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Alexis Klein, Valérie Deckert, Martina Schneider, Fabienne Dutrillaux, Arlette Hammann, et al.. α -Tocopherol Modulates Phosphatidylserine Externalization in Erythrocytes. Relevance in Phospholipid Transfer Protein-Deficient Mice. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 2006, 26 (9), pp.2160-2167. <10.1161/01.ATV.0000235699.98024.11>. <hal-04203058>

HAL Id: hal-04203058

<https://hal.science/hal-04203058v1>

Submitted on 11 Sep 2023

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α -Tocopherol Modulates Phosphatidylserine Externalization in Erythrocytes

Relevance in Phospholipid Transfer Protein–Deficient Mice

Alexis Klein, Valérie Deckert, Martina Schneider, Fabienne Dutrillaux, Arlette Hammann, Anne Athias, Naïg Le Guern, Jean-Paul Pais de Barros, Catherine Desrumaux, David Masson, Xian-Cheng Jiang, Laurent Lagrost

Objective—The aim of the present study was to assess the effect of α -tocopherol, the main vitamin E isomer on phosphatidylserine (PS) exposure at the surface of circulating erythrocytes, and to determine consequences on erythrocyte properties.

Methods and Results—In vitro α -tocopherol enrichment of isolated erythrocytes significantly decreased PS externalization as assessed by lower Annexin V-fluorescein isothiocyanate labeling. Plasma phospholipid transfer protein (PLTP) transfers vitamin E, and both α - and γ -tocopherol accumulated in circulating erythrocytes from PLTP-deficient homozygous (PLTP^{-/-}) mice as compared with wild-type mice. In agreement with in vitro studies, vitamin E–enriched erythrocytes from PLTP^{-/-} mice displayed fewer externalized PS molecules than wild-type controls (–64%, $P < 0.05$). The perturbation of phospholipid membrane asymmetry from PLTP^{-/-} erythrocytes was accompanied by impairment of their procoagulant properties, with a 20% increase in clotting time as compared with wild-type controls ($P < 0.05$). Less pronounced, however still significant, changes were observed in α -tocopherol content, procoagulant properties, and PS externalization in erythrocytes of PLTP-deficient heterozygotes. Finally, whole blood coagulation and circulating level of D-dimer, which reflects increased thrombus formation in vivo, were significantly decreased in PLTP^{-/-} mice compared with wild-type mice.

Conclusions—Vitamin E modifies PS externalization in circulating erythrocytes, thus modulating in vivo their PS-dependent procoagulant properties. (*Arterioscler Thromb Vasc Biol.* 2006;26:2160–2167.)

Key Words: antioxidants ■ lipoproteins ■ tocopherol ■ phospholipid transfer protein ■ mice

Under normal physiological conditions, phosphatidylcholine (PC) and sphingomyelin (SM) are predominantly present in the outer leaflet of the membrane bilayer, whereas polar phospholipids are mainly (phosphatidylethanolamine [PE]) or exclusively (phosphatidylserine [PS]) found in the inner leaflet.¹ Exposure of PS at the surface of cell membranes regulates numerous physiological processes in cellular control and signaling, including apoptosis² and coagulation.^{3–5} PS exposure in circulating cells^{3,6} or shed membrane microparticles⁴ provides a water/lipid surface for the interaction of coagulation factors, leading to an acceleration of thrombin formation. In sickle cell anemia, diabetes or under abnormal oxidative stress, erythrocytes display both a decrease in vitamin E content and an increase in PS externalization, causing an abnormal hypercoagulable state.^{7–10} However, the direct impact of vitamin E on PS externalization and related changes in blood coagulation has not been clearly established in vivo.

Plasma phospholipid transfer protein (PLTP), a member of the lipid transfer/lipopolysaccharide (LPS) binding protein gene family,¹¹ plays a key role in the transfer and distribution of α -tocopherol, the main vitamin E isomer, in a number of tissues.^{12,13} PLTP activity favors the net flux of α -tocopherol from circulating lipoproteins to tissues, such as vascular endothelium and liver, leading to a marked increase in their α -tocopherol content at the expense of circulating apolipoprotein B (apoB)-containing lipoproteins.^{12,13} In the present study, PLTP-deficient mice displayed significant alteration in blood vitamin E distribution, PS externalization, and coagulation.

