Translational Medicine
ISSN: 2161-1025

Tsao, et al., Transl Med (Sunnyvale) 2015, 5:3
DOI: 10.4172/2161-1025.1000150

Research Article Open Access

Fluorescent Determination of Secretory Phospholipase T ($sPLA_2$)-Mediated Human Serum Albumin Binding Activity with Membrane Phospholipids and Fatty Acids

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Abstract

Human serum albumin (HSA) is a complex protein with multiple functions and plays a key role in organ system homeostasis. Changes in albumin levels are associated with worsened outcome in critical illness. However, serum albumin functional activities cannot be easily determined to assess its capacities in illness. Two real-time and sensitive fluorescent liposome assays were developed to determine HSA binding activity, one for measuring the interaction activity between albumin and membrane phospholipids (PL), and one for measuring binding of albumin to fatty acid (FA). Secretory phospholipase A_2 ($sPLA_2$) was used as a mediator in the assay. The sensitive, yet simple fluorescent assays were specific for measuring the activities of serum albumin binding with PL and FA. Among heterogeneous forms of albumin, a small specific fraction of albumin (SFA) was the only portion that interacted with membrane PL whereas all fractions were capable of binding to FA. Oxidation of albumin or pre-binding of albumin with $sPLA_2$-generated FA and lysoPL (LPL) from liposome PL decreased albumin and SFA binding capacities. In the serum of patients with pneumonia and sepsis, the SFA activity was absent and the albumin-FA binding activity was 50% less than that of healthy subjects, even after adjusting for serum albumin content. This study shows that the SFA-PL and albumin-FA binding assays specifically determine albumin binding activities. These assays may be useful for monitoring the binding capacities of serum albumin under pathological conditions in critically ill patients.

Keywords: Albumin; Fatty acid; Fluorescence; Inflammation; Phospholipid; Secretory phospholipase A_2; Sepsis

Introduction

Albumin is the most abundant protein in the circulation and plays critically important roles in regulating the colloidal osmotic pressure of blood and in transporting a wide range of essential molecules throughout the body. The transport properties of human serum albumin (HSA) are predominantly due to its high ligand-binding affinity for various molecular moieties including fatty acids, thyroid hormones, bile salts, bilirubin, certain metal ions, and a wide variety of drugs that are poorly soluble in water [1]. Albumin functional activity is essential for maintaining normal tissue and organ homeostasis, but its function depends on both its concentration and its structure. Advancing age and, especially, critical illness can greatly affect albumin functional capacities due to declining albumin levels and structural modifications [2,3]. Albumin is also the major and predominant antioxidant in intra- and extra-vascular compartments. In healthy individuals HSA exists in the circulation as both reduced and oxidized forms with 70-80% of it in the reduced form. Under the conditions of oxidative stress, a common pathophysiological state in many disease states and advanced age, the redox balance of albumin shifts to more oxidized forms [4,5]. Oxidation can change albumin conformation, which can significantly affect albumin functional activity including its ligand-binding capacity [6-8].

Oxidative stress plays a key role in cell death [9,10]. During the process of cell death, apoptotic cell membranes become susceptible to hydrolysis catalyzed by the inflammatory response secretory phospholipase A_2 ($sPLA_2$) that releases membrane phospholipid (PL) breakdown products lysoPL (LPL) and fatty acids (FAs) [11]. Moreover, under oxidative stress, low-density lipoprotein (LDL) in the circulation [12] and lung surfactant PL in alveoli are also susceptible to hydrolysis by $sPLA_2$ to yield FAs and LPL [13]. The binding of excessive endogenous FA to albumin can induce conformational changes of albumin's binding sites and affect albumin's binding capacities with other ligands [14]. Albumin can also bind LPL [15], and in the circulation LPL is mainly transported by plasma albumin [16]. Whether binding of LPL to albumin impacts albumin's capacity to bind other ligands is not clear.

It has been suggested that HSA could be a useful global biomarker for assessing the state of oxidative stress in numerous diseases [4]. Although the HSA level can be readily determined to provide diagnostic and prognostic information for critical illness, changes in albumin functional activities that are independent of albumin quantity cannot be easily determined. The purpose of this study was to develop a sensitive, yet simple method to determine the HSA binding and transport activity in serum and plasma, to investigate the effect of oxidation and ligand binding on albumin functional activity and to determine the potential utility of the method in the clinical setting. For this purpose, we used a previously developed sensitive, continuous phospholipase A_2 (PLA_2) fluorescent assay [17] as a means to investigate the interactions between albumin and membrane phospholipids and fatty acids. It has been shown that serum albumin can modulate PLA_2 activity in the *in vitro* assays [18]. We speculated that the PLA_2 fluorescent assay might be able to yield important information on albumin, either in native structure or modified form, binding with LPL and FA generated by PLA_2 in the assay reaction. It is known that elevation of a small molecular weight species of PLA_2, secretory PLA_2-IIA ($sPLA_2$-IIA) in the circulation and hypoalbuminemia are both closely associated with sever sepsis [19-21].

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Received April 24, 2015; **Accepted** June 23, 2015; **Published** June 27, 2015

Citation: Tsao FHC, Xiang Z, Meyer KC (2015) Fluorescent Determination of Secretory Phospholipase A_2 ($sPLA_2$)-Mediated Human Serum Albumin Binding Activity with Membrane Phospholipids and Fatty Acids. Transl Med 5: 150. doi:10.4172/2161-1025.1000150

In this study the potential utility of the method in clinical application was also tested in the serum of patients with sepsis.

Materials and Methods

Materials

Porcine pancreatic $sPLA_2$ ($sPLA_2$-IB) in ammonium sulfate suspension, dioleoyl phosphatidylcholine (DOPC), phosphatidylglycerol (PG), fatty acid-free (FAF) albumin, H_2O_2 (30%), and catalase from *Aspergillus niger* in ammonium sulfate suspension were purchased from Sigma-Aldrich. Fluorescent bis-BODIPY® C_{11}-PC (1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-undecanoyl)-sn-glycero-3-phosphocholine) (BODIPY-PC) and C1-BODIPY® C12 (4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid) (BODIPY-FA) were purchased from Molecular Probes. All chemicals used in this study were reagent grade. Recombinant human $sPLA_2$-IIA was kindly provided by Dr. Wonhwa Cho in the Department of Chemistry, University of Illinois at Chicago, Illinois, USA and was previously used in the $sPLA_2$ assay [17].

