Does hemoglobin affect measures of mitochondrial respiration in red blood cells? Comment to "Increased retention of functional mitochondria in mature sickle red blood cells is associated with increased sickling tendency, hemolysis and oxidative stress"

We are intrigued by the Connes group's interesting study of the retention of mitochondria in mature red blood cells (RBC) in 61 patients with sickle cell anemia (SCA).[1] Using the sophisticated techniques of Image Stream (Amnis, MK II) and Mitotracker Red CMXRos Dye (Invitrogen) in combination with flow cytometry, they reported that a significant percentage of mature sickle cells in these patients retained mitochondria.[1] This presence of mitochondria in human erythrocytes is a unique phenomenon, heretofore considered to be a characteristic reserved for red cells in nonmammalian vertebrates (e.g., avians, fish, amphibians, reptiles).[2]

However, we were particularly fascinated by one of their primary aims; specifically, "to investigate the functionality of these mitochondria".[1] Mitochondrial function was assessed with high-resolution respirometry, which according to the authors, resulted in "detectable mitochondrial oxygen consumption in sickle mature RBC, but not in healthy RBC." The authors concluded that their data showed the presence of functional mitochondria in mature sickle RBC, "which could favor RBC sickling and accelerate RBC senescence, leading to increased cellular fragility and hemolysis".[1] The presence of functional mitochondria in human erythrocytes is an interesting observation with important implications. For example, the Connes group noted that the propensity of RBC to sickle when deoxygenated was greater in the SCA subgroup with a high percentage (13%) of mitochondria retained in mature RBC.[1] These are provocative findings with great potential for both increased understanding of basic mechanisms and application to practice.

Oxygen consumption rate (J_{O2}) is, of course, a valid indicator of mitochondrial function, particularly when flux is modified by agents known to inhibit or accelerate various steps in the oxidative pathway, as was done by the Connes group here.[1] However, in such assessments, care must be taken to ensure the validity and accuracy of the J_{O2} measurement itself. The Connes group used intact red cells in their polarographic measurement of RBC mitochondrial J_{O2},[1] basically following the methods of Sjövall *et al.*[3], and Stier *et al.*[2], with Stier being a co-author of this current study. Briefly, 100 μL of packed RBC was added to 1 mL of a potassium-based respiratory buffer (MiR05, see below), and transferred into an Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria) set at 37°C and containing another 1 mL of MiR05. Subsequently, a variety of standard mitochondrial respiratory measures were made, all based on J_{O2}. Of significant concern here is the O_2 associated with hemoglobin; i.e., oxyhemoglobin (HbO_2), in the red cells incubating in these respiration chambers. This store of O_2 was apparently ignored in the authors' calculation of J_{O2}. The errors introduced by this omission are potentially quite large and variable depending on hemoglobin content, O_2 binding parameters (e.g., P_{50}), the oxygen tension (PO_2) at which the analysis is being done, and the duration of a given assay. The discussion below will be restricted to a simple presentation of the overall problem. Basic concepts and details related to how the Oroboros respirometer calculates J_{O2} are clearly and completely described in the user's manual.

Clark type O_2 electrodes such as that used in the Oroboros instrument generate an electrical signal proportional to the oxygen tension, PO_2 (mmHg), which, in turn, is proportional to the concentration of dissolved O_2 ($nmolO_2 \cdot mL^{-1}$ or μM). As stated in Sander:[4] "... the amount of dissolved gas is proportional to its partial pressure in the gas phase. The proportionality factor is called Henry's law constant." The value of the Henry constant ($\mu mol \cdot mmHg^{-1}$); i.e., solubility, depends on temperature and other factors such as ionic strength. Pure water at 37°C exposed to (and in equilibrium with) room air at 1.0 atmosphere pressure contains about 213 μM of dissolved O_2 (213 $nmolO_2 \cdot mL^{-1}$).[4] If instead, MiR05 medium is the solution of interest, the higher ionic strength reduces O_2 solubility ("salting out"). In the Oroboros literature this is taken into account by the "FM" factor, which is 0.92 for MiR05. Accordingly, in our example: $[O_2]=0.92 * 213\ \mu M=196\ \mu M$ at 1.0 atmosphere and 37°C. This value is roughly similar to the initial $[O_2]$ shown in Figure 2 in Stier *et al.*.[2] If we now multiply 196 nmol $O_2 \cdot mL^{-1}$ by a total medium volume of 2.0 mL, we get 392 nmol O_2 in the respiratory chamber when the assay begins. Below we will compare this soluble O_2 mass to the O_2 mass bound to Hb.

