, will require further investigation.

These data demonstrate that PLA_2 fulfills several requisite criteria for mediators of circulatory shock as proposed by Lefer [6]. First, there is a demonstrable increase in circulating PLA_2 in response to bacteria or bacterial products. Second, exogenous PLA_2 was shown to cause hypotension in experimental animals and endogenous PLA_2 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 PLA_2 awaits the development of a selective and non-toxic inhibitor of circulating PLA_2 in man.

Acknowledgement

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