Human plasma and serum

Informed consent was obtained from all subjects or their authorized representatives for the collection of the blood samples, and all research protocols were approved by the Institutional Review Board of the University of Wisconsin School of Medicine and Public Health (Protocol HSC# 2002-617). Plasma and serum were isolated from blood samples obtained from adult healthy subjects (HS) and patients with pneumonia with sepsis. For isolation of plasma, blood was drawn into vacutainer tubes containing 15% (w/v) K3EDTA (BD, Franklin Lake, NJ, USA). The tubes were centrifuged at 2,000 × g for 20 min at 16°C to obtain plasma. For isolation of serum, peripheral blood was collected into tube containing clotting gel (Fisher Scientific) and allowed to clot for 30 min at room temperature. Serum was isolated after peripheral blood was centrifuged at 2,000 × g for 10 min after clotting had taken place. Each serum or plasma sample volume was approx. 5 ml and stored in 0.1 and 1 ml aliquot at -70°C. Most serum and plasma samples were assayed followed isolation. Samples were also assayed periodically after storage at -70°C in a time span up to five years.

Fluorescent assays

Fluorescent PC-labeled unilamellar liposome (flu-PC-UL) assay: To determine hydrophobic interactions between albumin (serum or plasma) and fluorescently labeled liposomes, we used unilamellar liposomes (UL) composed of 50% DOPC-50% PG labeled with BODIPY-PC as the substrates, which were prepared by the procedures described previously [17]. The newly prepared liposome solution (1 mg DOPC, 1 mg PG and 0.014 mg BODIPY-PC in 1 ml) was divided into five aliquots and stored at -20°C before use. The assay was conducted in a 3-ml quartz cuvette. Briefly, the procedure of the assay was performed by adding an amount of 0.01 M Tris-HCl buffer (pH 7.4) containing 10 mM $CaCl_2$ into the cuvette that would make up to a final volume of 3 ml after subtracting the volumes of other assay components. Then, an amount of 20 μg liposome phospholipids in 30 μl was added to the buffer followed by adding an amount of albumin, serum or plasma in a specified volume of μl. The reaction was initiated by adding 10 μl of $sPLA_2$ (50 ng) working solution. After each addition of the assay component, the reaction mixture was immediately mixed well by inversion of the parafilm-covered cuvette. After $sPLA_2$ was introduced into the assay mixture, the fluorescence intensity (FI) of the reaction was immediately determined at 488 nm excitation (slit 2.5) and 530 nm emission (slit 5.0) at 22°C using a luminescence spectrometer LS50B (Perkin-Elmer Instruments, Norwalk, CT). An initial reading was recorded as zero time and subsequent readings were taken every 5-10 sec for 2-4 min. The activity was expressed as FI vs. time (sec) after the initial reading was subtracted from each subsequent reading (ΔFI). In some studies the activity of the reaction was expressed as the initial rate of the reaction (e.g., FI/sec), which was determined from the reaction curve fitted with a second-order polynomial equation and the first-degree coefficient. The baseline was determined without the presence of any proteins in the assay mixture. Commercially available porcine pancreatic PLA_2 ($sPLA_2$-IB or briefly termed $sPLA_2$) was used as the enzyme source in most of the studies. The $sPLA_2$ working solution was freshly prepared by diluting 1 μl of Sigma-Aldrich PLA_2 product (2.5 μg) in 0.5 ml 0.01 M Tris-HCl, pH 7.4 for each day's assays. As specified, in certain experiments recombinant human $sPLA_2$-IIA was used as the enzyme source. Assays performed in a well of a 96-well microplate utilized 1/10th of each assay component used in the cuvette assay. The plate reader was an accessary attached to the LS50B luminescence spectrometer.

Fluorescent FA-labeled unilamellar liposome (flu-FA-UL) assay: In some assays liposomes labeled with fluorescent probe BODIPY-FA were used as the substrates. The BODIPY-FA-labeled liposomes were prepared and stored by the same procedures described for BODIPY-PC labeled liposomes. In a 1 ml of liposomes it contained 1 mg DOPC, 1 mg PG, and 0.016 mg BODIPY-FA. The stock solution of BODIPY-FA was prepared by dissolving 1 mg BODIPY-FA in 1 ml of methanol. The assay using BODIPY-FA liposomes as substrates (20 μg flu-FA-UL) was performed similarly as that described for flu-PC-UL assay, except an amount of 200 ng $sPLA_2$ was used to initiate the reaction.

Determination of the assay specificity in serum

To determine that the flu-PC-UL assay was specific for albumin in the serum, 5 ml of serum from a pool of four HS was applied to a Sephadex G100 (Pharmacia) column (2.6 × 55 cm) and the column was eluted with Tris-EDTA-NaCl buffer (0.01 M Tris-HCl, 5 mM 2-mercaptoethanol, 1 mM EDTA and 0.15 M NaCl, 0.02% NaN_3, pH 7.4). Each fraction was collected in 2 ml aliquots per tube. Protein in each fraction was determined by absorbance at 280 nm. An aliquot of 40 μl or 150 μl of each fraction collected from the column was used for the flu-PC-UL assay in a cuvette. Fractions that generated negative FI were pooled, equilibrated with 0.01M Tris-HCl, pH 7.4, concentrated to 1 ml and applied to a high performance liquid chromatography (HPLC) anionic exchange MonoQ column (5 x 50 mm) (Pharmacia). The column was first eluted for 10 min with 0.01 M Tris-HCl buffer, then with 25% of 1 M NaCl for 100 min, 50% of 1 M NaCl for 30 min, and finally with 100% of 1 M NaCl for 10 min. The flow rate was 1 ml/min, and the collected fraction volume was 1 ml per fraction tube. The protein concentration in each fraction was determined by absorbance at 280 nm. An aliquot of either 100 μl or 400 μl of each fraction was employed for the flu-PC-UL assay in a cuvette. The MonoQ column fractions that produced negative FI were pooled, concentrated and applied to a reverse phase HPLC Vydac C4 column (4.6 × 250 mm) (Separations Groups). The column was eluted with a gradient of solvent A (0.1% trifluoroacetic acid, TFA) and solvent B (90% acetonitrile in 0.088% TFA) at a flow rate of 1 ml/min. The elution program was set up as: 0-10 min 0% B, 10-25 min 0-30% B, 25-65 min 30-70% B, 65-80 min 70-100% B. Protein concentration in each fraction was determined by absorbance at 215 nm and 280 nm. Each protein fraction was subjected to the flu-PC-UL assay. The purity of the isolated protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% SDS Ready Gel and Mini-PROTEAN 3 Cell Assembly

Unit (Bio-Rad) under denaturing conditions. Proteins on the SDS gel were visualized by Coomassie brilliant blue staining.

