Redox Biology 2 (2014) 656–660

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Method

A method for assessing mitochondrial bioenergetics in whole white adipose tissues

Kimberly J. Dunham-Snary[a,1], Michael W. Sandel[b,1], David G. Westbrook[a], Scott W. Ballinger[a,*]

[a]Department of Pathology, Division of Molecular and Cellular Pathology, University of Alabama at Birmingham, Birmingham, AL 35294, United States of America
[b]Department of Biostatistics, Section on Statistical Genetics, The University of Alabama at Birmingham, Birmingham, AL 35294, United States of America

ARTICLE INFO

Article history:
Received 21 March 2014
Received in revised form 14 April 2014
Accepted 15 April 2014

Keywords:
Mitochondrion
White adipose tissue
Bioenergetics

ABSTRACT

Obesity is a primary risk factor for numerous metabolic diseases including metabolic syndrome, type II diabetes (T2DM), cardiovascular disease and cancer. Although classically viewed as a storage organ, the field of white adipose tissue biology is expanding to include the consideration of the tissue as an endocrine organ and major contributor to overall metabolism. Given its role in energy production, the mitochondrion has long been a focus of study in metabolic dysfunction and a link between the organelle and white adipose tissue function is likely. Herein, we present a novel method for assessing mitochondrial bioenergetics from whole white adipose tissue. This method requires minimal manipulation of tissue, and eliminates the need for cell isolation and culture. Additionally, this method overcomes some of the limitations to working with transformed and/or isolated primary cells and allows for results to be obtained more expediently. In addition to the novel method, we present a comprehensive statistical analysis of bioenergetic data as well as guidelines for outlier analysis.

Introduction

Metabolic syndrome is a growing epidemic in the United States and worldwide. Currently, approximately 34% of the US population are living with this diagnosis [1,2] and it is expected that this number will continue to increase. By 2030, it is anticipated that the financial burden of healthcare for patients requiring treatment for diabetes, heart and stroke will be $48–66 billion annually [3]. One of the hallmarks of metabolic syndrome is central obesity, an increase in visceral white adipose tissue (WAT) around the midsection. Central obesity not only contributes to metabolic syndrome but also increases individual risk for type II diabetes mellitus (T2DM) and cardiovascular disease (CVD) [4].

WAT has classically been viewed as a storage organ, containing metabolic substrates in the form of triglycerides as well as insulation and cushioning for the internal organs [5]. A more contemporary perspective that has surfaced over the last decade is one of WAT being an endocrine organ and a major factor in metabolism and homeostasis [6–9]. For example, in addition to triglyceride storage, WAT secretes numerous hormones and cytokines (adiponectin, leptin, TNF-α, etc.) [9]. Coupled with this viewpoint of WAT is the hypothesis that numerous cardiometabolic pathologies have mitochondrial dysfunction as a contributing factor [10–13]. Mitochondria are central to metabolism, yet prone to damage that can adversely affect their function [14,15]. The assessment of mitochondrial function in WAT has been identified as a key target for T2D and obesity [16] and current studies utilize both isolated adipocyte mitochondria [17], immortalized adipocyte cell lines [18], and isolated primary cells [19]. Isolation of mitochondria from WAT requires multiple steps and results in measures of organelle function in the absence of its native cellular environment. Also, functional measures of immortalized/isolated cells that have been manipulated to allow for multiple passages may substantially alter aspects of mitochondrial function. A method for direct assessment of mitochondrial function from whole WAT would provide an alternative or complementary approach for exploring adipose bioenergetics. In this regard, methods for mitochondrial bioenergetic measurement from whole adipose tissue have been previously reported [20,21] but have certain limitations in that certain parameters of mitochondrial function could not be defined.

A methodology herein is provided that utilizes minimal amounts of tissue and increased number of timepoint collections to ensure tissue equilibration. This approach allowed the measurement of additional parameters of mitochondrial function including ATP-linked, maximal oxygen consumption and reserve capacity. Additionally, a reproducible data filtering method was employed to account for among replicate variance often seen when analyzing whole tissue

[1] These authors contributed equally to this work.

* Correspondence to: Department of Pathology, Division of Molecular and Cellular Pathology, University of Alabama at Birmingham, Birmingham, BMR2 530, 1720 2nd Avenue South, Birmingham, AL 35294-2180, United States of America.
E-mail address: sballing@uab.edu (S.W. Ballinger).

2213-2317/$ - see front matter © 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-SA license (http://creativecommons.org/licenses/by-nc-sa/3.0/).
http://dx.doi.org/10.1016/j.redox.2014.04.005

samples (relative to isolated cells or mitochondria), presumably due to differential absorptive properties of whole tissue versus isolated cells. Therefore, the utility of the methodology and approach presented should aide those interested in evaluating raw bioenergetic data obtained from whole tissues including WAT.

For these studies, mitochondrial bioenergetics was compared between two WAT depots, inguinal (iWAT) and epididymal (eWAT) from C57BL6/J mice; a mouse model classically used as a control for cardiovascular and metabolic disease studies.