Methods

Mice

PLTP-deficient homozygous mice (PLTP^{-/-}), PLTP-deficient heterozygous mice (PLTP^{+/-}),¹⁴ and wild-type (WT) mice in an homogenous C57BL/6 background were fed a standard chow diet

Original received March 14, 2006; final version accepted June 14, 2006.

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Arterioscler Thromb Vasc Biol. is available at <http://www.atvbaha.org>

DOI: 10.1161/01.ATV.0000235699.98024.11

and had free access to water and food (A03 diet; Safe, France). All experiments involving animals were approved by the Ethical Committee on the Use of Laboratory Animals of the University of Burgundy, and they were performed in accordance with institutional guidelines.

Blood Sampling and Preparation of Lipoproteins and Erythrocytes

Mice were anesthetized with intraperitoneal injection of sodium pentobarbital (75 mg/kg body weight). Blood collected on sodium citrate (1/9, vol/vol) (Sigma, St Louis, Mo) via a heart puncture was centrifuged (10 minutes, 2000g at 4°C), and lipoproteins were further isolated from plasma by sequential ultracentrifugation as described earlier.¹² Erythrocytes were washed with 0.15 mol/L sodium chloride, supplemented with 0.5% pyrogallol (wt/wt; Sigma) for tocopherols quantitation. Isolated erythrocytes were free of other circulating cells as checked by optical microscopy, and they were counted by using the Malassez cell method.

In Vitro α -Tocopherol Loading of Erythrocytes

Freshly isolated erythrocytes from WT mice were incubated for 3 hours at 37°C with either 0.15 mol/L sodium chloride (control incubation), very-low-density lipoprotein (VLDL), or low-density (LDL)+intermediate density (IDL) lipoproteins isolated ultracentrifugally from a plasma enriched with DL- α -tocopherol (Calbiochem, San Diego, Calif) in the presence of butylated hydroxytoluene (BHT) (2 mg/mL).

α - and γ -Tocopherol Quantitation in Isolated Lipoproteins and Erythrocytes

Tocopherols were extracted and quantified by high-performance liquid chromatography as previously described.¹² Because tocopherol in plasma is almost exclusively located in lipoproteins,¹² tocopherol contents of total plasma were calculated as the sum of the contents of VLDL+IDL+LDL+high-density lipoprotein (HDL).

Quantitation of PS Externalization in Erythrocytes

Annexin V is a phospholipid-binding protein with a strong affinity for PS. PS exposure in erythrocytes was evaluated using commercially available Annexin V–fluorescein isothiocyanate (FITC) kit according to the instructions of the manufacturer (Bender MedSystems, San Bruno, Calif), and fluorescence was monitored on a FACScan Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ). Acquisition was performed on 10 000 events per sample, and data analysis was conducted by using the CellQuest program (Becton Dickinson). Results were expressed as the percentage of labeled erythrocytes in the total erythrocyte population or as the mean specific fluorescence intensity, which reflects the quantity of PS present at the surface of labeled erythrocytes.

Analysis of Phospholipid Species

Phospholipids were analyzed on a Hypersil Si 2 \times 200 mm column (Interchim, Montluçon, France) according to Becart et al.¹⁵ In lipoproteins, the abundance of PC, SM, PE, PS, and lysophosphatidyl choline (LPC) was expressed as compared with total lipids.¹²

Analysis of Cholesterol Derivatives

Gas chromatography/mass spectrometry (GC/MS) quantitation of sterol trimethylsilyl ethers of cholesterol and its derivatives was performed according to Deckert et al.¹⁶ Concentrations were determined from the ratio of the peak area of individual compounds to the peak area of the epicoprostanol for cholesterol or 19-hydroxycholesterol for cholesterol oxides.

Oxidant Level in Erythrocytes

Erythrocytes (10⁸ cells/mL) were incubated at 37°C with dichlorofluorescein (DCF) diacetate (DCFH-DA) (0.2 mmol/L; Sigma), and fluorescence corresponding to the formation of DCF by intracellular oxidant was monitored for 20 minutes on a Victor Wallac

spectrofluorometer (PerkinElmer, Courtaboeuf, France) (excitation wave length, 485 nm; emission wave length, 530 nm).

Conjugated Diene Formation in ApoB-Containing Lipoproteins

ApoB-containing lipoproteins (VLDL+IDL+LDL) were oxidized with copper sulfate as previously described.¹² Formation of conjugated dienes was monitored at 234 nmol/L over a 10-hour period.