The SDS gel protein band of the purified protein isolated from HPLC Vydac C4 column was also excised and placed into a 0.5 ml microcentrifuge tube for peptide sequence determination. The gel was treated in 100 µl of 25 mM NH_4HCO_3 in 50% acetonitrile to remove the Coomassie brilliant blue stain. The de-colorized gel was dried in a vacuum centrifuge, and the protein was reduced in 100 mM dithiothreitol followed by modification with 55 mM iodoacetamide. The protein was then digested with trypsin (20 µl of 0.006 mg/ml) (sequencing grade-modified, Promega) at 37°C for 24 hr. The peptides were collected by washing the gel with deionized water followed by washing with 5% trifluoroacetic acid and 50% acetonitrile. The washes were combined and dried in a vacuum centrifuge. The dried peptides were used for mass and peptide sequence determinations using the methods of "matrix-assisted laser desorption ionization" (MALDI) and tandem mass spectrometry (MS/MS) methods using the TOF instruments conducted at the University of Wisconsin Biotechnology Center (UWBC) on campus.

Treatment of serum and albumin with H_2O_2 or $sPLA_2$-liposome mixture

An aliquot of 10 µl of HS serum was incubated with 4 mM H_2O_2 or specified amount of H_2O_2 in a final volume of 0.1 ml in 0.01 M Tris-HCl buffer, pH 7.4 at 37°C for 5 or 30 min. The H_2O_2 reaction was stopped by adding 1 µl of 8 units of catalase in Tris-HCl buffer at 37°C for 2 min, similar to that previously described [22]. The solution was centrifuged in an Eppendorf centrifuge for 1 min to collapse the air bulbs and placed on ice. An aliquot of 10 µl was used as the serum source for the microplate flu-PC-UL and flu-FA-UL assays. Controls were performed in parallel as specified. To treat serum in $sPLA_2$-liposome mixture, non-fluorescent unilamellar liposomes (nf-UL) were prepared by the same procedure as for fluorescently labeled liposomes without the fluorescent probe. The stock solution of nf-UL was stored at -20°C before use. Treatment of serum with nf-UL was conducted in 10 µl Tris-CA^{2+} assay buffer containing 1 µl serum, specified amount of nf-UL, and 2.5 ng $sPLA_2$ in 0.5 µl. The mixture was incubated at 37°C for 5 or 30 min. Following incubation, the mixture was cooled on ice and used for the microplate flu-PC-UL and flu-FA-UL assays. Specified controls were performed in parallel.

Results

Flu-PC-UL assay of albumin, serum, and plasma

In the flu-PC-UL cuvette assay in the presence of $sPLA_2$ and absence of albumin, a moderate time-dependent increase in fluorescence intensity (FI) was observed (Figure 1A). As we previously demonstrated that increase in FI with time in the $sPLA_2$ reaction was due to the release of fluorescently labeled FA and lysoPC from BODIPY-PC imbedded in liposomes hydrolyzed by $sPLA_2$ [17]. Interestingly, the presence of 0.08 mg albumin in the $sPLA_2$ reaction mixture significantly increased

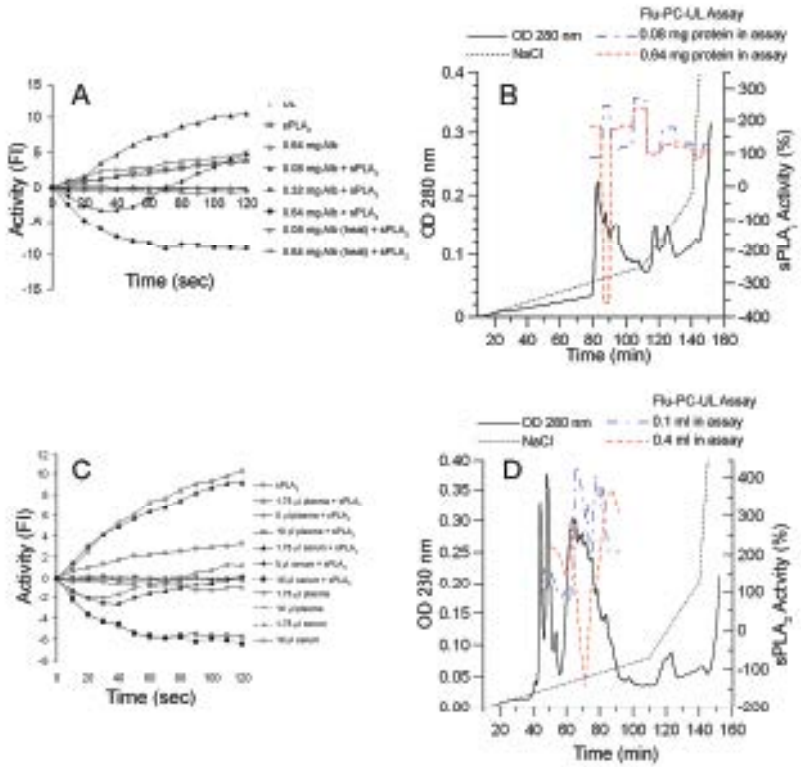

Figure 1: Flu-PC-UL assay of albumin binding activity and assay specificity. (A) Flu-PC-UL assay of FAF-albumin in the assay. The assay was conducted in a 3 ml cuvette containing 50 ng of $sPLA_2$ in the absence or presence of human FAF-albumin (Alb) (0.08, 0.32, or 0.64 mg). (B) HPLC anionic exchange MonoQ column chromatography of FAF-albumin. Two protein concentrations, 0.08 mg (blue) and 0.64 mg (red), were used for the assay and the activity determined from total FI (TFI) within 2 min of reaction as shown in Figure (A) was expressed as percentage of $sPLA_2$ TFI designated as 100%. (C) Flu-PC-UL assay of serum and plasma in a cuvette. The assay was conducted in a 3 ml cuvette containing 50 ng of $sPLA_2$ in the absence or presence of human serum or plasma (1.75, 5, or 10 µl). (D) HPLC MonoQ column chromatography of serum proteins. An aliquot of 0.1 ml (blue) or 0.4 ml (red) of each fraction was employed for the flu-PC-UL assay in a cuvette as described in (B). The units of column fractions used for the assay in Figures 1B and 1D correspond to that in Figures 1A and 1C respectively.

