PLATELET BIOENERGETIC SCREEN IN SICKLE CELL PATIENTS REVEALS MITOCHONDRIAL COMPLEX V INHIBITION WHICH CONTRIBUTES TO PLATELET ACTIVATION

Short title: Platelet mitochondria in Sickle Cell Disease

Nayra Cardenes¹,#, Catherine Corey¹#, Lisa Geary¹, Shilpa Jain¹,², Sergey Zharikov¹, Suchitra Barge¹, Enrico M. Novelli¹,³ and Sruti Shiva¹,⁴*

¹Vascular Medicine Institute, ²Division of Pediatric Hematology-Oncology, Children’s Hospital of Pittsburgh, ³Departments of Medicine and ⁴Pharmacology & Chemical Biology, University of Pittsburgh, Pittsburgh, PA

# These authors made equal contribution

*Corresponding Author:

Sruti Shiva, PhD
Department of Pharmacology & Chemical Biology
Vascular Medicine Institute
BST E1242
University of Pittsburgh
Pittsburgh, PA 15261
Fax: (412) 648-3046
Tel: (412)383-5854
e-mail: sss43@pitt.edu
KEY POINTS

- Sickle cell patients show mitochondrial dysfunction (complex V inhibition, oxidant formation) which is associated with platelet activation.

- Complex V inhibition is induced by hemolysis and causes platelet activation, which is attenuated by mitochondrial therapeutics

ABSTRACT

Bioenergetic dysfunction, while central to the pathogenesis of numerous diseases, remains uncharacterized in many patient populations due to the invasiveness of obtaining tissue for mitochondrial studies. While platelets are an accessible source of mitochondria, the role of bioenergetics in regulating platelet function remains unclear. Herein, we validate extracellular flux analysis in human platelets and utilize this technique to screen for mitochondrial dysfunction in Sickle Cell Disease (SCD) patients, a population with aberrant platelet activation of an unknown mechanism and in which mitochondrial function has never been assessed. We identify a bioenergetic alteration in SCD patients characterized by deficient complex V activity, leading to decreased mitochondrial respiration, membrane hyperpolarization and augmented oxidant production compared to healthy subjects. This dysfunction correlates with platelet activation and hemolysis \textit{in vivo} and can be recapitulated \textit{in vitro} by exposing healthy platelets to hemoglobin or a complex V inhibitor. Further, reproduction of this dysfunction \textit{in vitro} activates healthy platelets, an effect prevented by attenuation of mitochondrial hyperpolarization or by scavenging mitochondrial oxidants. These data identify bioenergetic dysfunction in SCD patients for the first time and establish mitochondrial hyperpolarization and oxidant generation as potential pathogenic mechanism in SCD as well as a modulator of healthy platelet function.
INTRODUCTION

The mitochondrion is an integral regulator of cellular function in most cell types. Beyond maintenance of energy homeostasis, the electron transport chain (ETC) regulates cellular fate through the initiation of apoptosis and dynamically produces reactive oxygen species (ROS) to mediate redox signaling. While it is now well established that altered bioenergetics contribute to the pathogenesis of a wide range of diseases in which the primary cause is non-mitochondrial, the exact function of the mitochondrion in many cell types, particularly circulating cells, remains elusive. Further, bioenergetics remain uncharacterized in many patient populations due to the requirement for viable intact human tissue to accurately measure mitochondrial function.

Platelets are an easily accessible source of human mitochondria and prior studies have measured ETC function in these thrombotic mediators as a surrogate for bioenergetic function in other organs\(^1\), \(^2\). Identification of specific mitochondrial alterations in platelets from patients with a variety of pathologies including Parkinson’s Disease\(^1\), \(^3\), \(^4\), sepsis\(^2\), \(^5\), and Type II Diabetes\(^6\), \(^7\), have established that platelets can be utilized as biomarkers for systemic mitochondrial dysfunction. However, the exact role of bioenergetics in regulating platelet thrombotic function is less clear. Studies of healthy platelets show that mitochondria supply a fraction of the ATP required for alpha granule secretion during platelet aggregation\(^8\), \(^9\). Additionally, the loss of mitochondrial membrane potential (ΔΨ) and increased membrane permeability initiate platelet phosphatidyl serine exposure and regulate coagulation\(^10\). Emerging *in vitro* data now suggest a role for augmented ΔΨ in regulating platelet sensitivity to thrombotic stimuli\(^11\), \(^12\). However, the contribution of mitochondrial hyperpolarization to platelet activation in a patient population with known platelet dysfunction has not been assessed.

