

Association of Type II Secretory Phospholipase A<sub>2</sub> and Surfactant Protein D with the  
Pulmonary Oxygenation Potential in Patients with Septic Shock during Polymyxin-B  
Immobilized Fiber-Direct Hemoperfusion

Yoriko Ishibe, Shigehiro Shibata, Gaku Takahashi, Yasushi Suzuki, Yoshihiro Inoue,  
Shigeatsu Endo

Department of Critical Care Medicine, School of Medicine, Iwate Medical University,  
Morioka, Japan;

Iwate Prefectural Advanced Critical Care and Emergency Center, Morioka, Japan.

Corresponding author: Yoriko Ishibe, MD

Department of Critical Care Medicine, Iwate Medical University,

19-1 Uchimaru, Morioka 020-8505, Japan

Email: yorikoi7702@yahoo.co.jp

Telephone number: 019-651-5111

Fax number: 019-651-5151

Running head: Type II Secretory PLA<sub>2</sub> and SP-D during PMX-DHP

## **Abstract**

The present study was undertaken to analyze the association of type II secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>-II) and surfactant protein D (SP-D) with the pulmonary oxygenation potential in patients with septic shock during polymyxin-B immobilized fiber-direct hemoperfusion (PMX-DHP). The study was conducted in 25 patients with acute lung injury (ALI) or acute respiratory distress syndrome (ARDS). PMX-DHP lowered the blood endotoxin level in all patients. Following PMX-DHP, there were decreases from day 0 → day 1 → day 2 in both the mean plasma sPLA<sub>2</sub>-II level (340 → 260 → 189 ng/mL) and plasma SP-D level (483 → 363 → 252 ng/mL). The PaO<sub>2</sub>/FiO<sub>2</sub> ratio (P/F ratio) rose (210 → 237 → 262) in all patients. Upon the onset of ALI or ARDS, there was a significant negative correlation between the sPLA<sub>2</sub>-II level and the P/F ratio. Furthermore, there was a significant positive correlation between the sPLA<sub>2</sub>-II and TNF- $\alpha$  levels. The results suggest that as the blood endotoxin levels were lowered by the PMX-DHP, the inflammatory reactions were suppressed, with suppressed formation of sPLA<sub>2</sub>-II and improved pulmonary oxygenation potential. The results also suggested possible involvement of TNF- $\alpha$  in the production of sPLA<sub>2</sub>-II.

**Key Words:** Sepsis, acute respiratory distress syndrome (ARDS), blood purification

## INTRODUCTION

Systemic inflammatory reactions triggered by severe infections or stresses such as surgery, trauma, burn and severe pancreatitis are collectively called “systemic inflammatory response syndrome (SIRS)” [1]. Acute lung disorder in SIRS is characterized by pulmonary edema formation associated with increased pulmonary vascular permeability, which causes sharp hypoxemia. In the presence of sepsis, endotoxin (ET) causes pooling of neutrophils in the lungs mediated by cytokines such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-6 and IL-8, exerting its activity synergistically with complementary systems, resulting in the formation of harmful substances by neutrophils and macrophages, and the onset of acute lung injury (ALI)/ acute respiratory distress syndrome (ARDS) [2].

Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) are widely distributed enzymes that exist in many isoforms, including the 14-kDa secretory PLA<sub>2</sub> (sPLA<sub>2</sub>) and the 85-kDa cytosolic PLA<sub>2</sub> [3]. Currently, at least 11 mammalian isoforms of sPLA<sub>2</sub> are identified [4], and type II secretory phospholipase A<sub>2</sub> (sPLA<sub>2</sub>-II) is detected at high concentrations in areas of inflammation [4, 5]. Therefore, sPLA<sub>2</sub>-II may be an acute phase reactant by inflammatory response and/or tissue injury [6, 7]. Many cell types such as endothelial cells, smooth muscle cells, platelets, mast cells, neutrophils and hepatic cells secrete sPLA<sub>2</sub>-II and all these cells stimulated by proinflammatory cytokines may contribute to the elevation of circulating levels during acute phase responses [8, 9]. Indeed, elevation of serum sPLA<sub>2</sub>-II levels was observed in malaria [10], sepsis/septic shock [11-13], and after elective surgery [6]. Furthermore, sPLA<sub>2</sub>-II excreted into the alveoli by macrophages and mast cells [14] has been reported to play an important role in the development of respiratory disorders [15].

