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Oxygen release from hemoglobin has limited effects on mitochondrial respiration measured from red blood cells. Reply to the Comment on *“Increased retention of functional mitochondria in mature sickle red blood cells is associated with increased sickling tendency, hemolysis and oxidative stress”*

Antoine Stier^{1,2*}, Sofia Esperti^{3,4,5}, Elie Nader^{3,4}, Damien Roussel⁶, Philippe Connes^{3,4*}

¹ Université de Strasbourg, CNRS, Institut Pluridisciplinaire Hubert Curien, UMR7178, 67000 Strasbourg, France

² Department of Biology, University of Turku, Turku, Finland

³ Laboratoire interuniversitaire de Biologie de la Motricité (LIBM) EA7424, Team « Vascular Biology and Red Blood Cell » Université Claude Bernard Lyon 1, Université de Lyon, Lyon, France.

⁴ Laboratoire d'Excellence du Globule Rouge (Labex GR-Ex), PRES Sorbonne, 79015 Paris, France.

⁵ Erytech Pharma, 69008 Lyon, France.

⁶ Laboratoire d'Ecologie des Hydrosystèmes Naturels et Anthropisés, CNRS UMR 5023 Université Claude Bernard Lyon 1, Université de Lyon, Lyon, France.

* Corresponding authors: antoine.stier@iphc.cnrs.fr & philippe.connes@univ-lyon1.fr

Disclosures

No conflicts of interest to disclose

Contributions

AS collected and analyzed the data, and wrote the manuscript with input from PC, SE, EN and DR.

Main text

The measurement of mitochondrial aerobic metabolism from blood cells has gained in popularity over the past decade due to its low invasiveness^{1,2}. In non-mammalian vertebrates, red blood cells are nucleated and possess functional mitochondria³, which enables the assessment of mitochondrial respiration from small blood samples (as low as 20 μ L of whole-blood⁴). Recently, we have showed that human mature sickle red blood cells retain some functional mitochondria, which was associated with increased sickling tendency, hemolysis and oxidative stress⁵. Willis et al.⁶ recently questioned the methodology used to demonstrate

the functionality of the mitochondria retained in sickle red blood cells, since hemoglobin-O₂ dissociation could influence the oxygen consumption rate (JO₂) measured with high-resolution respirometry.

As rightly noted by Willis et al.⁶, the PO₂ within the *in-vitro* chamber declines over time due to oxygen consumption by the cells. Such a decline in PO₂ leads to the potential release of O₂ by hemoglobin, to an extent depending mostly on: 1. the change in PO₂, 2. the HbO₂ binding parameters, and 3. the amount of hemoglobin in the chamber. Willis et al.⁶ state that because the PO₂ is progressively falling, the errors will confound not only the absolute rates but also the relative differences between respiratory states (*i.e.* the proof of mitochondrial functionality we used in⁵). Such statement assumes that O₂ dissociation from hemoglobin will be higher at lower PO₂. However, using the same protocol as in previous study on avian red blood cells⁷ Fig. 1 shows that it would not be the case since non-mitochondrial JO₂ (after antimycin A addition) does not vary over a broad range of PO₂ (see also electronic supplementary material (ESM) S1 for raw data). At low PO₂, O₂ release from hemoglobin could have been anticipated as assumed by Willis et al.⁶, which would have led to decreased or even negative (*i.e.* O₂ release > O₂ consumed) JO₂.

Actually, the release of O₂ from hemoglobin will not directly depend on the PO₂, but on the instantaneous rate of change in PO₂ linked to oxygen consumption (*i.e.* JO₂ in a given state), and on the position in terms of absolute PO₂ on the hemoglobin-O₂ dissociation curve. As supposed by Willis et al.⁶ based on Stier et al.¹ and Esperti et al.⁵, our measurements on both avian species and human were conducted at high PO₂ (Fig 2A, see raw data in ESM S2), enabling to remain within the linear and almost flat portion of the hemoglobin-O₂ dissociation curve (Fig. 2B). Extracting the data from Abdu et al. (2008)⁸ and Powell (2015)⁹ for human and birds (Fig. 2B, ESM S3), respectively, enabled to calculate the change in hemoglobin saturation (%) linked to JO₂-induced changes in PO₂, and the associated release of O₂ for each respiratory state (see ESM S4 and S5 for calculations). Based on these calculations, we can note that the release of O₂ is relatively minor (difference between JO₂ and JO₂ corrected in Fig. 2C and 2D). While absolute respiration rates are affected to a minor extent (Fig. 2C & 2D), the relative differences between respiratory states are not, as demonstrated for instance by the similarity between OXPHOS coupling efficiencies calculated from raw vs. corrected JO₂ values (*i.e.* for Japanese quail: raw = 0.731 ± 0.013 vs. corrected = 0.730 ± 0.013 ; for human sickle red blood cells: raw = 0.437 ± 0.065 vs. corrected = 0.424 ± 0.061). Since O₂ release is proportional to JO₂ and

