Depletion of HDL_3 high density lipoprotein and altered functionality of HDL_2 in blood from sickle cell patients

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Impact statement

Our study adds to the growing evidence that the dysfunctional red blood cell (RBC) in sickle cell disease (SCD) affects the plasma environment, which contributes significantly in the vasculopathy that defines the disease. Remodeling of anti-inflammatory high density lipoprotein (HDL) to pro-inflammatory entities can occur during the acute phase response. SCD plasma is depleted of the pre-β particle (HDL_3), which is essential for stimulation of reverse cholesterol from macrophages, and the function of the larger HDL_2 particle is altered. These dysfunctions are exacerbated during vaso-occlusive episodes. Interaction of lipoproteins with endothelium increases formation of inflammatory mediators, a process counteracted by the heme-scavenger hemopexin. This links hemolysis to lipoprotein-mediated inflammation in SCD, and hemopexin treatment could be considered. The use of RBC concentrates in transfusion therapy of SCD patients underestimates the importance of the dysfunctional plasma compartment, and transfusion of whole blood or plasma may be warranted.

Abstract

In sickle cell disease (SCD), alterations of cholesterol metabolism is in part related to abnormal levels and activity of plasma proteins such as lecithin cholesterol acyltransferase (LCAT), and apolipoprotein A-I (ApoA-I). In addition, the size distribution of ApoA-I high density lipoproteins (HDL) differs from normal blood. The ratio of the amount of HDL_2 particle relative to the smaller higher density pre-β HDL (HDL_3) particle was shifted toward HDL_2. This lipoprotein imbalance is exacerbated during acute vaso-occlusive episodes (VOE) as the relative levels of HDL_3 decrease. HDL_3 deficiency in SCD plasma was found to relate to a slower ApoA-I exchange rate, which suggests an impaired ABCA1-mediated cholesterol efflux in SCD. HDL_2 isolated from SCD plasma displayed an antioxidant capacity normally associated with HDL_3, providing evidence for a change in function of HDL_2 in SCD as compared to HDL_2 in normal plasma. Although SCD plasma is depleted in HDL_3, this altered capacity of HDL_2 could account for the lack of difference in pro-inflammatory HDL levels in SCD as compared to normal. Exposure of human umbilical vein endothelial cells to HDL_2 isolated from SCD plasma resulted in higher mRNA levels of the acute phase protein long pentraxin 3 (PTX3) as compared to incubation with HDL_2 from control plasma. Addition of the heme-scavenger hemopexin protein prevented increased expression of PTX3 in sickle HDL_2-treated cells. These findings suggest that ApoA-I lipoprotein composition and functions are altered in SCD plasma, and that whole blood transfusion may be considered as a blood replacement therapy in SCD.

Keywords: Inflammation, acute phase proteins, sickle disease, lipoproteins, hemopexin

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Introduction

In SCD patients, cholesterol metabolism appears dysfunctional as evidenced by abnormal plasma cholesterol, triglycerides and fatty acid content.[1–8] The formation of cholesterol ester (CE) from cholesterol (C) is an essential step in the reverse cholesterol transport pathway. Lecithin:cholesterol acyl-transferase (LCAT) bound to ApoA-I lipoproteins cleaves the fatty acyl group from the sn-2 position of phosphatidylcholine (PC) and transfers it to C, producing both lysophosphatidylcholine (LPC) and CE.[9–15] The CE moves to the hydrophobic interior of the lipoprotein particle which grows from a very high density and CE-poor particle (HDL_3/pre-β HDL) to a lower density particle defined as HDL_2. Defects of the cholesterol esterification process in SCD blood are reflected in the difference of both the concentration and activity of several plasma proteins related to this process. Both levels of LCAT[16,17] and ApoA-I,[18–20] which stimulates LCAT activity, are lower in SCD plasma. This decreased abundance is also observed for the PC transfer protein (PCTP),[21] which can provide PC to the LCAT reaction. Serum amyloid A, which can inhibit activity of LCAT,[22] and the acute phase enzyme secretory phospholipase A_2 ($sPLA_2$), which can mobilize CE from lipoproteins,[23,24] are reported to be increased.[19,25] In addition to lower levels, the activities of LCAT, ApoAI, and of the RBC lysophospholipid re-acylating enzymes are altered.[17] The ability of HDL particles to exchange ApoA-I was impaired in SCD plasma and this worsened during

vaso-occlusive episodes (VOE) even after the patients received RBC concentrate transfusions.[17] The decrease in HDL-ApoA-I exchange rates correlated with the increased levels of the acute phase enzyme $sPLA_2$ during VOE.[17] Through its de-acylating action, $sPLA_2$ generates bioactive lysophospholipids, which are implicated in acute and chronic inflammation.[23,24] During the acute phase response, significant remodeling of anti-inflammatory HDL to smaller pro-inflammatory entities can occur,[26–28] and both ApoA-I and LCAT on HDL are replaced by other plasma proteins such as serum amyloid A (SAA) and $sPLA_2$. It appears that a similar remodeling of the HDL takes place in SCD plasma. The decreased ApoA-I and increased SAA levels in SCD plasma have been defined as novel biomarkers of acute painful episodes,[19] and an altered protein profile of lipoproteins was confirmed in sickle patients with pulmonary hypertension.[20,29] HDLs from SCD plasma were more sensitive to Cu^{2+}-induced oxidation as compared to HDL from normal individuals.[30] Pro-inflammatory HDL is associated with tricuspid regurgitant jet velocity and the increase of $sPLA_2$ is predictive of ACS.[25,31,32]

We show a change in the ratio of HDL_2/HDL_3 particles in ApoB-depleted SCD plasma samples. HDL_2 particles isolated from SCD plasma increased the expression of the acute phase protein PTX3 in HUVEC cells as compared to HDL_2 isolated from normal individuals. This stimulation of PTX3 expression by sickle HDL_2 was prevented by the addition of the heme-scavenger protein hemopexin. This hemopexin treatment had no significant effect on control HDL_2-treated cells. The altered function of the larger HDL particle in SCD plasma was confirmed by the increased antioxidant capacity of HDL_2. These findings indicate a significant imbalance of lipoprotein function, which may warrant transfusion of plasma in addition of RBC concentrates in SCD.

