Alterations of lecithin cholesterol acyltransferase activity and apolipoprotein A-I functionality in human sickle blood

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Abstract

In sickle cell disease (SCD) cholesterol metabolism appears dysfunctional as evidenced by abnormal plasma cholesterol content in a subpopulation of SCD patients. Specific activity of the high density lipoprotein (HDL)-bound lecithin cholesterol acyltransferase (LCAT) enzyme, which catalyzes esterification of cholesterol, and generates lysoPC (LPC) was significantly lower in sickle plasma compared to normal. Inhibitory amounts of LPC were present in sickle plasma, and the red blood cell (RBC) lysophosphatidylcholine acyltransferase (LPCAT), essential for the removal of LPC, displayed a broad range of activity. The functionality of sickle HDL appeared to be altered as evidenced by a decreased HDL–Apolipoprotein A-I exchange in sickle plasma as compared to control. Increased levels of oxidized proteins including ApoA-I were detected in sickle plasma. *In vitro* incubation of sickle plasma with washed erythrocytes affected the ApoA-I-exchange supporting the view that the RBC blood compartment can affect cholesterol metabolism in plasma. HDL functionality appeared to decrease during acute vaso-occlusive episodes in sickle patients and was associated with an increase of secretory PLA_2, a marker for increased inflammation. Simvastatin treatment to improve the anti-inflammatory function of HDL did not ameliorate HDL–ApoA-I exchange in sickle patients. Thus, the cumulative effect of an inflammatory and highly oxidative environment in sickle blood contributes to a decrease in cholesterol esterification and HDL function, related to hypocholesterolemia in SCD.

Keywords: Cholesterol esterification, acyltransferase, sickle disease, lipoproteins, oxidation

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Introduction

In sickle cell disease (SCD) patients, cholesterol metabolism appears dysfunctional as evidenced by abnormal plasma cholesterol content, triglycerides, and fatty acids.[1–8] Those defects are also reflected in the concentration of the HDL-bound proteins lecithin:cholesterol acyl-transferase (LCAT) and apolipoprotein A-I.[9–11] The decreased level of ApoA-I seems to be a biomarker for pulmonary hypertension risk in SCD.[12–15] A single nucleotide polymorphism of LCAT ($P < 5.10^{-8}$) linked to a variation in the concentration of serum lipids was reported in a genome-wide association study.[16] In plasma, LCAT is mainly bound to the HDL particle and cleaves the fatty acyl group from the sn-2 position of phosphatidylcholine (PC) and transfers it to cholesterol (C), producing both lysophosphatidylcholine (LPC) and cholesteryl ester (CE).[17–23] The CE moves to the hydrophobic interior of the lipoprotein particle, an essential step in the reverse cholesterol transport pathway. LPC has detergent-like characteristics and inhibits the cholesterol esterification reaction. Both LPC and LPA (lysophosphatidic acid), converted from LPC by plasma lysophospholipase D, are inflammatory and affect physiologic processes and their plasma concentrations need to be controlled.[17–19,24–26] LPC binds to plasma albumin,[18,19,27] and distributes into the red blood cell (RBC), which constitute an efficient recycling sink for LPC. Glycolysis provides energy to the RBC membrane-bound enzymes of the Lands pathway to re-acylate LPC to PC using acyl-CoAs formed from plasma fatty acids.[28,29] The RBC membrane is also a sink for cholesterol and PC available for LCAT.[10,30–33]

Our study indicates dysfunction of the membrane-bound RBC enzymes, the HDL-bound LCAT enzyme, and ApoA-I-HDL function in sickle blood. The activities of the lysoPL-acyltransferase enzymes of the sickle RBC and the sickle plasma LCAT were decreased. ApoA-I is the major protein component of HDL particles, and lipid-free ApoA-I can exchange spontaneously with HDL-bound ApoA-I, permitting *in vitro* quantitation of HDL–ApoA-I exchange as a measure of HDL particle functionality in human plasma.[34–37] HDL–ApoA-I exchange was reduced in sickle plasma, indicating dysfunctionality of the sickle HDL and this decrease was more pronounced during vaso-occlusive episodes (VOE) of sickle patients even after they received

RBC concentrate transfusion. Our data indicate that the highly oxidative environment of sickle blood induced sulfhydryl alterations of the essential components in both plasma and RBC to maintain CE formation. Altogether, dysfunction of RBC and plasma lipoproteins may lead to the imbalance in cholesterol metabolism observed in SCD patients. In addition, the dysfunction of the HDL–ApoA-I exchange in sickle plasma positively correlated to increased levels of acute-phase secretory phospholipase A_2 ($sPLA_2$) enzyme during acute inflammatory episodes.

