Accumulation of bioactive lipids during storage of blood products is not cell but plasma derived and temperature dependent

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BACKGROUND: Bioactive lipids (lysophosphatidylcholines [lysoPCs]) accumulating during storage of cell-containing blood products are thought to be causative in onset of transfusion-related acute lung injury through activation of neutrophils. LysoPCs are thought to be derived from cell membrane degradation products such as phosphatidylcholines (PC) by partial hydrolysis of PC, a process that is catalyzed by phospholipase A_2 (PLA_2).

STUDY DESIGN AND METHODS: We investigated the underlying mechanisms of lysoPC generation and its contribution to in vitro neutrophil-priming capacity during storage of red blood cells (RBCs), platelet (PLTs) concentrates, and cell-free plasma. Blood from healthy volunteers was drawn, processed, and stored according to Sanquin Blood Bank protocols.

RESULTS: Storage of RBCs in saline-adenine-glucose-mannitol (SAGM) did not result in accumulation of lysoPCs or neutrophil-priming capacity. Replacement of SAGM by plasma as RBC storage medium caused elevated lysoPC levels on Day 0, which did not further increase during storage. Cell-free plasma stored at 22°C showed accumulation of lysoPCs during storage, which was not present at 4°C. Addition of a soluble PLA_2 or cytosolic PLA_2 inhibitor did not prevent accumulation of lysoPCs in plasma. In PLTs, lysoPC accumulation during storage was plasma dependent, but lysoPCs did not explain the observed neutrophil-priming effect as preventing accumulation of lysoPCs by removing the plasma fraction did not prevent the neutrophil-priming capacity.

CONCLUSION: Accumulation of lysoPCs during storage is not cell but plasma derived and storage temperature dependent and does not explain the neutrophil-priming effect of aged products.

Transfusion-related acute lung injury (TRALI) is the number one cause of transfusion-related morbidity and mortality.[1-4] TRALI is thought to be a two-hit event.[5] The first event is an inflammatory condition of the patient (e.g., sepsis, recent surgery) causing sequestration and priming of neutrophils in the pulmonary compartment. The second event is the transfusion, containing either antibodies or bioactive lipids that have accumulated during blood storage, stimulating the primed neutrophils to release proteases. The result is endothelial damage, capillary leak, and extravasation of neutrophils. The two-event hypothesis is supported by experimental studies, in which bioactive lipids (lysophosphatidylcholines [lysoPCs]) as well as outdated

ABBREVIATIONS: $cPLA_2$ = cytosolic phospholipase A_2; HPLC-MS/MS = high-performance liquid chromatography tandem mass spectrometry; lysoPC(s) = lysophosphatidylcholine(s); PAF = platelet-activating factor; PC(s) = phosphatidylcholine(s); PLA_2 = phospholipase A_2; SAGM = saline-adenine-glucose-mannitol; SBB = Sanquin Blood Bank; $sPLA_2$ = soluble phospholipase A_2.

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blood products have been used to cause TRALI after a priming hit.[6,7] Also, observational studies report associations between prolonged storage of blood products and respiratory failure in patients after cardiac surgery or trauma.[8,9] Previous studies showed that the concentration of four lysoPCs (lyso-PAF and steatoyl-, palitoyl-, and oleoyl phosphocholine) increases during storage of red blood cells (RBCs) and platelet (PLT) concentrates.[10,11] These lysoPCs have been associated with TRALI as their infusion causes a release of components of the microbicidal arsenal resulting in pulmonary leakage through the alveolar–capillary membrane.[6,7,12-14] In line with this, a study of 10 TRALI patients linked the occurrence of TRALI with transfusion of blood products containing lipids with significant neutrophil-priming activity.[13] LysoPCs, also called lysolecithins, are a class of chemical compounds that are derived from phosphatidylcholines (PCs). They result from partial hydrolysis of PC, a process that is catalyzed by phospholipase A_2 (PLA_2). It could be speculated that difference in temperature storage may influence this enzymatic process. We recently showed that accumulation of lysoPCs occurs during storage of PLTs when blood banking processes are followed.[15] As lysoPCs are thought to play a key role in onset of TRALI via activation of the neutrophil, it is important to understand whether storage conditions influence lysoPC accumulating. Insight in the mechanism of lysoPC accumulation may aid in developing new manufacturing processes of blood products that prevent transfusion-related adverse events. In this study, we investigated mechanisms of accumulation of lysoPCs during storage of RBCs and PLTs, including storage in different media and temperature conditions. Furthermore, we tested the effect of soluble and cytosolic PLA_2 inhibitors on the accumulation of lysoPCs.