Sickle Cell Disease (SCD) is a homozygous recessive disorder caused by a single point mutation in the β-globin chain of hemoglobin A, resulting in mutant hemoglobin (HbS). While the
primary dysfunction in SCD patients is the hypoxic polymerization of HbS, leading to diminished erythrocyte deformability and impaired microvascular blood flow, it is well documented that these patients demonstrate characteristics of chronic hemostatic activation, including elevated levels of platelet activation\textsuperscript{13-16}. Though the molecular mechanism underlying this platelet dysfunction is unknown, platelet activation is associated with augmented erythrocytic hemolysis in these patients\textsuperscript{15}. Clinically, platelet activation contributes to both acute and chronic vascular complications, including vaso-occlusive crisis (VOC) and pulmonary arterial hypertension (PAH), through the secretion of vasoactive and mitogenic factors\textsuperscript{13, 14, 17-19}. Notably, though aberrations in mitochondrial redox signaling and bioenergetics have been implicated in the pathogenesis of both systemic\textsuperscript{20} and pulmonary\textsuperscript{21} vasculopathies, mitochondrial function has never been assessed in SCD patients.

The advent of extracellular flux (XF) analysis has enabled the high throughput assessment of bioenergetics in small numbers of intact live cells and this technology has recently been applied to human platelets\textsuperscript{6, 22, 23}. Herein, we further validate XF analysis and couple this technology with biochemical measures of mitochondrial ROS generation and platelet thrombotic function to screen SCD patients for bioenergetic dysfunction and simultaneously determine the contribution of the mitochondrial ETC to platelet activation in this population. We identify a specific bioenergetic alteration in SCD patients and provide \textit{in vivo} and \textit{in vitro} evidence that this specific alteration in the mitochondrial ETC may mechanistically underlie augmented platelet activation in this population. The implications of this data for normal platelet physiology as well as the mitochondrion as a potential SCD therapeutic target will be discussed.
METHODS

Materials
Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and antibodies from Becton Dickinson (San Jose, CA) unless otherwise noted.

Study Population
This study was approved by the Institutional Review Board of the University of Pittsburgh Medical Center (UPMC) and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki. The study group consisted of 24 adult patients with homozygous SCD (HbSS) in steady state selected from the UPMC hematology clinic and 19 African American control participants with no known hemoglobinopathy. None of the participants were on anti-coagulant/anti-platelet medication or received transfusions in the three months prior to blood draw. Laboratory characteristics are summarized in Table 1.

Platelet Isolation
Venous blood samples were collected in citrate by standard venous puncture and the first 2ml of blood discarded to avoid artificial activation. Platelets were isolated by differential centrifugation as previously described\textsuperscript{15}. Briefly, whole blood was centrifuged (150xg, 10min) in the presence of PGI\textsubscript{2} (1µg/ml) to obtain platelet rich plasma (PRP). Platelets were subsequently pelleted by centrifugation (1500xg, 10 min). The platelets were washed with Erythrocyte Lysis buffer containing PGI\textsubscript{2} and final samples resuspended in modified Tyrode’s buffer (20mmol/L HEPES, 128mmol/L NaCl, 12mmol/L bicarbonate, 0.4mmol/L NaH\textsubscript{2}PO\textsubscript{2}, 5mmol/L glucose, 1mmol/L MgCl\textsubscript{2}, 2.8mmol/L KCl, pH 7.4). Platelet purity was confirmed by flow cytometric measurement of CD41a expression.

Platelet Activation
Whole blood or platelets were incubated with phycoerythrin (PE)-labeled mouse anti-human CD41a antibody and APC-labeled mouse anti-human CD62P antibody (30 min; 25°C) to
measure surface p-selectin expression by flow cytometry (LSRFortessa with FASCDiva software; Becton Dickinson). FITC labeled PAC-1 was used in some studies to recognize activated glycoprotein IIb/IIIa and data is expressed as a percentage of total integrin binding. Platelets were identified by their characteristic light scatter and CD41a antibody binding. Activated platelets are reported as the percentage of 10,000 CD41+ platelets exhibiting APC-CD62P-fluorescence.

**Platelet Aggregation**

Platelet number was normalized (10^8) in platelet rich plasma and then the sample was treated with hemoglobin or ADP before aggregation was followed for 15 minutes using a single-channel aggregometer (Chrono-Log Corp). All data is expressed in percent aggregation at 15 minutes and is normalized to a standard deflection, corresponding to light transmission through platelet-poor plasma.

**Platelet Factor 4 release**

Healthy human platelets in Tyrode’s buffer were untreated or treated with ferrous human hemoglobin for 30 min and then the buffer subjected to an ELISA (Sigma-Aldrich; St. Louis, MO) to measure the release of human platelet factor 4.