Materials and methods

Measurement of plasma biomarkers

Blood was collected under Institutional Review Board approval from Children's Hospital Research Center Oakland. Total cholesterol content in plasma was measured with a colorimetric assay kit (cat. STA-384, Cell Biolabs, Inc.) according to the manufacturer instructions. Total cholesterol content in plasma of samples used for analysis of HDL–ApoA-I exchange was determined with the method of Van Stewart.[38] LCAT activity was determined with a fluorometric assay kit (Cell Biolabs, Inc., San Diego, CA) and LCAT concentration with an ELISA kit (Cell Biolabs, Inc., San Diego, CA). Plasma $sPLA_2$ level was quantified with the $sPLA_2$ (human Type IIA) ELISA Kit (Cayman Chemical, Ann Arbor, MI). Quantification of LPC in plasma was performed with a novel assay using fluorescently labeled C_{16}-CoA (N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)-methyl]amino palmitoyl Coenzyme A), Avanti Polar Lipids, Inc. (Alabaster, AL, USA)] in the presence of recombinant LPCAT1 enzyme.[39] Fluorescent products were extracted and analyzed by thin-layer chromatography as previously described.[39] Carbonyl group detection was performed with the oxidized protein western blot kit (Abcam, Cambridge, MA) according to the manufacturer instructions. Briefly, derivatized samples obtained from 17 sickle and two control plasma samples were separated on denaturing SDS-PAGE and transferred onto PVDF membrane. DNP proteins were detected with a polyclonal rabbit anti-DNP antibody, which was stained with an HRP-conjugated secondary antibody. The signal was quantified with Quantity One software (Bio-Rad). The value obtained with 10 μL of the DNP-protein marker provided in the kit was used to normalize the signals. Membranes were stripped and ApoA-I was detected with an anti-ApoA-I antibody and was used to localize the DNP-ApoA-I signal on the original membrane. For each plasma sample, the signal of DNP-ApoA-I relative to the signal of ApoA-I was calculated.

Measurement of erythrocyte biomarkers

Measurement of the hemoglobin F content was performed by quantitative high performance liquid chromatography. Quantification of reticulocytes was performed with ReticCount reagent (Becton-Dickinson, Franklin Lakes, NJ). HbF containing cells were determined by fluorescence-activated cell sorting (FACS) analysis of cells stained with an APC-conjugated mouse IgG1 monoclonal antibody raised against human fetal hemoglobin (ThermoFisher Scientific, Grand Island, NY) as previously described.[40] Measurement of lysoPL-acyltransferase activity of RBC membranes was performed on ghost membranes isolated from 100 μL of packed frozen RBCs, in the presence of [^{14}C]oleyol-CoA and lysoPC or lysoPE, as previously described.[28] Cholesterol and phospholipid content of the RBC membranes was determined according to the method of Zlatkis *et al.*[41] and Rouser *et al.*,[42] respectively.

Measurement of HDL–ApoA-I exchange

HDL–ApoA-I exchange was measured by the addition of spin-labeled ApoA-I to plasma. The exchange of the spin-labeled ApoA-I with plasma HDL results in an increase in the EPR signal.[35] This increase in signal (unquenching) reports on the functionality of the HDL particle. Routinely, 120 μL of fresh or frozen plasma was depleted of LDL/VLDL by the PEG method[35] (150 μL final volume) and 10–60 μL of PEG-treated plasma were further diluted in PBS containing 0.96% PEG-6000 to 150 μL. For each of the dilutions, 45 μL were mixed with 15 μL spin-labeled recombinant ApoA-I mutant form (3 mg/mL)[35] and samples were incubated for 15 min at 37°C. Analysis was performed with a Bruker eScan EPR spectrometer outfitted with a temperature controller (Noxygen) and with a JEOL-RE series EPR spectrometer at 37°C. The middle peak amplitude value of the nitroxide signal was expressed relative to the peak amplitude value of the cavity manganese reference (JEOL spectrometer) or proprietary instrument internal standard (Bruker spectrometer). Each measurement was triplicated and the mean of the ratios was plotted as a function of the volume of plasma analyzed. The slope values of the linear regression fits were calculated with GraphPad Prism. These values represent the plasma ApoA-I-unquenching capacity parameter (PUC) reported in this study and is related to the HDL–ApoA-I exchange. Percentage HDL–ApoA-I exchange (%HAE) values of the samples presented in Figure 3(c) were measured and calculated as previously described.[35]

Statistical analysis

Unless otherwise indicated, experimental measures were expressed as mean ± SD. Differences between group mean values were assessed for statistical significance by one-way ANOVA or by the unpaired *t*-test as appropriate, and difference of parameter values during acute phases was compared to baseline using paired *t*-tests. Pearson's correlation coefficient was used to assess the statistical significance of associations between parameters of interest. Statistical significances for comparisons are indicated on the figures or in the legend of the figure. Statistical analysis was performed with GraphPad Prism statistical software (version 6; GraphPad, San Diego, CA, USA).

Results

Analysis of the sickle RBC

Significant differences in the percentage of HbF-containing RBCs, and in the total amount of HbF were observed in a cohort of 534 sickle patients (Figure S1(a)).[43] Blood samples

with the highest amount of HbF were low in young erythrocytes (reticulocytes) and those with the highest in reticulocytes were low in HbF (Figure S1(b)). There was no significant difference in the molar cholesterol/phospholipid ratio of the sickle RBC membrane compared to normal (Figure S1(c)), but as observed by others,[44,45] a broader range was found in sickle RBC as compared to the narrower range observed in normal RBC. This result confirmed that the lipid composition of the sickle RBC membrane can be different from a normal RBC. The activity of the membrane-bound acyltransferase enzymes catalyzing the acylation of lysophospholipids (LPL) to phospholipids was also very heterogeneous for both LPE and LPC enzymes in the sickle RBC (Figure S1(d) and (e)). RBCs from blood with high reticulocyte counts displayed increased activity values suggesting that young cells have stronger re-acylation capacity than older RBCs (Figure S1(d)). The activity levels of the two enzymes were positively correlated (Figure S1(e)) indicating that alterations broadly affected membrane-bound enzymes of the sickle RBC.

