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Analyzing mitochondrial function in brown adipocytes with a bioenergetic analyzer

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Abstract

Brown adipocytes are a cell type with high mitochondrial content and bioenergetic capacity. A critical means to measure mitochondrial function, macromolecule fuel usage, and other important phenotypes is with a bioenergetic analyzer. Here, we describe how to isolate, culture, and differentiate brown preadipocytes into mature adipocytes. We also explain how to perform a mitochondrial (mito) stress test using the bioenergetic analyzer. The mito stress test is able to give researchers a plethora of insights into mitochondrial function including basal respiration, proton leak, ATP production, maximal respiration, and reserve capacity, making it a powerful tool for analyzing brown adipocytes.

Keywords

brown adipose tissue; Seahorse; mitochondrial function; energy metabolism

1 Introduction

In 2009, several seminal studies demonstrated that adult humans possess brown adipose tissue (BAT) (1–3). Since then, laboratories around the world have focused on using activated BAT as a therapeutic strategy to combat the ever-increasing rates of obesity and metabolic syndrome. The rationale for BAT's use is that it has a high degree of energy expenditure due to its unique expression of uncoupling protein 1 (UCP1), it acts as a metabolic sink, and it secretes metabolically beneficial batokines (4–6). Since many of the effects of BAT activity also overlap with improved aging (7), there is growing interest in using BAT as a means alter lifespan and healthspan. Indeed, several long-lived animals have increased BAT activity (8–12).

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9.Before seeding on a Seahorse plate, the wells should be coated with 0.2% gelatin for approximately 10 minutes. Before seeding, aspirate the gelatin.

10.Seeding cells is an extremely important part of performing Seahorse, meaning that particular attention should be used in this step. Seed 50,000 preadipocytes per well. Since Seahorse analyzes cells only at the bottom of the well, it is advisable to seed the cells in approximately 150 μl, and to bring the total volume up to 500 μl later in the afternoon after the cells have adhered to the plate.

Since respiration and mitochondrial function are critically intertwined with both aging and brown adipocyte function, reliably and reproducibly measuring respiration in cells is critical. This chapter is designed to give an overview of using a bioenergetic analyzer to measure respiration in brown adipocytes using a mitochondrial (mito) stress test (13). A mito stress test consists of measuring basal respiration, respiration after the addition of oligomycin (an ATP synthase inhibitor), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, an uncoupling agent), and antimycin A (inhibits the oxidation of ubiquinone). Due to the actions of these drugs in the mitochondria, researchers can use the mito stress test to calculate basal respiration, proton leak, ATP production, maximal respiration, and reserve capacity as described in Section 3.4. Along with details on performing a mito stress test with mature adipocytes, we provide details on how to isolate preadipocytes from mice (which can be a powerful tool for knockout or transgenic mouse lines of interest), as well as details about culturing preadipocytes and differentiating them into mature adipocytes.

2 Materials

2.1 Preadipocyte isolation and culture reagents

1. Isolation buffer that consists of 123 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 5 mM glucose, 100 mM HEPES, 1% Penicillin/Streptomycin (P/S), and 4% bovine serum albumin (BSA) in ddH_2O (filter through a 0.22 μM filter and stored in 20 ml aliquots at −20°C) (**See** Note 1).
2. Primary cell culture media that consists of 20% fetal bovine serum (FBS), 20 mM HEPES, and 1% P/S in DMEM with high glucose (DMEM-High) (filter through a 0.22 μM filter and store at 4°C) (**See** Note 2).
3. 0.25% Trypsin-EDTA.
4. Phosphate-buffered saline (PBS).
5. Induction media that consists of 2% FBS, 20 nM insulin, 1 nM T_3, 0.125 mM indomethacin, 5 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) in DMEM-High (filter through a 0.22 μM filter) (**See** Note 3).
6. Differentiation media that consists of 2% FBS, 20 nM insulin, and 1 nM T_3 in DMEM-High (filter through a 0.22 μM filter and store at 4°C for up to one month).