Methods

Acquisition and preparation of adipose tissue

12-Week old male C57BL6/J mice were anesthetized by step-wise chloral hydrate overdose and euthanized via removal of the heart and exsanguination. The Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham approved all procedures involving animals. Inguinal (iWAT) and epididymal (eWAT) adipose depots were excised and placed in small petri dishes with "rinse media" comprised of DMEM with 25 mM glucose and 25 mM HEPES. Tissue was gently agitated to remove hair and blood and placed in clean rinse media. Each fat pad was sampled six times using a 2 mm UniCore Harris Punch (Whatman, Piscataway, NJ), resulting in 2 mg tissue samples. Attention was made to avoid any visible microvasculature during sampling and tissue punches were taken from the same section of tissue as close together as possible. Tissue punches were kept in fresh rinse media until application to an XF24 Islet Capture Microplate (Seahorse Bioscience, North Billerica, MA).

Application of tissue to assay plate

An XF24 Islet Capture Microplate Screen was loaded onto the Screen Applicator Tool and the tool was positioned with the screen facing up. A total of 4 mg of tissue (2 tissue punches) from each fat pad were placed onto the screen with forceps, resulting in three tissue replicates per fat pad. The screen was then snapped into the appropriate well of the Islet Microplate. Rinse media was added to each well after application of the tissue and screen. After applying all tissue samples and rinse media, each well was rinsed two additional times. After removal of the final rinse, running media (DMEM with 25 mM glucose and without HEPES) was added and removed to remove any remaining rinse media. Finally, 450 μL of running media was added to all sample and control wells.

Mitochondrial effectors

After a series of titration experiments for each mitochondrial effector, the following working concentrations were determined to elicit maximal responses: 8 μg/mL oligomycin, 8 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), 12 μM antimycin A (AA), and 3 μM rotenone (Rote). The effects of these mitochondrial inhibitors on oxygen consumption rate (OCR – pmol oxygen per minute) were assessed approximately 1 h after commencement of basal oxygen consumption measurements. After multiple test experiments, the number of basal measurements was increased to both allow for tissue settling and equilibration on the islet capture microplate.

XF24 Extracellular Flux Analyzer raw data output was transformed to OCR using the "Level (Direct) Akos" algorithm in a middle point mode, as previously described [22]. Cellular bioenergetic parameters including ATP- and non-ATP- linked oxygen consumption as well as maximal oxygen consumption and reserve capacity were calculated as previously described [23]. Briefly, ATP-linked oxygen consumption was calculated as the difference between basal oxygen consumption and OCR measured after the addition of oligomycin to inhibit complex V (ATP synthase). Non-ATP- linked oxygen consumption was the remainder of the basal OCR (minus the AA/Rote minimum rate which is defined as non-mitochondrial oxygen utilization). Maximal OCR was determined by subtracting the AA/Rote minimum reading from the maximal reading following addition of FCCP. Reserve capacity (or, spare respiratory capacity) is calculated by subtracting the basal oxygen consumption rate (respiration rate prior to addition of compounds) from the maximal oxygen consumption after the addition of FCCP (potential maximum respiratory capacity). It is hypothesized that reserve capacity exists as a compensation mechanism for cells when presented with increased workload or as a stress response [23]. A sample OCR trace from WAT is shown in Fig. 1.

Data summarization and exploratory observations

Raw OCR trace files were grouped according to eWAT and iWAT, and visualized using Microsoft Excel 2011. Trace data from each sample was divided into five time periods, from which seven OCR variables were calculated (basal, mitochondrial, non-mitochondrial, ATP linked, non-ATP linked, maximum, and reserve capacity) (Fig. 1). Each OCR measurement consisted of 3 min of mixing; 2 min wait time and 3 min of continuous measuring of O_2 levels. OCR values corresponding to the equilibration period were ignored, due to physiological acclimation of the tissues to the well microenvironment. OCR values and slopes for each time period were summarized as the mean value across measurements, and the coefficient of least-squares regression, in order to assess the distribution of variance among samples.

Quality control

Exploratory observations of reserve capacity revealed a high degree of variance among OCR values within eWAT and iWAT data sets (Table 1). Reserve capacity was selected as the first variable to analyze as a result of the maximal oxygen consumption being the most discriminatory during analysis of experiment success. To test whether this variance represented biological (among sample) variance, or methodological imprecision (among replicates), single factor ANOVAs were conducted on both groups using individual mouse identification number as the explanatory categorical variable. For this and all subsequent statistical analyses, a significance level (α) of 0.05 was assumed. The ANOVA failed to detect a significant individual (mouse) effect on reserve capacity in the eWAT ($p > 0.4$) and iWAT ($p > 0.4$) groups (Fig. 2). The effect of well position on the XF-24 plate was also evaluated as a possible source of measurement bias, and an ANOVA was conducted on pooled trace data using well number as the explanatory variable. This analysis also failed to detect a significant effect on reserve capacity ($p > 0.8$). Together, the ANOVA results suggested that a majority of among-replicate variance in basal slope could not be explained by the well position on the XF-24 plate or variation among animals. Therefore it was surmised that the majority of observed variance is the product of some unanticipated source of methodological imprecision, which is a potential source of noise that may impede detection of real biological patterns.

Consequently, it was necessary to filter data according to a classification method that would differentiate acceptable and unacceptable samples. Replicate traces for each mouse were ranked by reserve capacity, and pooled by rank (e.g. "maximum", "median", and "minimum"). Reserve capacity values were pooled according to rank to compare distribution parameters (symmetry, kurtosis, skew, etc.). Exploratory analyses identified a heavy tailed distribution and unacceptable coefficient of variation for pooled reserve capacity data, which was likely due to bias in the minimum group (Table 2). This suggested that a majority of samples with the lowest reserve capacity measurements were affected by nonrandom effects, interpreted here as interference from unidentified non-biological factors. Right skewed data presents a challenge for certain classification schemes,