Phospholipase A2 Levels in Acute Chest Syndrome of Sickle Cell Disease

By Lori A. Styles, Casper G. Schalkwijk, Anton J. Aarman, Elliott P. Vichinsky, Bertram H. Lubin, and Frans A. Kuypers

Acute chest syndrome (ACS) is associated with significant morbidity and is the leading cause of death in patients with sickle cell disease (SCD). Recent reports suggest that bone marrow fat embolism can be detected in many cases of severe ACS. Secretory phospholipase A2 (sPLA2) is an important inflammatory mediator and liberates free fatty acids, which are felt to be responsible for the acute lung injury of the fat embolism syndrome. We measured sPLA2 levels in 35 SCD patients during 20 admissions for ACS, 10 admissions for vaso-occlusive crisis, and during 12 clinic visits when patients were at the steady state. Eleven non-SCD patients with pneumonia were also evaluated. To determine if there was a relationship between sPLA2 and the severity of ACS we correlated sPLA2 levels with the clinical course of the patient. In comparison with normal controls (mean = 3.1 ± 1.1 ng/mL), the non-SCD patients with pneumonia (mean = 68.6 ± 82.9 ng/mL) and all three SCD patient groups had an elevation of sPLA2 (steady state mean = 10.0 ± 8.4 ng/mL; vaso-occlusive crisis mean = 23.7 ± 40.5 ng/mL; ACS mean = 336 ± 209 ng/mL). In patients with ACS sPLA2 levels were 100-fold greater than normal control values, 35 times greater than values in SCD patients at baseline, and five times greater than non-SCD patients with pneumonia. The degree of sPLA2 elevation in ACS correlated with three different measures of clinical severity and, in patients followed sequentially, the rise in sPLA2 coincided with the onset of ACS. The dramatic elevation of sPLA2 in patients with ACS but not in patients with vaso-occlusive crisis or non-SCD patients with pneumonia and the correlation between levels of sPLA2 and clinical severity suggest a role for sPLA2 in the diagnosis and, perhaps, in the pathophysiology of patients with ACS.

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MATERIALS AND METHODS

Patients. The sPLA2 level was determined in 35 SCD patients. Patients ranged in age from 1 to 20 years (mean = 11 years). All patients had a diagnosis confirmed by standard electrophoresis and isoelectric focusing methods. There were 30 patients with hemoglobin SS, two with hemoglobin SC, and three with hemoglobin S-β thalassemia. Serum sPLA2 levels were measured during 20 admissions for ACS and 10 admissions for VOC. Four patients were tested during more than one hospitalization. Eleven SCD patients had PLA2 levels drawn during a comprehensive health care visit in the sickle cell clinic when there was no evidence of illness. There was no overlap in patients between the steady state and ACS groups. Secretory PLA2 levels were also drawn in 19 normal controls and in 11 children without SCD who were admitted to the hospital with pneumonia.

Hospitalizations. Acute chest syndrome was defined as the development of a new infiltrate on chest radiography in combination with fever, respiratory symptoms, or chest pain. Patients admitted with ACS were treated following a standard protocol that included hydration at one and one-quarter times maintenance, parenteral cefuroxime and oral erythromycin, arterial blood gas monitoring, and daily complete blood counts. Tranfusion was used at the attending physician’s discretion based on the patient’s clinical course. Intravenous narcotics and nonsteroidal antiinflammatory medications were used to treat accompanying pain events.