2.2 Mito stress test reagents

1. XF calibrant solution.
2. XF assay medium that consists of DMEM-High without sodium bicarbonate at pH 7.4 (filter through a 0.22 μM filter and store at 4°C (**See** Note 4).

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1. Before using the isolation buffer, thaw and add collagenase 1 (1.5 mg/ml final concentration).
2. If you are using an immortalized cell line, you can culture the preadipocytes in culture media containing DMEM-High with 10% FBS and 1% P/S.
3. If the preadipocytes are not fully differentiating, 1 μM rosiglitazone can be added to the induction media. Likewise, the amount of serum can be increased up to 10%.

3. Oligomycin.
4. FCCP.
5. Antimycin A.
6. NaOH.
7. BCA Protein Assay Kit.

2.3 Equipment

1. Laminar flow hood.
2. Humidified CO_2 incubator.
3. Serologic pipettes.
4. Aspiration pipettes.
5. Light microscope with hemocytometer.
6. 12-well culture plates.
7. 10-cm culture plates.
8. 24-well Seahorse cell culture microplates.
9. 24-well Seahorse sensor cartridges.
10. Seahorse XFe24 Analyzer.

3 Methods

3.1 Isolation of brown preadipocytes

1. Remove interscapular BAT from p1-p2 pups (**See** Note 5).
2. Mince tissue in 500 μl of sterile PBS using scissors.
3. Pipette tissue into 500 μl of isolation buffer in a sterile Eppendorf tube.
4. Vortex for 10 seconds.
5. Place Eppendorf tubes (sealed with parafilm) into a shaking water bath at 90 rpm for 35 minutes. Every 5-7 minutes, vortex tubes for 10 seconds.
6. Filter digested tissue through a 100 μM filter into a fresh 15 ml tube (**See** Note 6).
7. Centrifuge at 1200 rpm for 4 minutes at room temperature.
8. Resuspend cells in 3 ml of pre-warmed primary cell media, and plate in a 12-well plate. Place cells in a humidified CO_2 incubator.

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4. It is recommended that assay media contain the same components/nutrients as culture media, but without FBS and sodium bicarbonate. The pH of this media is critical to the assay.
5. Pups can be taken just before birth (e19-e20). It is not advisable to take tissue from pups older than p2.
6. To increase the cell yield, wash the Eppendorf tube with primary cell media, and filter into the 15 ml tube.

3.2 Culture of brown preadipocytes and differentiation into mature brown adipocytes

1. Aspirate media and wash cells with PBS (**See** Note 7).
2. Aspirate PBS, and add enough trypsin to cover the cells. Place cells in a CO_2 incubator for ~4 minutes.
3. Neutralize trypsin by adding culture media. Split cells into a 10-cm plate to pass, or onto a 24-well Seahorse cell culture microplate to perform a mito stress test (**See** Notes 8–11).
4. Once the cells have reached 100% confluence in the 24-well Seahorse cell culture microplate (which should occur 1-2 days after seeding), aspirate the media, wash the cells with PBS, and add 500 μl of induction media to each well (**See** Note 12). This is referred to as Day 0.
5. On Day 2, remove the induction media from each well, and replace it with 500 μl of differentiation media.
6. On Day 4, remove the differentiation media from each well, and replace it with 500 μl of fresh differentiation media. Repeat this step on Day 6. On Day 8, the cells will be fully mature, (**See** Note 13) and ready for a mito stress test.

3.3 Mito stress test

1. A day before the mito stress test, hydrate an XFe24 sensor cartridges by adding 1 ml of XF calibrant solution to each well. Store the XFe24 sensor cartridges overnight at 37°C without CO_2 (**See** Note 14).
2. Setup the mito stress test protocol for the optimal time of oxygen consumption rate (OCR) measurements, which varies based on the cell's respiratory capacity. Basal measurements, and measurements after each drug injection, should be measured at least 3 times for cells, and preferentially more for *ex vivo* tissue analysis.
3. Aspirate media from the Seahorse cell culture microplate, and wash the cells with prewarmed seahorse assay media.
4. Add 450 μl of assay media to each well. Incubate the Seahorse cell culture microplate at 37°C without CO_2 for 45–60 min to allow the medium temperature and pH to reach equilibrium.

