Diabetic Complications Consortium

Application Title: Cellular Bioenergetics as a Predictor of Diabetic Nephropathy

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1. Project Accomplishments:

- Established Bioenergetic Measurements in Leukocytes and Platelets in 35 Chronic Kidney Disease Patients.
- Developed the Bioenergetic Health Index (BHI) as a novel measure of the bioenergetic function of DKD patients.
- Demonstrated significant differences in Bioenergetics and BHI in DKD patients compared to healthy controls.
- Published 3 articles describing the methods and concepts developed during the course of the project.

Specific Aims:

Specific Aim 1: Determine the Bioenergetic Profiles in monocytes, platelets, and lymphocytes from patients with chronic kidney disease (DKD) Stage, 3b, compared to Stages 4 and 5.

Results: During the course of the study we were able to recruit 35 patients, to date, as they have presented to the DKD clinic as shown in Table 1. The majority of these were Stage 3 and were stratified according to their glomerular filtration rate. From blood samples the platelets, monocytes, lymphocytes and neutrophils were isolated and their bioenergetic profiles determined. No Stage 5 patients were recruited.

Table 1: Patient Demographics for DKD

Stage 3a	Stage3b	Stage 4	Age	Sex	Race
11	9	15	62 ± 0.8	F11, M24	AA 14 EA 21

Establishing and Interpreting the Cellular Bioenergetic Profile

The bioenergetic profiles were measured from the patients using the cellular mitochondrial function assay or stress test shown in Figure 1. Parameters from the cellular mitochondrial function assay (Figure 1) give insights into different aspects of mitochondrial function and below we discuss how these can be used to calculate the Bioenergetic Health Index or **BHI**. An important aspect of these mitochondrial parameters that can be measured from this assay is that they are potentially interactive, and taken together, can serve as a sensitive indicator of the response of cells to oxidative stress and the changing metabolic programs associated with their role in inflammation.

Basal oxygen consumption rate (OCR): The first measurement is the basal oxygen consumption rate (OCR) measured in the cells prior to injection of mitochondrial

inhibitors. Changes in basal OCR in the DKD patients relative to normal subjects can be interpreted with the information obtained from the rest of the profile.

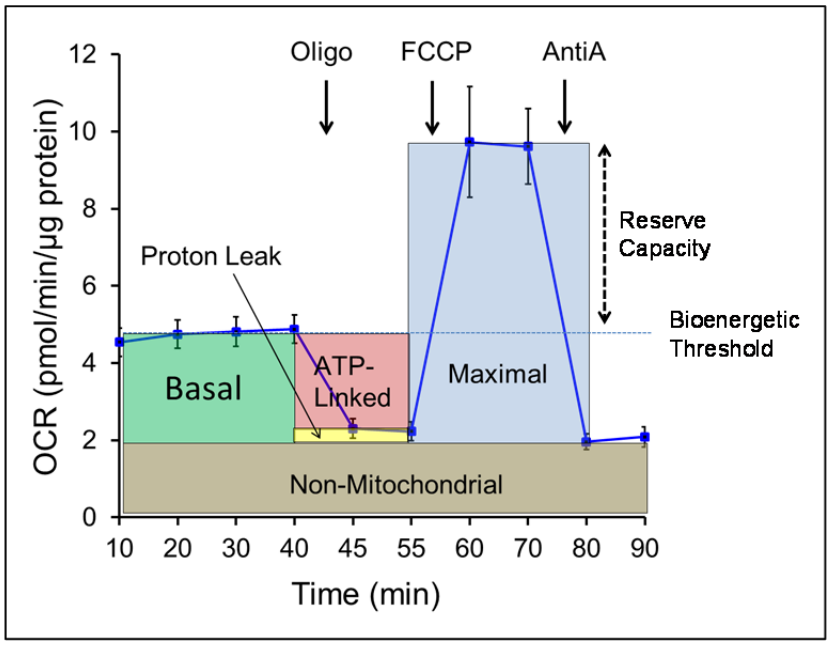

Figure 1: Cellular Mitochondrial Profile in Human Monocytes. *This assay defines cellular mitochondrial function using the well-defined inhibitors, oligomycin (Oligo), FCCP and antimycin A (AntiA). The interpretation of the different parameters defined by the assay is described in the accompanying text. Data is typically normalized to total protein or cell number in each well. n = 3-5, mean ± SEM.*