FI (Figure 1A). However, when albumin was increased to 0.32 mg, FI decreased in the first 60 s to a level below the baseline and then gradually increased to the level above the baseline. The later increase in FI was likely produced by $sPLA_2$ which overpassed albumin effect in the reaction. With increasing the amount of albumin to 0.64 mg, FI was reduced far below the baseline during the 2-min reaction period (Figure 1A). Without $sPLA_2$, albumin alone in the assay did not significantly change the FI as compared to the substrate background. The results of Figure 1A and the following FI vs. time activity figures were representative examples of more than two assays.

After treating albumin in boiling water for 5 min, albumin completely lost its effects on increasing or decreasing FI. Although normal albumin showed a single protein band on the SDS gel (data not shown), it yielded multiple protein fractions after albumin solution was passed through the anionic MonoQ column (Figure 1B). Two fractions of sequential tubes were pooled, equilibrated in 0.01 M Tris-HCl (pH 7.4) and condensed to 0.5 ml to be used for the flu-PC-UL assay. The activity of the reaction was presented as total ΔFI obtained from the reaction curve as shown in Figure 1A and the activity of $sPLA_2$ alone in the assay was designated as 100% (Figure 1B). Presence of 0.08 mg of each combined MonoQ fractions in the assay increased $sPLA_2$ activity (Figure 1B blue). However, the presence of 0.64 mg of each combined fractions in the assay, only the fractions eluted between 87 and 92 min from the column generated negative FI values or negative % $sPLA_2$ activity (Figure 1B red). These fractions were designated as "Specific Fraction of Albumin" (SFA). The large dip down (negative value) of the activity (Figure 1B red) also suggests that full amount of protein used in the assay came from SFA.

Similarly, in the flu-PC-UL cuvette assay, healthy subject (HS) serum or plasma increased FI in small volume (e.g., 1.75 µl) but decreased FI to negative values in 10 µl during 2 min of the reaction (Figure 1C). An amount of 5 µl serum or plasma yielded negative FI in the first 60 sec but then gradually recovered to the baseline level. Serum or plasma in 10 µl generated a negative FI reaction curve similar to that produced by high concentration of albumin. The results of 20 µl serum or plasma were similar to 10 µl (not shown). Serum or plasma alone in the assay mixture did not significantly change the FI value as compared to the baseline. The activities of serum and plasma in the flu-PC-UL assay were the same (Figure 1C). Storage of serum or plasma on ice for more than 5 h or at -70°C for more than a year had no effect on the activity. The assay efficacy was further demonstrated by assaying six serum samples from different healthy subjects on different days. The activity expressed as total FI obtained from adding each time point FI value from the reaction curve generated by 10 µl serum (such as that shown in Figure 1C) was -56.74 ± 3.61 (mean ± SD, n = 6), and activity from the curve generated by 1.75 µl was 60.25 ± 5.72 (mean ± SD, n = 6).

Similar to albumin (Figure 1B), most serum fractions collected from MonoQ column showed $sPLA_2$ activity (%) increasing in the presence of 0.1 ml of the fractions eluted between 60 and 90 min (Figure 1D blue). However, only small fractions collected between 65 and 75 min decreased the $sPLA_2$ activity to yield negative ΔFI in the presence of 0.4 ml of each fraction in the assay (Figure 1D red). Further purification of this group of proteins by HPLC Vydac C4 column showed that a single protein (named Protein-I) with apparent molecular weight of 65 kDa (Figure 2A and insert) exhibited similar activity patters in the flu-PC-UL assay (Figure 2B) to Figures 1A and 1C. Protein-I itself had no effect on FI in the assay. After treating Protein-I in boiling water for 5 min, Protein-I lost all of its effects on increasing or decreasing FI in the assay. The determined several peptide amino acid sequences of Protein-I were

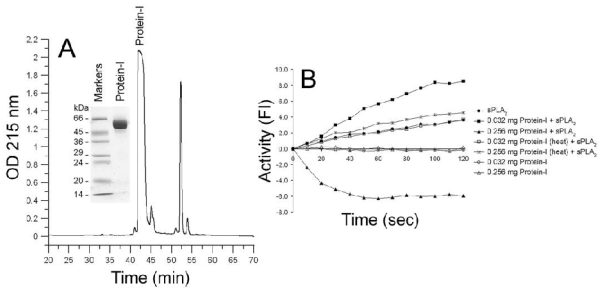

Figure 2: HPLC Vydac C4 column chromatography and SDS gel image of Protein-I. (A) Reverse phase HPLC Vydac C4 column chromatography. The MonoQ column fractions that produced negative FI (Figure 1D red fractions between 65 and 89 min) were pooled, concentrated and applied to a reverse phase HPLC Vydac C4 column. The purity of Protein-I eluted between 42 and 45 min from Vydac C4 column was analyzed by SDS gel electrophoresis (A). (B) Flu-PC-UL assay of Protein-I concentrated from the peak shown in (A).

identical to the matched human serum albumin peptide sequences at the numeric number positions (Table 1). Therefore, Protein-I was albumin and the flu-PC-UL assay was specific for albumin.

Flu-FA-UL assay of albumin, serum and plasma

In the flu-FA-UL assay, $sPLA_2$ did not produce significant FI in the assay mixture because BODIPY-FA was not $sPLA_2$ substrate (Figure 3A). Similarly, in absence of $sPLA_2$, the presence of albumin, serum or plasma in the assay mixture did not cause significant changes in FI either. However, when $sPLA_2$ was added to the reaction mixture containing plasma, serum or albumin, a time-dependent increase in FI was observed (Figure 3A). Assays of serum with liposomes made of 50% DOPC-50% PG yielded about 20-30% higher activity than with 100% PG liposomes (Figure 3B). Liposomes made of 100% DOPC did not produce any activity in the assay. Similar to the flu-PC-UL assay, the flu-FA-UL assay activity (total FI) obtained from the reaction curve (Figure 3A) generated from six serum samples of healthy subjects was 195.26 ± 4.10 (mean ± SD, n = 6).

In both flu-PC-UL and flu-FA-UL assays the reaction activity was mainly dependent on three assay components, the UL substrate, serum or plasma (albumin), and $sPLA_2$. We found that the activity in the serum or plasma stored at -70°C (without being subjected to multiple episodes of freezing and thawing) was stable for more than five years. The pancreatic $sPLA_2$ from Sigma-Aldrich was used as the enzyme source for the assay because it was commercially available and relatively stable for over two years at 4°C. However, it was necessary to check the enzyme activity periodically to ensure the consistency of the activity. The amount of $sPLA_2$ (50 ng) used in the flu-PC-UL assay was kept at a level that yielded 4-5 FI at 2 min of the reaction to yield the optimal negative activity as shown in Figures 1A and 1C; thus, the volume of $sPLA_2$ used for the assay might need adjustment. In the flu-FA-UL assay a higher amount of $sPLA_2$ (200 ng) was needed to produce the optimal albumin activity. Although freshly prepared liposomes were better substrates, it was difficult to make liposomes in less than 0.5 ml with using our equipment and unreasonable to treat it as disposable agent. We found that both flu-PC-UL and flu-FA-UL substrates were stable for at least one month at -20°C.

