Increased retention of functional mitochondria in mature sickle red blood cells is associated with increased sickling tendency, hemolysis and oxidative stress

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Supplemental materials

Increased retention of functional mitochondria in mature sickle red blood cells is associated with increased sickling tendency, hemolysis and oxidative stress.

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Materials and methods

Patients, study design and sample collection

Fresh peripheral blood samples from 61 SCA patients (HbSS genotype, 29 men and 32 women, adults and children at steady state, 23.2 ± 14.2 yrs, non-transfused) and from 7 to 10 healthy race-matched donors (AA) were collected in EDTA tubes and analyzed by flow cytometry in a delay of less than 5 h after sampling. The presence of glucose-6-phosphate dehydrogenase (G6PD) deficiency was analyzed as previously described.[1] Forty-nine percent of patients with SCA were from Central Africa, 24.5% from West Africa, and 26.5% from North Africa. All patients were regularly followed at the Hospitals of Lyon (France) and clinical data were collected by physicians. The study was conducted in accordance with the guidelines set by the Declaration of Helsinki, and all subjects gave informed written consent before their participation. The study was approved by the Regional Ethics Committees (L16-47, CPP Sud-Est IV, Hospices Civils de Lyon).

Samples preparation for Image Stream

An amount of $5x10^6$ RBCs from 3 SCA patients 3 and healthy donors were added into a 96-well plate and washed three times with HEPES buffer at 600g for 2 minutes at RT. Then, RBCs were incubated in 100 µL of prediluted Mitotracker Red CMXRos Dye (Invitrogen, M46752) (1:200 dilution), 30 min in the dark at RT, to label active mitochondria. After incubation, samples were washed twice in HEPES buffer, the supernatant was removed and the cells were fixed with 50µL of 0.2% glutaraldehyde for 20 minutes. After fixation, RBCs were washed twice with HEPES buffer at 600g for 2 minutes at RT. Images were recorded with Image Stream (Amnis, MK II) and results were analyzed using IDEAS software version 6.2.

Samples preparation for Flow cytometry analysis

Blood samples were centrifuged at 1,000g for 5 minutes at room temperature (RT). The supernatant and the buffy coat were removed, RBCs pellets were washed twice with PBS 1X (1:1) and resuspended at 0.2 % Ht in the appropriate staining buffer according to manufacturer instructions. All the flow cytometry stainings were performed on 100 µl of RBCs suspension, for 20 min in the dark at room temperature (RT). After incubation, samples were washed, resuspended in their respective staining buffer and analyzed by flow cytometry (MACSQuant 16, Miltenyi). Gating was performed on 100.000 RBCs per condition using the Flowlogic software. Unstained sickle RBCs were used as negative controls.

Characterization of RBCs retaining mitochondria

The percentage of sickle RBCs retaining mitochondria in SCA patients was determined by flow cytometry after performing a double staining using MitoTracker$^{(R)}$ Deep Red probe (MTKdr, Sigma-aldrich, 5 nM final concentration) and an anti-CD71 antibody (1:50 dilution, Miltenyi 130-115-070), to determine the percentages of reticulocytes and mature RBCs containing mitochondria. The percentage of positive events was recorded to quantify mitochondria^{+} and CD71^{-} RBCs (Figure 1C). Triple staining on blood samples from 10 SCA patients was performed with an anti-CD235a (1:400 dilution, Miltenyi 130-120-473), an anti-CD41 (1:50 dilution, Miltenyi 130-124-910) antibodies and mitotracker deep Red probe, to gate on the RBC+/platelet- population.

RBC senescence markers, ROS and intracellular Ca^{2+}

The percentage of RBC exposing PS at the outer membrane leaflet of RBCs and the anti-phagocytic CD47 antigen levels were determined using Annexin-V-PE (1:11 dilution, Miltenyi 130-118-363) on RBCs resuspended in Annexin binding buffer (Miltenyi 130-092-820) and anti-CD47-PE antibody (1:100 dilution, Miltenyi 130-101-348), respectively. Intracellular ROS were determined using 2',7'–dichlorofluorescin diacetate (DCFDA, 20 µM final concentration Sigma-Aldrich, Saint-Quentin-Fallavier, France). A double staining for mitochondria retention (Mitotracker Deep Red, 5nM final concentration) and 1) intracellular Ca^{2+} (Fluo3 AM, 10 µM final concentration, ThermoFicher, F1242), 2) CD47 (anti-CD47-PE antibody, 1:100 dilution, Miltenyi 130-101-348), 3) PS (Annexin-V-PE, 1:11

dilution, Miltenyi 130-118-363) or 4) intracellular ROS (DCFDA, 20 µM final concentration Sigma-Aldrich, Saint-Quentin-Fallavier, France), was also performed to compare intracellular Ca^{2+}, RBC senescence markers (CD47 and PS), and intracellular ROS between mature RBCs containing mitochondria to those without. The samples were analyzed by flow cytometry (MACSQuant 16, Miltenyi). The percentage of positive events was recorded to quantify RBCs exposing PS. The Mean Fluorescence Intensity (MFI) was recorded to quantify CD47 expression, Ca^{2+} and ROS levels.