Materials and methods

Measurement of plasma biomarkers

Blood was collected under the Institutional Review Board approval from the Children's Hospital Research Center Oakland (CHRCO). Steady-state (baseline) samples were obtained either prior a vaso occlusive crisis event during a routine visit to the clinic or at least three weeks after a crisis event. Patients with confirmed SCD (Hb SS, Hb SC, Hb Sβ) with no underlying chronic inflammatory diseases other than SCD were included in the study. Patients undergoing chronic transfusions or transfused in the previous four weeks were excluded. Hydroxy urea treatment was not an exclusion criterion. Total cholesterol content in plasma of samples was determined with the method of Van Stewart.[33] HDL-ApoA-I exchange was measured as previously described.[17]

Lipoprotein isolation

ApoA-I lipoproteins were isolated by sedimentation ultracentifugation with serial density gradients of potassium bromide (KBr) made in saline as described by McPherson et al.[34] Centrifugation steps were performed in 1.5 mL tubes in a TLA100.2 rotor at 10°C for 3 h at 100,000 *g*. Frozen plasma samples were thawed on ice and 650 μL was brought to a density of 1.063 g/L by the addition of 50.6 mg of KBr. The plasma was transferred to a centrifuge tube and was overlaid with 650 μL of a KBr/saline solution at a density of 1.063 g/L. After centrifugation, the top layer containing LDL and VLDL was discarded. The crude HDL fraction was collected and the volume was adjusted to 450 μL with KBr/saline with a density of 1.063 g/L. Crude HDL was then brought to a density of 1.125 g/L with 52 mg of KBr and was overlaid with 650 μL of KBr/saline at 1.125 g/L. After centrifugation, the top layer, representing the HDL_2 fraction, was collected and saved. The underlying crude HDL_3 fraction was collected and the volume was adjusted to 800 μL with KBr/saline at 1.125 g/L. Crude HDL_3 was then brought to a density of 1.210 g/L with 82 mg of KBr and was overlaid with 500 μL of KBr/saline at 1.210 g/L. After centrifugation, the top layer, representing the pre-β (HDL_3) fraction, was collected and saved. The factions were then dialyzed for 20 h at 4°C against PBS pH 7.4 supplemented with 10 mM EDTA. The protein concentration was determined with Bradford dye using BSA as a standard.

Non-denaturing gel electrophoresis

Non-denaturing gel electrophoresis (NDGE) separation of lipoproteins was performed as described by Gambert et al.[35] with a polyacrylamide gradient gel 2.5–18% made in 19 mM Tris-HCl pH8.7 with 30 mM glycine and 7 g/L saccharose. Twenty microliters of ApoB-depleted plasma[17] was mixed with 20 μL of loading buffer (23.2 mg/mL Saccharose with 5 mg/mL Sudan Black made in ethylene glycol) and loaded in each lane. Samples were run in the same Tris–glycine–saccharose buffer for 18 h at 4°C at 60 V. The High Molecular Weight Calibration kit for electrophoresis (GE Healthcare) was used to estimate the size of the bands. Quantification was performed with Quantity One software (Bio-Rad). ApoA-I analysis of the fast migrating (HDL_3) and slow migrating (HDL_2) was performed by elution of the protein from the gel. Bands were cut and transferred in a tube with SDS-PAGE loading buffer and left overnight at 4°C. Acrylamide gel pieces were removed by centrifugation and eluted proteins were denatured by boiling for 4 min and loaded on a SDS-PAGE 12% gel. Following separation, proteins were either stained with GelCode Blue or transferred onto nitrocellulose membrane and stained with a goat polyclonal anti-ApoA-I antibody (Abcam).

Measurement of DCFH oxidation

Dichlorofluorescein fluorescent assays were adapted from the method described by LeBel and colleagues.[36] A 1 mg/mL stock solution of the non-fluorescent 2′,7′-dichlorofluorescin diacetate (DCFHDA) was made daily in methanol and protected from light. A 125 μL DCFHDA aliquot was de-esterified by alkaline treatment[36,37] by addition of 500 μL of freshly made 0.01 N NaOH and incubation for 30 min at room temperature, protected from light. NaOH was neutralized by addition

of 2.5 mL of 25 mM sodium phosphate buffer pH 7.4, yielding a 40 μM stock solution of 2′,7′-dichlorofluorescin (DCFH). Oxidation of DCFH by reactive oxygen species generates the fluorescent species 2′,7′-chlorofluorescein (DCF) (excitation 498 nm/emission 522). In absence of alkaline treatment, DCFHDA is resistant to oxidation. Reactions were performed in 96-well plates in 100 μL Tris-HCl 40 mM pH 7.4 with 1 μM DCFH at 37°C. Samples were incubated with $CuCl_2$, H_2O_2 or buffer (as indicated in legends of the figures) for 1 h at 37°C before addition of DCFH. In experiments with human hemopexin, the protein was added during the pre-incubation period. Fluorescence was read every 10 min for up to 2 h (see figures) at 37°C with a microplate reader (Synergy H1, BioTek, Winooski, VT, USA).