Peptide Number	Observed m/z	Expected Mass	Calculated Mass	Peptide Amino Acid Sequences	Matched Amino Acid Positions in Albumin Protein Sequence*
1	927.49	926.49	926.49	YLYEIAR	162-168
2	1000.61	999.61	999.60	QTALVELVK	550-558
3	1149.63	1148.62	1148.61	LVNEVTEFAK	66-75
4	671.82	1341.62	1341.63	AVMDDFAAFVEK	570-581
5	820.47	1638.92	1638.93	KVPQVSTPTLVEVS	438-451
6	955.97	1909.93	1909.92	RPCFSALEVDETYV	509-522
7	682.36	2044.07	2044.09	VFDEFKPLVEEPQN	397-410

* From human albumin amino acid sequence reported in GenBank/NCBI with accession No. AAA98797.

Table 1: Amino acid sequences of trypsin in-gel digested Protein I peptides determined by tandem mass spectrometry (MS/MS). Protein I was isolated from serum from normal volunteers.

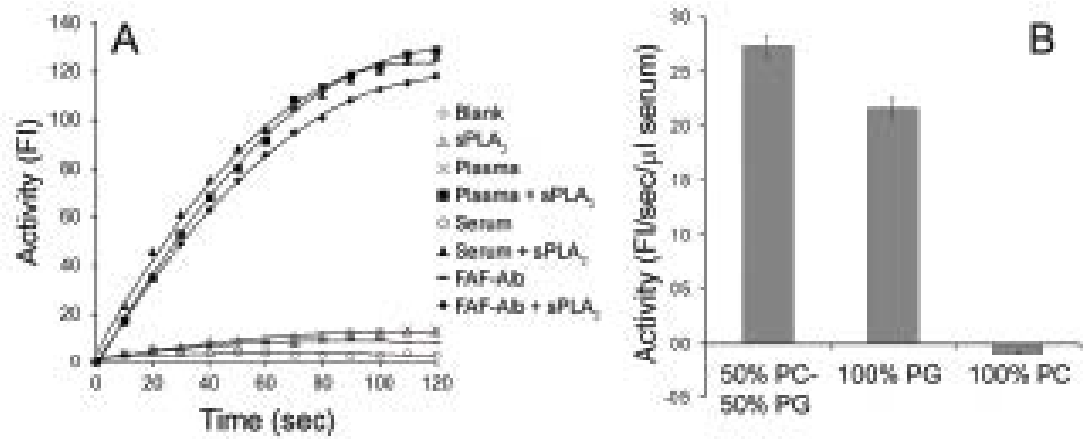

Figure 3: Flu-FA-UL assay of albumin binding activity. (A) The assay was conducted as that described in Figure 1A, except flu-FA-UL was used as the substrate. An aliquot of 10 µl of serum or plasma or 0.64 mg of FAF-Alb was used in the assay in a cuvette. The reaction was induced by adding 200 ng $sPLA_2$. (B) Three groups of flu-FA-UL with different phospholipid compositions (50% DOPC-50 %PG, 100% PG, 100% DOPC) were used as substrates. The assay was conducted in a 96-well microplate. The amounts of flu-FA-UL, serum and $sPLA_2$ were 1/10th of that used in the 3 ml cuvette assay. The data of FI/sec was determined from the reaction curve fitted with a second-order polynomial equation, and the first-degree coefficient was taken to be the initial rate of reaction and expressed as mean± SEM (n=3).

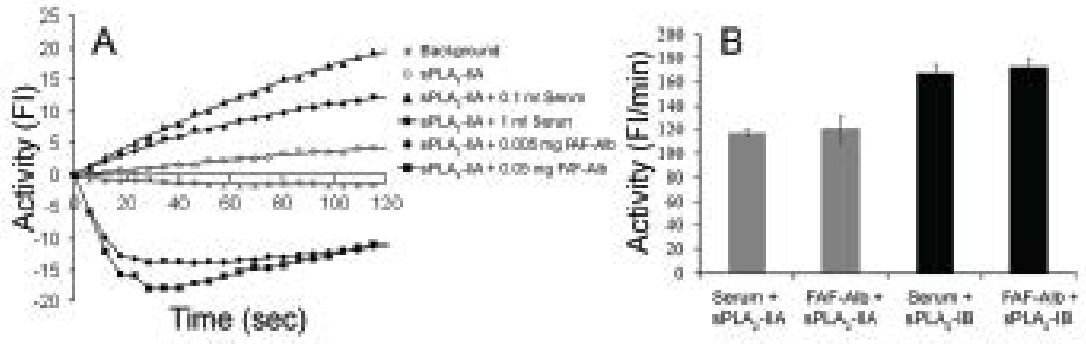

Figure 4: Effects of human $sPLA_2$-IIA on albumin binding activity. (A) Effect of recombinant human $sPLA_2$-IIA (5 ng) on albumin binding activity in serum (0.1 µl or 1 µl) or in FAF-albumin (5 µg or 50 µg) determined by the flu-PC-UL assay in 96-well microplate. (B) Comparison of the FA binding activity in serum (1 µl) and FAF-albumin (50 µg) mediated by $sPLA_2$-IIA (20 ng) and $sPLA_2$-IB (20 ng) determined by the flu-FA-UL assay in 96-well microplate. The activity (FI/min) was determined as that described in Figure 3B and represents as mean ± SEM of duplicate assays.

Determination of $sPLA_2$-IIA-mediated albumin activity

In the flu-PC-UL assay, recombinant human $sPLA_2$-IIA induced serum and albumin to yield the activity patterns similar to that mediated by $sPLA_2$-IB (Figure 4A). Likewise, both $sPLA_2$-IIA and $sPLA_2$-IB induced serum and albumin activities similarly in the flu-FA-UL assay (Figure 4B).