Lung surfactant is a physiologically active substance synthesized and secreted by/from type II alveolar cells, which line the alveolar lumen. It prevents alveolar collapse at the end of expiration [16]. Surfactant-induced injuries have also been reported to cause lung disorders [17]. We previously reported simultaneous elevation of the plasma sPLA<sub>2</sub>-II and surfactant protein-D (SP-D) levels in patients with ALI or ARDS [18-20]. Recently, elevation of the blood levels of surfactant was demonstrated in patients with ARDS, which likely serves as the cause or a prognostic determinant of ARDS [21, 22].

Polymyxin-B immobilized fiber-direct hemoperfusion (PMX-DHP) has been reported as a useful modality for treating endotoxemia through adsorption of ET [23]. Blood purification using an extracorporeal hemoadsorption cartridge was developed to eliminate ET from peripheral blood circulation. The cartridge for PMX was produced on polystyrene fibers by immobilizing polymyxin B that had interacted with the lipid A portion of ET [24]. We have applied PMX-DHP to many patients with septic shock complicated by endotoxemia and demonstrated its usefulness [25]. Following PMX-DHP, the PaO<sub>2</sub>/FiO<sub>2</sub> ratio (P/F ratio) has been reported to increase, indicating improved respiratory status [25]. Previous reports have demonstrated the efficacy of PMX-DHP in improving the blood pressure and the pulmonary oxygenation potential [26, 27]. To date, however, the mechanisms underlying the ability of PMX-DHP to improve the pulmonary oxygenation potential have not been clarified. The present study was designed to analyze the association of sPLA<sub>2</sub>-II and SP-D levels with pulmonary oxygenation potentials in patients with endotoxemia accompanied by shock during PMX-DHP.

## **SUBJECTS AND METHODS**

This study was carried out with the approval of the Iwate Medical University Ethics Committee, after obtaining informed consent from each patient (or his/her family member). A total of 25 patients with septic shock (ET level  $\geq 1.1$  pg/mL, systolic blood pressure  $< 90$  mmHg) who underwent PMX-DHP during the four-year period between 2005 and 2009 were enrolled in the study. The duration of individual PMX-DHP sessions ranged from 2 to 6 hours, depending on the condition in individual cases. If no elevation of blood pressure was observed at 2 hours, the PMX-DHP session was extended to a maximum of 6 hours. Each patient underwent a maximum of two sessions of PMX-DHP. In cases where the systolic blood pressure showed no tendency to fall on the day after the first session of PMX-DHP, as compared with the level recorded immediately after the first session, the second session of PMX-DHP was withheld. In cases where the systolic blood pressure tended to fall further on the day after the first session of PMX-DHP, a second session of PMX-DHP was applied, even if the blood ET level had fallen to  $< 1.1$  pg/mL.

The diagnosis of sepsis was based on the criteria established by the members of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference Committee [1]. ALI/ARDS was diagnosed in accordance with the criteria proposed by the American-European Consensus Conference [28]. Indicators of severity used in this study include acute physiology and chronic health evaluation score (APACHE II score) [29] and sequential organ failure assessment score (SOFA score) [30]. These diagnoses and scorings were made jointly by doctors qualified as both infection control doctors and specialists in critical care medicine, and a clinical research coordinator.

All of the subjects were provided with the routine treatments for septic shock. In relation to the treatment of septic ALI/ARDS, our facility has not adopted the use of low tidal volumes [31]. Our routine management includes the following. In patients showing poor improvement of the P/F ratio, continuous infusion of methylprednisolone for 48–72 hours (0.83 mg/kg/h) is applied, if deemed appropriate by the attending physician [32]. Each patient is placed in the prone position twice a day, in the morning and in the afternoon (30 minutes each time). In addition, sivelestat sodium hydrate, a polymorphonuclear elastase inhibitor available for use only in Japan [33], is used in all cases.

PMX-DHP (Toray Medical Co., Ltd., Tokyo, Japan) was used to remove ET from the blood, with nafamostat mesilate (Torii Pharmaceutical Co., Ltd., Tokyo, Japan) used as the anticoagulant. The PaO<sub>2</sub>/FiO<sub>2</sub> ratio (P/F ratio) served as an indicator of the pulmonary oxygenation potential.