Intracellular glutathione

Reduced (GSH) and total (GSSG+GSH) intracellular glutathione was measured using the luminescence-based assay GSH-Glo glutathione Assay (Promega, V6912). Briefly, RBCs pellets were mixed by inversion in GSH-Glo Reaction buffer (1:5 dilution) to facilitate lysis and centrifuged at 10,000 g for 15 min at 4°C. The supernatant was collected and diluted (1:25 dilution) in deionized water; the reducing agent TCPE was added (30 min in the dark at RT) to reduce the oxidized GSSG and measure the total GSH. 100µl of GSH-Glo reagent (containing Luciferin NT substrate and glutathione S transferase) were added and the samples were incubated for 30 min in the dark at RT. The luminescence was measured using the Synergy Microplate readers. Total GSH corresponds to the measure of the plate containing the reducing agent TCEP. GSSG was calculated subtracting the concentration of GSH (GSH-Glo plate) from the total GSH (obtained with TCEP). The ratio GSH/GSSG was obtained by dividing the GSH concentration by the GSSG concentration.

RBC deformability in Oxygen gradient and determination of the Point of Sickling

Oxygen gradient ektacytometry was performed using the Oxygenscan protocol of the LORRCA Maxsis (Mechatronics, The Netherlands) to measure RBC deformability in oxygen gradient, as previously described.[2,3] Briefly, 50 µl of blood from SCA patients, standardized to 200 x 10^6 RBC count, were resuspended in 5 mL of Oxy-Iso polyvinylpyrrolidone (PVP, Mechatronics, The Netherlands) medium with a mean viscosity of 28–30 cP and osmolality of 282–286 mOsm/kg. The suspension was sheared at 30 Pa and 37°C in a Couette system. The oxygen partial pressure (pO2) was gradually decreased from 160 mmHg to 20 mmHg (deoxygenation) in 20 minutes and then returned to normoxic values in 5

minutes. The diffraction pattern was analyzed and recorded by the computer to calculate an Elongation Index (EI) reflecting RBC deformability. The maximum RBC deformability (EImax) reached in normoxia and the oxygen pressure at which RBCs start to sickle (Point of sickling, PoS) were determined. Measurements were performed less than 4 h after blood sampling.[4] EImax was also determined in 10 race-matched healthy donors: there is no PoS and no EImin in healthy subjects as hemoglobin is normal and does not polymerize under hypoxia.

Hematological and Biochemical Parameters

Fetal hemoglobin was determined by capillary electrophoresis (Capillarys 2 device, Sebia, France), while mean cell volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and percentages of reticulocytes were determined using a hematology analyzer (Advia, Siemens, Rungis, France). Bilirubin levels were determined by standard biochemical method.

Mitochondrial Superoxide production

Mitochondrial superoxide production in sickle RBCs was assessed using the MitoSOX Red mitochondrial superoxide indicator (Invitrogen, M36008). RBCs were stained with 5µM of MitoSOX Red, 30 min at 37°C under shaking. After incubation, samples were washed and resuspended in PBS 1X. 100µL of the RBCs suspensions were analyzed by flow cytometry (MACSQuant 16, Miltenyi). The remaining RBCs suspensions already loaded with MitoSOX were incubated for 30 min at 37°C under shaking with 5µM of Antimycin A, an inhibitor of the Respiratory Complex III, which increases the production of superoxide anion from mitochondria. After incubation, the RBCs suspensions were analyzed again by flow cytometry (MACSQuant 16, Miltenyi) and gating was performed on 100,000 RBCs per condition.

Percoll gradient separation of mature RBCs

Blood samples from 8 patients and 5 healthy controls were centrifuged at 1,000 g for 5 minutes at room temperature. The supernatant and the buffy coat were removed, RBCs pellets were washed twice with PBS 1X (1:1) and resuspended at 50 % Ht. Analysis of the residual presence of platelets and white blood

cells showed very low values in both patients and controls suspensions (less than $0{,}3x10^3$). Then, thirty mL of Percoll solution (Percoll, Sigma-Aldrich P1644, 3M NaCl, 0.2 Phosphate solution, 0.5 EDTA, 40 g/l Glucose, PMSF protease inhibitor (Thermo Fisher 36978, H20) were added in 50 mL Falcon tubes. Four mL of RBCs at 50% Ht were placed on the top of Percoll solution, without mixing the two phases. RBCs were centrifuged at 18,000g for 30 min at 4°C with 5 acceleration and 5 brake. The 1st layer containing reticulocytes was removed, the 2nd layer containing mature RBCs was washed twice in a 15 mL tube with 14 mL of PBS 1X. The presence of reticulocytes among the isolated RBCs was assessed by Flow Cytometry by using an anti-CD71 antibody (1:50 dilution, Miltenyi 130-115-070).