Vaso-occlusive crisis was defined as an admission for pain, which required parenteral narcotics and indicated no cause for pain other than SCD. Patients admitted with VOC were treated following a...
with 150 PL PBS containing 30 mg/mL bovine serum albumin (BSA) for 30 minutes at room temperature. Samples diluted in PBS, 1 mg/mL Tween 20 and 2 mg/mL gelatin (PTG) were incubated in the wells for 1 hour, and after washing, the wells were incubated for 1 hour with the detecting antibody, which was biotinylated and diluted 1:1000 in PTG. Thereafter the wells were incubated for 30 minutes with streptavidin-horseradish peroxidase conjugate, diluted 1:1000 in PTG. The plate was washed and the whole complex was incubated with the chromogenic substrate 3,3',5,5' tetramethyl benzidine (0.1 mg/mL), 30 µg/mL H2O2 in 0.1 mol/L sodium acetate buffer pH 5.5 for 8 minutes. The reaction was stopped by adding an equal volume of 1 mol/L H2SO4 to each well and the absorbance was read at 490 nm in a microtiter plate reader (EAR 400, SLT-Lab Instruments, Austria). Results were compared with those obtained with cultured medium from Hep G2 cells stimulated with human interleukin-6.14 The amount of sPLA2 in this cultured medium was assessed by comparison with purified recombinant human sPLA2 (kindly provided by Dr H.M. Verheij, Department of Enzymology and Protein Engineering, University of Utrecht, Utrecht, the Netherlands). The lower limit of detection was approximately 1 ng/mL and the inter-measurement variability on a single sample was up to 10% to 15%.

Secretory PLA2 concentration as measured with ELISA in plasma was shown to have an excellent linear correlation with sPLA2 activity (r² = 0.953), confirming that the sPLA2 found in the plasma is in an active form (Fig 1). Virtually identical results were found when either plasma or serum was used. Hence, sPLA2 concentration data was used to analyze the relationship between the presence of active sPLA2 and ACS. Nineteen normal (hemoglobin AA) controls also had PLA2 determination to confirm that assay values were in the expected range reported in other series.

Statistical evaluation. Statistical evaluation was performed using a nonparametric procedure for the four patient groups (ACS, VOC, SCD at steady state, and non-SCD with pneumonia) using the Kruskal-Wallis One Way Analysis of Variance on Ranks. Dunn’s Method was used to determine if individual group medians were significantly different.

### RESULTS

Compared with values obtained from control patients, mean sPLA2 concentrations were elevated in all three SCD patient groups studied (ACS, VOC, and steady state) and in the non-SCD group with pneumonia (Table 1). Steady state SCD patients had a mean sPLA2 level of 10.0 ± 8.4 ng/mL (median = 9 ng/mL), which was three times higher than values in normal controls (mean = 3.1 ± 1.1 ng/mL, median = 3.1 ng/mL). Sickle cell disease patients with VOC had a similar threefold to fivefold elevation above normal controls. The sPLA2 concentration of patients with VOC (mean = 23.7

<table>
<thead>
<tr>
<th>Patient Groups</th>
<th>Mean (median) (ng/mL)</th>
<th>Range (ng/mL)</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steady state SCD (n = 11)</td>
<td>10.0 ± 8.4 (9.1)</td>
<td>1.1-28.5</td>
<td>&lt;.05</td>
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<tr>
<td>VOC (n = 10)</td>
<td>23.7 ± 40.5 (8.7)</td>
<td>1.8-134.6</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>ACS (n = 20)</td>
<td>336 ± 209 (289)</td>
<td>12-725</td>
<td>—</td>
</tr>
<tr>
<td>Pneumonia (n = 11)</td>
<td>68.6 ± 82.9 (38)</td>
<td>6-267</td>
<td>&lt;.05</td>
</tr>
</tbody>
</table>

* P values are for differences between ACS and the other patient groups using analysis of variance techniques (see the Materials and Methods section). All other comparisons are not significant.