HUVEC culture and PTX3 mRNA quantification

Human Umbilical Vein Endothelial Cells (HUVEC) were obtained from the UC-Berkeley Cell Culture facility (Berkeley, CA, USA). Flasks and plates were coated with gelatin (0.1 mg/mL), and cells were grown in medium supplemented with endothelial cell growth factor as previously described.[38] HUVEC treatments with HDL were performed in 96-well plates (Nunclon, ThermoFisher) in duplicated wells. Cells were grown to about 70% confluency for 24 h in complete medium. To maintain uniformity of the treatment condition and to avoid difference of dilution of the culture medium as result of the addition of the different HDL samples, the stock of HDL particles was first diluted at twice the experimental concentration in a final volume of 25 μL PBS. The diluted HDL/PBS solution was then added to 25 μL of Opti-MEM. Before HDL treatment, the complete medium was replaced with serum-poor Opti-MEM and cells were incubated for 3 h. Culture medium was then removed from the wells and the 50 μL diluted HDL mixtures were then added to the wells. Untreated cells were obtained by adding 25 μL of PBS to 25 μL of medium per well. Experiments with human hemopexin (Hx) were performed as above with the addition of 25 μM Hx to the HDL/PBS mixture, resulting in a final concentration of 12.5 μM per well. After 4 h of incubation at 37°C in the presence of HDL, 150 μL of the RTL lysis reagent were added directly to the wells and total RNA was isolated according to the manufacturer's instructions (PureLink RNA Mini Kit, ThermoFisherScientific). Purified RNAs were treated with RNase-free DNaseI (TURBO DNase, ThermoFisherScientific), extracted with acidic phenol/chloroform and concentrated by ethanol precipitation. DNA-free RNA was suspended in RNase-free water and stored at −80°C. Synthesis of cDNA was performed with the RevertAid First Strand cDNA Synthesis kit in the presence of random hexamer primers (ThermoFisherScientific). Real-time PCR reactions were performed with human *PTX3* and *ACTB* gene specific PrimeTime qPCR Primers (IDT DNA), using iTAQ SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA, USA). Triplicated reactions were performed in 5 μL volumes in a 384-well plate and were run on an ABI7900HT instrument (Applied Biosystems, Foster City, CA, USA). Ct values of *PTX3* were normalized to the values obtained for the *ACTB* gene. Normalized values (ΔCt) obtained in cells without HDL was used as reference and the ΔΔCt method was used to determine the fold change in expression of *PTX3* in the HDL-treated HUVEC compared to the PBS-treated cells.

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 the difference of parameter values during acute phases was compared to baseline using paired *t*-tests. Statistical significances for comparisons are indicated on the figures or in the legend of the figures. Statistical analysis was performed with GraphPad Prism statistical software (version 6; GraphPad, San Diego, CA, USA).

Results

Altered HDL_2/HDL_3 distribution in SCD plasma

Lipoprotein migration profiles on non-denaturing gradient gel (NDGE) stained with Sudan Black (see the Materials and methods section) were analyzed from ApoB-depleted plasma samples of blood collected from non-sickle ('normal' controls) donors and from sickle cell disease patients who were not presenting with painful events (baseline). The lipoprotein profile of normal plasma shows a distribution of abundant fast migrating HDL_3/pre-β and of slower migrating larger HDL_2 (Figure 1(a) and (b)). Most baseline sickle samples were characterized by an increase in the intensity of the HDL_2 band relative to HDL_3 (Figure 1(a)). The HDL_2 signal in SCD plasma was significantly stronger than in normal, whereas the HDL_3 band was far less abundant in those samples as compared to normal. The mean value of the signal intensity of HDL_2 relative to HDL_3 (HDL_2/HDL_3 ratio) of the sickle cohort was 1.4 times higher than the mean value observed in normal (0.97 ± 0.06 vs. 0.68 ± 0.03; $P < 0.006$) (Figure 1(b)). Thus, in addition to lower total plasma cholesterol content and ApoA-I level, a significant distribution shift from HDL_3 to HDL_2 also appeared to occur in SCD.

Acute vaso-occlusive episodes exacerbate HDL_3 deficiency

Analysis of plasma samples from blood collected on the first day on which SCD patients presented with an acute vaso occlusive event (VOE) confirmed the altered lipoprotein distribution observed at baseline (mean HDL_2/HDL_3 ratio 1.10 ± 0.15) (Figure 1(b)). For five patients, analyzed at baseline and day 1 of VOE, the proportion of HDL_2 relative to HDL_3 further increased on the first day of the crisis event ($P = 0.005$) (Figure 1(c)). We previously showed that ApoA-I exchange rates were lower in SCD plasma as compared to normal and this exchange rate further decreased during VOE.[17] Thus, the difference in the relative amount of HDL_2/HDL_3 particles provides additional evidence of alterations that affect ApoA-I lipoproteins, in SCD plasma, which are further exacerbated during inflammatory events.

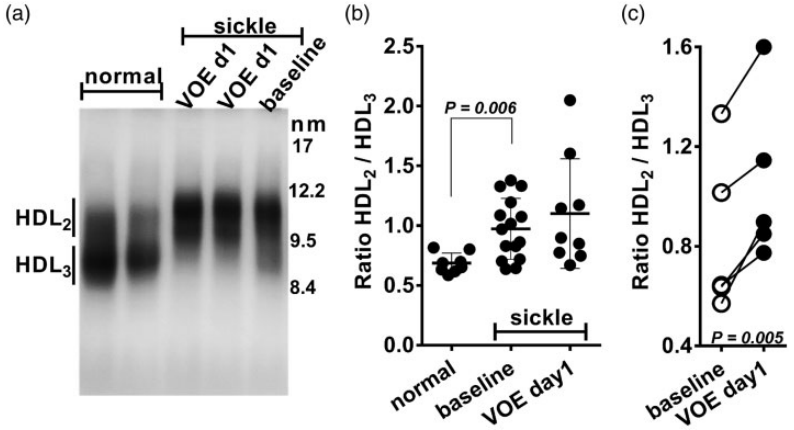

Figure 1 Non-denaturing gel electrophoretic lipoproteins separation. Twenty microliters of ApoB-depleted plasma mixed with 20 μL of saccharose loading buffer containing 5 mg/mL of the lipid stain Sudan Black were loaded in each lane of a polyacrylamide 2.5–18% gradient gel. Samples were run in a Tris–glycine–saccharose system (see the Materials and methods section) for 18 h at 4°C at 60 V. (a) A representative image of a gel with two normal plasma samples, one SCD plasma sample of a patient at baseline, and two samples of patients at day 1 of a VOE onset. The position of the High Molecular Weight Calibration kit for electrophoresis (GE Healthcare) bands is shown on the right. (b) Quantification of the amount of HDL_2 relative to HDL_3, in plasma samples of eight normal plasmas, 15 SCD plasmas at baseline and 9 SCD plasmas at day 1 of a VOE are shown. (c) The HDL_2/HDL_3 ratio was analyzed for five of the sickle patients at baseline (open circles) and the matching sample during VOE (filled circles). Two-tailed *P* values were calculated with GraphPadPrism software and are indicated on the panels