Measurement of Clotting Times

Externalized PS responsible for the procoagulant properties of erythrocytes was evaluated through the activation of coagulation by Russell's Viper Venom (RVV) (ICN Biomedicals, Aurora, Ohio) in vitro. With isolated erythrocytes, clotting time of human poor-platelet plasma activated by RVV was measured as described by Zwaal et al.¹⁷ in a ST-art4 automate (Diagnostica Stago, Asnières, France). For whole blood, clotting time was measured after addition of RVV to mouse blood samples. In both cases, clotting time, corresponding to the time required for the formation of a fibrin clot was determined by the arrest of a magnetic sphere.

Analysis of D-Dimer Level in Mouse Plasma

D-Dimer was assayed by using a commercially available ELISA kit (Asserachrom D-Di; Diagnostica Stago).

PLTP Activity Measurements

PLTP activity was measured on fasting plasma using a commercially available PLTP activity kit, as described previously,¹⁸ and expressed as initial phospholipid transfer rate (ie, increase in fluorescence per second: arbitrary units [AU] per second).

Statistical Analysis

Results are expressed as mean \pm SEM. The statistical significance of difference between data means was determined with the nonparametric Mann–Whitney test.

Results

In Vitro α -Tocopherol Enrichment of Isolated Erythrocytes Reduces Phosphatidylserine Externalization

A moderate, 2-fold increase in α -tocopherol content was obtained by incubating erythrocytes with the α -tocopherol-enriched IDL+LDL fraction, with a more pronounced, 7-fold increase with the α -tocopherol-enriched VLDL fraction (final protein concentration of either preparation, 1 mg/mL; Figure 1A). Moderate enrichment produced only a small, nonsignificant tendency toward a reduction in total erythrocyte labeling, however, with a significant reduction when mean specific fluorescence intensity (reflecting PS externalization at the surface of positive cells) was measured ($P<0.05$ versus erythrocytes incubated alone). Reduction in PS exposure was even more pronounced when a greater vitamin E enrichment was achieved, with marked, significant decreases in both the percentage of labeled cells (Figure 1B) and mean specific fluorescence intensity (Figure 1C). In all cases, differences in PS exposure did not relate to differences in cell integrity, with hemolysis remaining constantly below 2% (data not shown).

PLTP Deficiency As a Model of Vitamin E Accumulation in Mouse Erythrocytes

In accordance with earlier work,¹² α -tocopherol contents of total plasma from PLTP^{-/-} and WT mice did not differ (Table

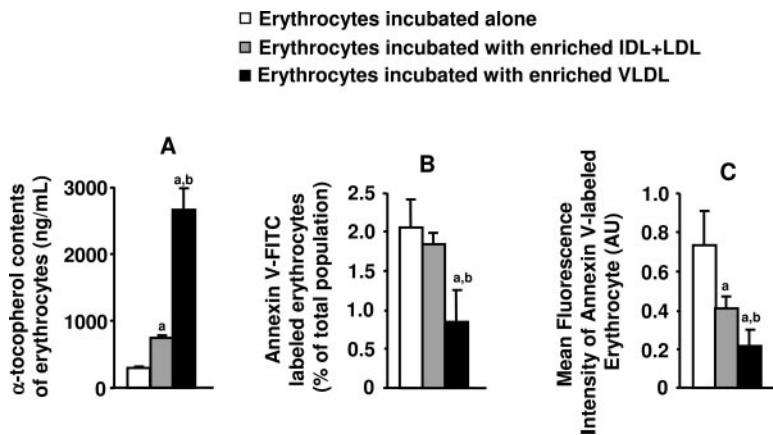


Figure 1. Effect of enrichment of erythrocytes with α -tocopherol on PS externalization. Erythrocytes were incubated alone (open bars), with enriched IDL+LDL (gray bars), or with α -tocopherol-enriched VLDL (black bars) (see Methods). Data are means \pm SEM of $n=6$ distinct mice. Significance of difference from incubation without IDL+LDL or VLDL: ^a $P<0.05$. Significance of difference from incubation with enriched IDL+LDL: ^b $P<0.05$.