LCAT is damaged in sickle plasma

Depending on the presence of ApoA-I and of LDL, the acyl-CoA independent acyl-transferase LCAT can generate different acylated products.[22,23,26,46,47] In the presence of ApoA-I, LCAT esterifies cholesterol by transferring the sn2-acyl from PC to form cholesterol ester (CE). However, in the absence of a lipophilic acyl-acceptor, it transfers the acyl group to water and acts like a PC hydrolyzing phospholipase A_2. In the presence of LDL, the enzyme can transfer an acylgroup from PC to LPC or trans-esterify two LPC molecules. In plasma, LCAT is mainly bound to HDL particles and the LCAT levels positively correlate to total cholesterol content in normal blood.[48] To gain insight on the ApoA-I-dependent activity of LCAT,[26] the LCAT activity was measured by the phospholipase activity of the enzyme (see ''Materials and method'' section).

Compared to normal plasma, LCAT activities were lower in sickle plasma (Figure 1(a)). We confirmed that in sickle plasma, LCAT concentrations were also lower than in normal blood[1,3–6,8,9] but the decrease in activity was greater than could be accounted for by the lower concentration in sickle plasma resulting in a significantly lower LCAT specific activity compared to normal (Figure 1(a)). A positive association between the LCAT amount and the plasma total C concentration was observed but there was no association between activity of the enzyme and plasma total C (Figure 1(b)).

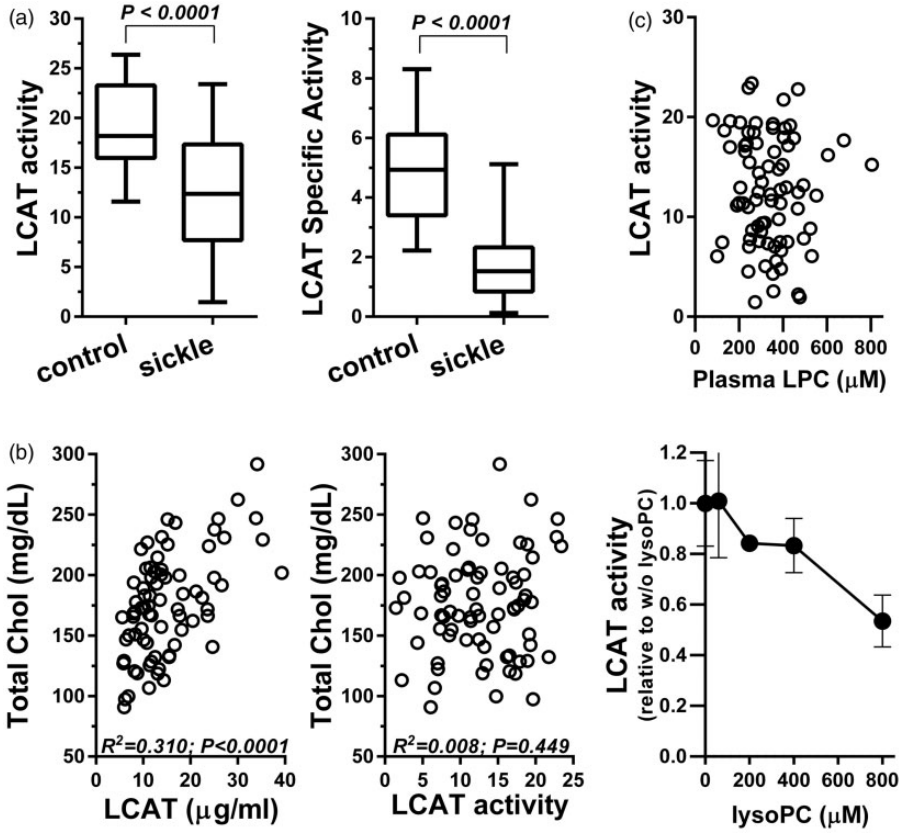

Figure 1 LCAT dysfunction in sickle plasma. (a) Activity and amount of plasma LCAT were determined in 33 controls and in 78 sickle plasma samples shown in Figure S1(d). LCAT specific activity was calculated from the activity and amount measured in each sample. (b) Total plasma cholesterol content was determined and values were plotted as a function of LCAT amount and LCAT activity obtained in those samples. (c) LPC amount in the sickle plasma samples was determined and LCAT activity is plotted as function of the amount of LPC measured in those samples. To determine the dependency of the lipase activity of LCAT for LPC, LCAT activity in a control plasma sample was determined in presence of increasing concentration of LPC. Error bars represent the standard deviations of three measurements. Pearson correlation coefficients and two-tailed P values were calculated with GraphPadPrism software and are indicated on the panels