**Oxygen Consumption Rate (OCR)**

Platelet number was determined spectrophotometrically at 800nm as described in 24. Platelets (50 million/well) were loaded in unbuffered DMEM into each well of a XF24 microplate to measure OCR by XF analysis (XF24, Seahorse Biosciences; Billerica, MA). The plate was subsequently centrifuged (1500xg, 4 min) to form a monolayer in the well. XF analysis commenced after equilibration of the plate (10 min; 37°C). For bioenergetic profile measurement, platelets were consecutively treated with oligomycin A (2.5µmol/L), Carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone (FCCP; 0.7µmol/L) and Rotenone (10µmol/L).
Optimal concentration of each modulator was determined in concentration response experiments. For measurement of OCR by Clark electrode, isolated platelets were suspended in unbuffered DMEM in a Clark-type electrode containing chamber (Instech; Plymouth Meeting, PA) and OCR measured in the absence and presence of FCCP (0.7 µmol/L).

**Measurement of ΔΨ**

Platelets were incubated with 500nmol/L JC-1 (Molecular Probes, Eugene, CA) for 5 min and then subjected to 2 color flow cytometry to quantify the proportion of cells that contain JC-1 green monomer (indicative of low ΔΨ) or red dimer (indicative of hyperpolarization). The ratio of red to green fluorescence was calculated and expressed on a percentage scale in which 100% was oligomycin (0.7µmol/L) treated platelets for maximal ΔΨ and 0% was FCCP (0.7µmol/L) treated platelets.

**Mitochondrial ROS generation**

Platelets were incubated with MitoSOX™ (Invitrogen, Carlsbad, CA; 5µM; 10 min), washed and fluorescent intensity (510/580 nm) measured kinetically. In select experiments, H$_2$O$_2$ generation was quantified by measuring the rotenone sensitive oxidation of Amplex Red as previously described$^{25}$.

**ETC complex expression and activity assays**

Complex expression was measured by Western blot. Antibodies to mitochondrial ETC and integrin αIib were purchased from Mitosciences (Eugene, OR) and Santa Cruz Biotech (Dallas, TX) respectively. Densitometry was performed on a LICOR system and complex expression normalized to integrin αIib. The enzymatic activity of complexes I-V and citrate synthase was measured by spectrophotometric kinetic assays after several cycles of platelet freeze/thaw as previously described$^{25}$. 
**Isolation of human hemoglobin**

Hemoglobin was isolated from the blood of healthy subjects by hypotonic lysis followed by dialysis as previously described\(^2\). The concentration of hemoglobin was measured by visible absorption spectroscopy as previously described\(^2\) and ferrous oxygenated Hb was used unless otherwise noted.

**Measurement of plasma hemoglobin**

The concentration of free hemoglobin in SCD plasma was measured by a chemiluminescence nitric oxide (NO) Analyzer (GE analytical) by assessing the consumption of NO by plasma samples and compared to a standard curve derived from the consumption of NO by known concentrations of hemoglobin as previously described\(^2\).

**Statistics**

Individual group samples were compared by Student’s t-test while multiple comparisons were made by appropriate ANOVA. Correlations were performed by 2-tailed nonparametric Spearman correlations (due to non-Gaussian distribution of data) and linear regression analysis with 95% confidence interval; \(P\) values less than 0.05 were considered significant. Data are presented as the means ± SEM unless otherwise specified.

**RESULTS**

**Validation of human platelet bioenergetic measurements by XF analysis**

We first sought to validate the use of XF analysis as a screening tool for human platelet bioenergetics. Platelets were isolated from a fresh blood draw from healthy African American subjects and seeded for XF analysis at a density of 50 X 10\(^6\) platelets/well. Measurement of OCR in the absence of treatment showed that basal OCR was stable for 2 hours (Figure 1A). Further, measurement of platelet activation prior to and after XF analysis demonstrated that
seeding and XF measurement did not stimulate platelet activation (Figure 1B). To generate a bioenergetic profile, basal OCR was first measured. The complex V inhibitor oligomycin (2.5µmol/L) was then administered to quantify the OCR not linked to ATP synthesis. The resulting rate is indicative of proton leak across the inner mitochondrial membrane. Next, the uncoupler FCCP (0.7µmol/L) was added to stimulate maximal respiratory rate. Finally, the complex I inhibitor, rotenone (10µmol/L), was administered to determine the non-mitochondrial OCR by the platelets. Consistent with prior studies\textsuperscript{6, 22}, platelets responded to all pharmacological agents, demonstrating fully functional mitochondria and generating a measurable bioenergetic profile. Notably, platelets possessed a significant rate of non-mitochondrial OCR (Figure 1A,C). This non-mitochondrial OCR was inhibited by treatment of the platelets with allopurinol (100 µmol/L) and indomethacin (100 µmol/L), inhibitors of the oxygen consuming enzymes xanthine oxidoreductase and cyclooxygenase respectively (Figure 1C).