1). In contrast, absolute α -tocopherol content of HDL was significantly decreased in PLTP^{-/-} mice compared with WT mice, whereas absolute α -tocopherol contents of VLDL and IDL+LDL were significantly increased ($P<0.01$ in all cases;

Table 1). As shown by comparing α -tocopherol to total lipid ratios, only PLTP^{-/-} IDL+LDL were actually enriched with α -tocopherol as compared with WT counterparts ($P<0.05$) (Table 1). PLTP deficiency produced a significant 63%

TABLE 1. α - and γ -Tocopherol Distribution in Blood From PLTP^{-/-} and WT Mice

	WT	PLTP ^{-/-}	<i>P</i>
<i>n</i>	9	9	
Plasma tocopherol content			
α -Tocopherol, ng/mL	901 \pm 70	901 \pm 123	NS
α -Tocopherol-to-total lipid ratio, ng/mg	1901 \pm 194	2250 \pm 240	NS
γ -Tocopherol, ng/mL	17 \pm 2	29 \pm 7	<0.05
γ -Tocopherol-to-total lipid ratio, ng/mg	35 \pm 6	58 \pm 8	<0.05
Lipoprotein tocopherol content			
α -Tocopherol contents, ng/mL of plasma			
VLDL	156 \pm 27	477 \pm 61	<0.005
IDL+LDL	155 \pm 16	241 \pm 23	<0.01
HDL	590 \pm 57	183 \pm 59	<0.005
α -Tocopherol-to-total lipid ratio, ng/mg of lipids			
VLDL	473 \pm 36	492 \pm 52	NS
IDL+LDL	655 \pm 86	991 \pm 110	<0.05
HDL	773 \pm 83	767 \pm 89	NS
γ -Tocopherol contents, ng/mL of plasma			
VLDL	4 \pm 1	19 \pm 5	<0.005
IDL+LDL	2 \pm 1	6 \pm 1	<0.005
HDL	11 \pm 1	4 \pm 1	<0.001
γ -Tocopherol-to-total lipid ratio, ng/mg of lipids			
VLDL	10 \pm 2	17 \pm 2	<0.05
IDL+LDL	11 \pm 2	24 \pm 4	<0.01
HDL	15 \pm 2	17 \pm 3	NS
Erythrocyte tocopherol content			
α -Tocopherol contents, ng/mL	850 \pm 49	1138 \pm 66	<0.01
α -Tocopherol contents, ng/g of hemoglobin	2234 \pm 138	3020 \pm 187	<0.01
<i>n</i>	30	30	
γ -Tocopherol contents, ng/mL	16 \pm 2	21 \pm 2	<0.05
γ -Tocopherol contents, ng/g of hemoglobin	40 \pm 5	56 \pm 5	<0.05
<i>n</i>	20	23	

Tocopherols were assayed as described in Methods. Data are means \pm SEM.

TABLE 2. Detailed Phospholipid Composition of Lipoproteins and Erythrocytes From WT and PLTP^{-/-} Mice

	WT	PLTP ^{-/-}	P
n	8	9	
Phospholipid-to-total lipid ratio in lipoproteins, μ g/mg			
Phosphatidylcholine			
VLDL	309 \pm 21	268 \pm 31	NS
IDL+LDL	544 \pm 100	479 \pm 79	NS
HDL	564 \pm 88	470 \pm 76	NS
Sphingomyelin			
VLDL	14.4 \pm 1.8	9.4 \pm 1.4	<0.05
IDL+LDL	29.1 \pm 2.3	22.4 \pm 1.7	<0.05
HDL	26.2 \pm 2.0	27.8 \pm 3.1	NS
Phosphatidylethanolamine			
VLDL	5.0 \pm 1.0	5.4 \pm 1.5	NS
IDL+LDL	4.5 \pm 1.1	5.1 \pm 1.1	NS
HDL	7.9 \pm 3.1	8.4 \pm 2.5	NS
Phosphatidylserine			
VLDL	Non Detectable	Non Detectable	
IDL+LDL	Non Detectable	Non Detectable	
HDL	Non Detectable	Non Detectable	
Phospholipid-to-cholesterol ratio in erythrocytes, μ g/ μ g			
n	7	8	
Phosphatidylcholine	2.18 \pm 0.08	2.09 \pm 0.14	NS
Sphingomyelin	0.62 \pm 0.04	0.70 \pm 0.11	NS
Phosphatidylserine	0.40 \pm 0.04	0.44 \pm 0.03	NS
Phosphatidylethanolamine	0.46 \pm 0.04	0.46 \pm 0.03	NS