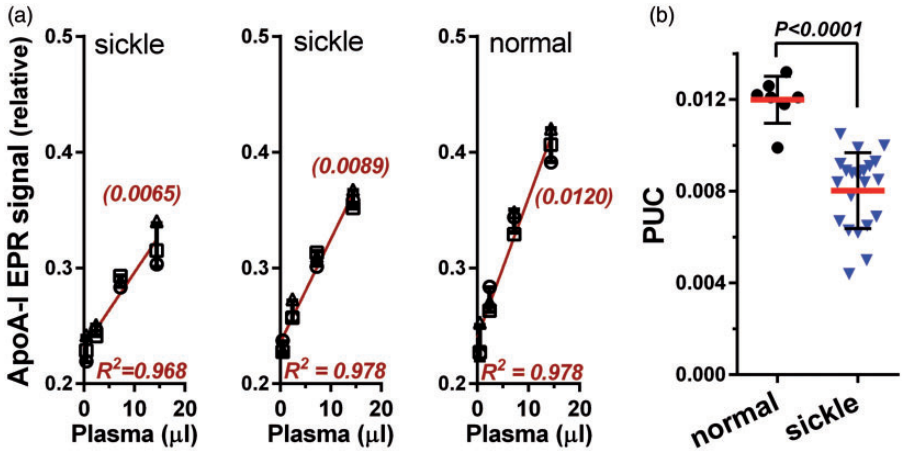

Figure 2 EPR measurement of the HDL–ApoA-I exchange of sickle plasma. The EPR nitroxide signals, relative to an instrument reference peak, obtained by addition of lipid-free ApoA-I-spin labeled probe[35] mixed with an increasing volume of plasma are shown on the y-axis of the three graphs in (a). For each sample, measurements were performed in triplicate with four volumes of plasma from 0.6 to 14.4 μL (shown on the x-axis). The slopes of the linear fit, defined as the plasma unquenching capacity (PUC) of the sample, were calculated with GraphPadPrism software. Results obtained for two sickle plasma samples and one normal sample are presented in (a). Analysis of seven normal and 21 sickle samples are shown in (b). Error bars represent the standard deviations of three measurements. Pearson correlation coefficients and two-tailed P values were calculated with GraphPadPrism software and are indicated on the panels. (A color version of this figure is available in the online journal.)

In the absence of albumin, the esterase activity of LCAT was reported to be strongly inhibited by its by-product LPC.[17,19,20] Sickle plasma contained significant amounts of LPC (340 μM ± 130) (Figure 1(c)) that is inhibitory to the cholesterol esterification activity of LCAT.[19] However, its lipase ability, as measured by the release of the acyl chain from the sn-2 position, is much less sensitive to LPC (Figure 1(c)). Consistent with this finding, the LCAT-lipase activity values did not correlate to the amount LPC present in sickle plasma (Figure 1(c)). Hence, despite that such concentrations of plasma LPC could inhibit the esterification of cholesterol, the measurement of lower LCAT-lipase enzyme activity in sickle plasma compared to normal was not the result of high concentrations of LPC in those samples. LCAT carries two critical cysteine residues sensitive to sulfhydryl alterations.[49–51] Our findings provide evidence that the decrease in LCAT activity was a combination of lower protein concentration and of structural damage affecting the enzyme activity in sickle plasma.

HDL–ApoA-I dysfunction in sickle plasma

Apolipoprotein A-I is the major protein component of the HDL particle. ApoA-I is conformationally dynamic and lipid-free ApoA-I exchanges with ApoA-I bound to HDL particles, a process critical to HDL formation, maturation, and reverse cholesterol transport.[34–37]

Lipid-free ApoA-I undergoes significant conformational changes when exchanging onto HDL particles. The addition of a nitroxide spin label permits this change to be monitored by EPR.[34,35] When plasma is mixed with a lipid-free ApoA-I-spin labeled probe, the exchange with HDL will unquench the EPR signal of the spin probe.[34,35] In this study, we measured HDL–ApoA-I exchange at several plasma concentrations, by measuring the EPR signal obtained in the presence of increasing volumes of plasma (Figure 2). The slope of the linear regression fit of the EPR signal versus plasma volume defined the ApoA-I-PUC. Using 0.6–14.4 μL of plasma, measurements were highly reproducible for both normal and sickle plasma (Figure 2(a)). Compared to normal, PUC values for sickle plasma were significantly different and lower (Figure 2(b)). A strong positive correlation ($R^2 = 0.743$, $P < 0.0001$) was also observed between the ApoA-I concentration and the HDL–ApoA-I exchange rate parameter (%HAE,[34,35]) in sickle plasma (Figure 3(a)). As measured by the amount of carbonyl groups, an increased level of oxidized proteins (including ApoA-I) was detected in sickle plasma (Figure 3(b)). A negative correlation was detected between the PUC of plasma and the total amount of carbonyl groups in the samples ($R^2 = 0.257$) (data not shown). A more significant association ($R^2 = 0.69$) was obtained between the amount of oxidized ApoA-I and PUC in two-thirds of the samples (Figure 3(c), group b). This agrees with previous findings that oxidized HDL-bound ApoA-I exchanges more slowly.[35,36] These observations indicated that lower ApoA-I levels and sulfhydryl alterations affecting ApoA-I function resulted in the observed changes in most samples, but that additional plasma factors present at the time of blood collection may also alter the exchange rate of ApoA-I.

Acute VOEs exacerbate ApoA-I-HDL dysfunction

Blood from sickle patients was collected at different times with respect to the onset of an acute vaso-occlusive event (VOE). PUC values at crisis were compared to values obtained from samples collected either before or a month after the event (baseline). As anticipated, PUC values were already low at baseline, but ApoA-I-HDL function further declined during VOE (Figure 4(a)). In most patients, PUC values rapidly decreased at onset of VOE (Figure 4(b) and (c)). Following the initial decline, PUC either started to increase or further decrease and in most patients PUC values were still lower at day 3 compared to baseline.