Basal and maximal respiratory rates were dependent on platelet seeding density and showed a linear response between 5-100 X 10\textsuperscript{6} platelets/well (Figure 1D). To compare the accuracy of XF analysis with a conventional method of respirometry, basal and maximal OCR was measured by a Clark-type oxygen electrode and XF analysis in platelets isolated from the same individual. While no significant difference was found in the basal and maximal OCR obtained by the differing methods when normalized to platelet number, measurement by conventional electrode required significantly greater numbers of platelets (8X10\textsuperscript{8}) than XF analysis (25X10\textsuperscript{6}) to obtain reliable rates (Figure 1E).

**SCD patient platelets have altered bioenergetics**

We next used platelet XF analysis to screen SCD patients (n=24) for altered bioenergetics compared to healthy African American control subjects (n=19) (Figure 2A; See Table 1 for clinical characteristics). While platelets from both groups demonstrated robust responses to
each mitochondrial modulator, SCD patient platelets exhibited a significantly greater non-mitochondrial OCR (25.3 ±1.9 pmol/min/10^6 cells) than controls (16.4±1.1 pmol/min/10^6 cells; **Figure 2B**). This rotenone insensitive OCR was completely inhibited by allopurinol (100µM) and indomethacin (100µM; data not shown), consistent with prior reports of increased activity of oxygen consuming enzymes in SCD[^28]. When normalized for differences in non-mitochondrial OCR, platelets from SCD patients showed a significantly lower rate of basal respiration in (64 ±6.5 pmol/min) as well as decreased OCR due to proton leak (17.4 ± 2.2 pmol/min) than healthy subjects (92.3±5.1 and 25.7 ±1.9 pmol/min), with no significant difference in maximal OCR (99.9 ± 6.5 vs 107 ±13 pmol/min) (**Figure 2C**).

Since decreased proton leak can result in increased ΔΨ, we next assessed ΔΨ and found that platelets from SCD patients were significantly more hyperpolarized than those from controls (**Figure 2D**). Mitochondrial hyperpolarization can result in increased ETC reduction, propagating electron leak and subsequent oxidant generation. Using mitoSOX, we next demonstrated that mitochondria in platelets from SCD patients generated ~2.4-fold greater concentrations of mitoSOX oxidation products than healthy platelets, consistent with increased mitochondrial oxidant production (**Figure 2E**). Measurement of Amplex Red oxidation showed that SCD platelets generated significantly more hydrogen peroxide (H2O2) than controls (**Figure 2F**).

To determine whether these bioenergetic and redox changes were due to a specific enzymatic alteration, we next examined individual enzymatic components of the ETC in platelets from SCD patients and controls. While the protein expression of complexes I, II, IV and V were similar in both groups (**Figure 3A-B**), the specific enzymatic activity of complex V was significantly decreased in SCD patients versus controls (16.7 ± 2.3 vs. 25.2 ± 2.5 nmol/min/mg) (**Figure 3C**). Notably, though complex V activity was decreased, platelet ATP generation rate was not significantly different (**Figure 3D**). This was potentially due to a statistically significant (p<0.05) upregulation of glycolysis (measured as the extracellular acidification rate; ECAR) in the
platelets isolated from SCD patients (5.4 ± 0.9 mpH/min/10^6 cells) compared to that of healthy controls (3.2± 0.4 mpH/min/10^6 cells).

Complex V is a point of proton re-entry from the mitochondrial inter-membrane space to the matrix and inhibition of this activity could decrease basal OCR, increase ΔΨ and ROS generation. To test whether this occurs in platelets, healthy human platelets were treated in vitro with a low dose of oligomycin (0.4 µmol/L), which inhibited complex V activity by 30 ± 7%. Measurement of OCR showed that this treatment decreased basal OCR while having no effect on maximal OCR (Figure 3E). Further, ΔΨ and oxidant production were augmented by 1.6 ± 0.1 and 2.1 ± 0.2 fold respectively (Figure 3E-F), generating a similar profile to that observed in SCD platelets. Collectively, these data demonstrate that SCD patients harbor a bioenergetic aberrancy characterized by decreased respiration, increased ΔΨ and augmented mitochondrial oxidant generation and this is potentially caused by deficient complex V activity.