MATERIALS AND METHODS

Storage-related biochemical changes in human RBCs

Healthy adult volunteers (n = 9) donated 1 unit of whole blood (500 mL), collected in citrate-phosphate-dextrose (CPD; 70 mL) and stored for 12 to 18 hours at 20 to 22°C. RBCs were prepared by centrifugation for 8 minutes at $2800 \times g$. After removal of plasma and buffy coat, 110 mL of the standard storage medium saline-adenine-glucose-mannitol (SAGM) was added via the in-line leukoreduction filter to the RBCs, which were subsequently leukoreduced by filtration. The RBCs were stored at 2-6°C according to Sanquin Blood Bank (SBB) standards. Supernatants were collected after preparation of the products and on Days 7, 21, 35, and 42 prepared by centrifugation for 10 minutes at $14{,}500 \times g$ at 4°C to remove cells and acellular debris. Aliquots of supernatants were stored at –80°C for analysis of lysoPC and PC and in vitro neutrophil-priming capacity.

Storage-related biochemical changes in human RBCs using adaptive storage solution

To study the effect of storage solution on accumulation of lysoPCs and in vitro neutrophil-priming capacity, we compared two types of storage solutions for RBCs: 1) the conventional storage solution SAGM as described above (n = 9) and 2) CPD plasma with added adenine as storage solution (n = 4), to mimic plasma from collections using CPD-A1 as anticoagulant. Supernatants were collected after preparation of the products and on Days 35 and 42.

Storage-related biochemical changes in human PLTs

Platelet concentrates in plasma were prepared from pooled buffy coat (n = 5 per pool) as previously described.[16] The concentrates were filtered through a leukoreduction filter (Compostop CS, type T3995, Fresenius Kabi, Emmer-Compascuum, the Netherlands) with a polyvinylchloride-citrate container connected to the outlet of the filter. The PLTs (n = 6) were stored at 22 ± 2°C horizontally shaking with one cycle per minute (Helmer Labs, Inc., Noblesville, IN), according to SBB standards. Supernatants were collected after preparation of the products and on Days 5, 7, and 9.

Storage-related biochemical changes in human PLTs using adaptive storage solutions

To study the effect of storage solution on accumulation of lysoPCs and in vitro neutrophil-priming capacity we compared three types of storage solutions for PLTs: 1) we used the conventional storage solution of 100% plasma from one of the five buffy donors as described (n = 6); 2) we centrifuged the PLTs and replaced part of the plasma by SSP+ up to a final resuspension mixture of 65% SSP+ and 35% plasma (n = 3); and 3) we centrifuged the PLTs and replaced as much as possible of the plasma by SSP+, resulting in a resuspension mixture of 95% SSP+ and 5% plasma (n = 3). Plasma stored at 22°C served as a control. Supernatants were collected after preparation of the products and on Days 5 and 7.

Storage-related biochemical changes in human plasma using different storage conditions and the effect of PLA_2 inhibitors

In additional studies, cell-depleted plasma (using Haemonetics apheresis plasma) and plasma obtained directly after whole blood donation (n = 6) was stored at 4°C (comparable to storage of RBCs) and at 22°C (comparable to storage of PLTs). As the formation of lysoPCs results from partial hydrolysis of PCs that removes one of the fatty acid

groups, which is generally the result of the enzymatic action of PLA_2, we tested whether soluble (s)PLA_2 or cytosolic (c)PLA_2 is essential in formation of lysoPCs during storage of products in plasma by adding a $sPLA_2$ or $cPLA_2$ inhibitor to the plasma during storage. We investigated the effect of two different $sPLA_2$ inhibitors in two different concentrations ((c2NapA)LS(2NapA)R [10 and 62 μmol/L], Category Number 525145, Calbiochem, San Diego, CA; and thioetheramide-PC [40 and 100 μmol/L], Category Number 62750, Cayman Chemical, Ann Arbor, MI) and two different $cPLA_2$ inhibitors in two different concentrations (AACOCF3 [20 and 50 μmol/L], Calbiochem; aristolochic acid [40 and 100 μmol/L], Sigma, St Louis, MO) on the formation of lysoPCs during storage (n = 3, for each concentration).[17] Supernatants were collected after preparation of the products and on Days 5 and 7.

Lipid extraction and lysoPC and PC measurement

Lipid extraction of supernatant from stored RBC and PLT supernatant was performed using Bligh and Dyer method. In short, 3 mL of $CHCl_3$:MeOH (1:2) was added to 100 μL of sample and 100 μL of internal standard solution (lysoPCs 14:0, 2.5 nmol; and PC 28:0, 10 nmol). Quantities of 700 μL of 0.5% HAc, 1 mL $CHCl_3$, and 800 μL of 0.5% HAc were added. After each step samples were mixed vigorously for 30 seconds. The final mixture was centrifuged for 10 minutes at 1892 × *g* at room temperature. After centrifugation, the lower layer of $CHCl_3$ was separated. This step was repeated two times by adding 1 mL of $CHCl_3$. The separated $CHCl_3$ layers were combined and dried (N_2, 30°C). Samples were dissolved in 150 μL 25% of $CHCl_3$/MeOH/H_2O/NH_3 (50/45/5/0.01 vol/vol/vol/vol) for further analysis.