### Table 1. Secretory PLA2 Levels in Sickle Cell Disease

![Graph](Fig 1. Correlation of sPLA2 activity and protein concentration. Correlation between sPLA2 activity as measured by hydrolysis of radiolabeled phosphotidylethanolamine, and sPLA2 protein concentration as measured by ELISA in 28 plasma samples of SCD patients (r² = 0.953). AU, arbitrary units.)
Acute chest syndrome patients had a mean sPLA2 level of 336 ± 209 ng/mL (median = 289 ng/mL), which was 100 times greater than normal controls and 35 times greater than in samples from SCD patients in the steady state (Fig 2). In 18 of the 20 ACS episodes there was a history of vaso-occlusive pain preceding or accompanying ACS. Both patients without a history of pain were under 3 years of age and one of these was the only ACS patient without a significant elevation of sPLA2 above baseline (12 ng/mL). Phospholipase A2 levels in ACS patients were also significantly elevated above non-SCD pneumonia patients (mean = 68.6 ± 82.9 ng/mL, median = 38 ng/mL).

In the 15 SCD patients followed with serial sPLA2 measurements, sPLA2 levels seemed to parallel their clinical course. Seven patients with VOC were followed with sequential sPLA2 levels and four of these went on to develop ACS. In all four of these patients, sPLA2 levels rose abruptly with the development of ACS and then decreased as the patient clinically improved (Fig 3). In the three patients who did not develop ACS, sPLA2 levels remained low. The remaining eight patients were admitted with a diagnosis of ACS. Sequential evaluation of sPLA2 concentration in these patients documented that sPLA2 levels were highest with the onset of ACS and declined as the patient recovered.

Secretory PLA2 levels were highest in patients with clinically more severe lung disease as assessed by arterial blood gas results and the need for transfusion. Arterial blood gas measurements in room air were performed on 15 ACS patients. Comparisons of ACS patients with and without significant hypoxia (PaO2 <70 and ≥70 mm Hg) and with and without increased alveolar-arterial O2 gradients (>30 and ≤30 mm Hg) revealed an excellent correlation between elevated sPLA2 clinical severity (Fig 4).

Secretory PLA2 concentration was also compared in the transfused versus untransfused patient groups. One patient from the transfused group was removed from the analysis because he was transfused secondary to aplastic crisis and not due to pulmonary disease. Also, one severely alloimmunized patient was removed from the analysis because, despite...
severe hypoxia, he could not be transfused secondary due to a lack of compatible blood. Secretory \( \text{sPLA}_2 \) levels were significantly higher in the group needing transfusion, suggesting a relationship between \( \text{sPLA}_2 \) concentration and clinical severity (Fig 4).

DISCUSSION

Elevated levels of \( \text{sPLA}_2 \) have been reported in ARDS, sepsis, multi-organ dysfunction, and arthritis. Secretory \( \text{PLA}_2 \) is felt to be an important mediator of inflammation in these conditions as it can hydrolyze arachidonic acid from the sn-2 position of phospholipids providing the essential substrate for a number of eicosanoids. Secretory \( \text{PLA}_2 \) is upregulated in response to proinflammatory cytokines such as tumor necrosis factor and interleukin-1. In animal models, \( \text{PLA}_2 \) administered intravenously or instilled intratracheally, produces diffuse ARDS-like changes including diffuse alveolar edema and an inflammatory cell influx. This same lung injury can be prevented by pretreatment with inhibitors of \( \text{PLA}_2 \). In humans, \( \text{sPLA}_2 \) is increased in patients with ARDS and has been shown to correlate with outcome, severity of lung injury, and alveolar-arterial oxygen gradient.

We found dramatically elevated levels of \( \text{sPLA}_2 \) in SCD patients with ACS. Similar elevations were not seen in SCD patients with VOC alone and the presence or absence of fever with pain crisis did not alter this result. Despite the fact that most of these ACS patients were not seriously ill, their \( \text{sPLA}_2 \) levels were similar to that found in critically ill patients with ARDS and sepsis. Additionally, \( \text{sPLA}_2 \) levels in the ACS group were nearly five times greater than levels in non-SCD patients with pneumonia and suggest that \( \text{sPLA}_2 \) elevation is not just a secondary marker for lung damage.