Erythrocytes enhanced HDL remodeling

A remodeling from HDL_3 to HDL_2 during incubation of serum at 37°C was reported.[35] However, these changes were slow as they took more than 12 h to become apparent. We reported that ApoA-I exchange rates in SCD plasma (defined as the Plasma Unquenching Capacity (PUC) parameter) was affected during incubation for 1–2 h with washed RBC at 37°C.[17] The HDL_2/HDL_3 distribution was analyzed after incubation of SCD or normal plasma with washed RBC for a relatively short period of 1 h at 37°C. Figure 2 shows that incubation of normal plasma with saline or with normal RBC did not change the HDL_2/HDL_3 ratio. In contrast, the HDL_2/HDL_3 ratio increased in the two SCD plasmas incubated in the presence of RBC. The shift from HDL_3 to HDL_2 was noticeable after 30 min and further increased within 1 h in SCD plasma. The PUC parameter decreased in the two SCD plasma samples, whereas values were stable in the RBC and saline-treated normal plasma. The enlargement of the ApoA-I lipoprotein from the small HDL_3 to the larger HDL_2 particle and concurrent slowing of ApoA-I exchange rates appears to indicate a relation between size and the ability of ApoA-I to exchange with the particle.

The high HDL_2/HDL_3 ratio with low PUC values found in SCD plasma supports the notion that this correlation observed during this *in vitro* incubation may reflect *in vivo* conditions. This is further supported by the increase in the HDL_2/HDL_3 ratio and the lowering of PUC values during VOE (Figure 1).[17] Together, these observations suggest that measurement of the PUC parameter could represent a new diagnostic tool to rapidly detect plasma lipoprotein imbalance in SCD and perhaps in the early phase of a VOE.

Dichlorofluorescein fluorescence assays

The cell permeable di-acetate form of dichlorofluorescin (DCFHDA) has been used to measure reactive oxygen species (ROS) in cells. DCFHDA is cleaved by cellular esterases and the trapped hydrolyzed form (DCFH) becomes highly fluorescent once oxidized to dichlorofluorescein (DCF) (review in Gomes et al.[39]). A similar approach was reported to measure ROS, and quantify the pro-inflammatory HDL level, and a pro-inflammatory/anti-inflammatory HDL index in plasma,[30,40–43] and we aimed to use this approach to assess ROS and the inflammatory index in SCD plasma. As described by Navab et al.,[42] DCFHDA was dissolved in fresh methanol and incubated for 30 min at room temperature and an aliquot was then added to plasma and fluorescence was detected. However, as previously established by others,[36,37] we confirmed that DCFHDA dissolved in methanol was stable, that it did not release DCFH and did not generate a fluorescence signal, even under oxidizing conditions (Figure 3(a)). Removal of the two acetyl group by alkaline treatment generated DCFH, which was slowly oxidized in air and more rapidly in the presence of Cu^{2+}, and generated a fluorescence signal (Figure 3(a)). Oxidized lipids, such as POVPC,[44] were reported to oxidize DCFHDA.[42] Under similar experimental conditions, we failed to detect an increase in DCF fluorescence in the presence of POVPC even when the dye was appropriately de-esterified from DCFHDA to DCFH (Figure 3(a)). Because incubation of DCFHDA with plasma produced a signal,[30,40–43] we concluded that esterase activities present in plasma must have released DCFH *in situ*. To detect plasma esterase activities independently of ROS-induced fluorescence, we incubated the oxidized di-acetate form of DCFH (DCFDA), dissolved in methanol, with plasma under similar condition as were reported to monitor DCFHDA fluorescence.[30,40–43] As shown in Figure 3(b), incubation of DCFDA with plasma rapidly resulted in a fluorescence signal indicative of a de-esterification reaction releasing the fluorescent DCF. To confirm that the increase in fluorescence was the result of the plasma-mediated released

