Phospholipase A2 Levels in Acute Chest Syndrome of Sickle Cell Disease

By Lori A. Styles, Casper G. Schalkwijk, Anton J. Aarsman, 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 mg/mL; ACS mean = 336 ± 209 mg/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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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-LabInstruments, Austria). Results were compared with those obtained with cultured medium from Hep G2 cells stimulated with human interleukin-6. 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 1. Secretory PLA2 Levels in Sickle Cell Disease

<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>
</tr>
<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.

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**protocol that included intravenous hydration, not to exceed one and one-half times maintenance, intravenous narcotics, and nonsteroidal antiinflammatory drugs. If fever developed, patients were evaluated with chest radiography, blood and urine cultures, and intravenous cefuroxime was started.**

For the non-SCD patients admitted with pneumonia, diagnosis was established with a chest radiograph demonstrating an infiltrate in combination with symptoms of respiratory distress and hypoxia. Clinical and laboratory data were collected on all hospitalized SCD patients including history of preceding or accompanying pain, PaO2 on room air arterial blood gas, transfusion history, and the presence or absence of fever. Arterial blood gas measurements were determined using an AVL 995 (AVL Scientific Corp, Roswell, GA). Alveolar-arterial oxygen gradient was calculated from room air arterial blood gas values according to the following formula: (A - a) PaO2 = (713 × FiO2) - (PaCO2 × 1.2) - PaO2. All sPLA2 levels were measured using the method described below. Fifteen SCD patients had two or more sPLA2 level determinations during a single hospital admission. In the patients that were followed with sequential sPLA2 levels from before the onset of ACS through convalescence, the sample with the highest sPLA2 value was used in the calculation of statistical significance.

**Secretory PLA2 activity and concentration.** Phospholipase A2 activity was measured with 1-acyl-2-[14C]linoisoyl-sn-glycerol-3-phosphoethanolamine, prepared as described by Van den Bosch et al.‡ as substrate. Enzymatic activity was assayed by incubating 0.2 mmol/L radioactive substrate (specific radioactivity 3,000 dpm/nmol) in 0.2 mol/L Tris/HCl (pH 8.5). 10 μmol/L Ca2+ and 5 μL plasma in a final volume of 200 μL. After 30 minutes at 37°C, reactions were stopped by extracting the liberated 14C-labeled fatty acid by a modified Dole-extraction procedure and the radioactivity was determined by liquid scintillation counting. Secretory phospholipase A2 antigen levels in plasma were determined with a sandwich enzyme-linked immunosorbent assay (ELISA) modified from Smith et al.†† Two different monoclonal antibodies against human sPLA2 (kindly provided by Dr F.R. Taylor Jr, Oklahoma Medical Research Foundation, Oklahoma City) were used as coating and detecting antibodies, respectively. Microtiter plates (Nunc-Immuno Plate MaxiSorb™; A/S Nunc, Roskilde, Denmark) were coated with the first antibody (100 μL, 2.5 μg/mL) in phosphate-buffered saline (PBS) for 16 hours at 4°C. After washing, the wells were blocked with 150 μL 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-LabInstruments, Austria). Results were compared with those obtained with cultured medium from Hep G2 cells stimulated with human interleukin-6. 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%.

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PHOSPHOLIPASE A₂ IN ACUTE CHEST SYNDROME

Fig 2. sPLA₂ levels in different SCD patient groups. Comparison of mean sPLA₂ levels in three different groups of SCD patients and in non-SCD patients with pneumonia. ACS, n = 20; VOC, vaso-occlusive crisis, n = 10; steady state SCD patients at the time of routine comprehensive health care visit, n = 11; non-SCD pneumonia patients, n = 11.

± 40.5 ng/mL, median = 8.7 ng/mL) was not significantly different from the value found in SCD patients in the steady state. Four of the ten patients with VOC had fever during hospitalization. Comparison of the febrile and afebrile groups showed no difference in sPLA₂ concentration (P = .45).

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 PLA₂ levels were significantly higher in the group needing transfusion, suggesting a relationship between sPLA₂ concentration and clinical severity (Fig 4).

**DISCUSSION**

Elevated levels of sPLA₂ have been reported in ARDS, sepsis, multi-organ dysfunction, and arthritis. Secretory PLA₂ 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 PLA₂ is upregulated in response to proinflammatory cytokines such as tumor necrosis factor and interleukin-1. In animal models, PLA₂ 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 PLA₂. In humans, PLA₂ 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 sPLA₂ 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 sPLA₂ levels were similar to that found in critically ill patients with ARDS and sepsis. Additionally, sPLA₂ levels in the ACS group were nearly five times greater than levels in non-SCD patients with pneumonia and suggest that sPLA₂ elevation is not just a secondary marker for lung damage.

As in ARDS, sPLA₂ concentration in patients with ACS correlated with several measures of clinical severity. The correlation between sPLA₂ 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 sPLA₂ coincided with the onset of ACS and levels declined as the patient improved. In total, these results suggest that there is a relationship between sPLA₂ and ACS.

The detection of fat embolism and elevated levels of sPLA₂ 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 sPLA₂ 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 sPLA₂ levels with the results of BAL, in the future, could also help document a relationship between sPLA₂ and fat embolism. Implicating sPLA₂ in the pathophysiology of ACS and fat embolism has clinical importance that sPLA₂ may be useful as an early marker for ACS. In the vaso-occlusive crisis patients followed sequentially, sPLA₂ levels seemed to increase in the 2 to 3 days before ACS. These data are preliminary, however, and sequential sPLA₂ levels on a large number of SCD patients admitted with pain will be necessary to further evaluate sPLA₂ usefulness as a predictor for ACS. If sPLA₂ proves accurate in predicting ACS, therapies such as transfusion or sPLA₂ inhibitors could be considered earlier in disease.

Because of the association of sPLA₂ with rheumatoid arthritis and sepsis, there is already considerable interest in the development of PLA₂ inhibitors.

Our study also demonstrated elevated sPLA₂ levels in SCD patients at baseline compared with normal controls. If bone marrow fat embolism is a stimulus for sPLA₂, increased baseline sPLA₂ levels may reflect ongoing or intermittent leakage of marrow fat intravascularly. Alternatively, higher levels of sPLA₂ at baseline may reflect a disturbed balance of inflammatory mediators. In support of this latter hypothesis, reports in SCD patients have documented elevations of endotoxin, tumor necrosis factor and interleukin-1, all known to upregulate sPLA₂. Other studies have also reported that SCD patients often have altered levels of lipid mediators related to the action of sPLA₂, including prostaglandins, leukotrienes, and thromboxanes.

In summary, we documented dramatically elevated levels of sPLA₂ in association with ACS. The rise in sPLA₂ coincided with the onset of ACS and the degree of sPLA₂ elevation correlated with several measures of clinical severity. In addition to documenting high levels of sPLA₂ 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 PLA₂ 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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