As in ARDS, \( \text{sPLA}_2 \) concentration in patients with ACS correlated with several measures of clinical severity. The correlation between \( \text{sPLA}_2 \) and arterial-alveolar gradient is particularly relevant as Emre et al. recently reported this to be the strongest predictor of clinical severity in ACS. In the ACS patients followed sequentially, the increase in \( \text{sPLA}_2 \) coincided with the onset of ACS and levels declined as the patient improved. In total, these results suggest that there is a relationship between \( \text{sPLA}_2 \) and ACS.

The detection of fat embolism and elevated levels of \( \text{sPLA}_2 \) in ACS suggests a causal relationship between free fatty acids, fatty acid-derived lipid mediators, and ACS. Since vaso-occlusive crisis can result in the intravascular release of bone marrow fat and lead to pulmonary fat embolism, this may trigger the upregulation of \( \text{sPLA}_2 \) and generate more free fatty acids, either systemically or locally in the lung. While the pulmonary toxicity of increased free fatty acids in an in vitro setting, as well as in animal models, is well established, documenting the toxic effects of free fatty acids in vivo has been more difficult. A recent report, however, indicates that both palmitic and oleic acid levels as well as total free fatty acids are elevated in ACS but not in vaso-occlusive crisis and further supports the above hypothesis. Bronchoalveolar lavage (BAL) has recently been demonstrated to be a safe and effective means to detect pulmonary fat embolism. The ACS patients in this study did not undergo BAL but correlating \( \text{sPLA}_2 \) levels with the results of BAL in the future, could also help document a relationship between \( \text{sPLA}_2 \) and fat embolism. Implicating \( \text{sPLA}_2 \) in the pathophysiology of ACS and fat embolism has clinical importance in that \( \text{sPLA}_2 \) may be useful as an early marker for ACS. In the vaso-occlusive crisis patients followed sequentially, \( \text{sPLA}_2 \) levels seemed to increase in the 2 to 3 days before ACS. These data are preliminary, however, and sequential \( \text{sPLA}_2 \) levels on a large number of SCD patients admitted with pain will be necessary to further evaluate \( \text{sPLA}_2 \) usefulness as a predictor for ACS. If \( \text{sPLA}_2 \) proves accurate in predicting ACS, therapies such as transfusion or \( \text{sPLA}_2 \) inhibitors could be considered earlier in disease. Because of the association of \( \text{sPLA}_2 \) with rheumatoid arthritis and sepsis, there is already considerable interest in the development of \( \text{sPLA}_2 \) inhibitors.

Our study also demonstrated elevated \( \text{sPLA}_2 \) levels in SCD patients at baseline compared with normal controls. If bone marrow fat embolism is a stimulus for \( \text{sPLA}_2 \), increased baseline \( \text{sPLA}_2 \) levels may reflect ongoing or intermittent leakage of marrow fat intravascularly. Alternatively, higher levels of \( \text{sPLA}_2 \) at baseline may reflect a disturbed balance of inflammatory mediators. In support of this latter hypothesis, reports in SCD patients have documented elevations of endotoxins, tumor necrosis factor and interleukin-1, all known to upregulate \( \text{sPLA}_2 \). Other studies have also reported that SCD patients often have altered levels of lipid mediators related to the action of \( \text{sPLA}_2 \), including prostaglandins, leukotrienes, and thromboxanes.

In summary, we documented dramatically elevated levels of \( \text{sPLA}_2 \) in association with ACS. The rise in \( \text{sPLA}_2 \) coincided with the onset of ACS and the degree of \( \text{sPLA}_2 \) elevation correlated with several measures of clinical severity. In addition to documenting high levels of \( \text{sPLA}_2 \) in ACS, we found that SCD patients have elevated levels at baseline compared with normal controls. The significance of this is unknown at present but may reflect an altered regulation of the inflammatory system. Secretory \( \text{PLA}_2 \) may be useful in identifying patients at risk for ACS and provide justification for the evaluation of additional therapies to treat this complication.

ACKNOWLEDGMENT

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