Cholesterol and phospholipid species were assayed as described under Methods. Data are means \pm SEM.

increase in total plasma concentration of γ -tocopherol to plasma total lipid ratios as compared with WT controls (Table 1). The absolute γ -tocopherol content of PLTP^{-/-} HDL was significantly decreased, whereas absolute γ -tocopherol contents of PLTP^{-/-} VLDL and IDL+LDL were significantly increased (Table 1), with significant rises in γ -tocopherol-to-total lipid ratio in VLDL and IDL+LDL.

Interestingly, not only the tocopherol contents of lipoproteins but also the tocopherol contents of circulating erythrocytes were modified in PLTP^{-/-} mice. As for IDL+LDL, absolute tocopherol contents were higher in PLTP^{-/-} erythrocytes than in WT erythrocytes, and concordant conclusions were drawn when data were expressed as tocopherol to hemoglobin ratio (Table 1). Observed changes in tocopherol distribution occurred in the absence of alteration in the overall phospholipid content of erythrocytes. Whereas in

accordance with earlier data,¹⁹ the SM content of apoB-containing lipoproteins was significantly lower in PLTP^{-/-} mice than in WT mice ($P < 0.05$; Table 2), phospholipid compositions of erythrocyte membranes from PLTP^{-/-} and WT mice were identical (Table 2).

In Vivo Vitamin E Accumulation Modifies Phosphatidylserine Externalization in Circulating Erythrocytes of PLTP^{-/-} Homozygotes

Erythrocytes from PLTP^{-/-} mice displayed a decreased procoagulant activity, as indicated by a significant 20% ($P < 0.05$) increase in the RVV clotting time induced by PLTP^{-/-} isolated erythrocytes as compared with RVV clotting time induced by WT isolated erythrocytes (Figure 2A), indicating a decrease in PS exposure at the surface of erythrocytes from PLTP-deficient homozygotes. Flow cytom-

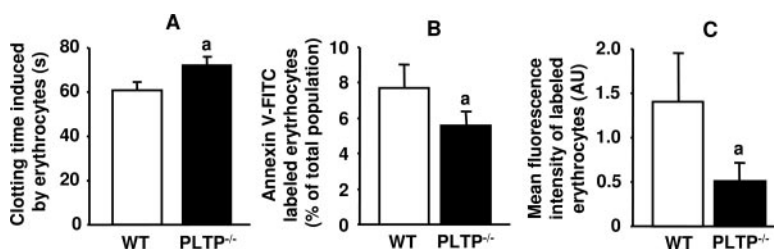


Figure 2. Variation of procoagulant properties and PS externalization in erythrocytes from PLTP^{-/-} and WT mice. A, Clotting time was measured as described in Methods. Data are means \pm SEM of n=15 mice in each group. B and C, Annexin V-FITC labeling assayed as described in Methods. Data are means \pm SEM of n=18 distinct mice. ^a $P < 0.05$.

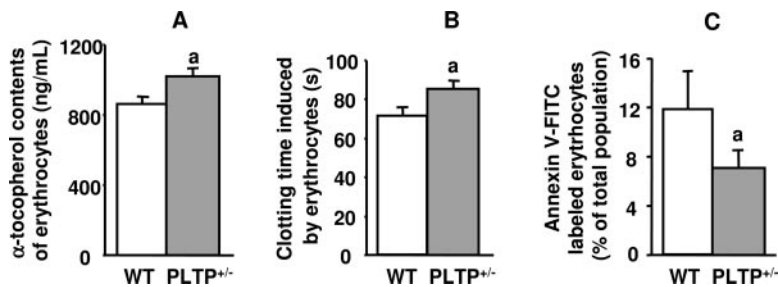


Figure 3. α -Tocopherol content, procoagulant properties, and PS externalization in erythrocytes from PLTP^{+/-} mouse heterozygotes. α -Tocopherol (A), procoagulant properties (B), and Annexin V-FITC labeling (C) were assayed on isolated erythrocytes from WT or PLTP^{+/-} mice as described in Methods. Data are means \pm SEM from n=11 mice for WT and n=14 mice for PLTP^{+/-} mice. ^aP<0.05.

etry analysis confirmed significant abnormalities in PS distribution between the inner and the outer leaflet of PLTP^{-/-} erythrocyte membranes. Indeed, fewer erythrocytes from PLTP^{-/-} mice were labeled with Annexin V-FITC as compared with WT counterparts ($P<0.05$; Figure 2B), and mean specific fluorescence intensity in labeled erythrocytes was significantly lower in PLTP^{-/-} erythrocytes than in WT erythrocytes ($P<0.05$; Figure 2C). In all cases, hemolysis remained constantly below 2% (data not shown).