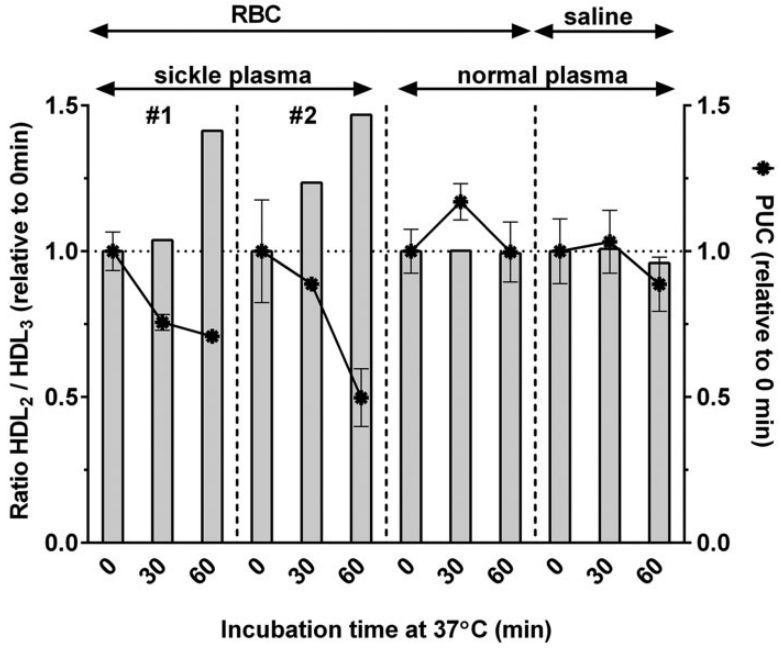

Figure 2 HDL distribution and HDL-ApoA-I exchange of SCD plasma. Five hundred microliters of fresh washed RBC were mixed with 900 μL of normal or SCD plasma and incubated at 37°C. At the indicated time, 420 μL of the mixture was transferred to a new tube and centrifuged at low speed to remove the RBC. The plasma (300 μL) was collected and stored at −80°C until analysis. Quantification of the relative amount of HDL_2/HDL_3 was performed as described in Figure 1 and is shown on the left axis. The EPR nitroxide signals, relative to an instrument reference peak, obtained by addition of lipid-free ApoA-I-spin labeled probe[65] mixed with an increasing volume of ApoB-depleted plasma (0.6 μL to 14.4 μL) were performed in triplicate as previously described.[17] The plasma unquenching capacity (PUC) of the samples was calculated with GraphPadPrism software and is shown on the right axis. The results are presented as relative to the value obtained with the plasma sample mixed with RBC (or saline) without incubation (t = 0 min)

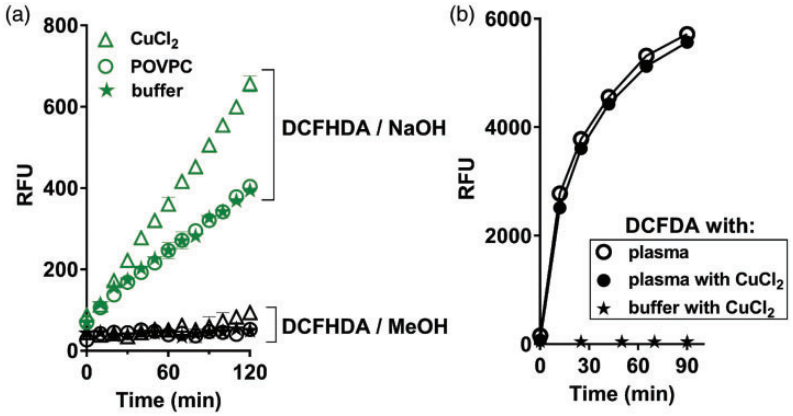

Figure 3 Dichlorofluorescein assay. Results obtained with the di-acetate esterified reduced form of DCFH (DCFHDA) and of the di-acetate esterified oxidized form of DCF (DCFDA) are shown on panels (a) and (b), respectively. A stock solution of DCFHDA was prepared in methanol as described in the Materials and methods section and was either diluted in water (DCFHDA/MeOH; black symbols) or treated with NaOH (0.01 N) for 30 min (DCFHDA/NaOH; green symbols). The chemical was then added at a final concentration of 1 μM in 100 μL Tris-HCl 40 mM pH 7.4. Real-time fluorescence measurements were performed in 96-well plates at 37°C. Assays in panel (a) were performed in buffer alone (asterisk), buffer with 20 μM $CuCl_2$ (triangle) or with 50 μg/mL POVPC (circle). Assays in panel (b) were performed with plasma (0.4 μg cHDL) (open circle), plasma with 5 μM $CuCl_2$ (filled circle) or buffer with 5 μM $CuCl_2$ (asterisk). (A color version of this figure is available in the online journal.)

of DCF and not of plasma-ROS, addition of Cu^{2+}, which oxidized DCFH (Figure 3(a)), was not able to generate a fluorescent product in the absence of plasma and did not increase the signal in the presence of plasma. Thus, esterase activities that release DCF(H) from DCF(H)DA were present in plasma, and also appear to be present in the HDL fraction.[30] Hence, reports that use DCFHDA, without prior release of DCFH,[30,40–43] provide information on two distinct reactions in plasma: de-esterification of DCFHDA and oxidation of DCFH. This obviously implies a problem with the interpretation of the results. Plasma samples with different esterase activities would generate a different rate of increase in fluorescence values and will lead to erroneous interpretation on ROS amount, proHDL level and the pro/anti-inflammatory HDL index of those samples. Careful re-interpretation of those results appears therefore warranted.

Measurement of the antioxidant capacity of HDLs

DCFH diluted in buffer and exposed to air resulted in significant oxidation over time, a process that was enhanced in presence of Cu^{2+} and hydrogen peroxide (H_2O_2) (Figure 4). Thus, independently of the presence of plasma, both H_2O_2 and Cu^{2+} can directly oxidize DCFH in the presence of air. This further complicates interpretation of experiments that aim to assess the sensitivity of HDL and the rate of its oxidation by indirect measurement of DCF fluorescence. Under the three oxidizing conditions, plasma reduced the formation of DCF, which confirmed the antioxidant potential of plasma (Figure 4(a)). Figure 4(b) shows that using the Cu^{2+}-mediated oxidation assay, no difference was observed between the SCD and normal plasma samples. This finding confirmed previous observations that pro-inflammatory HDL levels are not elevated in SCD as compared to normal.[31]

Increasing concentrations of HDL_2 and HDL_3 particles isolated from SCD and normal plasma were tested in a Cu^{2+}-mediated oxidation assay (Figure 5). At the lowest concentration tested, 0.05 μg/mL, HDL_2 and HDL_3 had no detectable effect and values obtained in their presence were similar to those obtained in their absence (ratio of ≈1). However, at higher concentrations, HDL_3 from normal and from SCD blood protected DCFH from oxidation. A ratio of <1 obtained with HDL_3 from SCD plasma and normal plasma seems to indicate similar antioxidant properties of those particles (Figure 5(d)). At a concentration of 0.2 μg/mL, a difference was detected between HDL_2 isolated from normal plasma in comparison to HDL_2 from SCD plasma (Figure 5(c) and (d)). SCD plasma HDL_2 prevented oxidation (mean ratio = 0.76 ± 0.17), whereas HDL_2 from normal plasma displayed no such capacity