Blood samples for measurement of the plasma levels of ET, sPLA<sub>2</sub>-II, SP-D and TNF- $\alpha$  were obtained in heparinized endotoxin-free syringes. Each blood sample was immediately centrifuged at 3,000 rpm for 40 seconds and the supernatant was removed from blood samples to obtain platelet-rich plasma (PRP). ET was measured immediately after obtaining the PRP sample. The PRP samples that were unused for the ET measurement were stored at -80°C until required for later simultaneous measurements of sPLA<sub>2</sub>-II, SP-D and TNF- $\alpha$ . The plasma ET level was measured by a high-sensitivity method (ET-specific turbidimetric time assay) using a Toxinometer<sup>®</sup> ET-500 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) [34, 35], and the cutoff level for the diagnosis of endotoxemia was set at 1.1 pg/mL [35]. sPLA<sub>2</sub>-II was measured using an immunoradiometric assay (Shionogi Research Institute, Osaka,

Japan) [36], and the normal range of this parameter was set at  $<3.7$  pg/mL. Plasma SP-D was measured using enzyme-linked immunosorbent assay (ELISA) (Teijin Institute for Bio-medicine, Tokyo, Japan), and the cutoff level was set at 109.8 ng/mL. Plasma TNF- $\alpha$  was also measured using ELISA (TFB, Tokyo, Japan), and the limit of detection of this assay was 3 pg/mL.

Values are expressed as mean  $\pm$  SD. The significances of differences were tested by an unpaired t-test. Pearson's formula was employed for the analysis of correlations. In all tests,  $P<0.05$  was regarded as denoting statistical significance.

## **Results**

PMX-DHP was performed on 25 patients, of whom 10 patients received one session of PMX-DHP, and the remaining 15 received 2 sessions. There were 16 males and 9 females, aged  $71.9 \pm 7.8$  years (Table I). The underlying disease was peritonitis in 12 cases, burn in 4 cases, pneumonia in 3 cases, urinary tract infection in 3 cases, multiple trauma in 2 cases, and soft tissue infection in 1 case (Table I). The APACHE II score was  $31.5 \pm 6.0$  and the SOFA score was  $12.1 \pm 4.1$  at the time of entry into the study.

All patients emerged from the shock state following the PMX-DHP. The plasma ET level on Day 0 (immediately before the first session of PMX-DHP) was  $12.4 \pm 23.5$  pg/mL, while that immediately after the end of the first session of PMX-DHP was  $1.6 \pm 2.9$  pg/mL. The ET level on Day 1 was  $0.9 \pm 1.0$  pg/mL (in the case of patients who received a second session of PMX-DHP, the ET level immediately before the second session). The plasma ET level immediately after the end of the second session of PMX-DHP was  $0.3 \pm 0.2$  pg/mL. Thus, the ET level decreased significantly after the

first session of PMX-DHP as compared with the level recorded before the first session, or after the second session of PMX-DHP as compared with the level recorded before the second session.

The plasma sPLA<sub>2</sub>-II level on Day 0 (immediately before the first session of PMX-DHP) was  $340.0 \pm 150.7$  ng/mL. It decreased to  $259.9 \pm 120.4$  ng/mL on Day 1 (immediately before the second session of PMX-DHP). On Day 2, it decreased further to  $189.0 \pm 73.4$  ng/mL. Thus, the plasma sPLA<sub>2</sub>-II levels on Day 1 and Day 2 were significantly lower than the level on Day 0 (Fig. 1).

The plasma SP-D level on Day 0 (immediately before the first session of PMX-DHP) was  $483.3 \pm 290.0$  ng/mL. The level on Day 1 was  $362.8 \pm 199.0$  ng/mL (in patients who received a second session of PMX-DHP, the level immediately before the second session). On Day 2, it decreased further to  $251.6 \pm 117.0$  ng/mL. Thus, the plasma SP-D levels on Day 1 and Day 2 were significantly lower than the level on Day 0 (Fig. 2).

The plasma TNF- $\alpha$  level on Day 0 (immediately before the first session of PMX-DHP) was  $183.6 \pm 120.6$  pg/mL. On Day 1, it decreased to  $112.4 \pm 56.8$  pg/mL (in patients who received a second session of PMX-DHP, the level immediately before the second session). On Day 2, it decreased further to  $69.7 \pm 44.0$  pg/mL. Thus, the plasma TNF- $\alpha$  levels on Days 1 and 2 were significantly lower than the level on Day 0 (Fig. 3).