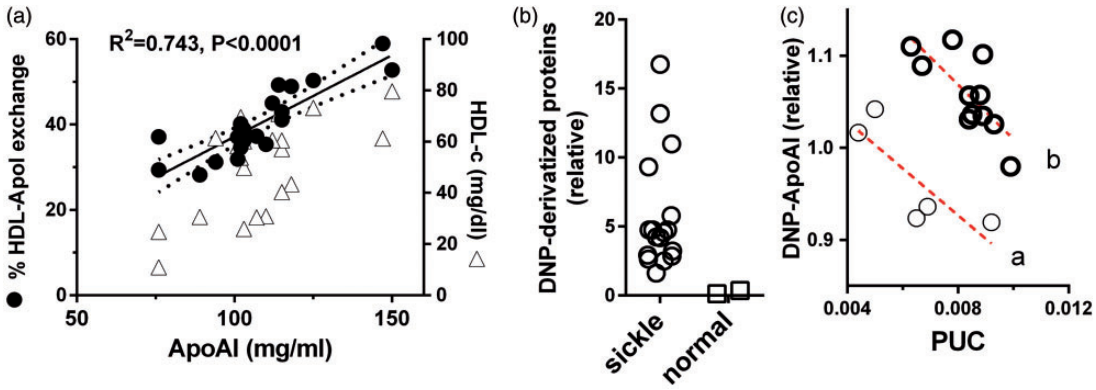

Figure 3 Protein oxidation levels in sickle plasma. ApoA-I level, HDL-cholesterol and their HDL–ApoA-I exchange activity, as defined as % HDL–ApoA-I exchange,[35] were determined in 22 sickle plasma samples (a). The protein oxidation level in plasma was assayed by measuring the amount of carbonyl groups detected by derivatization of plasma protein side chains with the reagent 2,4-dinitrophenylhydrazone (DNPH) as described in the ''Materials and method'' section. Derivatized samples obtained from 17 sickle and two control plasma samples were analyzed. The DNP signal was quantified and was expressed relative to the total amount of protein (b). Membranes were stripped and the DNP-ApoA-I signal relative to the signal of ApoA-I, detected with an anti-ApoA-I antibody, was plotted as a function of the unquenching capacity (c). Pearson correlation coefficients and two-tailed P values were calculated with GraphPadPrism software. By visual inspection, two sub-populations were arbitrarily defined (a and b) with a R^2 value of 0.552 ($P < 0.01$) and 0.690 ($P < 0.1$), respectively. (A color version of this figure is available in the online journal.)

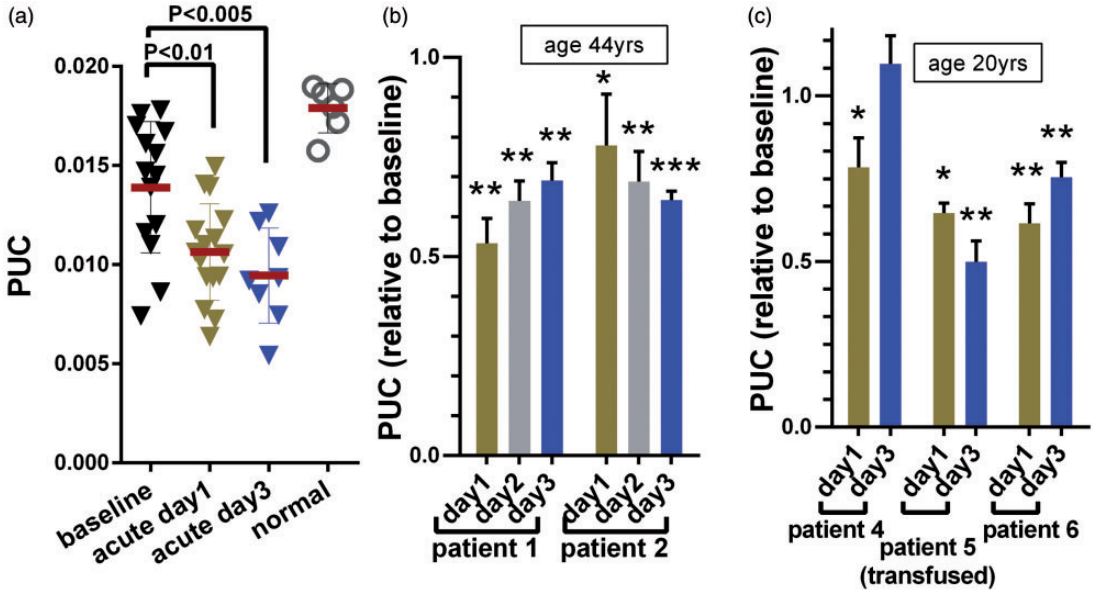

Figure 4 HDL functionality decreases during acute vaso-occlusive crisis. Measurements were performed as described in Figure 2 from plasma collected at baseline and at day 1, 2, and 3 during acute VOE. Analysis of the cohort is presented in (a) and results obtained for two patients aged 44 years (b) and three patients of an average age of 20 years (c) are also shown. The patient receiving red cells concentrate transfusion on day 1 is indicated on (c). Note that these measurements were performed with a JEOL EPR spectrometer, with a different internal manganese standard as the measurements presented in Figure 2 resulting in a different relative signal. Values obtained with normal plasma were included for reference in (a). Error bars represent the standard deviations of three measurements. Two-tailed P values were calculated with GraphPadPrism software (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$). (A color version of this figure is available in the online journal.)