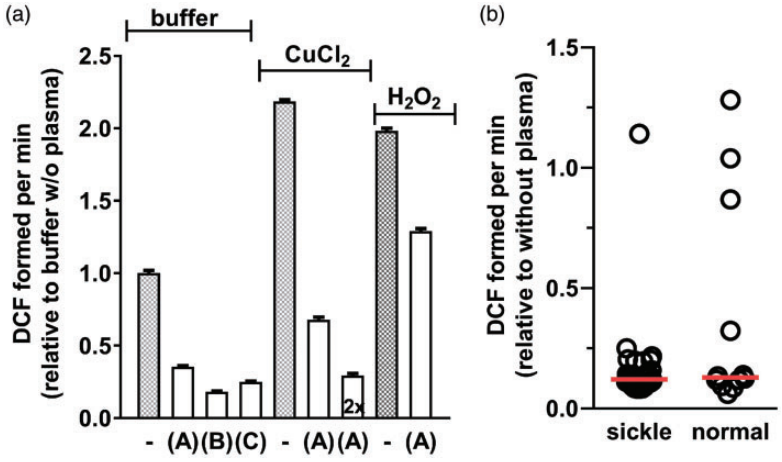

Figure 4 Reducing potential of plasma. Measurements were performed with DCFH in 100 μL Tris-HCl 40 mM pH 7.4 at 37°C. (a) Reactions were performed with three plasma samples (A, B, C) and with plasma sample (A) in presence of 5 μM $CuCl_2$ or 100 μM H_2O_2. One assay was performed with twice the amount of sample (indicated by 2×). (b) Reactions were performed with SCD ($n = 31$) and normal ($n = 12$) plasma samples in the presence of 5 μM $CuCl_2$. The slopes of the linear fit of DCF fluorescence values as a function of time were calculated with GraphPadPrism software. Data are presented relative to the values obtained in buffer in the absence of plasma in panel A and relative to the values obtained with 5 μM $CuCl_2$ in the absence of plasma in panel B. Error bars represent the standard deviations of three measurements. The median of the values for normal (0.1295) and SCD (0.1218) is indicated by a red line. (A color version of this figure is available in the online journal.)

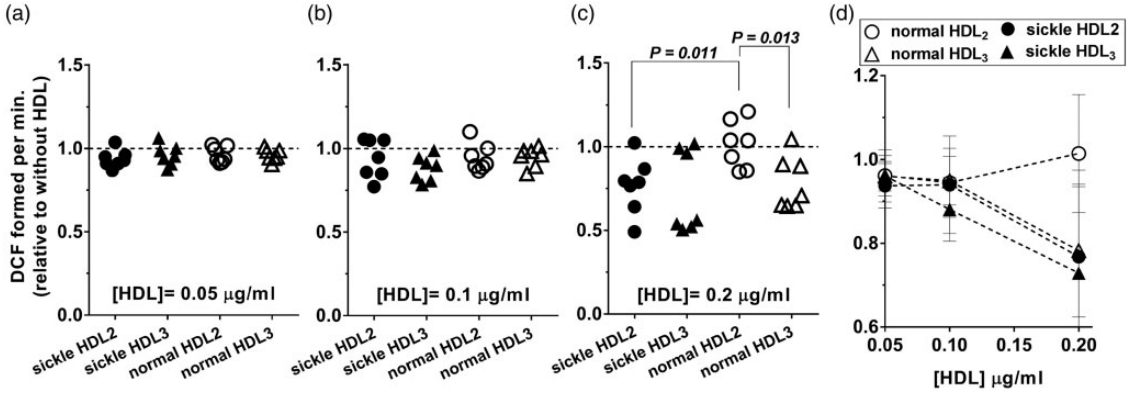

Figure 5 Anti-oxidant potential of HDL_2 and HDL_3. HDL_2 and HDL_3 were isolated from SCD and control plasma and assayed for their ability to prevent (<1) or enhance (>1) oxidation of DCFH. Measurements were performed in triplicate as described in Figure 4 in the presence of $CuCl_2$ with HDL particles at a final concentration of 0.05–0.1–0.2 μg/mL (panels a, b and c). In panel (d), the mean (±SD) of the values obtained in each of the four groups (sickle HDL_2, sickle HDL_3, normal HDL_2, normal HDL_3) were calculated and plotted as a function of the HDL concentration. Note that at the highest concentration tested of 0.2 μg/mL, HDL2 isolated from control plasma was neither anti- or pro-oxidant (ratio of 1) but that sickle HDL2 was as effective as the control HDL3 particles in preventing DCFH oxidation (<1)

(mean ratio = 1.01 ± 0.14). The ratio of <1 obtained with the more abundant sickle HDL_2 particles further confirmed the difference of lipoproteins in SCD blood as compared to normal.[19,20]

Up-regulation of acute phase PTX3 by sickle HDL_2

To further assess the extent of the difference of properties of SCD plasma HDL_2 as compared to normal plasma, their capacity to modulate gene expression in HUVEC was tested. As previously shown,[38] we confirmed that mRNA levels of the acute phase protein long pentraxin 3 (PTX3) were increased after exposure of the cells to HDL_2 isolated from normal plasma (Figure 6(a)). Quantification of *PTX3* mRNA shows a dependency to the concentration of HDL2, with a maximum fold change reached at 50 μg/mL after 4 h of treatment. The relative values ranged from 1.5 to 3.0 with a mean of 2.17 ± 0.61 (Figure 6(a)). HUVEC exposed to sickle HDL_2 displayed a similar concentration-dependent expression profile and reached a similar level (mean of 2.29 ± 0.46). Significantly higher *PTX3* mRNA levels were detected with sickle HDL_2 at the non-optimum concentrations of 30 and 200 μg/mL (Figure 6(b) and (c)). No significant difference was detected between normal and SCD HDL_3.

