Chapter 8
Studies of Thermogenesis and Mitochondrial Function in Adipose Tissues

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Summary Brown and white adipose tissues in mammals have a number of similar properties, such as lipid storage and adipokine production, but also distinctive properties. The energy-storing white adipose tissue has few mitochondria and low oxidative capacity. The heat-producing brown adipose tissue has a high density of mitochondria and high oxidative capacity. Mitochondrial function can be investigated in cells and organelles isolated from both brown and white adipose tissues. This chapter describes methods for successful isolation of suitable preparations of adipose tissues and their subsequent use. Questions concerning thermogenic capacity of the tissues, their potential influence on whole body metabolism, and specific properties of the mitochondria and their mode of function may be addressed using these methods.

Key words White adipose tissue; brown adipose tissue; mitochondria; thermogenesis; respiration; membrane potential.

1 Introduction

Adipose tissues in mammals are distinguished as being brown or white. Brown adipose tissue functions to produce heat and, thus, has a high oxidative capacity, evidenced by the extraordinarily high density of mitochondria in the cells. White adipose tissue is primarily an energy-storing tissue with low oxidative capacity. However, studies of metabolic activity are relevant for both tissues.

Because thermogenesis (heat production) is the function of brown adipose tissue, it would be a natural choice to measure this directly. However, it has only been done a few times, mainly because of technical limitations (microcalorimeters are still not widespread equipment in biological laboratories). However, it has been calculated that respiratory determinations are indeed satisfactory measures of heat production ***(1)*** (this is probably true for most mammalian organs with

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good blood supply). It is therefore a routine procedure to perform respiratory measurements and equate the result with that of thermogenesis and metabolic activity in general.

Respiratory measurements can be performed on isolated mitochondria, isolated cells, and on tissue pieces. To obtain sound values, it is essential that the measurements are made under the most optimal conditions possible. This includes provision of an adequate oxygen supply throughout the experiment and also the use of a substrate for respiration that is not limiting.

Because of the requirement for sufficient oxygen, tissue pieces can often be problematic, as oxygen supply may be limited by the diffusion of oxygen through the piece of tissue. Dispersed cells and mitochondria can be more easily oxygenated but are obviously more artificial in other respects.

The supply of a suitable substrate for respiration is often difficult. To estimate maximal capacity, the rate of substrate supply must exceed that of the ongoing respiration. For brown adipose tissue, respiration is uncoupled from phosphorylation under conditions when thermogenesis is activated, and the rate is thus limited by the capacity of the uncoupling protein (UCP1) or by the respiratory chain. In white adipose tissue, respiration is normally coupled to ADP phosphorylation, and the rate is therefore determined by the rate of utilization of ATP. Choice of a nonoptimal substrate, the transport of which is rate-limiting, can provide spurious results, leading to erroneous conclusions.

Isolated mitochondria from brown adipose tissue are relatively easy to study, because the mature cells contain such high mitochondrial density that the mitochondrial population isolated after homogenization of whole tissue is statistically representative for the mature adipocytes. For white adipose tissue, the mitochondrial density in the adipocytes is low; a mitochondrial preparation from total tissue may therefore not be representative for white adipocyte mitochondria and it can therefore be necessary to isolate mitochondria from isolated cells, which leads, however, to very low yields. Mature adipocytes can be conveniently isolated from both tissues based on Rodbell's classical collagenase digestion procedure ***(2)***, as the fat-containing cells readily float and can thus be separated from tissue debris in aqueous media.

2 Materials

2.1 Isolation of Mitochondria

1. 0.25 *M* sucrose (*see* **Note 1**).
2. 100 m*M* KCl containing 20 m*M* K-TES, pH 7.2.
3. Bovine serum albumin, (fraction V), fatty-acid-free. Dissolve in 0.25 *M* sucrose to a concentration of 0.3%.
4. Glass homogenizer with tight-fitting Teflon pestle.
5. Small glass hand homogenizer.

6. High-speed centrifuge with fixed angle rotor, tube size ≈ 50 mL.
7. Gauze.

2.2 Isolation of Adipocytes

1. Krebs/Ringer phosphate buffer with the following composition (in m*M*): Na^+ 148, K^+ 6.9, Ca^{2+} 1.5, Mg^{2+} 1.4, Cl^- 119, SO_4^{2-} 1.4, $H_2PO_4^-$ 5.6, HPO_4^{2-} 16.7, glucose 10, fructose 10. Include 4% crude bovine serum albumin. Adjust pH with Tris-OH or HCl to 7.4.
2. Krebs/Ringer bicarbonate buffer with the following composition (in m*M*): Na^+ 145, K^+ 6.0, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, SO_4^{2-} 1.2, HCO_3^- 25.3, $H_2PO_4^-$ 1.2, glucose 10, fructose 10, and fatty-acid-free bovine serum albumin 4%. Bubble the buffer with 5 % CO_2 in air at 37°C and adjust the pH with Tris-OH or HCl to 7.4; keep the buffer at 37°C and continuously bubble with a small stream of 5 % CO_2 in air until use.
3. Crude and fatty-acid-free bovine serum albumin (Fraction V).
4. Crude collagenase (Type I, Clostridiopeptidase A, EC 3.4.24.3).
5. Water shaker at 37°C.
6. Silk filter cloth (Joymar Scientific, Hicksville, NY).