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7. Primary cells are cold and aspirant sensitive. Always pre-warm media, and use a hand pipette to slowly aspirate. It is ok to use an aspirator with immortalized cells.
8. When passing preadipocytes, splitting the cells 1:10 or 1:20 is optimal, depending on the cell line. Preadipocyte subculture should remain below confluence.
11. It is advisable to leave several wells empty as "blank" controls. To accurately reflect differences between wells on the edges of the plate and in the middle of the plate, we recommend leaving wells A1, B4, C3, and D6 empty.
12. Induction media should be made fresh the day it is used. It is ok to make the differentiation media that will be used during the experiment on the same day, and leave it at 4°C until use.
13. A plate of mature adipocytes should be covered by cells with multilocular lipid droplets. This can be seen using a light microscope, or by performing Oil red O staining. Further testing of differentiation can be performed by examining the expression of differentiation markers such as AP2, FABP4, and PPAR-γ. Cell lines all differ. Differentiation may be complete on Day 7 instead of Day 8.
14. At least hydrate the XFe24 sensor cartridges for 4 hr. If the cartridge is going to be hydrating for more than 16 hours, wrap it to avoid evaporation.

5. Prepare 10 μM oligomycin, 10 μM FCCP, and 50 μM antimycin A in assay media (**See** Note 15).

6. Load 50 μL of oligomycin, 56 μL of FCCP, and 62 μL of antimycin A into port A, B, and C on XFe24 sensor cartridges.

7. Insert the cartridge plate into the XFe24 analyzer and start running the protocol. After the calibration step is complete, replace the calibrating plate with the Seahorse cell culture microplate, and continue the protocol.

8. After equilibrating the temperature of the cells, basal OCR levels are determined, followed by measurements of these rates in response to each drug injection (**See** Note 16).

9. Once the run finishes (**See** Note 17), aspirate the assay media and add 100-200 μl of 50 mM NaOH to lyse the cells (**See** Note 18). Perform a BCA protein assay to determine the protein concentration for each well.

3.4 Data analysis and interpretation

1. Using the Wave software, normalized OCR (pmole oxygen/minute/μg protein) will be automatically calculated by entering the protein concentration of each well. This can be exported to Excel.

2. Non-mitochondrial respiration is the oxygen consumption contributed from a subset of cellular enzymes (e.g., oxygenase) after inhibition of mitochondrial respiration by antimycin A. By subtracting non-mitochondrial respiration, researchers can accurately measure mitochondrial respiration.

3. Basal mitochondrial respiration can be calculated by subtracting the basal OCR by non-mitochondrial respiration.

4. Basal mitochondrial respiration can be divided into two parts, ATP coupled and ATP uncoupled (proton leak) respiration. The decrease of OCR after injecting oligomycin is the OCR from ATP production. The remaining basal respiration not coupled to ATP production is the proton leak respiration (**See** Note 19).

5. Maximal mitochondrial respiration can be calculated by subtracting OCR after adding FCCP by non-mitochondrial respiration.

6. Reserve capacity can be calculated by subtracting maximal OCR from basal OCR.

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15. Effective drug concentrations might differ between primary adipocytes, immortalized adipocytes, and between cell lines. Titrating to find the effective concentration of each drug is recommended. This is especially true FCCP, which has a narrow window of an effective concentration.
16. The final concentration of oligomycin, FCCP, and antimycin A will be 1 μM, 1 μM and 5 μM, respectively.
17. Check cell morphology for each well, and determine if all of the drugs were completely injected into each well.
18. Homogenate cell lysate thoroughly and take representative sample for BCA assay.
19. UCP1 contributes partially in proton leak respiration. Using UCP1 knockout cells, or a UCP1 activator, can measure UCP1-dependent respiration more accurate.

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