**Mitochondrial dysfunction is associated with platelet activation and hemolysis**

SCD patients exhibit augmented platelet activation compared to healthy subjects^{13-15}. This phenomenon was corroborated in our cohort in which SCD patient platelets showed a significantly greater percentage of activated platelets (21 ± 6.9%; n=24) than controls (11.0 ± 2.3%; n=19; p=0.029; Table 1). Thus, we next sought to determine whether the bioenergetic dysfunction identified in SCD platelets was associated with aberrant platelet activation. We found a significant positive correlation between two markers of platelet activation (surface p-selectin and activated glycoprotein IIb/IIIa) and the degree of mitochondrial hyperpolarization or H_2O_2 production in SCD patients (Table 2; Supplemental Figure 1). These data suggested that platelet mitochondrial dysfunction is associated with increased platelet activation in SCD.

SCD patients experience high rates of intravascular hemolysis and this hemolysis has been associated with platelet activation in these individuals^{15, 29}. Consistent with published studies, a
significant positive correlation was found between the concentration of plasma free hemoglobin
and the percentage of activated platelets (surface p-selectin expression) in our SCD cohort
(r=0.49; p=0.04). Notably, strong correlations were also observed between all markers of
mitochondrial dysfunction (ΔΨ, H₂O₂ production and complex V activity) and plasma cell free
hemoglobin (a marker of hemolysis) in these subjects (Table 3; Supplemental Figure 2). Collectively, these data suggested an association between hemolysis, mitochondrial dysfunction
and platelet activation in SCD patients.

Bioenergetic dysfunction: a mechanistic link between hemolysis and platelet activation

We next performed a series of in vitro experiments to determine whether hemolysis causes the
platelet mitochondrial dysfunction and platelet activation observed in SCD patients. Healthy
human PRP was exposed to increasing concentrations of oxygenated hemoglobin (0-50µmol/L)
and platelet mitochondrial function was measured concomitantly with platelet thrombotic
function. Consistent with prior studies, platelet activation was significantly increased after
hemoglobin exposure (Figure 4A-B). Further, hemoglobin induced platelet aggregation in a
concentration dependent manner, though to a much lesser extent than 20µM of the classic
agonist ADP (Figure 4C). Hemoglobin mediated stimulation of alpha granule secretion was also
confirmed by the measurement of a 2.1± 0.3 fold increase in platelet factor 4 release by the
hemoglobin treated platelet in comparison to untreated controls. Measurement of complex V
activity and bioenergetics in these platelets showed a hemoglobin-dependent decrease in
complex V activity (Figure 4D) and basal OCR (Figure 4E), as well as an increase in ΔΨ
(Figure 4E) and H₂O₂ production (Figure 4F), recapitulating the bioenergetic alteration
observed in SCD patients. Importantly, partial inhibition of complex V with oligomycin
(0.4µmol/L; Figure 4D) also resulted in platelet activation (Figure 4A). These data demonstrate
that partial inhibition of complex V pharmacologically or by hemoglobin exposure is sufficient to
increase ΔΨ, augment ROS generation and activate platelets.
**Attenuation of hyperpolarization or ROS scavenging prevents platelet activation**

To directly determine whether mitochondrial hyperpolarization and H$_2$O$_2$ generation induced by complex V inhibition causes platelet activation, healthy human PRP was incubated with hemoglobin (25µmol/L) or oligomycin (0.4µmol/L) in the absence and presence of the mitochondrially-targeted ROS scavenger mitoTEMPO (100 µmol/L), or a low concentration of the mitochondrial uncoupler FCCP (0.3µmol/L) to partially decrease ΔΨ. As expected, MitoTEMPO treatment had no effect on ΔΨ (not shown) but significantly decreased H$_2$O$_2$ concentration (**Figure 5A**). Consistent with the fact that hyperpolarization induces ROS generation, partial attenuation of ΔΨ by FCCP (**Figure 5B**) also decreased hemoglobin or oligomycin-dependent H$_2$O$_2$ generation (**Figure 5A**). Importantly, both mitoTEMPO and FCCP significantly decreased oligomycin or hemoglobin-induced platelet activation (**Figure 5C**). Collectively, these data demonstrate that complex V inhibition leading to mitochondrial hyperpolarization induces subsequent ROS production which ultimately causes platelet activation.

**DISCUSSION**

This study represents the first direct examination of mitochondrial function in SCD patients and provides evidence that bioenergetic dysfunction mechanistically contributes to SCD-induced platelet activation. The data herein identify a specific bioenergetic alteration in this cohort induced by free hemoglobin, characterized by inhibited complex V activity, which leads to increased ΔΨ and augmented oxidant generation. This bioenergetic dysfunction is associated with enhanced platelet activation *in vivo*. Further, partial inhibition of complex V in healthy platelets *in vitro* recapitulates the bioenergetic dysfunction observed in SCD patients and results
in platelet activation, establishing a causal relationship between this bioenergetic alteration and platelet activation.