Effects of H_2O_2 or $sPLA_2$-nf-UL treatment on serum albumin activity

Under maximal treatment of serum in 4 mM H_2O_2 at 37°C for 30 min, albumin activity decreased about 50% in both flu-PC-UL and flu-FA-UL assays (Figures 5A and 5B, respectively). The flu-FA-UL assay clearly showed that the effect of H_2O_2 on albumin activity was dependent

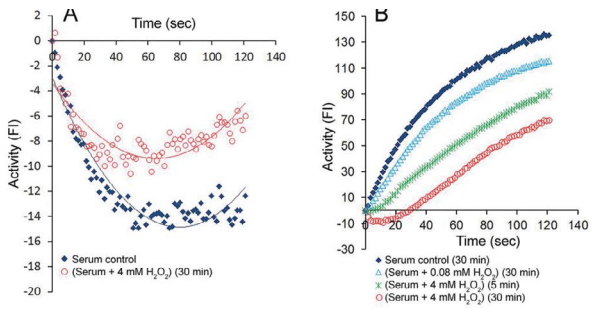

Figure 5: Effects of oxidation on serum albumin binding capacity. A reaction mixture of 0.1 ml containing 10 µl HS serum or 0.5 mM FAF-albumin in the presence or absence of H_2O_2 was incubated at 37°C for up to 30 min. An aliquot of 10 µl of the reaction mixture was added to a microplate well which contained assay buffer and liposome substrate, followed by adding $sPLA_2$. The solution was well mixed after each addition. The albumin activity was determined by the flu-PC-UL (A) or flu-FA-UL (B) assay.

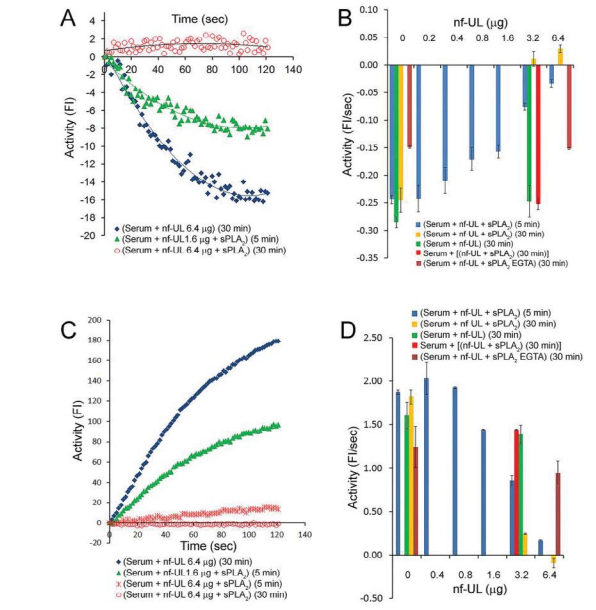

Figure 6: Effects of bound ligands on serum albumin binding capacity. An amount of 1 µl HS serum was mixed with a specified amount of nf-UL and 2.5 ng $sPLA_2$ in 10 µl assay buffer and incubated at 37°C for 5 or 30 min. Following incubation, the mixture was applied to a 96-well microplate well for flu-PC-UL assay and flu-FA-UL assay (examples of the real-time activity expressed as FI vs. Time are shown in A and C, respectively). More studies of the effects of different amounts of nf-UL on the serum albumin activity and controls are shown in Figure 6B (flu-PC-UL assay) and Figure 6D (flu-FA-UL assay). Each assay was conducted in duplicate. The results of the columns in (B) and (D) are mean ± SEM.

on the time of treatment (5 min vs. 30 min) and H_2O_2 concentration (0.08 mM vs 4 mM) (Figure 5B). Pre-incubation of serum in a mixture containing $sPLA_2$ and nf-UL markedly reduced albumin activities. Examples of the activity presented as FI vs. time are shown in Figures 6A (flu-PC-UL assay) and Figure 6C (flu-FA-UL assay), and the activities presented as FI/sec are shown in Figure 6B and 6D, respectively. In both assays albumin activity was inhibited by pre-incubation of serum with $sPLA_2$-nf-UL mixture in an nf-UL dose-dependent and incubation time dependent manner. Pre-incubation of serum with $sPLA_2$ or nf-UL alone for up to 30 min had no significant effect on the albumin activity. Also, addition of a 30-min pre-incubated mixture of nf-UL and $sPLA_2$ into the assay had little effect on the serum albumin activity, suggesting that pre-binding of $sPLA_2$-generated LPL and FA to albumin caused albumin activity decrease. The presence of EGTA in the pre-incubation mixture of serum, nf-UL, and $sPLA_2$ prevented albumin activity decrease. This is because that EGTA, a calcium chelating agent inhibited $sPLA_2$ catalytic activity that is calcium-dependent. EGTA alone might also affect serum albumin activity and lowered the albumin activity to a certain extent. Interestingly, nf-UL at less than 3.2 µg affected albumin activity more in the flu-PC-UL assay than in the flu-FA-UL assay. At 3.2 µg nf-UL with 30 min incubation treatment, albumin activity was completely depleted and positive FI was produced in the flu-PC-UL assay (Figure 6B), whereas the flu-FA-UL assay still showed certain amount of albumin-FA binding capacity (Figure 6D). At 6.4 µg nf-UL with 30 min incubation treatment, albumin activity was completely depleted in both assays. The low amount of $sPLA_2$ in the incubation mixture had no significant effect in the assays. Treatment of FAF-albumin with H_2O_2 or nf-UL-$sPLA_2$ mixture had similar inhibitory effects on FAF-albumin activity (data not shown).

Comparison of the albumin activity in the serum from healthy subjects and patients with sepsis

The flu-PC-UL assay was conducted in a 3-ml cuvette and the flu-FA-UL assay in a 0.3 ml microplate well under optimal assay conditions. In the flu-PC-UL assay in the presence of exogenous $sPLA_2$ the average albumin activity in six healthy subjects (HS) serum samples was -0.127 ± 0.007 (n=6) (mean ± SEM), whereas in the serum from three patients with sepsis (SEPSIS) was 0.077 ± 0.002 (n=3) ($p<0.4 \times 10^{-6}$) (Figure 7A solid circle). The serum albumin levels (g/dL) of 6 HS and 3 SEPSIS samples were 4.0 ± 0.1 and 2.8 ± 0.4 (mean ± SEM), respectively. Normalization of the albumin activity with serum albumin content did not change the discrepancy much (Figure 7C). In the absence of exogenous $sPLA_2$, there was no significant amount of albumin activity in the HS and SEPESIS serum samples (Figure 7A open circle). The serum albumin activity determined by the flu-FA-UL assay was 2.040 ± 0.064 (n=6) for HS and 1.101 ± 0.155 (n=3) for SEPSIS ($p<0.02$) (Figure 7B solid diamond). Normalization of the activity for albumin content did not significantly change the activity pattern (Figure 7D). In the absence of exogenous $sPLA_2$, SEPSIS serum also displayed albumin activity whereas HS serum did not show any activity in the flu-FA-UL assay (Figure 7B open diamond).