The effect of Hemopexin on HDL properties

The *PTX3* gene was identified as a gene marker of iron-overload.[45] Hemolysis, cell free hemoglobin, and heme play important roles in SCD pathology, and hemopexin (Hx) – the plasma heme-scavenger protein[46,47] – is low in SCD plasma.[48] HDL-induced *PTX3* expression was investigated in the presence of Hx which is reported to bind to the HDL in SCD.[49] Hx added during exposure of HUVEC to isolated HDL particles (Figure 7) prevented up-regulation of *PTX3* by sickle HDL_2 and mean relative values dropped from 1.2 ± 0.2 to 0.6 ± 0.2 (Figure 7(a)). DCFH oxidation assays were performed to assess the effect of Hx on the anti-oxidant potential of sickle HDL. Although plasma protected DCFH from Cu^{2+}-mediated oxidation (Figure 5), oxidation was increased in the presence of Hx in a concentration-dependent fashion (Figure 7(b)). This pro-oxidant activity was also observed with isolated HDL particles from SCD plasma (Figure 7(c)). These findings suggested that binding of Hx to HDL also complexed Cu^{2+} to the lipoprotein resulting in increased oxidation. Hx has a high affinity to several cations[50] and the decrease expression of iron-induced *PTX3* in HUVEC could have involved scavenging of iron by Hx, limiting the response of the cells to sickle HDL.

Discussion

A point mutation in the beta globin gene results in a dysfunctional red blood cell and leads to the vasculopathy that defines SCD. Hemoglobin polymerization under low oxygen conditions, altered cell–cell interactions in blood, intravascular hemolysis, and an increased inflammatory state are all aspects of the pathology of SCD that affects all organs.[51–53] Temporal vascular obstruction results in painful vaso-occlusive events and results in ischemia reperfusion injury.[54] Inflammation and increased levels of ROS can be predicted to result in high level of pro-inflammatory HDL.[30,40–43] However, our study confirmed that pro-inflammatory HDL levels were not different in SCD plasma as compared to control plasma.[31] As we show in our study, the use of DCFHDA, to define a pro-inflammatory/anti-inflammatory HDL index in plasma,[30,40–43] has a number of pitfalls. These findings also suggest that other studies that use this approach should be re-examined carefully. The ratio of HDL_3/HDL_2 was altered in SCD plasma. Our data also indicate that HDL_2 in SCD has different

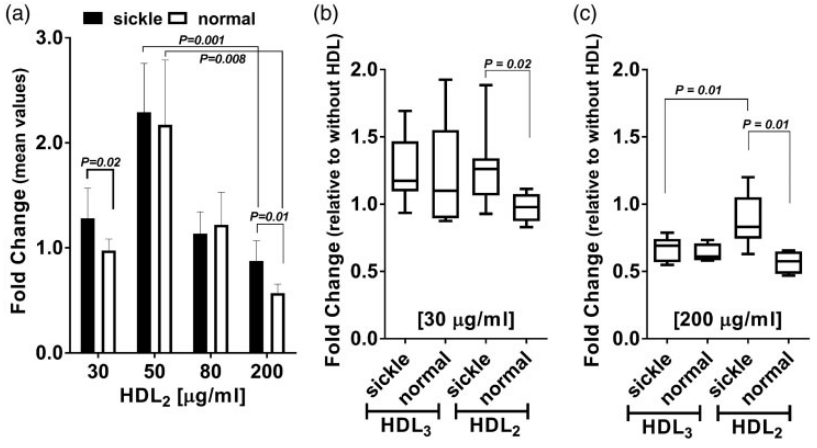

Figure 6 *PTX3* mRNA quantification in HDL-treated HUVEC. Human umbilical vein endothelial cells were grown in complete medium to 70% confluence, and then cultured for 3 h in serum-poor medium prior co-culture with HDL diluted in serum-poor medium at the indicated concentration (see the Materials and methods section). HDL particles were isolated from SCD and normal plasma as shown in Figure 5. Total RNAs of treated cells were isolated and expression of *PTX3* mRNA was quantified. Fold change of expression is presented as the ratio of the level of values obtained in the presence of HDL relative to those obtained in their absence. The bar graph in panel A shows the mean of the fold change values obtained with the samples in the normal ($n = 5$) or sickle ($n = 8$) cohort at different HDL_2 concentration (30–50–80–200 μg/mL). Error bars represent the standard deviation of the mean. Panels (b) and (c) show the fold change of expression of each sample obtained after treatment with HDL_3 and HDL_2 particles isolated from normal and SCD plasma at a concentration of 30 μg/mL and 200 μg/mL, respectively. The means and error bars, representing the min-max in each treatment group, are also shown. Two-tailed *P* values were calculated with GraphPadPrism software