High-performance liquid chromatography tandem mass spectrometry

The relative concentrations of lysoPC and PC species in supernatant of RBCs and PLTs and in plasma samples were determined using high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS). Ten microliters of extracted lipid sample was injected on the HPLC-MS/MS system. Chromatographic separation was achieved on a modular HPLC system (Surveyor, Thermo Finnigan, San Jose, CA) consisting of a cooled autosampler (T = 12°C), a low-flow quaternary MS pump and analytical HPLC column (LichroSpher Si60, 2 × 250 mm column, 5-μm particle diameter; Merck, Darmstadt, Germany). Samples were eluted with a flow rate of 300 μL/min and a programmed linear gradient between Solution B (chloroform : methanol, 97:3, vol/vol) and Solution A (methanol : water, 85:15, vol/vol); A and B contained 1 and 0.1 mL of 25% (vol/vol) aqueous ammonia per liter of eluent, respectively. The gradient was as follows: T = 0 to 10 minutes, 20% A to 100% A; T = 10 to 12 minutes, 100% A; T = 12 to 12.1 minutes, 100% A to 0% A; and T = 12.1 to 17 minutes, equilibration with 0% A. Total run time, including the equilibration, was 17 minutes. A splitter between the HPLC and MS was used for the introduction of the eluent in the MS by 75 μL/min.

MS/MS analyses were performed on a triple quadrupole mass spectrometer (TSQ Quantum AM, Thermo Finnigan, Waltham, MA) operated in the positive ion electrospray ionization mode. The skimmer offset was set at 10 V; spray voltage was 3600 V and the capillary temperature was 300°C. In the optimized MS/MS experiments, argon was used as collision gas at a pressure of 0.07 Pa and a collision energy of 40 V. The parent ion scan of m/z 184.1 (m/z 400-m/z 1000, 1 sec) was used for the quantization of the following precursor ions: m/z 468.3 (lysoPC 14:0, I.S.), m/z 496.3 (lysoPC 16:0), m/z 524.3 (lysoPC 18:0/ platelet-activating factor [PAF] 16:0), m/z 522.4 (lysoPC 18:1), m/z 482.4 (LysoPAF 16:0), m/z 510.4 (LysoPAF 18:0), m/z 508.4 (LysoPAF 18:1), m/z 678.4 (PC 28:0, I.S.), m/z 758.4 (PC 34:2), m/z 782.4 (PC 36:2).

Neutrophil-priming assay

Blood was collected from healthy volunteers as described previously.[18] The neutrophils were isolated using standard techniques including 1.076 g/mL Percoll gradient centrifugation and NH_4Cl/$KHCO_3$ lysis of RBCs. Neutrophils (1.0×10^6/mL) were incubated with supernatant for 30 minutes at 37°C and thereafter activated with formyl-methionyl-leucylphenylalanine. We used H_2O_2 release as a measure of neutrophil priming measured with the fluorescent dye Amplex Red in the presence of horse radish peroxidase. Buffer and 20 ng/mL *Escherichia coli* lipopolysaccharide (LPS)/LPS-binding protein (LPB; Sigma) served as negative and positive controls, respectively.

Statistical analyses

Sample size was based on previous studies investigating lysoPC accumulation and neutrophil priming.[6,15,19] Data are expressed as mean ± SD or mentioned otherwise. Comparisons between the groups were performed using a t test, Mann-Whitney U test, Kruskal-Wallis test, or one-way analysis of variance (ANOVA), followed by post hoc Dunnett's, depending on data distribution. A p value of less than 0.05 was considered significant. Statistical analyses were performed with computer software (SPSS 12.0, SPSS, Chicago, IL; and Prism 4.0, GraphPad Software, San Diego, CA).

RESULTS

LysoPC accumulation and neutrophil-priming capacity during storage of RBCs in SAGM solution

Several previous studies reported accumulation of lysoPC in stored RBC blood products.[6,10] In line with our previous results with human RBC products,[19] the concentrations of lysoPC did not increase in human RBC products stored up to 42 days in SAGM with minimal plasma content (Table 1, first block). In line with these results, the concentration of PCs, the biochemical precursor of lysoPCs, remained unchanged during storage (Table 1). Supernatant of RBCs stored in SAGM did not have neutrophil-priming capacity, not even after 35 and 42 days of storage (Fig. 1B).