ATP-linked OCR and Proton Leak: After basal measurements are recorded, cells are exposed to oligomycin; an inhibitor of the ATP synthase. By inhibiting proton flux through this enzyme, the increased proton gradient across the mitochondrial inner membrane prevents electron transport through Complexes I-IV. Oxygen consumption then decreases accordingly. The remaining rate of mitochondrial respiration represents proton leak; i.e., protons pumped during electron transport that result in oxygen consumption but not ATP production. An increase in the ATP-linked OCR would indicate an increase in ATP demand, whereas a decrease would indicate either low ATP demand, a lack of substrate availability, and/or severe damage to oxidative phosphorylation, which would impede the flow of electrons and result in a lower OCR.

An increase in apparent proton leak could be due to a number of factors including increased uncoupling protein (UCP) activity, damage to the inner mitochondrial membrane and/or ETC complexes. This results in the leakage of protons into the matrix and oxygen consumption in the absence of normal proton translocation across the inner mitochondrial membrane by Complexes I, III, and IV, a process known as electron slippage. Increased calcium transport can also manifest as a change in proton leak. We have also shown that oxidative stress modifies the bioenergetic parameters and also increase ATP-linked oxygen consumption and proton leak.

Maximal OCR and Reserve Capacity: An uncoupler, such as FCCP (p-trifluoromethoxy carbonyl cyanide phenyl hydrazone), is next used to estimate maximal respiration; however, respiratory substrates are provided by cellular metabolism, which can be physiologically limiting. A high FCCP-stimulated OCR compared to basal OCR indicates that the mitochondria are using less than the maximal rate of electron

transport that can be supported by substrate supply from the cells. As shown in Figure 2, basal respiration can be considered a threshold below which the cell cannot sustain oxidative phosphorylation to meet energy demand. In support of this, we have demonstrated with mitochondrial inhibitors that reserve capacity is decreased by oxidative stress and if this threshold activity cannot be met, glycolysis is then stimulated to meet the energetic needs of the cell. The difference between the basal and maximal respiration is called the spare or reserve bioenergetic capacity. The reserve capacity concept is well established in the literature. For example, it has been shown in the heart that under an increased work load in the physiological range, mitochondria have a substantial "reserve capacity", which is depleted under conditions of severe stress including pressure overload or ischemia. More recently, we have shown that under conditions of oxidative stress the reserve capacity is depleted, and if the threshold for the basal respiration is breached then cell death occurs.

Whether cells can utilize the maximal electron transport activity for ATP synthesis will depend on the capacity of the components of oxidative phosphorylation system, including the ATP synthase, which may be limiting. Taken together, it is clear that reserve bioenergetic capacity is a cell- and context- dependent parameter intimately linked to bioenergetic health whether it is utilized for ATP synthesis or other mitochondrial functions. Importantly, a low maximal capacity could indicate decreased substrate availability or that mitochondrial mass or integrity is compromised. From a translational perspective, bioenergetic alterations in monocytes and lymphocytes are also linked to their changing biology during the progression of the inflammatory process.

Non-mitochondrial OCR: This parameter is an index of oxygen consuming processes which are not mitochondrial. In leukocytes, non-mitochondrial OCR is typically attributed to enzymes associated with inflammation, including cyclooxygenases, lipooxygenases and NADPH oxidases, and regarded as negative indicators of bioenergetic health. We have shown that non-mitochondrial OCR varies, and typically increases in the presence of stressors, including ROS and RNS and it is well established that mitochondria are a target for the deleterious effects of these reactive intermediates.