Evidence for a Vitamin E/Phosphatidylserine Phenotype in PLTP^{+/-} Heterozygotes

PLTP^{+/-} heterozygotes with only a 35% decrease in plasma PLTP activity were compared with WT controls (plasma PLTP activity: 21.7 ± 0.6 AU/sec in PLTP^{+/-} mice [n=12] versus 33.3 ± 1.6 AU/sec in WT controls [n=7], $P<0.0005$). As shown in Figure 3A, PLTP^{+/-} mice showed significant, $\approx 20\%$ increases in α -tocopherol content of erythrocytes as compared with WT mice ($P<0.05$ in both cases). As observed above in PLTP^{-/-} homozygotes (Table 1 and Figure 2A), the accumulation of α -tocopherol in erythrocytes from PLTP^{+/-} mice led to a significant increase in RVV clotting time of poor-platelet plasma ($P<0.05$; Figure 3B). Similar to what has been observed in PLTP^{-/-} homozygotes (Figure 2), there was a trend, however

nonsignificant in this case, toward a decrease in mean specific fluorescence intensity of labeled PLTP^{+/-} erythrocytes (0.7 ± 0.3 AU) as compared with WT controls (1.4 ± 0.5 AU). However, fewer PLTP^{+/-} heterozygous erythrocytes were labeled with Annexin V-FITC ($P<0.05$; Figure 3C).

In Vivo Markers of Blood Coagulation Are Decreased in PLTP^{-/-} Mice

Clotting time measured in whole blood samples was significantly higher in PLTP^{-/-} mice (73.3 ± 3.2 seconds) than in WT mice (59.0 ± 6.1 seconds, $P<0.05$). The level of D-dimer, which is known to reflect increased thrombus formation in vivo,²⁰ was significantly lower in plasma of PLTP^{-/-} mice (16 ± 2 ng/mL plasma) than in plasma of WT mice (32 ± 3 ng/mL plasma, $P<0.005$).

Vitamin E-Related Effects on Blood Coagulation in PLTP^{-/-} Mice Are Not Associated With Changes in Oxidation Status

Although apoB-containing lipoproteins from PLTP^{-/-} mice displayed a higher resistance to copper-mediated oxidation in vitro, the level of LPC (a product of phosphatidylcholine oxidation) and the oxysterol to cholesterol ratios in homologous lipoproteins (VLDL, IDL+LDL or HDL) from PLTP^{-/-}

TABLE 3. Oxidation Markers in Lipoproteins and Erythrocytes From PLTP^{-/-} and WT

	WT	PLTP ^{-/-}	P
Lipoproteins			
n	8	10	
Lag time of diene formation in apoB-containing lipoproteins, min	33 ± 5	91 ± 13	<0.005
Oxysterol-to-cholesterol ratio, ng/ μ g			
VLDL	4.1 ± 0.72	3.1 ± 0.7	NS
IDL+LDL	1.1 ± 0.2	0.8 ± 0.1	NS
HDL	0.8 ± 0.3	0.9 ± 0.3	NS
LPC-to-total lipids ratio, μ g/mg			
VLDL	14.9 ± 2.8	15.7 ± 3.6	NS
IDL+LDL	29.4 ± 3.6	26.0 ± 2.2	NS
HDL	49.8 ± 6.0	43.1 ± 2.6	NS
Erythrocytes			
n	6	6	
Initial rate of DCF formation in erythrocytes, nmol/L per sec	0.82 ± 0.11	0.85 ± 0.08	NS
n	7	8	
Oxysterol-to-cholesterol ratio, ng/ μ g	6.64 ± 1.45	6.87 ± 1.35	NS
LPC-to-cholesterol ratio, μ g/ μ g	0.11 ± 0.01	0.10 ± 0.01	NS

DCF formation, oxysterol-to-cholesterol ratio, LPC and diene formation were measured as described under Methods. Data are means \pm SEM.

and WT mice were identical (Table 3). In addition, the initial rate of DCFH-DA oxidation as well as the LPC content and the oxysterol-to-cholesterol ratio in circulating erythrocytes was not modified by PLTP deficiency (Table 3).