TABLE 1. LysoPC concentrations (μmol/L) in human RBCs stored in SAGM or plasma*

	Day		
RBC storage medium	0	35	42
SAGM (n = 9 batches)			
LysoPC			
16:0	12.2 ± 2.7	9.2 ± 3.7	9.2 ± 4.0
18:1	3.1 ± 0.6	2.8 ± 0.9	2.6 ± 0.8
LysoPC/PAF			
18:0/16:0	0.7 ± 0.1	0.6 ± 0.2	0.5 ± 0.1
LysoPAF			
16:0	0.5 ± 0.1	0.5 ± 0.2	0.5 ± 0.2
18:0	0.3 ± 0.1	0.2 ± 0.2	0.2 ± 0.1
18:1	0.7 ± 0.1	0.6 ± 0.2	0.5 ± 0.1
PC			
34:2	24.3 ± 4.7	23.4 ± 5.7	23.4 ± 5.8
36:4	9.2 ± 2.1	8.7 ± 2.6	8.7 ± 2.1
Plasma (n = 4 batches)			
LysoPC			
16:0	82.9 ± 11.3[a]	75.1 ± 15.1[a]	79.4 ± 19.8[a]
18:1	22.5 ± 2.6[a]	21.7 ± 1.2[a]	22.0 ± 2.2[a]
LysoPC/PAF			
18:0/16:0	2.9 ± 0.3[a]	3.1 ± 0.8[a]	3.6 ± 1.3[a]
LysoPAF			
16:0	2.1 ± 0.4[a]	2.0 ± 0.5[a]	2.0 ± 0.6[a]
18:0	1.0 ± 0.2[a]	1.1 ± 0.2[a]	1.3 ± 0.4[a]
18:1	2.9 ± 0.3[a]	3.1 ± 0.8[a]	3.6 ± 1.3[a]
PC			
34:2	77.5 ± 14.0[a]	74.5 ± 9.0[a]	73.1 ± 15.1[a]
36:4	22.2 ± 3.5[a]	19.4 ± 3.7[a]	20.9 ± 5.2[a]

* Data are presented as mean ± SD. No increase in lysoPC during storage, ANOVA repeated measurement, followed by post hoc Dunnett's, nonsignificant. RBCs stored in plasma during all times have a higher concentration of lysoPCs in the supernatant compared to RBCs stored in SAGM. [a]$p < 0.01$, Mann-Whitney test.

In line with our previous studies,[7,11] the concentration of lysoPCs increased in human PLT products stored in 100% plasma for 5 and 7 days when compared to fresh PLTs (Table 2, first block), with a concomitant decrease in PC concentrations ($p < 0.01$ for all, Table 2). Supernatant of fresh PLTs had significant neutrophil-priming capacity compared to buffer control (H_2O_2 release 200 ± 4.0 [Fig. 1C] vs. 100 ± 1.0 pmol/mL [Fig. 1A]; $p < 0.01$). Storage further enhanced neutrophil-priming capacity, because supernatant of PLTs stored for 5, 7, and 9 days in 100% plasma had higher in vitro neutrophil-priming capacity compared to supernatant of fresh PLTs stored in 100% plasma (H_2O_2 release 260 ± 4.0, 290 ± 3.0, and 292 ± 2.0 vs. 200 ± 4.0 pmol/mL; $p < 0.05$ and $p < 0.01$, respectively; Fig. 1C).

LysoPC accumulation during storage of human RBCs in 100% plasma

The results of the first experiments revealed that lysoPCs only accumulated

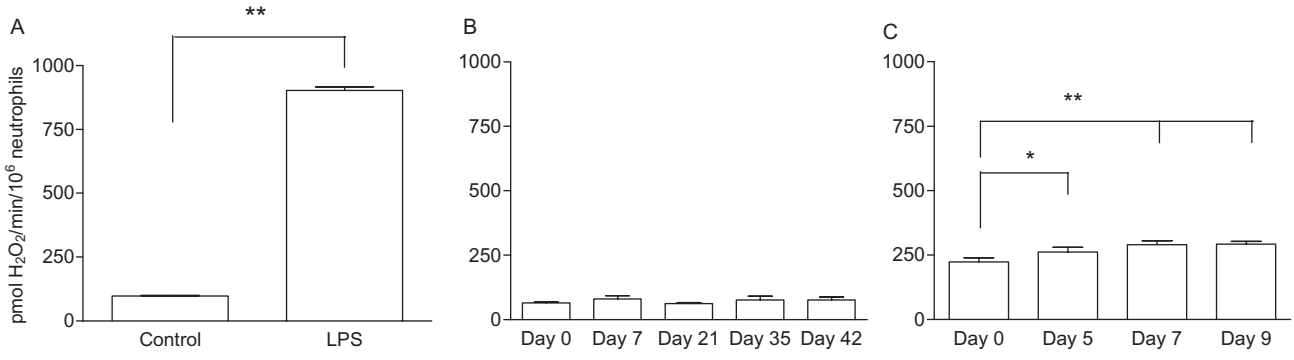

Fig. 1. (A) H_2O_2 release when neutrophils were incubated with buffer solution (negative control) or *E. coli* LPS/LPB 20 ng/mL (positive control). (B) H_2O_2 release when neutrophils were incubated with supernatant of human RBCs stored in SAGM of different storage time points up to 42 days (n = 5, NS). (C) H_2O_2 release when neutrophils were incubated with supernatant of human PLT concentrates stored in 100% plasma at 22°C of different storage time points up to 9 days (n = 5). *$p < 0.05$, **$p < 0.01$, ANOVA repeated measurement, Dunnett's post test. Data are presented as mean and SD.