Discussion

In the flu-PC-UL assay with $sPLA_2$ alone, the increase in FI was due to the release of fluorescently labeled lysoPC (LPC) and FA from BODIPY-PC embedded in liposomes catalyzed by $sPLA_2$ [17]. With the presence of a small amount of albumin, serum or plasma, the augmented FI was due to removal of $sPLA_2$-liberated fluorescent FA and LPC by albumin from liposomes, which consequently accelerated the $sPLA_2$ activity [18]. When a large amount of albumin, serum or plasma was present in the assay, negative FI was obtained due to fluorescence quenching that was likely caused by a coating of albumin on the surface of liposomes, which blocked fluorescence emission from BODIPY-PC in liposomes. An extensive degree of albumin coating might also prevent $sPLA_2$ from gaining access to the bilayer membrane interface to hydrolyze membrane PL, and that notion seems consistent with the findings of the inhibition of $sPLA_2$ by high concentration of albumin using the end-point determination methods [18]. This study shows that albumin actions on liposomes were facilitated by $sPLA_2$. The $sPLA_2$-mediated albumin-liposome interaction was also supported by the flu-FA-UL assay. In this assay flu-FA-UL was inert to $sPLA_2$ because FA is

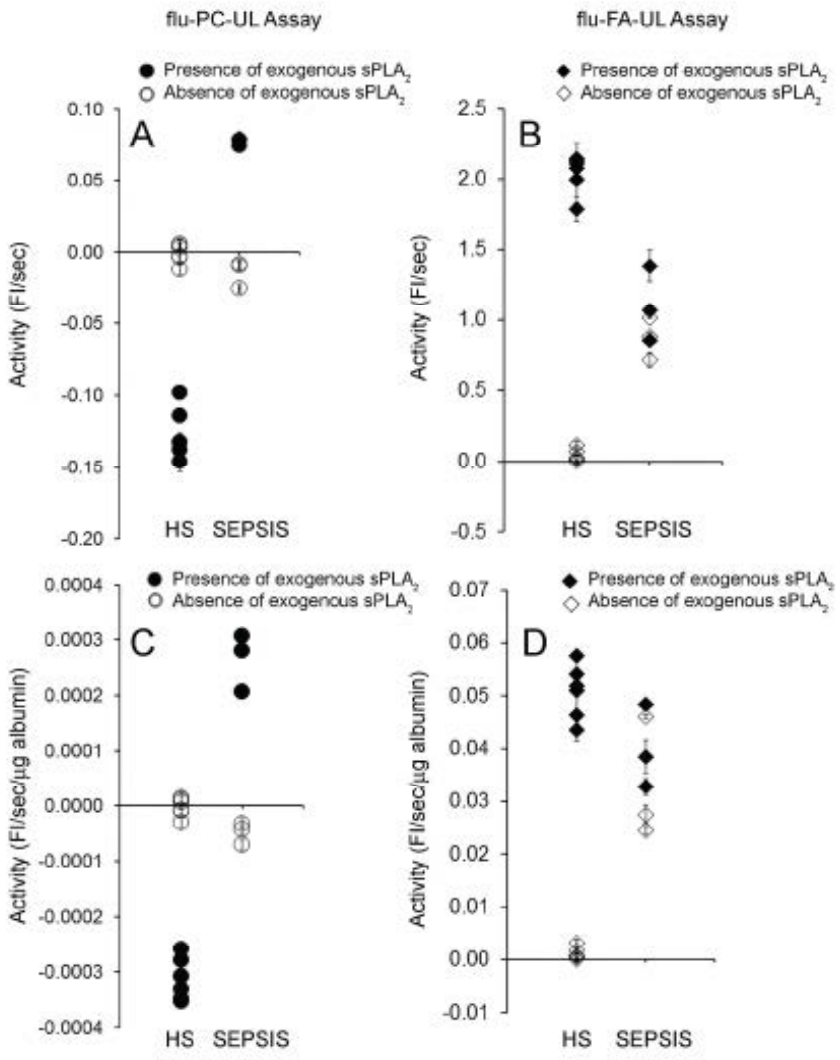

Figure 7: Determination of albumin activity in the serum of healthy subjects and patients with sepsis. (A) Albumin activity in the serum from 6 healthy subjects (HS) and 3 patients with sepsis (SEPSIS) determined by the flu-PC-UL assay. The assay was conducted in a cuvette and the albumin activity is expressed as the mean ± SEM of the initial rate derived from the reaction curve as described in Figure 3B. Each individual serum sample was assayed in duplicate. (B) Albumin activity in the HS serum and SEPSIS serum determined by the flu-FA-UL assay in a microplate. (C) The activity shown in (A) was normalized to µg serum albumin. (D) The activity shown in (B) was also normalized to µg serum albumin.

a $sPLA_2$ reaction product. Flu-FA-UL was also inert to albumin, serum or plasma. However, the co-presence of albumin (serum or plasma) and $sPLA_2$ in the reaction mixture yielded a time-dependent increase in FI. Based on the nature of the BODIPY-FA probe, FI increases if the probe is removed from liposome membranes. Thus, in this assay $sPLA_2$ drives albumin to the liposome membrane vicinity so albumin can bind and remove BODIPY-FA from liposomes. The flu-FA-UL assay also showed that $sPLA_2$-induced albumin-PL interaction required negatively charged PL such as PG in the liposomes; liposomes composed of 100% neutral PC were inert to the interactions. This is consistent with the action of PLA_2 on membranes in which membrane surface electrostatic forces are required to allow PLA_2 to facilitate its actions on the membranes [23]. Without liposome membranes, BODIPY-FA alone could not serve as a substrate in the assay. These conditions (including the requirement of calcium ion in the assay) are all essential for the $sPLA_2$ activity, suggesting that $sPLA_2$ activity, regardless of whether the enzyme type is $sPLA_2$-1B or $sPLA_2$-IIA, is the driving force of albumin-PL and albumin-FA interactions in the assays. Because $sPLA_2$-IB and $sPLA_2$-IIA are structurally alike [24] and also catalytically similar in the flu-PC-UL and flu-FA-UL assays, the commercially available, inexpensive and relatively stable pancreatic $sPLA_2$-IB appears to be an ideal enzyme source for the flu-PC-UL and flu-FA-UL assays. Our extensive analysis of serum components confirmed that albumin was the only constituent in the human serum (plasma) that possessed the ability to interact with liposomes mediated by $sPLA_2$. Thus, the flu-PC-UL and flu-FA-UL assays are specific for serum albumin.