Analysis of mitochondrial respiration in mature RBCs from healthy controls and patients with SCA

We used a high-resolution respirometry protocol (Oxygraph-2k high-resolution respirometer, Oroboros Instruments, Innsbruck, Austria) for intact blood cells adapted from Sjövall et al[5] and Stier et al[6] Immediately before the start of the mitochondrial measurements, Percoll-isolated RBCs stored for < 1 hours in PBS at 4°C were centrifuged at 800 *g* for 2 min to pellet the cells. The supernatant was removed and 100µL of packed cells was re-suspended in 1mL of respiratory buffer MiR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose, free fatty acid bovine serum albumin (1 g/L), pH 7.1) pre-warmed at 37°C, and transferred in the respirometry chamber of one Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria) set at 37°C and containing 1mL of Mir05. Endogenous O_2 consumption was then recorded after approximately 10 min of stabilization. ATP-dependent O_2 consumption was inhibited by adding oligomycin (2.5µM final), an inhibitor of ATP synthase. The mitochondrial uncoupler FCCP (carbonyl cyanide-p-trifluoro-methoxyphenyl-hydrazone) was then titrated in 0.05 µM steps until the maximal uncoupled O_2 consumption was reached. Finally, mitochondrial O_2 consumption was fully inhibited by adding antimycin A (2.5 µM). Consumption of O_2 for each step was then extracted using DatLab version 7.0 software. RBCs from 8 SCA patients and 5 healthy controls after the Percoll separation were used for these experiments.

Statistical analysis

Statistical analyses were performed using Graphpad Prism (version 9.0; Graphpad software, San Diego, California, USA) and *R* v.4.0.2 (http://www.R-project.org/) for mitochondrial respiration. No criteria or threshold exist in the literature to decide which patient has high or low mitochondria retention into mature RBCs. Indeed, patients were divided into tertiles according to the percentages of mature RBCs containing mitochondria in the whole RBC population: a group with "high" percentage of RBC containing mitochondria, a group with "intermediary/moderate" levels and a group with a "low" percentage of RBC containing mitochondria. A one-way Anova analysis followed by post-hoc Newman-Keuls comparisons were used to compare the different biological markers between the three groups. An unpaired student t-test was used to compare the Ca^{2+}, CD47, ROS levels and percentages of RBCs with externalized PS levels between mitochondria positive or negative RBCs and a paired student t-test was used to compare mitochondrial superoxide anion^{+} RBCs before and after incubation with Antimycin A. Mitochondrial respiration was analyzed using a linear mixed model with patient status (SCA patient vs. healthy control), respiratory state (*i.e.* endogenous, ATP-inhibited, uncoupled, non-mitochondrial) and their interaction as fixed factors, while including patient identity as a random effect to account for repeated measurements (post-hoc with Tukey's adjustment). Data are expressed as means ± SD. A p value less than 0.05 was considered as statistically significant.

References

1. Esperti S, Boisson C, Robert M, et al. Does G6PD deficiency cause further damage to red blood cells of patients with sickle cell anaemia? Br J Haematol. 2022 Oct;199(2):289-291.
2. Boisson C, Rab MAE, Nader E, et al. Effects of Genotypes and Treatment on Oxygenscan Parameters in Sickle Cell Disease. Cells. 2021;10(4).
3. Rab MAE, Kanne CK, Bos J, et al. Oxygen gradient ektacytometry-derived biomarkers are associated with vaso-occlusive crises and correlate with treatment response in sickle cell disease. Am J Hematol. 2021;96(1):E29-E32.
4. Baskurt OK, Boynard M, Cokelet GC, et al. New guidelines for hemorheological laboratory techniques. Clin Hemorheol Microcirc. 2009;42(2):75-97.
5. Sjovall F, Ehinger JK, Marelsson SE, et al. Mitochondrial respiration in human viable platelets--methodology and influence of gender, age and storage. Mitochondrion. 2013;13(1):7-14.
6. Stier A, Romestaing C, Schull Q, et al. How to measure mitochondrial function in birds using red blood cells: a case study in the king penguin and perspectives in ecology and evolution. . Meth Ecol Evol. 2017;8:1172-1182.