TABLE 2. LysoPC concentrations (μmol/L) in human PLT concentrates stored in 100% plasma, 35% plasma:65% SSP, or 5% plasma:95% SSP*

	Day		
PLT storage medium	0	5	7
Plasma (n = 6 batches)			
LysoPC			
16:0	97 ± 6.7	155 ± 22.4[a]	169 ± 37.7[a]
18:1	17 ± 1.5	29 ± 6.2[a]	31 ± 8.2[a]
LysoPAF			
16:0	2.4 ± 0.1	3.3 ± 0.2[a]	3.6 ± 0.9[a]
18:0	3.1 ± 0.4	4.9 ± 0.6[a]	5.2 ± 1.5[a]
18:1	1.1 ± 0.2	1.7 ± 0.3[a]	2.0 ± 0.7[a]
LysoPC/PAF			
18:0/16:0	3.1 ± 0.4	4.9 ± 0.6[a]	5.2 ± 1.5[a]
PC			
34:2	100 ± 32.9	92 ± 32.0[a]	83 ± 31.1[a]
36:4	32.6 ± 13.5	30.7 ± 13.9[b]	28.5 ± 14.0[a]
65% SSP† (n = 3 batches)			
LysoPC			
16:0	40 ± 2.2	44 ± 3.3	42 ± 1.3
18:1	9.6 ± 2.4	10.2 ± 2.0	9.8 ± 1.4
LysoPAF			
16:0	1.0 ± 0.2	1.1 ± 0.1	1.0 ± 0.1
18:0	1.4 ± 0.1	1.7 ± 0.2	1.3 ± 0.2
18:1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
LysoPC/PAF			
18:0/16:0	1.4 ± 0.1	1.7 ± 0.2	1.3 ± 0.2
PC			
34:2	49 ± 4.6	36 ± 3.7[b]	35 ± 5.2[b]
36:4	16.1 ± 0.2	12.0 ± 2.0[b]	12.6 ± 1.7[b]
95% SSP† (n = 3)			
LysoPC			
16:0	13 ± 2.8	13 ± 3.2	13 ± 4.0
18:1	3.3 ± 1.3	3.2 ± 1.3	3.2 ± 1.2
LysoPAF			
16:0	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
18:0	0.6 ± 0.2	0.5 ± 0.1	0.7 ± 0.2
18:1	0.2 ± 01	0.2 ± 0.1	0.3 ± 0.2
LysoPC /PAF			
18:0/16:0	0.6 ± 0.2	0.5 ± 0.1	0.7 ± 0.2
PC			
34:2	24 ± 3.1	22 ± 3.8[b]	21 ± 3.7[b]
36:4	8.0 ± 0.2	7.4 ± 0.6	7.5 ± 0.8

* Data are presented as mean ± SD. [a]$p < 0.01$ and [b]$p < 0.05$, compared to Day 0 PLTs (ANOVA repeated measurement, followed by post hoc Dunnett's).

† $p < 0.05$, PLTs stored in 65% SSP and PLTs stored in 95% SSP compared to PLTs in plasma for all measurements and PLTs stored in 95% SSP compared to PLTs stored in 65% SSP (ANOVA followed by Bonferroni's posttest).

during storage of PLTs and not during storage of RBCs. This raised the question whether the accumulation of lysoPCs may be cell type or plasma dependent. When RBCs were stored in plasma, the lysoPC concentration was significantly higher on Day 0 compared to RBCs stored in SAGM (Table 1, second block), although the concentration of lysoPC did not further increase during storage.

LysoPC accumulation and neutrophil-priming capacity during storage of PLTs using different additive solution

The previous results suggest that plasma is essential in the process of lysoPC formation during storage of PLTs. We investigated whether using different mixtures of plasma and additive solutions (ASs) reduced lysoPC concentration in PLTs and neutrophil-priming capacity during storage. In PLTs on Day 0, replacement of plasma with 35% plasma and 65% SSP as AS resulted in a significant decrease in lysoPCs and PC levels (Table 2, second block). During storage, there was some decrease in PC levels, but no increase in lysoPCs. When plasma was nearly eliminated as AS using 95% SSP mixture, levels of lysoPCs were even lower (Table 2, third block).

Supernatant of fresh PLTs showed significant neutrophil-priming capacity, irrespective of the AS used compared to the buffer control (H_2O_2 release 185 ± 59 [100% plasma], 221 ± 13 [35% plasma and 65% SSP], and 201 ± 20 [5% plasma and 95% SSP] pmol/mL vs. 100 ± 1 [buffer control]; $p < 0.01$; Figs. 2A and 2C-2E). Of interest, supernatant of PLTs stored in 5% plasma:95% SSP for 7 days resulted in a higher neutrophil priming in vitro compared to supernatant of PLTs stored in 35% plasma:65% SSP or in 100% plasma despite a lower lysoPC concentration (528 ± 12 pmol/mL vs. 334 ± 14 and 290 ± 15 pmol/mL; $p < 0.01$; Figs. 2C-2E).