2.3 Oxygen Electrode

1. Clark type oxygen probe. Available from, e.g., Rank Bros. or Hansatech. Purchased as a complete system with measuring chamber and magnetic stirrer. Alternatively, particularly suitable for small samples, the Oroboros Oxygraph 2k.
2. PowerLab 4/30 data acquisition and analysis system with ChartPro software for Windows or Macintosh.
3. Chart recorder (optional).

3 Methods

3.1 Isolation of Brown Adipose Tissue Mitochondria

For a routine preparation of brown adipose tissue mitochondria, use five mice that have been living at normal animal facility temperatures (*see* **Note 2**). All procedures are conducted at 0–4°C.

1. The animals are anaesthetized for 1–2 min in 79% CO_2 and 21% O_2 and decapitated. Dissect out the periaortic, cervical, interscapular and axillary brown adipose tissue carefully into a small volume of ice-cold sucrose on a square of parafilm. Rinse in sucrose.

2. Absorb excess sucrose with a medical wipe, mince the tissue with scissors and homogenize the mince in approx. 40 mL of 250 m*M* sucrose solution (about 5% w/v), in a glass homogenizer with Teflon pestle. Five to six strokes are required.
3. Filter the homogenate through two layers of gauze and centrifuge (all centrifugation steps for 10 min) at 8500 *g*.
4. Discard the hard-packed fat layer and supernatant by rapidly inverting the tube and wipe the walls of the tube clean with a medical wipe.
5. Resuspend the pellet (containing cell debris, nuclei and mitochondria) in a small volume of sucrose and transfer to a clean tube. Dilute again to about 40 mL sucrose solution and centrifuge at 800 *g*.
6. Transfer the supernatant carefully to a clean tube. Discard the pellet (that contains debris and nuclei). Centrifuge the supernatant at 8500 *g*.
7. Resuspend the resulting mitochondrial pellet in 5 mL of sucrose solution with 0.3% fatty-acid-free bovine serum albumin and centrifuge at 8500 *g*.
8. Further wash the albumin-washed mitochondrial pellet by one of two procedures:
   a. For respiratory studies, resuspend the pellet in about 15 mL of the KCl-TES buffer. (*see* **Note 3**) Centrifuge the suspension at 8500 *g*.
   b. For other studies, resuspend the pellet in 15 mL of sucrose solution. Centrifuge the suspension at 8500 *g*.
9. Resuspend the resulting pellet in a minimal volume of the respective medium by hand homogenization.
10. Measure the protein concentration in the final, albumin-washed mitochondrial pellet and dilute the suspension with KCl-TES buffer (*see* **Subheading 2.1.**) or sucrose solution (*see* **Subheading 2.1.**) to a stock concentration of 10 to 20 mg per milliliter for storage on ice.

3.2 Isolation of Brown Adipocytes

For a routine preparation of brown adipocytes, 2 adult (10- to 30-wk-old) Syrian hamsters (*Mesocricetus auratus*) of either sex are used. The hamsters are kept at 20–22°C, one to three per cage, with food and water ad libitum (*see* **Note 4**).

1. The animals are anesthetized by 79% CO_2 and 21% O_2 and decapitated. Dissect out the cervical, interscapular and axillary brown adipose tissue into a small volume of ice-cold Krebs/Ringer phosphate buffer on a square of parafilm and carefully clean from contaminating tissues.
2. Place the brown adipose tissue in a polyethylene vial containing 3 mL Krebs/Ringer phosphate buffer with 4% crude bovine serum albumin and 0.83 mg/mL collagenase.
3. Preincubate the tissue for 5 min in a 1.7-Hz shaking water bath at 37°C. Add 7 mL of the buffer and vortex the vial for 5 s.