The present study suggests that mitochondrial dysfunction, through the stimulation of platelet activation, potentially contributes to SCD-induced vascular pathogenesis. In addition to overt thrombus formation, activated platelets contribute to both acute and chronic vasculopathy in SCD patients through the secretion of soluble vasoactive and mitogenic factors, promoting vascular damage, red cell adhesion to the endothelium and intimal hyperplasia. Consistent with this, augmented platelet activation observed basally in steady state SCD patients is further elevated during acute vaso-occlusive (VOC) crisis and correlates with the severity of PAH, a chronic vascular proliferative complication and leading cause of mortality in adult SCD patients. Though patients in VOC or with diagnosed PAH were excluded from this study, it will be important to determine whether complex V inhibition and the bioenergetic dysfunction observed is further exacerbated in these populations. It is also unclear whether the mitochondrial bioenergetic dysfunction is reversible, however the in vitro data presented show that scavenging of mitochondrial ROS or mild attenuation of ΔΨ prevents hemoglobin induced platelet activation. Recent clinical trials have demonstrated beneficial effects of antiplatelet therapies on pain events in SCD patients. While mitochondria have previously not been considered a therapeutic target in SCD, it is intriguing to speculate whether existing strategies to decrease mitochondrial ROS, such as administration of the mitochondrial antioxidant MitoQ, may have favorable effects on decreasing pain and decelerating PAH and thrombotic development in SCD.

Our data demonstrate that hemoglobin induces a shift in mitochondrial function resulting in the generation of oxidants. Notably, though oxidant production is increased by in vitro hemoglobin treatment and in SCD patients, ATP generation is not compromised. Comparison of H$_2$O$_2$ generation in control and SCD platelets shows that ~11% of basal oxygen consumption in
control platelets contributes to H$_2$O$_2$ production while in SCD platelets H$_2$O$_2$ accounts for ~20%.

Interestingly, though significantly more oxygen consumption is diverted to ROS generation in SCD platelets, ATP generation does not decrease significantly. Calculation of the phosphate: oxygen (P:O) ratio in the two platelet populations show a trend for increased efficiency in platelets from SCD patients compared to controls (2.96 ± 0.36) compared to controls (2.25 ± 0.29). While the mechanism underlying this maintenance of ATP generation is not completely clear, upregulation of glycolysis could contribute. Further, this type of shift may represent a signaling mechanism by which the mitochondrion generates ROS to modulate signaling outside the mitochondrion. Future studies will determine whether mitochondrial ROS contribute to platelet function beyond activation and aggregation. It is also conceivable that the platelets serve as a surrogate for mitochondria in other cell types and this type of metabolic shift underlies mitochondrial redox signaling in the vasculature. For example, in the context of SCD, mitochondrial H$_2$O$_2$ generation in vascular endothelial and smooth muscle cells has been implicated in pulmonary vascular remodeling$^{21,39}$. Further, mitochondrial H$_2$O$_2$ mediates the hypoxic stabilization of hypoxia inducible factor 1α (HIF-1α)$^{40}$, which could contribute to the aberrant normoxic stabilization of HIF-1α, which has been reported in SCD and implicated in the pathogenesis of vasculopathy$^{41-43}$.

Hemolysis and platelet activation are known to be closely associated in SCD patients$^{14,15,29}$. The current study confirms this relationship and extends it to include mitochondrial dysfunction as a mechanistic link. While our data demonstrate that exposure of healthy platelets to hemoglobin in vitro mimics the mitochondrial dysfunction observed in SCD platelets, it is unclear how hemolysis inhibits complex V function. No change in complex V protein expression was observed in SCD patients suggesting that heme-induced post-translational modification is likely responsible for this change in activity. While a number of post-translational modifications have been demonstrated to regulate complex V activity in other cell types, reports of enzymatic
inhibition by nitration, S-nitrosation and cysteine oxidation are particularly relevant in the context of platelets and hemolysis\textsuperscript{44, 45}. The ferrous (Fe$^{2+}$) heme contained in cell free hemoglobin is both a potent scavenger of nitric oxide (NO)\textsuperscript{46} and a catalyst for the generation of oxidants\textsuperscript{47, 48}. Prior studies have focused on the role of hemoglobin-mediated NO scavenging in hemolysis-induced platelet activation\textsuperscript{15}. However, it is conceivable that in the presence of superoxide generated by free hemoglobin, NO could be oxidized to yield species such as nitrogen dioxide (NO$_2^*$) and peroxynitrite (ONOO$^-$) that mediate nitration or S-nitrosation of complex V, which results in its inhibition\textsuperscript{44}. Current studies are aimed at investigating the mechanism of hemoglobin induced mitochondrial dysfunction and it will be important to determine whether this mechanism is present in hemolytic disorders beyond SCD.