A similar pattern was observed independently of the age of the patients. In one patient receiving transfusion of red cell concentrate, HDL function wasn't improved and the PUC value further decreased during the episode (Figure 4(c)). Consistent with inflammation during VOE, plasma $sPLA_2$ levels and WBC counts increased at day 1 and day 3 compared to baseline (Figure 5 and data not shown). A significant negative association ($R^2 = 0.766$, $P < 0.01$) of the increase in concentration of $sPLA_2$ and the decrease of PUC was determined in sickle plasma (Figure 5(c)). A weaker correlation was observed between WBC counts and PUC. Thus, in addition to oxidative damage affecting ApoA-I, inflammatory episodes in sickle patients appeared to negatively affect HDL function.

Simvastatin treatment does not ameliorate HDL–ApoA-I exchange

HDL particles can become pro-inflammatory during an acute phase or chronic systemic inflammatory condition.

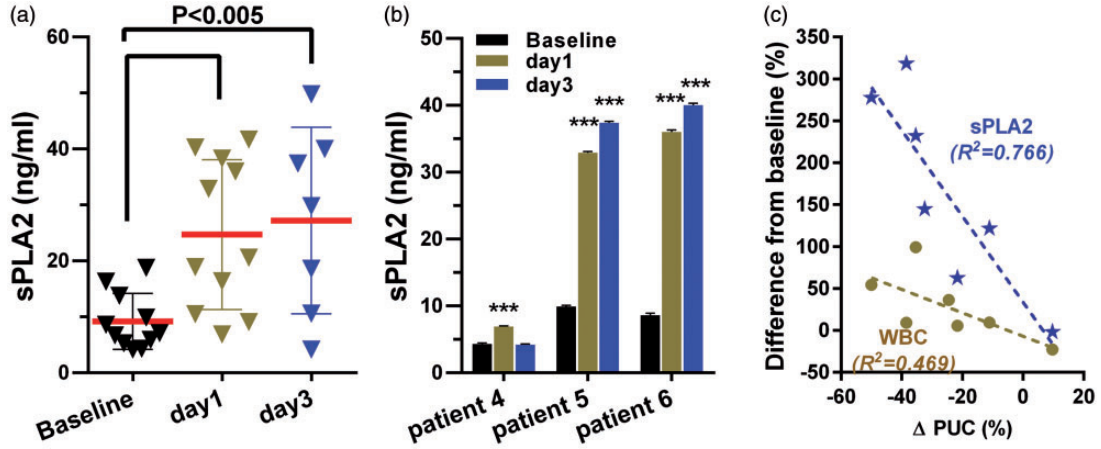

Figure 5 sPLA2 level increases during acute vaso-occlusion crisis. The concentration of $sPLA_2$ (in ng/mL) was determined by ELISA (see ''Materials and method'' section) in the plasma samples shown in Figure 4(a). Results obtained for the three patients shown in Figure 4(c) are presented in (b). The difference in $sPLA_2$ concentration (blue star) and in WBC counts (gold circle) at day 1 and day 3 into crisis relative to baseline were plotted as a function of the difference in PUC in crisis relative to baseline (c). Values are presented as % difference in (c). Error bars represent the standard deviations of three measurements. Pearson correlation coefficients and two-tailed P values were calculated with GraphPadPrism software. In (b), P values were < 0.0001 when indicated (***), and in (c), $P < 0.01$ for $sPLA_2$ and $P < 0.1$ for WBC