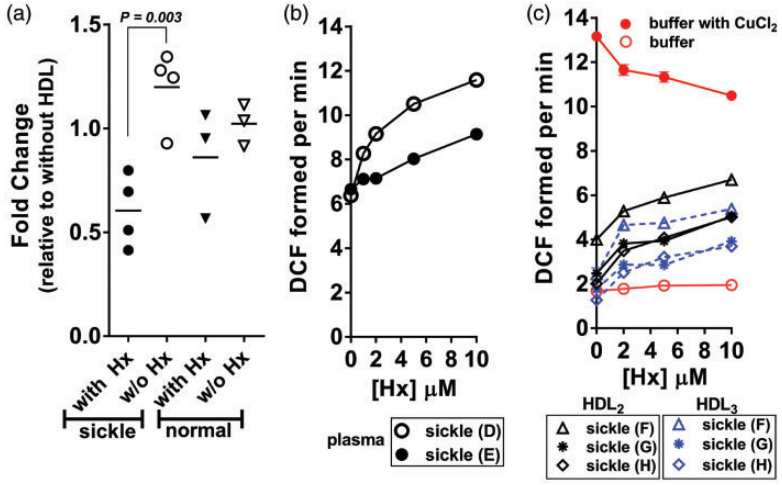

Figure 7 Hemopexin interference with HDL function. (a) *PTX3* mRNA expression was quantified as described in the legend of Figure 6 in HUVEC treated with HDL_2 particles isolated from normal or SCD plasma at a final concentration of 30 μg/mL in the absence or presence of 12.5 μM Hx. The effect of Hx on the reducing potential of plasma (panel B) and of HDL_2 and HDL_3 (panel C) was performed by monitoring the oxidation of DCFH in the presence of 20 μM $CuCl_2$ as described in the legend of Figure 4. Measurements were triplicated and performed with ApoB-depleted plasma (0.4 μg cHDL) (panel b) or with 0.2 μg/mL of HDL (panel c)

characteristics as compared to HDL_2 in normal plasma. We speculate that these HDL_2 alterations in SCD plasma could at least partially compensate for the depletion of the HDL_3 particles, which are usually associated with antioxidant properties.[55–59] As assessed by the DCF assay, the sickle HDL_2 particles showed an increased anti-oxidant capacity, different from normal HDL_2, and acted like HDL_3. These results could account for the findings of similar level of proHDL, as measured by the DCF assay, in SCD and in the normal population (Figure 4(b) and Ataga et al.[31]). This alteration of the HDL species distribution, and the decrease of the smaller HDL_3, accounts for the slower ApoA-I exchange rate (PUC) observed in SCD.[17] This finding suggests that PUC measurements could be used to monitor HDL changes that relate to changes in HDL size. The decreased levels of HDL_3 particles, which are essential to stimulate ABCA1-mediated cholesterol export, are likely to impair cholesterol efflux from macrophages in SCD.[60] In addition, lower HDL_3 levels could negatively impact the protection of endothelial cells from apoptosis induced by oxidized-LDL.[55] Long pentraxin 3 (PTX3), a member of the family that includes C-reactive protein (CRP), is produced in response to inflammatory stimuli and elevated levels were observed during VOE in SCD.[61,62] While at baseline the CRP levels are elevated in SCD, baseline PTX3 levels are reported to be similar in SCD and normal plasma.[61,62] However, during vaso-occlusive episodes, PTX3 levels increase, making it a predictive marker for mortality in acute chest pain,[63] and a severity marker of painful crisis in SCD.[62] The findings that sickle HDL_2 was more efficient in the induction of *PTX3* expression than HDL_2 isolated from normal plasma provided further evidence of altered function of the sickle lipoproteins. The mechanism by which HDL_2 acts to stimulate *PTX3* expression is not well understood. PTX3 is produced at the site of inflammation by several cell types, including mononuclear phagocytes, endothelial cells, dendritic cells and fibroblasts. Increased levels of sphingosine 1 phosphate (S1P) in SCD[64] may play

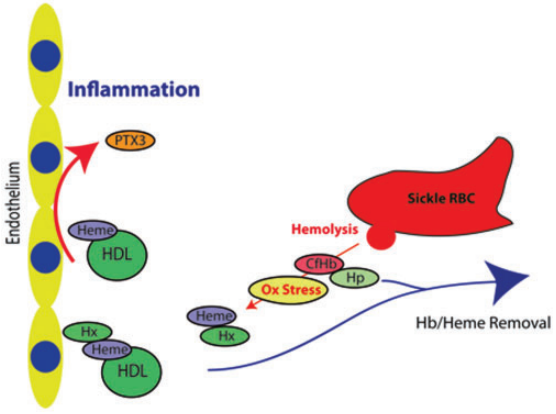

Figure 8 Working model. The high level of intravascular hemolysis in SCD leads to increased cell-free hemoglobin (CfHb) and consequently an increase of heme released from hemoglobin. Both haptoglobin (Hp) and hemopexin (Hx) are taxed to remove these redox active agents. The steady-state concentration of these compounds is found to be low in sickle cell plasma. As a result, heme bound to HDL is not efficiently Hx bound and initiates inflammatory reactions, including the formation of PTX3

a role in *PTX3* induction by activation of the S1P-receptor.[38] A role for S1P bound to lipoproteins has been proposed.[38] The *PTX3* gene was also identified as a marker of iron-overload in SCD.[45] The apparent shielding of sickle HDL_2 by Hx seemed to indicate that induction of *PTX3* expression was related to the presence of heme in the sickle lipoprotein (Figure 8). The heme-bound Hx-HDL complex is normally rapidly removed in blood. In SCD plasma, the increased presence of cell free hemoglobin and heme, which are continuously released from hemolyzing sickle erythrocytes, could potentially overwhelm the pathways that remove heme from plasma. Lower levels of Hx in SCD plasma and the interaction between endothelial cells and heme mediated by the sickle HDL could lead to the release of increased amounts of acute phase proteins, such as PTX3,

and aggravate or prolong inflammatory episodes in SCD.[47,50,61,63]

In conclusion, our findings support the assessment that in addition to the abnormal RBC, the plasma lipoprotein compartment of sickle blood is also significantly altered. Enzymes and structural proteins related to the formation and growth of ApoA-I lipoproteins are impaired and HDL function is altered. Based on these findings, blood transfusions, a well accepted treatment of SCD patients, may have to be re-evaluated since replacing only the red blood cells may underestimate the importance of the altered composition of the plasma compartment. Moreover, addition of Hx may provide an additional option to reduce inflammatory pathways by lowering the burden of cell-free heme.

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 and SKL conducted the experiments; and ES, SKL and FAK wrote the manuscript.

DECLARATION OF CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

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