The Calculation of BHI:

The equation used to calculate BHI in this study is shown below and captures positive aspects of bioenergetic function (reserve capacity and ATP linked respiration) and contrasts these with potentially deleterious aspects (non-mitochondrial oxygen consumption and proton leak). The first term in the numerator is the reserve capacity. The larger the value for reserve capacity the more effectively mitochondria can meet both the ATP needs of the cell and increased energetic demand and ionic or metabolic stress.

Equation 1:

$$\text{BHI} = \frac{(\text{Reserve Capacity})\,(\text{ATP linked})}{(\text{Non-mitochondrial})(\text{Proton Leak})}$$

The second term in the numerator, ATP-linked respiration, is a measure of the capacity of the cell to meet its energetic demands (**Figure 1**). For the denominator, the proton leak decreases mitochondrial efficiency with respect to ATP generation and is then a negative term. The final term in the denominator is the non-mitochondrial respiration. Non-mitochondrial oxygen consuming processes are not well defined but in these cells they are predominantly those that originate from pro-oxidant and pro-inflammatory enzymes such as cyclooxygenases, cytochrome P450s or NADPH oxidases. Since increased activity of these processes can damage mitochondria then we propose that BHI will decrease under conditions of inflammation.

In general, defects in the electron transport chain will result in a lower BHI because of lower reserve capacity, ATP linked respiration or increased uncoupling. It is important to note that cells which show a decrease in both reserve capacity and an increase in proton leak and non-mitochondrial respiration can still potentially provide sufficient ATP to meet the metabolic demands of the cell but less efficiently. For this reason, BHI has prognostic value because it can identify a progressive deterioration in bioenergetic health before the threshold at which failure to meet energy demand occurs.

Monocyte Bioenergetics is impaired in DKD patients:
As shown in **Figure 2**, diabetics with DKD have altered bioenergetic responses in monocytes compared to healthy individuals. Specifically, DKD patients have a lower maximal respiratory response to FCCP and a lower reserve capacity compared to healthy volunteers. These data support the primary hypothesis of the pilot project that the metabolic effects of renal dysfunction are reflected in cellular bioenergetics.

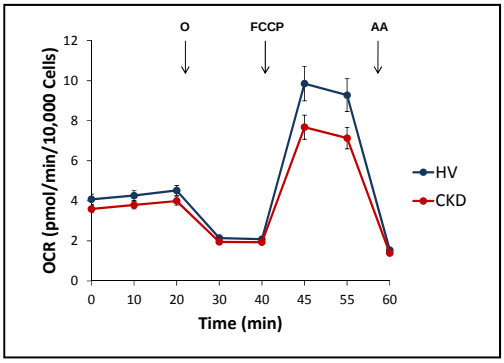

Figure 2: Cellular bioenergetics in isolated monocytes from healthy human volunteers and DKD patients. *(A) The oxygen consumption rate (OCR) of monocytes seeded at 150,000 cells per well in 96-well Seahorse plates. Following plating, cells were allowed to equilibrate for 30 min at 37°C prior to measuring basal OCR and the effects of the mitochondrial inhibitors: oligomycin (O), FCCP (F), and antimycin A (A) on OCR. panel A for each individual healthy volunteer or DKD patient. Results are mean ± SEM, n=3-6 per group, *p<0.05 compared to healthy volunteers.*

<u>BHI is depressed in monocytes from patients with DKD:</u> The data from Figure 1 was used to calculate the BHI in monocytes and was found to be significantly lower in the DKD patient population (stages 3-4 combined) compared to healthy controls (**Figure 3A**). At this point in time we do not have enough patients in this cross-sectional study to determine whether BHI is significantly different between Stage 3, 3b or 4. However, we could divide the patients with stage 3 or 4 into 2 groups; those with a high BHI (10-25) and those with low BHI (1-10). Next we used the HbA1c levels in

each diabetic patient as an assessment of diabetic control. Typically the higher the HbA1c levels are the worse the state of the disease.