While the precise signaling pathway between mitochondrial ROS and platelet activation is unknown, several studies support the role of oxidants in activating platelets\textsuperscript{10-12, 50, 51}. Specifically, collagen-induced platelet activation has been shown to involve the production of H$_2$O$_2$, which modulates activation of the phospholipase C signaling cascade\textsuperscript{51}. Further, mitochondrial ROS generation sensitizes platelets to activation mediated by other physiological stimuli\textsuperscript{11, 12}. However, the role of mitochondrial $\Delta \Psi$ in regulating platelet activation is less clear. Platelet activation has been linked to both mitochondrial hyperpolarization and depolarization\textsuperscript{10-12, 52}. This discrepancy is likely due to differences in time course or strength of stimulation across studies as demonstrated by a study in which stimulation of platelets with opsonized zymosan induced mitochondrial hyperpolarization stimulated platelet activation followed temporally by depolarization leading to phosphatidyl serine externalization\textsuperscript{11}. Based on this idea, it is conceivable that small dynamic changes in $\Delta \Psi$ cause mild activation of platelets that remain in circulation, while persistent mitochondrial damage leads to depolarization and cell death. This is consistent with the fact that, as demonstrated here, mild hyperpolarization can sustain mitochondrial oxidative phosphorylative function, as well as with studies demonstrating that
depolarization is integral in mitochondrial permeability transition pore assembly and phosphatidyl serine externalization\textsuperscript{10,52}, components of both strongly activated platelets and the apoptotic cascade.

In conclusion, this study demonstrates that SCD patients possess a specific profile of mitochondrial dysfunction and suggests that this alteration in bioenergetics is stimulated by hemolysis and propagates elevated platelet activation. The data herein show that mitochondrial alterations cause platelet activation in vitro and demonstrate the relevance of this pathway \textit{in vivo}. Collectively, these data advance the understanding of the role of mitochondria in platelet function and suggests that the study of mitochondria in SCD and other hemolytic disease warrants further study.

**ACKNOWLEDGEMENTS**

This work was supported by the Institute of Transfusion Medicine, the Hemophilia Center of Western Pennsylvania, the NIH (1R01HL096973), and the AHA (09SDG2150066).

**AUTHORSHIP CONTRIBUTIONS**

NC, CC, LG, and SZ collected and analyzed data. SJ, SB, and EMN recruited subjects, obtained written consent and obtained blood samples. SS and NC planned the study, interpreted results and wrote the manuscript.

**DISCLOSURES**

The authors have no conflicts of interest to disclose.
REFERENCES


Table 1: Characteristics of SCD patients and control subjects.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SCD</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>24</td>
<td>19</td>
</tr>
<tr>
<td>Male; Female</td>
<td>10;14</td>
<td>8;11</td>
</tr>
<tr>
<td>Age, y</td>
<td>34±11</td>
<td>38±12</td>
</tr>
<tr>
<td>Activated platelets (%)</td>
<td>21 ±3.9%</td>
<td>11±2.3</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>9±2</td>
<td></td>
</tr>
<tr>
<td>Cell Free Hb (µM)</td>
<td>0.75±0.22</td>
<td></td>
</tr>
<tr>
<td>Platelet count (x10^9/L)</td>
<td>327±133</td>
<td></td>
</tr>
<tr>
<td>Reticulocyte (%)</td>
<td>8±5</td>
<td></td>
</tr>
<tr>
<td>Lactate dehydrogenase (U/L)</td>
<td>352±184</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Platelet activation is associated with mitochondrial dysfunction in SCD

<table>
<thead>
<tr>
<th>Platelet Activation Marker</th>
<th>Mitochondrial Dysfunction Marker</th>
<th>r</th>
<th>P</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface p-selectin</td>
<td>ΔΨ</td>
<td>0.47</td>
<td>0.04</td>
<td>0.025 to 0.764</td>
</tr>
<tr>
<td></td>
<td>H₂O₂ generation</td>
<td>0.48</td>
<td>0.03</td>
<td>0.049 to 0.760</td>
</tr>
<tr>
<td></td>
<td>Complex V activity</td>
<td>-0.42</td>
<td>0.06</td>
<td>-0.73 to 0.022</td>
</tr>
<tr>
<td>Activated GPIIb/IIa (%)</td>
<td>ΔΨ</td>
<td>0.53</td>
<td>0.02</td>
<td>0.112 to 0.799</td>
</tr>
<tr>
<td></td>
<td>H₂O₂ generation</td>
<td>0.46</td>
<td>0.04</td>
<td>0.013 to 0.759</td>
</tr>
<tr>
<td></td>
<td>Complex V activity</td>
<td>-0.45</td>
<td>0.04</td>
<td>-0.75 to -0.014</td>
</tr>
</tbody>
</table>