influenced by hemoglobin content (that does not vary between the different respiratory states), it is not surprising from our perspective that the relative differences between respiratory states are not influenced by O₂ release from hemoglobin, as long as the assay is conducted within the linear and almost flat part of the Hb-O₂ dissociation curve (Fig. 2B).

As rightly pointed out by Willis et al.⁶, issues can arise when two groups have different Hb contents and/or O₂ binding kinetics. This is however unlikely to confound the results presented in Esperti et al.⁵ because O₂ binding kinetics does not vary between healthy and sickle red blood cells in normoxic conditions⁸ (> 92.5% saturation, PO₂ > 65 mm Hg), and Hb content (and thus potential O₂ release) is lower in sickle cell patients than healthy individuals¹⁰.

Willis et al.⁶ also rightly questioned the choice of Mir05 as a respiratory medium and the lack of exogenous substrate (*i.e.* glucose) when assessing mitochondrial respiration of 'intact' red blood cells. Respiration of intact blood cells can for instance be conducted in PBS or plasma¹¹, but our own experience with avian blood cells shows that mitochondria loose functionality along the assay with PBS, as evidenced by a FCCP-induced respiration being lower than the endogenous respiration, which does not happen when using Mir05. Mir05 also enables to first measure the endogenous respiration and then to permeabilize the red blood cells for more detailed investigation¹². From our perspective, refraining from using exogenous substrates enables the measurement of mitochondrial respiration rates being more closely related to the *in-vivo* physiology, where substrates are usually not at saturating levels. Using the subject's own plasma¹¹, whenever possible, is likely the best way to obtain the more meaningful information about *in-vivo* mitochondrial metabolism. Regarding the amount of red blood cells used in respirometry assays, Willis et al.⁶ also question the precision of pipetting packed red blood cells, which we have questioned before (see¹). Counting red blood cells is likely the best approach possible (as done in^{4,12}).

Around 75% of the patients included in our study⁵ were under hydroxyurea therapy, and all patients from the mitochondrial respiration experiments received this treatment. Hydroxyurea has recently been shown to promote erythroid differentiation by accelerating maturation processes¹³, which may impact the degree of mitochondrial retention into mature red blood cells. To answer this question, a study would be needed to test the degree of mitochondrial retention into mature red blood cells and their functionality before and after hydroxyurea therapy.

In light of the excellent comment by Willis et al.⁶, here are some recommendations that should be useful to ensure the best accuracy when measuring mitochondrial respiration from red blood cells: 1. provide the range of PO₂ at which JO₂ measurements are conducted; 2. ensure that measurements are conducted at a PO₂ being within a linear and almost flat part of the Hb-O₂ dissociation curve; 3. check for potential bias between experimental groups in terms of Hb content or O₂ binding kinetics; 4. measure Hb in individual samples to correct JO₂ if deemed necessary based on theoretical expectations (*e.g.* if Hb content is high and variable between samples) or if a difference exists between groups in terms of Hb content.

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Figure legends

Fig. 1: Relationship between non-mitochondrial JO_2 and PO_2 in Japanese quail (*Coturnix japonica*) red blood cells measured *in-vitro* with high-resolution respirometry (N = 2 biological replicates per PO_2 ; mean \pm SD). Measurements were conducted according to the methodology described in Stier et al.⁷, and the various PO_2 at which non-mitochondrial JO_2 was measured were achieved by letting intact red blood cells consume more or less O_2 within the chamber before adding antimycin A.

Fig. 2: Relationship between PO_2 and JO_2 in red blood cells of Japanese quail (blue) and human with sickle cell disease (red): A. Raw JO_2 measurements and the associated PO_2 during measurement; B. Hemoglobin-oxygen dissociation curves, redrawn from Powell⁹ and Abdu et al.⁸; C. Comparison of raw JO_2 and JO_2 corrected for O_2 release by hemoglobin for Japanese quails; D. Comparison of raw JO_2 and JO_2 corrected for O_2 release by hemoglobin for human sickle red blood cells. N = 8 biological replicates for each species, data from Stier et al.⁷ and Esperti et al.⁵ re-analyzed (mean \pm SE).