Discussion

The present study shows a key role of cell-incorporated vitamin E in modifying PS externalization and blood coagulation *in vivo*. Firstly, PS externalization in erythrocytes of WT mice is significantly reduced in cells incubated *in vitro* with vitamin E-enriched lipoproteins as compared with cells incubated alone. Secondly, complete as well as partial PLTP deficiencies in mouse are proven for the first time to constitute relevant, yet unrecognized means to produce significant alteration in the vitamin E content of circulating erythrocytes. Thirdly, a direct relationship between vitamin E content and PS exposure in erythrocytes could be brought in PLTP-deficient mice, independently of changes in the overall phospholipid content of the cells. Fourthly, the high-vitamin E/low-PS exposure phenotype of circulating PLTP^{-/-} erythrocytes decreased whole blood coagulation. Fifthly, levels of D-dimer (ie, a circulating marker of thrombus formation²⁰) were lower in PLTP^{-/-} mice than in WT mice. Finally, the present study brings a new argument in favor of a potent, non-antioxidative function of vitamin E because changes in PS externalization and coagulant properties in erythrocytes were observed despite no change in their oxidized lipid contents.

Complete PLTP deficiency was previously shown to be associated with the accumulation of α -tocopherol in apoB-containing lipoproteins (mainly LDL) as compared with WT mice.¹² Here we show for the first time that PLTP determines not only the plasma distribution of α -tocopherol but also of γ -tocopherol. In absolute terms, γ -tocopherol is much less abundant than α -tocopherol in circulating lipoproteins, and observed differences in γ -tocopherol distribution in PLTP^{-/-} mice as compared with WT mice were of much lower magnitude than differences in α -tocopherol, suggesting that α -tocopherol transfer may actually be of higher biological relevance. In addition, it is shown in the present study that PLTP is also a key factor in determining the net α - and γ -tocopherol content of erythrocytes, an observation that extends the previously recognized ability of PLTP to promote *in vitro* the rapid exchange of radiolabeled α -tocopherol between lipoproteins and blood cells.²¹ Whereas a number of tissues, including liver and vascular wall, were shown to contain fewer vitamin E molecules in the PLTP-deficient state,^{12,13} the present study indicates that circulating erythrocytes behave rather as circulating apoB-containing lipoproteins, cumulating vitamin E in the PLTP-deficient state. Interestingly, vitamin E enrichment of PLTP^{-/-} erythrocytes occurred in the absence of significant alterations in the overall cellular content in phospholipids which are other amphipathic compounds known to be transferred by PLTP.¹¹ In other words, PLTP deficiency appeared as a relevant and convenient mean to modify selectively the vitamin E content of circulating erythrocytes.

A major point of the present work was the significant decrease in phosphatidylserine externalization in plasma membrane of PLTP^{-/-} erythrocytes. This was found to occur in parallel with vitamin E enrichment, and a significant reduction in PS externalization was obtained *in vitro* through transfer of vitamin E to isolated erythrocytes. Most interestingly, the vitamin E-mediated changes in PS externalization occurred independently of its antioxidant properties, with no alteration in the production of oxidized lipids. Although observed loss of phospholipid asymmetry in erythrocytes has often been related to oxidative events,^{8,10} controlled *in vitro* oxidation of erythrocytes failed to reproduce PS externalization in isolated erythrocytes,²² suggesting indeed that the loss of phospholipid asymmetry, as observed for instance in thalassemia and sickle cell anemia,⁵ is not simply attributable to increased levels of oxidative stress. Moreover, some of the effects of vitamin E, such as inhibition of smooth muscle cell proliferation through modulation of protein kinase C activity,²³ were proven to be mediated independently of its antioxidant properties. Recently, vitamin E was shown to act as a membrane-stabilizing agent in erythrocytes, independently of its antioxidant properties.²⁴ Thus, it is tempting to speculate that the maintenance of membrane phospholipid asymmetry observed in the present study is another reflect of the membrane stabilizing properties of vitamin E.