The P/F ratio on Day 0 (immediately before the first session of PMX-DHP) was  $210.0 \pm 51.8$ . On Day 1, the P/F ratio rose to  $236.8 \pm 48.9$  (in patients who received the second session of PMX-DHP, the ratio immediately before the second session). On Day 2, the ratio rose further to  $262.2 \pm 52.1$ . Thus, the P/F ratios on Day 1 and Day 2 were significantly higher than the ratio on Day 0 (Fig. 4).



Prior to the PMX-DHP, there was a significant positive correlation between the plasma TNF- $\alpha$  level and plasma sPLA<sub>2</sub>-II level (Fig. 5), and also between the plasma sPLA<sub>2</sub>-II level and plasma SP-D level (Fig. 6); conversely, there was a significant negative correlation between the plasma sPLA<sub>2</sub>-II level and the P/F ratio (Fig. 7), and also between the plasma SP-D level and the P/F ratio (Fig. 8).

The overall 30-day mortality rate was 8%. The overall 60-day, 90-day and 180-day mortality rates were 12%, 20% and 20%, respectively.

## **Discussion**

This study showed that PMX-DHP decreased the plasma levels of ET, sPLA<sub>2</sub>-II, SP-D and TNF- $\alpha$  which had been elevated in septic shock patients with ALI/ARDS and increased P/F ratio, and also found that a significant positive correlation between the plasma sPLA<sub>2</sub>-II level and plasma SP-D level. However, we also found a significant negative correlation between the plasma sPLA<sub>2</sub>-II level, the plasma SP-D level and the P/F ratio.

We previously reported that in patients with septic ALI/ARDS, the plasma levels of sPLA<sub>2</sub>-II and SP-D, in addition to those of the inflammatory cytokines (e.g., TNF- $\alpha$ , IL-8), were elevated [18-20]. While many open questions remain with regard to the actions of SP-D, SP-D may play important roles in septic ALI/ARDS. Because SP-D-knockout mice showed abnormal accumulation of surfactant [37], and because accumulation of the surfactant lipid was suppressed when specific SP-D was expressed in the lungs of SP-D-deficient mice [38], it seems possible that SP-D plays an important role in surfactant metabolism. SP-D also plays an important role in the early mechanism of prevention of ET-induced lung disorders. SP-D derived from the rat has been shown

to bind to the cell membrane receptor CD14 of ET in a concentration-dependent manner [39], and lung disorders induced by ET administration could be suppressed in mice showing excessive SP-D expression [40]. Furthermore, SP-D is considered to be involved in alveolar immune regulation mediated by macrophages. According to a previous report, surfactant suppresses the expression of secretory type PLA<sub>2</sub> from guinea pig alveolar macrophages [41]. It has also been reported that inactivation of the surfactant by PLA<sub>2</sub> causes respiratory disorders [42].

Although SP-D has been shown to appear in the blood at high levels in patients with lung diseases such as interstitial pneumonia and pulmonary alveolar proteinosis, the mechanism of appearance of SP-D in the blood also involves many open questions. Factors possibly involved include enhanced type II alveolar cell production, alveolar epithelial damage/alveolar membrane injury, and increased capillary permeability in septic ALI/ARDS. The finding of **simultaneous elevation of plasma sPLA<sub>2</sub>-II and SP-D concentrations** may suggest that **type II PLA<sub>2</sub> is an acute phase protein and the elevation of type II PLA<sub>2</sub> concentration by endotoxin and/or cytokines results in the injury of the lung tissue and the release of SP-D into the circulating blood**. This finding may be interpreted as indicating, conversely, that inhibition of sPLA<sub>2</sub>-II activity can suppress lung tissue damage and alleviate ALI/ARDS. The **elevation of plasma TNF- $\alpha$  concentration** reflects inflammatory reactions and suggests that this cytokine may be closely involved in the production of sPLA<sub>2</sub>-II. Therefore, PMX-DHP resulted in removal of ET and suppression of the inflammatory reactions involving cytokines, probably leading to suppression of sPLA<sub>2</sub>-II production and improvement of the pulmonary oxygenation potential. This may also be reflected by the finding of a reduction in plasma TNF- $\alpha$  level following PMX-DHP.