Serum albumin consists of heterogeneous forms because of its diverse ligand-binding moieties and the susceptibility of its thiol groups to oxidation. Although the SFA fraction was eluted from MonoQ column at a position similar to that of mercaptalbumin with free thiol groups [25], the SFA might have a PL specific binding site because this was the only albumin fraction that generated negative FI in the flu-PC-UL assay. Because SFA is a small fraction of total albumin mass, this

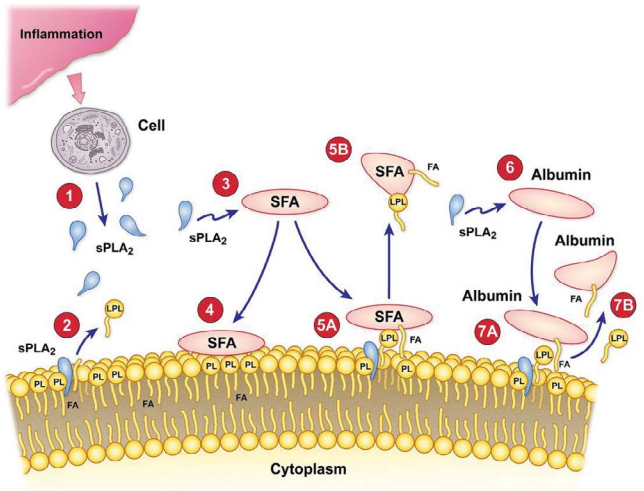

Figure 8: Illustration of the putative relationships among serum albumin, SFA, $sPLA_2$ and membranes at the inflammatory site. In conjunction with the recognized phenomena of inflammation including the release of $sPLA_2$ from cells upon activation (1) and $sPLA_2$-catalyzed breakdown of membrane PL to liberate LPL and FA (2), here we propose that $sPLA_2$ interacts with SFA (3) to drive SFA to the vicinity of membrane to bind PL (4) or to bind LPL and FA if the membrane contains $sPLA_2$-hydrolyzed PL breakdown products, FA and LPL (5A). SFA bound with LPL and FA may undergo conformational change and no longer be able to bind membrane PL and FA even in the presence of $sPLA_2$ (5B). $sPLA_2$ can also interact with other albumin fractions (6) to induce albumin to interact with membranes and bind FA, but not LPL generated from $sPLA_2$-hydrolyzed membrane PL (7A). Albumin-FA binding may also cause albumin conformational change and impair albumin's ability to bind other ligands (7B).

explains why a relatively large amount of albumin (serum or plasma) was needed to allow SFA to coat liposomes to produce negative FI. Although albumin alone can interact with model PL membranes under certain conditions [26], we could not detect any albumin-liposome interactions under the assay conditions without $sPLA_2$. Previous study has shown that serum albumin interacts with PL liposomes only at pH values below its isoelectric point [27]. The HSA isoelectric point is 5.2; this explains why under current assay conditions at pH 7.4, albumin does not interact with or bind liposome PL membranes without $sPLA_2$ which may facilitate a protein-protein interaction with albumin to mediate albumin-PL binding.

Treatment of serum with H_2O_2 under maximal conditions in this study impaired albumin activity by approximately 50%. In contrast, pre-incubation of serum with $sPLA_2$ and nf-UL under maximal conditions (e.g., with nf-UL at 3.2 μg or greater and 30 min incubation) completely depleted the albumin activity (Figures 6B and 6D). The explanation for this finding is that $sPLA_2$ hydrolyzed nf-UL PL and released FA and LPL during pre-incubation, which then became bound to albumin. When treated serum was introduced into the flu-PC-UL or flu-FA-UL assay mixture, the pre-bound FA and LPL decreased albumin's binding capacities in the assay. If albumin's binding sites were saturated with FA and LPL, this form of albumin's binding capacities with fluorescent PC (flu-PC-UL assay) or FA (flu-FA-UL assay) would be completely blocked (Figures 6B and 6D). The SFA-PL binding appears to be more sensitively affected by pre-incubation of serum with $sPLA_2$ and nf-UL than albumin-FA binding (Figures 6B and 6D). This is probably because albumin has multiple FA binding sites [28], whereas SFA is the only albumin fraction that binds LPL. We speculate that in synergy with $sPLA_2$, SFA could act as a scavenger to remove the potent bioactive LPL mediators from $sPLA_2$-degraded PL at the inflammatory sites [29]. It has been shown that some ligand-bound albumin molecules are more rapidly taken up by scavenger cells to clear sites of inflammation [30]. Our proposed relationships among $sPLA_2$, SFA (albumin), and membrane are depicted in Figure 8.

The two assays identified marked differences in the SFA and albumin activities in the serum between HS and patients with sepsis. Because the activity reduction in the patient sera was independent of serum albumin content, the diminished activity was likely due to albumin (SFA) modification. Although we did not analyze the specific modification of albumin (SFA) that occurred in patient sera, this study definitively demonstrated that oxidation and ligand binding could certainly impair albumin (SFA) activity. Total absence of SFA activity in patient sera (Figure 7A) shows a similar pattern of SFA saturated with LPL and FA (Figure 6B). This suggests that patient's SFA could be saturated with FA and LPL derived from dead cell membranes *in vivo*, and it is known that an overstressed inflammatory response in sepsis leads to extensive cell death [31]. Cell membranes of apoptotic cells could be further degraded by $sPLA_2$ [11], which were present in the sera from patients with sepsis (Figure 7B) [17]. Unlike SFA, the flu-FA-PC assay showed that serum from patients with sepsis still had approximately 50% of albumin-FA binding activity as compared to HS. This activity was likely due to binding by non-SFA albumin fractions. Although elevation of the $sPLA_2$ level in the peripheral blood circulation is an innate response to bacterial infection [32], a prolonged elevation of $sPLA_2$ coupled with the absence of SFA in the circulation may be comprise an important risk factor for poor patient outcome, such as the three septic patients succumbed to their illness soon after their serum samples had been obtained. We suggest that our assays may be useful for monitoring the serum albumin and SFA binding capacities in critical illness when deliveries of nutrients, essential elements and drugs that depending on albumin and SFA transport are needed for efficacy. Additionally, our assays may be useful for the detection of early stages of sepsis and potentially able to identify patients at increased risk of developing a sepsis syndrome.

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