Table 3: Mitochondrial dysfunction is associated with markers of hemolysis in SCD

<table>
<thead>
<tr>
<th>Marker of Hemolysis</th>
<th>Mitochondrial Dysfunction Marker</th>
<th>R</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Free Hemoglobin</td>
<td>ΔΨ</td>
<td>0.56</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>H₂O₂ generation</td>
<td>0.45</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Complex V activity</td>
<td>-0.54</td>
<td>0.02</td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1: Validation of XF analysis for human platelets. (A) Typical bioenergetic profile of platelets from a healthy African American subject. Arrows denote the addition of oligomycin (2.5 µmol/L), FCCP (0.7 µmol/L) and Rotenone (10 µmol/L) to black trace. Gray trace is same sample in the absence of modulators. All OCR rates are normalized to 10⁶ platelets. (B) Percent of activated platelets measured in five different samples prior to seeding in XF analyzer and after measurement. (C) OCR of healthy platelets in the presence of rotenone. Arrows denote the addition of Indomethacin (100 µmol/L) and Allopurinol (100µmol/L). (D) Basal (black squares) and maximal (open circles) OCR in increasing numbers of healthy human platelets. (E) Basal (black) and maximal (white bars) OCR in healthy platelets measured by XF analysis versus Clark electrode.

Figure 2: SCD platelets show bioenergetic dysfunction. (A) Representative platelet bioenergetic profile from a SCD patient (red) and healthy control (black). (B) Rotenone-insensitive OCR of control and SCD patient platelets. Bars represent mean ± SEM. (C) Quantification of each OCR component of bioenergetic profile of SCD patient and control platelets after correction for non-mitochondrial OCR (n=24 SCD; 19 Control). (D) Quantification of the percent of hyperpolarized mitochondria in platelets from SCD and control subjects (n=16 each group). (E) Representative MitoSOX fluorescence images from control and SCD platelets and relative fluorescence intensity of MitoSOX labeling in control and SCD platelets (n=18 each group). (F) Representative Amplex Red traces and quantification of H₂O₂ generation (normalized to 10⁶ cells) from control and SCD platelets (n=14 each).

Figure 3: SCD platelets have inhibited complex V activity. (A) Representative Western blots for complexes I, II, IV, V and integrin αIIb in 3 control and SCD subjects. (B) Densitometric quantification of several such blots in 12 control and SCD platelets. (C) Enzymatic activity of each ETC complex in platelets from SCD patients (black bars) and healthy controls (white bars; n=15). (D) ATP generation rate in SCD (black bars) and control (white bars) platelets (n=10 each). (E) Bioenergetic profile (basal and FCCP-induced OCR) for untreated (filled circles) or oligomycin-treated (open squares) platelets. (F) ΔΨ and MitoSOX fluorescence intensity of untreated (black bars) or oligomycin-treated (white) healthy platelets. n=5; *p<0.01.

Figure 4: Hemolysis induces platelet aggregation and mitochondrial dysfunction. (A-D) Platelet activation measured by surface p-selectin expression (A) or percent of activated glycoprotein IIb/IIIa (B), platelet aggregation 15 minutes after treatment (C) and complex V activity (D) in untreated (control) healthy platelets or those exposed to free hemoglobin or oligomycin (0.4µmol/L) or ADP (20µM; to stimulate aggregation). (E) Basal respiratory rate (solid line) and mitochondrial hyperpolarization (dashed line) in healthy platelets exposed to increasing concentrations of free hemoglobin. (F) H₂O₂ generation in hemoglobin treated platelets. n=5; *p<0.01 vs untreated.

Figure 5: Mitochondrial uncoupling and ROS scavenging attenuates platelet activation. H₂O₂ production (A), ΔΨ (B) and activation levels (C) in untreated (control) healthy platelets or those exposed to hemoglobin (25µmol/L) or oligomycin (0.4µmol/L) in the absence (white bars) or presence of FCCP (0.35µmol/L; gray bar) or mitoTEMPO(100µmol/L; black bar). Data are means ± SEM; n>4; p<0.01.
Figure 1

A

B

C

D

E

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Platelet bioenergetic screen in sickle cell patients reveals mitochondrial complex V inhibition which contributes to platelet activation

Nayra Cardenes, Catherine Corey, Lisa Geary, Shilpa Jain, Sergey Zharikov, Suchitra Barge, Enrico M. Novelli and Sruti Shiva