When gas exchange with the environment is prohibited in the Oroboros, O_2 consumption decreases the PO_2 hence proportionally decreasing the electrode signal. The progressive fall in PO_2 across time ($mmHg \cdot min^{-1}$), linked by the Henry constant ($\mu mol \cdot mmHg^{-1}$) to the corresponding fall in

[O_2] (μM or nmol O_2·mL^{-1}), along with the medium volume (mL), allows the calculation of J_{O2} (nmol O_2·min^{-1}). Such calculations have been routinely made and reported in a vast literature, which began rapidly expanding shortly after Leland Clark developed his O_2 electrode with a cellophane covering in the 1950s.[5]

However, the presence of an O_2 buffering molecule such as Hb in the respiratory chamber complicates the above straightforward and linear relationships in two ways: i) the Hb mass available to bind O_2 in each assay must be determined, and ii) the HbO_2 binding parameters must be evaluated. Obviously, accounting for the utilization of this O_2 pool will involve a non-linear relationship between the electrode signal and O_2 concentration, and thereby J_{O2}. We can approximate the Hb-bound O_2 by assuming a normal mean corpuscular hemoglobin concentration of 34 g·dL^{-1} (similar to the SCA patients in Figure 6C[1] if the 10-fold unit error is overlooked), and a Hb molecular weight of 67,000 g·mol^{-1}. With these values, the 100 μL of RBC in the assay contains a Hb mass of 0.507 μmol. At the onset of the assay, assuming 1.0 atmosphere barometric pressure, 0.2095 mole fraction of O_2 in dry air, and a 37°C assay temperature (thus 47.1 mmHg water vapor pressure at the air:water interface), gives a medium (MiR05) PO_2=(760-47.1)x0.2095=149 mmHg at the start of the assay. Thus, the Hb will initially be almost fully saturated, holding 4 O_2·Hb^{-1}x0.507 μmol Hb=2,030 nmol O_2, which is 5.2 times greater than the soluble O_2 in the medium (see above). As J_{O2} commences early in the assay the medium PO_2 will fall, drawing predominantly from the soluble pool. With time and ongoing J_{O2}, however, the continuous fall in PO_2 will elicit significant O_2 dissociation from Hb, which will dampen changes in PO_2 and result in an underestimation of the true JO_2 (if O_2 bound to Hb is ignored). Because in a given assay the PO_2 is progressively falling, the errors will confound not only the absolute rates but also the relative differences between what Stier *et al.*[2] call "routine, oxphos, leak, ETS, and non-mito" O_2 consumption. In a comparative study, like that of Esperti *et al.*[1], such errors may be amplified when the two RBC groups have different Hb contents and/or O_2 binding kinetics. In any case, standard polarographic procedures used to measure J_{O2} by red blood cells must be viewed with extreme caution.

In practice, the mitochondrial respiration measurements may be largely made at high partial pressures of O_2 where the oxyhemoglobin dissociation curve is quite flat.[6] However, a decrease of even 0.5% in percent saturation would release ≈5.2 nmol of O_2, an amount that would be about one-third of the decline in O_2 content occurring during "endogenous" mitochondrial function in king penguin RBC; see Figure 2 in Stier *et al.*[2] Of course, the further the PO_2 declines during the respiratory measurements, the closer the approach to a steeper portion of the HbO_2 dissociation curve. Accordingly, we propose that hemoglobin must be accounted for in quantitative measures of mitochondrial respiration in RBC.

We also noted that RBC were suspended in 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^{-1}], pH 7.1), a solution commonly used for studies of mitochondria, which are intracellular organelles.[1] Accordingly, we wondered i) whether a respiratory solution more closely resembling plasma might be more appropriate for RBC, and ii) whether glucose should be provided as a fuel.

Further, for quantitative mitochondrial respiratory analyses, it is important to be certain about the volume of RBC used in the assays. In this context, how an exact volume of RBC is assayed is unclear. If packed RBC are transferred, air displacement pipetting is error-laden, and if packed cells are resuspended, the amount of supernatant retained with prior centrifugation is uncertain.

Finally, we saw no mention of any pharmacotherapy for the SCA patients. Would any such treatments have potential ramifications for either the presence or function of mitochondria in the RBC of this group?

Once again, we acknowledge and appreciate the research of Esperti *et al.*,[1] but we also believe that their methods for mitochondrial respirometry deserve a candid discussion.

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Contributions

LBG conceived the idea. WTW, ACB, and LBG reviewed the literature, and discussed the basic principles. WTW and LBG performed the calculations. LBG wrote the first draft. WTW, ACB, and LBG reviewed and revised all subsequent drafts. WTW, ACB, and LBG read and approved the final version of the manuscript.

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