In the present study, 5 (20%) of the 25 patients died by Day 180. Three patients died of preexisting heart failure and 2 patients died of multiple organ failure. There were no deaths attributable to respiratory failure alone. The overall 30-day mortality rate was 8% (2/25), markedly lower than the rates reported from previous studies [28, 43, 44]. This result is similar to the results of our previous studies on the treatment of septic ALI/ARDS at our facility, although the timing differed slightly among the studies [45-47]. Thus, our studies to date have yielded reproducible results.

There are some limitations to our study. First, there was no control group. However, considering that PMX-DHP is expected to improve the blood pressure in septic shock patients, it would be ethically problematic to allocate patients with shock to a control group. **Second, causative agents were not identified in all patients. It has been reported that infection may be seldom confirmed microbiologically when treatment is started (even when microbiological tests are completed, culture-positive sepsis is observed in only 30% to 40% of cases) [48]. Therefore, culture-positives were not necessarily indispensable in sepsis definition [1, 48]. However, ET levels >1.1 pg/mL in this study suggested that septic shock was (at least in part) caused by Gram-negative bacteria and/or hypercytokinemia (e.g., increase in levels of TNF- $\alpha$  in Fig. 3) induced by Gram-negative bacteria. Indeed, all patients treated with PMX-DHP (which removes ET, a component of the outer membrane of Gram-negative bacteria) emerged and recovered from septic shock states. Further studies may be needed to elucidate this point. Third,** approximately 30% of patients underwent renal replacement therapy (RRT). However, fluid removal was not conducted within 2 days in hospital because RRT was instituted to improve circulatory failure and/or electrolyte imbalance. In our institution, fluid removal by RRT was applied in patients for whom oxygenation did not improve after

the second session of PMX-DHP. Therefore, it is unlikely that RRT would have improved oxygenation in patients enrolled in the present study.

## **Conclusions**

The present study results suggest that the ability of PMX-DHP to lower the blood sPLA<sub>2</sub>-II and SP-D levels may be among the factors that can explain these beneficial effects of PMX-DHP, and PMX-DHP applied in addition to other treatments may be able to contribute to improving the outcomes of septic ALI/ARDS treatment.

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## Figure Legends

**Figure 1.** Changes in the plasma sPLA<sub>2</sub>-II level during PMX-DHP. sPLA<sub>2</sub>-II: type II secretory phospholipase A<sub>2</sub>; PMX-DHP: polymyxin-B immobilized fiber-direct hemoperfusion.

**Figure 2.** Changes in the plasma SP-D level during PMX-DHP. SP-D: surfactant protein D; PMX-DHP: polymyxin-B immobilized fiber-direct hemoperfusion.

**Figure 3.** Changes in the plasma TNF- $\alpha$  level during PMX-DHP. PMX-DHP: polymyxin-B immobilized fiber-direct hemoperfusion.

**Figure 4.** Changes in the P/F ratios during PMX-DHP. P/F ratio: PaO<sub>2</sub>/FiO<sub>2</sub> ratio; PMX-DHP: polymyxin-B immobilized fiber-direct hemoperfusion.

**Figure 5.** Correlation between the plasma TNF- $\alpha$  level and plasma sPLA<sub>2</sub>-II level before the onset of PMX-DHP. sPLA<sub>2</sub>-II: type II secretory phospholipase A<sub>2</sub>; PMX-DHP: polymyxin-B immobilized fiber-direct hemoperfusion.

**Figure 6.** Correlation between the plasma sPLA<sub>2</sub>-II level and plasma SP-D level before the onset of PMX-DHP. sPLA<sub>2</sub>-II: type II secretory phospholipase A<sub>2</sub>; SP-D: surfactant protein D; PMX-DHP: polymyxin-B immobilized fiber-direct hemoperfusion.

**Figure 7.** Correlation between the plasma sPLA<sub>2</sub>-II level and the P/F ratio before the onset of PMX-DHP. sPLA<sub>2</sub>-II: type II secretory phospholipase A<sub>2</sub>; P/F ratio: PaO<sub>2</sub>/FiO<sub>2</sub> ratio; PMX-DHP: polymyxin-B immobilized fiber-direct hemoperfusion.

**Figure 8.** Correlation between the plasma SP-D level and the P/F ratio before the onset of PMX-DHP. SP-D: surfactant protein D; P/F ratio: PaO<sub>2</sub>/FiO<sub>2</sub> ratio; PMX-DHP: polymyxin-B immobilized fiber-direct hemoperfusion.