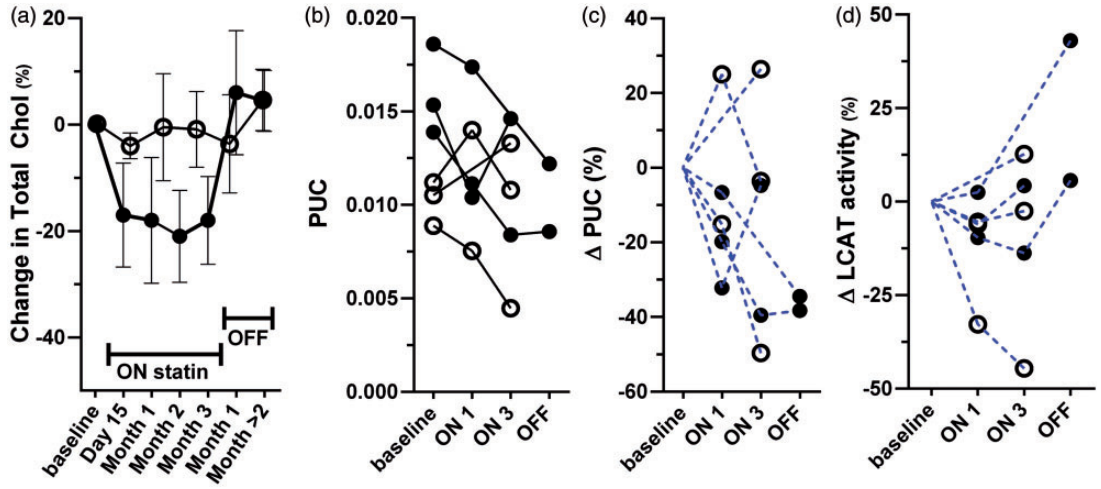

Figure 6 HDL function is not ameliorated by simvastatin treatment. Plasma total cholesterol content (a), PUC ((b) and (c)), and LCAT activity (d) were determined in samples collected from a cohort of patients taking orally a daily dose of simvastatin for a three-month period. Changes in plasma cholesterol content is presented relative to the value measured at baseline in (a). Difference in the values of PUC and in LCAT activity obtained during treatment relative to baseline is presented as % in (c) and (d). Baseline, simvastatin intake (ON), and drug withdrawal (OFF) periods are indicated. Data from three patients that show a decrease in cholesterol content during treatment are shown with filled circle and from three patients with steady cholesterol content are indicated in open circle. (A color version of this figure is available in the online journal.)

Inflammation causes significant changes in the protein and lipid composition of HDL, which can be remodeled from an anti-inflammatory to a pro-inflammatory particle.[52,53] In addition to their cholesterol lowering effect via inhibition of the HMG-CoA reductase enzyme, statin compounds have been reported to improve endothelial function and have been considered as therapeutic agents to reduce vascular dysfunction in SCD.[54–56] Statin drugs can also affect HDL remodeling and improve the anti-inflammatory potential of the HDL.[57,58] PUC values in blood collected from sickle patients taking a daily dose of simvastatin for up to three months were analyzed. In a subset of the patients, the plasma cholesterol level dropped within the first two weeks of drug intake and stayed low during the three-month treatment period (Figure 6(a)). Baseline levels were restored a month after withdrawal of simvastatin. However, in some patients, levels were not significantly altered and three samples in each of the two subpopulations were further analyzed. At baseline, the group that did not respond to simvastatin had a lower PUC as compared to those who responded (Figure 6(b)). A wide variability in the patients' response was detected and overall, drug intake did not appear to have improved HDL–ApoA-I exchange. With one exception, even after the three-month treatment, the values remained at baseline or were significantly lower (Figure 6(b) and (c)). Although cholesterol levels were

rapidly restored to baseline one month after drug withdrawal, PUC values stayed low. Similarly, simvastatin had various effects on the activity of LCAT and did not correlate with the cholesterol response in the patients (Figure 6(d)).

In summary, simvastatin treatment did not improve HDL–ApoA-I exchange rates, and consistent with our previous observations (see above), other plasma factors appeared to have lowered the unquenching capacity of sickle plasma during the drug intake period.

The RBC compartment can affect HDL functionality in plasma

HDL remodeling can be affected by the enzymes of the RBC membranes. As presented in Figure 4(c), the PUC value did not improve and further decreased in the patient receiving RBC concentrate during acute VOE. To investigate the potential role of the RBC compartment on the PUC value, sickle plasma samples were incubated *in vitro* with washed normal RBC at 37°C for up to 2 h. After removal of the RBCs, the transfer rates of plasma samples were determined (Figure 7(a)). Incubation with normal RBCs resulted in an increase of PUC of the treated sickle plasma. Incubation of three different sickle plasma samples (A, B, and C) with the RBC of the sickle sample A (RBC A) resulted in different outcomes (Figure 7(b)). Incubation with one plasma sample had no effect (plasma C), but RBC of sample A incubated with plasma B and with the matched plasma (A) significantly further decreased the transfer rate (Figure 7(b)). The variety of the responses following incubation with RBCs strongly suggests that the erythrocyte blood compartment is one of several factors affecting HDL functionality in sickle blood.

Discussion

In SCD, plasma cholesterol levels can be lower than in normal blood. Levels of the apolipoprotein A-I are also decreased and could be predictive of pulmonary arterial hypertension (PAH) risk and of painful events.[12–15] We show that in sickle plasma, the ability of the HDL particle to exchange with exogenously added lipid-free ApoA-I was reduced compared to normal blood and rapidly declined during VOE. We confirmed that levels of the acute phase enzyme $sPLA_2$ were elevated rapidly during VOE. A negative correlation of the increase of $sPLA_2$ with a decrease in HDL–ApoA-I exchange rate was established. $sPLA_2$ mobilizes cholesterol from the HDL and over-expression of $sPLA_2$ leads to a decrease in plasma total cholesterol, increased catabolism of CEs, and a decrease of HDL size.[59,60] The acute phase response can also affect the composition of the HDL particles and results in a significant remodeling of anti-inflammatory HDL to smaller pro-inflammatory entities.[52,53] In particular, both ApoA-I and LCAT are being replaced by other plasma proteins such as serum amyloid A (SAA) and $sPLA_2$.[57,61] In plasma, HDL is the major substrate for $sPLA_2$, which generates bioactive LPLs implicated in acute and chronic inflammation.[59,60] In SCD, pro-inflammatory HDL is associated with tricuspid regurgitant jet velocity and the increase of $sPLA_2$ is predictive of ACS.[62–64]

The concentration and the activity of the plasma cholesterol esterifying enzyme, LCAT, were also lower in sickle plasma. As established by a net decrease in the specific activity of the enzyme, the decreased activity of LCAT was not solely the consequence of the lower amount of protein and of the presence of an inhibitory level of LPC, but indicated damages affecting LCAT function. Expression of