It must be emphasized that the pathophysiological relevance of the low-vitamin E/high-PS externalization duality is sustained by the greater exposure of PS on the surface of thalassemic or sickled red blood cells⁵ that are known to be vitamin E deficient.^{25,26} In diabetes, vitamin E-deficient blood cells were also proven to display increased passive transmembrane lipid movement with the appearance of PS in the outer leaflet of plasma membrane.²⁷ In these pathologies, decreased vitamin E content of erythrocytes and increased PS externalization at their surface is known to be associated with an abnormal hypercoagulable state.⁷⁻¹⁰ Interestingly, and beyond its procoagulant property, it has been clearly established that expression of PS at the surface of erythrocytes leads to their faster removal by splenic macrophages through specific cell-ligand interactions and, finally, to anemia as it is known to be the case in sickle cell disease, thalassemia, and chronic renal failure.⁵ It is known to occur even with small amounts of cell-associated PS.²⁸ In further support of fewer PS molecules exposed in PLTP^{-/-} erythrocytes, PLTP^{-/-} mice in the present study displayed significantly greater hematocrit as compared with controls (+13%, $P < 0.05$; data not shown). The latter point comes in further support of the *in vivo* relevance of PLTP in increasing externalized PS molecules.

Erythrocytes contribute significantly to the control of hemostasis and thrombosis.⁶ High procoagulant activity associated with loss of lipid asymmetry has been reported in a number of membrane bilayers, including glucose-treated erythrocytes,¹⁰ sickled cells,²⁹ apoptotic cells,³⁰ and shed membrane microparticles as those derived from the atherosclerotic plaque.⁴ Interestingly, PS exposure under

pathological conditions occurs frequently in erythrocytes, and it has often been referred to as an "apoptotic-like" even.⁵ Thus, and as proposed for circulating shed membrane particles, it is tempting to speculate that vitamin E-poor membranes in the blood compartment, as those provided by erythrocytes in the presence of elevated PLTP activity, might constitute a prothrombotic surface in vivo. In support of the biological relevance of the hypocoagulability observed in the PLTP-deficient state in our study, the circulating levels of D-dimer were decreased in PLTP^{-/-} mice compared with controls. D-Dimer, a product of the breakdown of crosslinked fibrin accumulates under conditions of increased thrombus formation,²⁰ and its reduction in PLTP^{-/-} mice compared with WT mice indicates an hypocoagulable state in vivo.

Determination of the vitamin E content of erythrocytes might be actually a major function of PLTP. Indeed, and unlike plasma HDL-cholesterol level, which was found to be dramatically decreased only in PLTP-deficient homozygotes, and not in PLTP-deficient heterozygotes,¹⁴ vitamin E content and procoagulant properties of circulating erythrocytes were abnormal not only in animals with complete PLTP deficiency but also in animals with partial PLTP deficiency. It is worthy to note that the accumulation of vitamin E in apoB-containing lipoproteins caused by PLTP deficiency was shown to be even greater in hyperlipidemic mice. For instance, PLTP deficiency produced nearly 2-fold increases in the α -tocopherol-to-lipid ratio in LDL from LDL-receptor knocked-out mice or apoB/CETP transgenic mice, with even a greater 4-fold rise in the α -tocopherol-to-lipid ratio in VLDL from apoE-knocked out mice.¹² Thus, a more pronounced effect of PLTP deficiency on vitamin E content of erythrocyte and blood coagulation may be expected in a hyperlipidemic context. Coagulation is a key event in the acute phase of atherothrombosis, and the effect of PLTP-mediated erythrocyte vitamin E enrichment on coagulation might thus contribute to the positive and independent relationship that has been reported between plasma PLTP activity and the incidence of coronary artery disease in humans.³¹ Interestingly, reported increases in plasma PLTP mass and activity levels in type 2 diabetic patients and coronary patients vary from +14% up to +45% of control values.^{31,32} Whether vitamin E transfer activity of PLTP might contribute significantly to the increased atherothrombotic risk in those patients will deserve further attention.

Acknowledgments

We thank Françoise Vaizan for excellent technical assistance.

Sources of Funding

This work was supported by the Université de Bourgogne, the university hospital CHU-Dijon, the Conseil Régional de Bourgogne, INSERM (Institut National de la Santé Et de la Recherche Médicale), and the Fondation de France.

Disclosures

None.

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