LysoPC accumulation and neutrophil-priming capacity during storage of plasma at 22 and 4°C

Plasma stored at 22°C showed a similar lysoPC accumulation as PLTs stored in plasma. Concentrations of lysoPCs were already increased after 5 days of storage compared to fresh plasma, with a concomitant decrease in PC concentrations ($p < 0.05$ for all; Table 3, first block). Additional experiments with cell-free plasma showed similar results (data not shown). Plasma stored at 4°C showed similar lysoPC changes as RBCs stored in plasma. There was a high level of lysoPCs on Day 0, without further increase during storage (Table 3, second block).

Supernatant of fresh plasma had significant neutrophil-priming capacity compared to buffer control (H_2O_2 release 270 ± 7.0 pmol/mL vs. 100 ± 1.0 pmol/mL; $p < 0.01$; Figs. 2A and 2B). Surprisingly, however, supernatant of plasma stored for 7 days at 22°C showed no increase in neutrophil priming in vitro compared to fresh

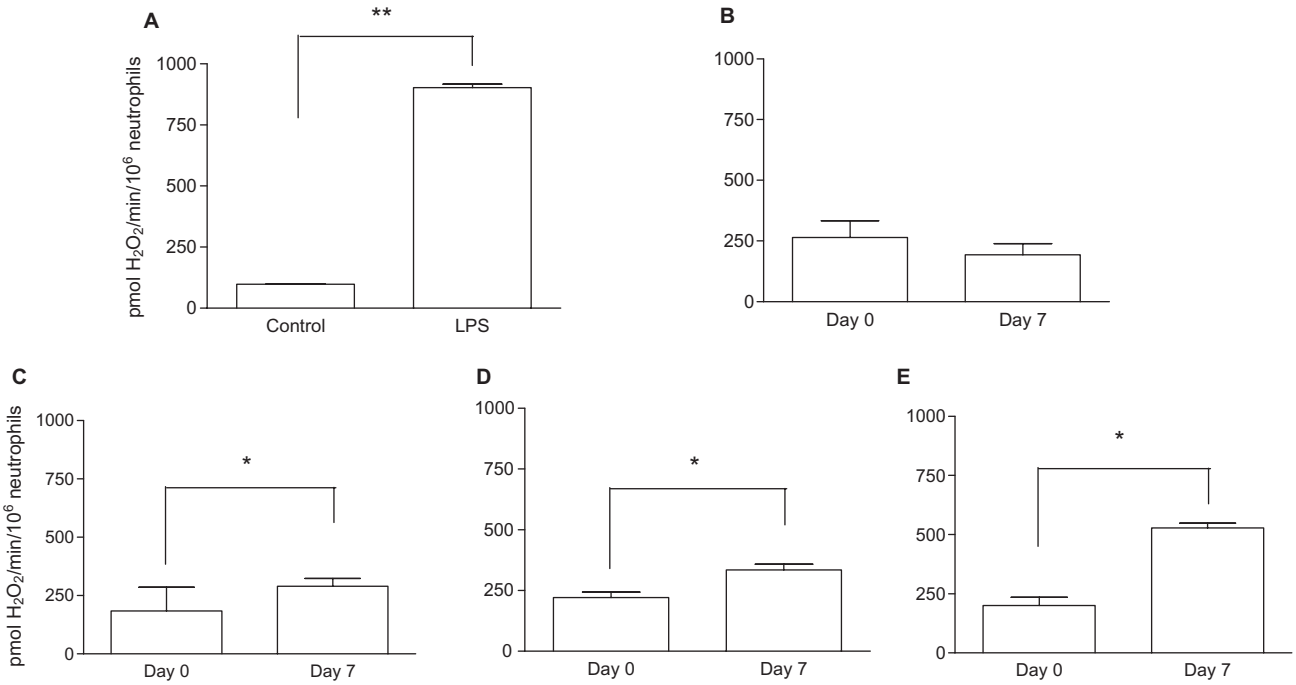

Fig. 2. (A) H_2O_2 release when neutrophils were incubated with buffer solution (negative control) or *E. coli* LPS/LPB 20 ng/mL (positive control). (B) H_2O_2 release when neutrophils were incubated with supernatant of cell-depleted plasma obtained directly from whole blood donation, stored at 22°C of different storage time points (Days 0 and 7). (C) H_2O_2 release when neutrophils were incubated with supernatant of PLT concentrates stored in 100% plasma of different storage time points (Days 0 and 7). (D) H_2O_2 release when neutrophils were incubated with supernatant of PLTs stored in 35% plasma:65% SSP of different storage time points (Days 0 and 7). (E) H_2O_2 release when neutrophils were incubated with supernatant of PLTs stored in 5% plasma:95% SSP of different storage time points (Days 0 and 7; n = 3 batches for each measurement). *p < 0.05, **p < 0.01, paired t test. Data are presented as mean and SD.

plasma, despite an increase in lysoPC concentration (Fig. 2B).