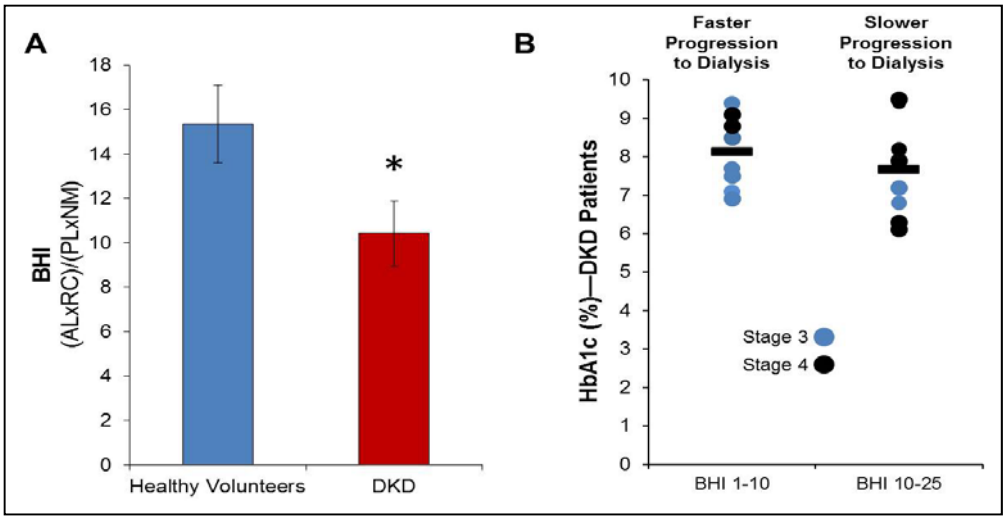

Figure 3: The BHI as a potential marker of disease progression in healthy volunteers and DKD patients. *Using the equations for BHI described above, the BHI was calculated for 26 healthy subjects and 19 DKD patients with stage 3-4 renal disease. (A) The mean BHI value for each group is shown. Data presented as mean±s.e.m. *P≤0.05 significantly different from healthy control group. (B) HbA1c levels plotted according to BHI values in DKD patients. The hypothesis is that the patients with lower BHI will have a faster progression of the disease and the need for dialysis. Data presented as mean±s.e.m., n=16 DKD.*

As shown in **Figure 3B,** HbA1c was not significantly different for patients with high or low BHI. Also shown in this Figure are the distribution of patients with Stage 3 and 4 renal diseases between high and low BHI. As we expected patients with Stage 3 and 4 DKD were distributed between both the high and low BHI group. The hypothesis is that those patients with lower BHI will progress to renal failure more rapidly. Since this is a cross-sectional study we do not have the data for BHI and the rate of progression which will be the purpose of the current proposal. ***The data support the feasibility that we can test the hypothesis that patients with a low BHI will progress to dialysis faster than those with a High BHI which will serve as the basis of an application for a longitudinal study to the NIH.***

We will continue to collect patients to increase the power of the study and prepare a manuscript for submission once this is achieved.

2. Publications:

1. Kramer, P.A., Saranya Ravi, Balu K. Chacko, Michelle S. Johnson and Victor M. Darley-Usmar. (2014) A review of the Mitochondrial and Glycolytic Metabolism in Human Platelets and Leukocytes; Implications for their use as Bioenergetic Biomarkers. Redox Biology 2:206-210.
2. Ravi S, Mitchell T, Kramer PA, Chacko B, Darley-Usmar VM. (2014) Mitochondria in monocytes and macrophages-implications for translational and basic research. Int J Biochem Cell Biol. 53:202-7.
3. B.K. Chacko, P.A. Kramer, S. Ravi, M.S. Johnson, R.W. Hardy, S.W. Ballinger, V.M. Darley-Usmar. (2013) Methods for defining distinct bioenergetic profiles in platelets, lymphocytes, monocytes, and neutrophils, and the oxidative burst from human blood, Laboratory investigation. 93(6):690-700. (most read article in Clinical Science and Featured in the New Scientist).