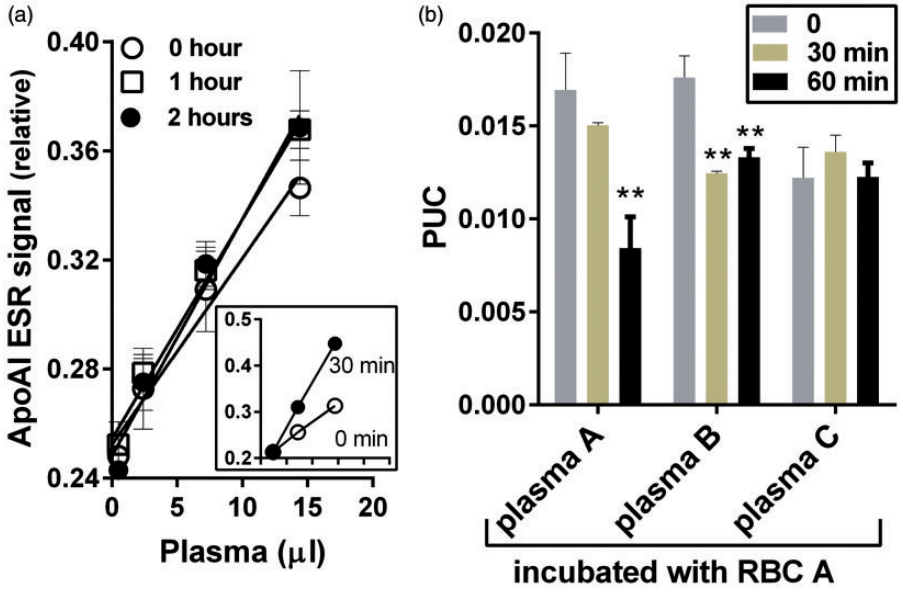

Figure 7 Plasma incubation with RBCs affects HDL function. Sickle plasma was incubated at 37°C with freshly collected RBCs from 0 to 2 h, as indicated. Samples were collected at the different time points and RBCs were removed by low-speed centrifugation. Plasma was saved and EPR signal of the ApoA-I probe was measured as described in Figure 4. Inset in (a) shows the result obtained for a different plasma/RBC set. Results obtained following incubation of three different plasma samples (A, B, and C) with the same RBC sample (matching plasma sample A) for 30 and 60 min are presented in (b). Error bars represent the standard deviations of three measurements. Two-tailed P values were calculated with GraphPadPrism software (**, $P < 0.005$ in (b))

the PC transfer protein is lower in sickle blood than in normal which could decrease the availability of PC to LCAT.[65] High level of SAA inhibits activity of LCAT, which would further decrease cholesterol esterification.[66] The re-acylating enzymes of the RBC Lands pathway were also damaged. A defect in the sickle RBC re-acylation capability could account for the accumulation of LPC in plasma. The level of LPLs would further increase with elevated $sPLA_2$ and exacerbate inflammation during VOE. Given the sensitivity to oxidative damage of the RBC re-acylating enzyme and of LCAT,[49–51] detection of increased levels of oxidized proteins in sickle plasma provides strong evidence that the highly oxidative environment of sickle blood negatively affects cholesterol esterification in sickle plasma. We determined that the oxidization level of ApoA-I was negatively correlated to the ApoA-I unquenching capacity of sickle plasma. This finding is consistent with the slower HDL–ApoA-I exchange rate observed when either lipid-free ApoA-I or HDL–bound ApoA-I was oxidized before measurement.[35,36]

SCD can be considered as a chronic vasculopathy characterized by endothelial injury. Independent of their lipid lowering effects, the pleiotropic effects of statins on vasculopathies relate to activation of NOS activity, and attenuate the oxidative, inflammatory, and thrombotic pathways that lead to endothelial injury.[67–70] In that context, the lack of improvement of HDL–ApoA-I exchange in sickle patients under simvastatin therapy, which can ameliorate the relative proportion of anti-inflammatory to pro-inflammatory HDL,[58] supports the view that the oxidative environment of sickle plasma is a major contributor to HDL dysfunction in SCD. Another statin drug, atorvastatin, also had a limited effect in sickle patients.[55]

The lower ApoA-I level would further hinder the beneficial effect of statin on HDL function in SCD. In support of our findings suggesting remodeling of anti- to pro-inflammatory HDL in sickle plasma, the decreased ApoA-I and the increased SAA levels in plasma have been defined as novel biomarkers of acute painful episodes in SCD.[13] HDLs from sickle plasma were more sensitive to Cu^{2+}-induced oxidation than HDL from normal.[71] An altered apolipoprotein profile was identified by mass spectrometry analysis in sickle patients with pulmonary hypertension.[14,15] Sickle patients had lower ApoA-I and higher SAA levels than normal, but the PAH patients had lower and higher levels, respectively, than the sickle population as a group. Those findings provide more evidence of HDL dysfunction in the vasculopathy of SCD.

Overall, we propose that the combination of increased $sPLA_2$ level during acute phase, dysfunction of the cholesterol-esterification system and of ApoA-I remodeling of the HDL particle, contributes to the plasma cholesterol abnormality detected in sickle blood. Complications related to these defects might participate in the development of vascular endothelial dysfunction in SCD.

Authors' contributions: All authors participated in the interpretation of the studies and analysis of the data and review of the manuscript; ES designed the study; ES, MSB, MB, and SKL conducted the experiments; ES, SKL, and FAK wrote the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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