Effect of addition of PLA_2 inhibitors on lysoPC accumulation

Because the formation of lysoPCs results from partial hydrolysis of PCs, which removes one of the fatty acid groups, which is generally the result of the enzymatic action of PLA_2, we tested whether soluble (s)PLA_2 or cytosolic (c)PLA_2 is essential in formation of lysoPCs during storage of products in plasma by adding a $sPLA_2$ or $cPLA_2$ inhibitor to the plasma during storage. Addition of a $sPLA_2$ inhibitor to plasma did not prevent an increase in lysoPC concentration compared to plasma stored under the same conditions but without the $sPLA_2$ inhibitor (Table 3, only shown for highest concentration of thioetheramide-PC; Table 3, second and fourth block). Additional experiments with $cPLA_2$ inhibitor also did not prevent an increase in lysoPC concentration compared to plasma stored without $cPLA_2$ inhibitor (data not shown). Storage temperature did not influence this effect.

DISCUSSION

We describe the effect of different storage solutions and temperature conditions on lysoPC accumulation and neutrophil priming, using a clinical protocol for the preparation and storage of blood products according to SBB standards. In this study, the main findings are as follows: 1) accumulation of lysoPCs is not cell derived, but plasma derived; 2) accumulation of lysoPCs during storage is temperature dependent and absent at a storage temperature of 4°C; and 3) neutrophil priming in vitro by aged PLTs is lysoPC independent because lowering the lysoPC level did not prevent neutrophil priming.

Previous studies showed that accumulation of lysoPCs occurs during storage of RBCs and that these lipids may play a key role in the onset of neutrophil priming and/or activation in the pathogenesis of TRALI.[6,10,20] The association of aged RBCs and transfusion-related morbidity and mortality has been described in observational studies.[9,21] It should be noted, however, that both preclinical and clinical studies are mainly performed in the United States.[6,9,10,20,21] Indeed, clinical and

TABLE 3. Storage-related changes (μmol/L) in lysoPCs in plasma stored at different temperatures and with addition of $sPLA_2$ inhibitor*

	Day		
Plasma storage temperature	0	5	7
22°C (n = 6)			
LysoPC			
16:0	102 ± 8.1	146 ± 14.2[a]	140 ± 30.6[a]
18:1	24.2 ± 2.1	30.0 ± 4.7[a]	29.0 ± 6.4[b]
LysoPAF			
16:0	2.5 ± 0.4	3.2 ± 0.4[a]	3.0 ± 0.7[b]
18:0	3.5 ± 0.6	4.8 ± 0.6[a]	4.5 ± 0.9[a]
18:1	1.2 ± 0.2	1.5 ± 0.2[a]	1.4 ± 0.2[b]
LysoPC/PAF			
18:0/16:0	3.5 ± 0.6	5.2 ± 0.5[a]	4.7 ± 1.0[a]
PC			
34:2	122 ± 20.8	91 ± 7.3[a]	106 ± 10.5
36:4	55 ± 14.2	42 ± 4.5[b]	47 ± 5.9
4°C (n = 6)			
LysoPC			
16:0	102.3 ± 7.0	97 ± 2.5	106 ± 9.4
18:1	22.5 ± 2.9	21.0 ± 2.4	22.5 ± 1.6
LysoPAF			
16:0	2.6 ± 0.3	2.3 ± 0.3	2.4 ± 0.4
18:0	3.5 ± 0.3	3.4 ± 0.5	3.4 ± 0.6
18:1	1.4 ± 0.3	1.2 ± 0.1	1.2 ± 0.2
LysoPC/PAF			
18:0/16:0	3.5 ± 0.5	3.4 ± 0.5	3.6 ± 0.7
PC			
34:2	125 ± 20.2	113 ± 16.6	114 ± 14.0
36:4	58 ± 11.5	52 ± 8.9	49 ± 5.9
22°C with $sPLA_2$ inhibitor (n = 3)			
LysoPC			
16:0	94 ± 5.3	146 ± 5.9[a]	142 ± 15.3[a]
18:1	23.0 ± 1.0	29.0 ± 3.6	26.0 ± 7.1
LysoPAF			
16:0	2.6 ± 0.1	3.4 ± 0.5[a]	3.2 ± 0.2[a]
18:0	3.5 ± 0.7	5.1 ± 0.8[a]	4.5 ± 0.6[a]
18:1	1.3 ± 0.3	1.6 ± 0.3[a]	1.6 ± 0.4[a]
LysoPC/PAF			
18:0/16:0	3.5 ± 0.4	5.7 ± 0.5	5.5 ± 1.5
PC			
34:2	147 ± 16.8	84 ± 7.0[a]	91 ± 18.0[a]
36:4	67 ± 5.0	40 ± 23[a]	41 ± 9.5[a]
4°C with $sPLA_2$ inhibitor (n = 3)			
LysoPC			
16:0	96 ± 5.1	98 ± 4.9	99 ± 5.6
18:1	22.3 ± 3.5	23.3 ± 3.1	23.0 ± 2.7
LysoPAF			
16:0	2.4 ± 0.2	2.3 ± 0.2	2.4 ± 0.1
18:0	3.8 ± 0.9	3.4 ± 0.4	3.3 ± 0.2
18:1	1.4 ± 0.3	1.2 ± 0.1	1.3 ± 0.2
LysoPC/PAF			
18:0/16:0	3.5 ± 0.3	3.6 ± 0.3	3.5 ± 0.2
PC			
34:2	122 ± 6.4	112 ± 20.0	110 ± 12.5
36:4	62 ± 6.0	49 ± 6.7	46 ± 3.1[b]

* Data are presented as mean ± SD (plasma without $sPLA_2$ inhibitor, n = 6 batches; plasma with $sPLA_2$ inhibitor, thioetheramide-PC, 100 μmol/L, n = 3 batches). Similar results were found with the lower concentration of thioetheramide-PC (40 μmol/L); the two concentrations of the other $sPLA_2$ inhibitor used, (c2NapA)LS(2NapA)R (10 and 62 μmol/L); and the two different $cPLA_2$ inhibitors in two different concentrations, AACOCF3 (20 and 50 μmol/L) and aristolochic acid (40 and 100 μmol/L; data not shown). [a]$p < 0.01$ and [b]$p < 0.05$, compared to Day 0 plasma (ANOVA repeated measurement, followed by post hoc Dunnett's).

preclinical studies outside the United States did not show this effect,[22-24] which may suggest that there is a difference in product preparation or storing processes of RBCs. Of note, the absence of lysoPC accumulation was not caused by the shorter storage time of RBCs in the Netherlands compared to the United States (35 days vs. 42 days). Therefore, our results suggest that the percentage of plasma present in the storage medium is important in formation and accumulation of lysoPCs, a process that is temperature dependent and does not occur at 4°C.

We showed that lysoPCs still accumulate during storage when the supernatant is immediately cell depleted on Day 0. This suggests that the lysoPCs are mainly formed from PCs, which are already present in plasma and not from PCs resulting from cell degradation during storage. Furthermore, we showed that lysoPC accumulation is storage temperature dependent, which suggests an enzymatic process. For this reason we investigated the effect of a $sPLA_2$ and $cPLA_2$ inhibitor on plasma. However, no effect was seen on the increase of lysoPCs in plasma stored at 22°C, independent of the type $sPLA_2$ or $cPLA_2$ inhibitor or concentration used (additional data not shown). An explanation for this result is tempting. First, besides PLA_2, PLA_1 is also able to partial hydrolyze PCs, which results in removal of one of the fatty acid groups causing the formation of lysoPC.[25,26] Inhibition of PLA_1 may be one of the candidates for further research. Second, the $sPLA_2$ inhibitors and $cPLA_2$ inhibitors used may not have been specific or strong enough; although in previous experiments these agents were shown to be effective, we did not confirm their inhibitor activity in this study.[17]

We confirm that supernatant of aged PLTs is able to prime neutrophils in vitro.[15] However, replacing plasma for SSP mixtures decreased lysoPC levels while in vitro neutrophil-priming capacity increased, suggesting that the neutrophil-priming capacity of aged products is lysoPC independent. In line with this, plasma stored for 7 days

showed an increase in lysoPC concentration in the absence of an increased neutrophil priming. This finding raises the question whether in our products, lysoPCs are merely a marker or a mediator of product deterioration and transfusion-related morbidity and mortality. We recently showed that supernatant of aged products caused lung injury in an in vivo "two-hit" animal transfusion model,[15,19] irrespective of the presence of lysoPC. Other factors that may play a role are biochemical changes in the supernatant such as an increase in potassium, decrease in pH, accumulation of proinflammatory cytokines, and procoagulant activity by accumulation of microparticles with phosphatidylserine exposure.[27,28]

Our data may have relevance for manufacturing processes, suggesting that storage conditions have significant effect on accumulation of bioactive lipids and in vitro neutrophil-priming capacity. Whether elimination of plasma as resuspension medium for cellular products prevents adverse effects of transfusion, however, remains to be determined. Also, considerable differences in manufacturing and storage protocols exist between blood banks (e.g., Dutch RBC additives are hypertonic and most US RBC additives are isotonic, although AS-1 is also hypertonic). Our results suggest that these differences may have clinical relevance because the use of different storage additives results in different levels of neutrophil priming.

In conclusion, we showed that in vitro neutrophil priming and accumulation of lysoPCs during storage are absent in RBCs, which may be due to low storage temperature. Furthermore we showed that lysoPC accumulation is not cell but plasma derived and storage temperature dependent. In PLTs, lysoPC accumulation during storage is not the explanation for the observed in vitro neutrophil-priming effect, because prevention of accumulation and/or removing of the lysoPCs did not decrease the in vitro neutrophil-priming capacity.

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CONFLICT OF INTEREST

None.

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