4. Filter the contents of the vial onto silk cloth, discard this first filtrate. Transfer the tissue pieces collected on the silk to a small volume of Krebs/Ringer phosphate buffer on a square of parafilm and mince with scissors. Incubate this mince in 3 ml fresh, albumin- and collagenase-containing buffer as above for 25 min, with 5 s vortexing every fifth minute.
5. Add 7 mL of buffer, and vortex the vial for 15 s, and filter the contents as above. Collect the filtrate and centrifuge it (5 min, 65 *g*). Discard the infranatant by suction with a Pasteur pipet with plastic tubing on the tip connected to a water suction pump (*see* **Fig. 8.1**), add 10 mL of buffer and allow the cells to stand at 4°C.
6. Incubate the tissue pieces remaining on the silk filter as above for 15 min, and collect the cells; the tissue pieces now remaining can be incubated for 10 min and the cells collected, in order to increase the yield.
7. Discard the infranatants in all three tubes and combine the cells. Add 10 mL of buffer, and centrifuge the cells as above for 2 min.
8. Discard the infranatant and count the cells in a Bürker chamber. Store the cells on ice until use, at a concentration of 1–3 × 10^6 cells/mL; very little deterioration of cell response is observed during a working day. This is only valid for hamster cells. Cells from rats, and particularly from mice, break easily and should be aliquoted into Eppendorf tubes, kept at room temperature and used immediately (*see* **Notes 5** and **6**).

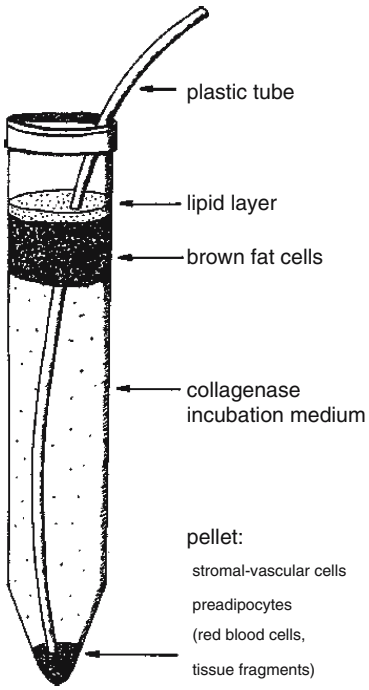

Fig. 8.1 The separation of mature brown adipocytes from lipid droplets and other constituents

9. Hamster cells can be stored overnight at 4°C in 10 ml of KRPB, in which case the cells are washed again the next day by centrifugation (*see* **Notes 5** and **6**).
10. It may be noted that undifferentiated preadipocytes are present in the discarded infranatant. These cells can be collected, cultured in primary culture and differentiated into mature brown adipocytes by the procedures described earlier ***(3)***.

3.3 Measurement of Respiratory Rate

The rate of oxygen consumption of both isolated brown-fat mitochondria and cells can be readily measured polarographically with a Clark-type oxygen probe ***(4)***. Such a probe (obtainable from, e.g., Rank Bros., Hansatech or Oroboros) determines oxygen concentration in aqueous solutions. The current produced by the electrode is proportional to the oxygen tension in the solution. The electrode chamber must be continuously stirred (most practically magnetically). Preferably, the electrode chamber must also be temperature-controlled (e.g., by circulating water from a water bath). Calibrate as follows:

1. Fill the electrode chamber with distilled water at the experimental temperature, do not add any lid (to allow equilibration with atmospheric oxygen), and allow the output to stabilize. At 37°C, this corresponds to 217 nmol O_2/mL (*see* **Note 7***)*; at 25°C (a traditional but clearly less physiological temperature classically used for mitochondrial experiments), this corresponds to 253 nmol O_2/mL.
2. Add a few crystals of sodium dithionite to the solution; this reduces all oxygen and thus provides for determination of zero oxygen level.
3. Remove the calibration solution, carefully wash the chamber well with distilled water and add the relevant buffer. Note that the output may not fully return to the level it had with distilled water; this is correct, as salt-containing media dissolve less oxygen than distilled water.
    a. For mitochondrial studies, use a medium consisting of 100 m*M* KCl, 20 m*M* K-TES, 4 m*M* KH_2PO_4, 2 m*M* $MgCl_2$, 1 m*M* EDTA, final pH 7.2. For introduction of energy conservation and choice of substrate, *see* **Notes 8** and **9**.
    b. For cell studies, use the Krebs/Ringer bicarbonate buffer, bubbled with CO_2, as described previously *(see* **Note 10**).
4. Add 0.2–0.5 mg mitochondrial protein or 50,000–80,000 cells per ml buffer in the electrode chamber. Close the chamber and allow the system to stabilize. Make further additions with a Hamilton syringe through a small hole in the cover of the chamber. Note that an addition artifact may voluntarily be produced by allowing the syringe to momentarily stop the magnetic stirring. Note also that the addition of ethanol (as solvent for some compounds) may lead to a small baseline shift.

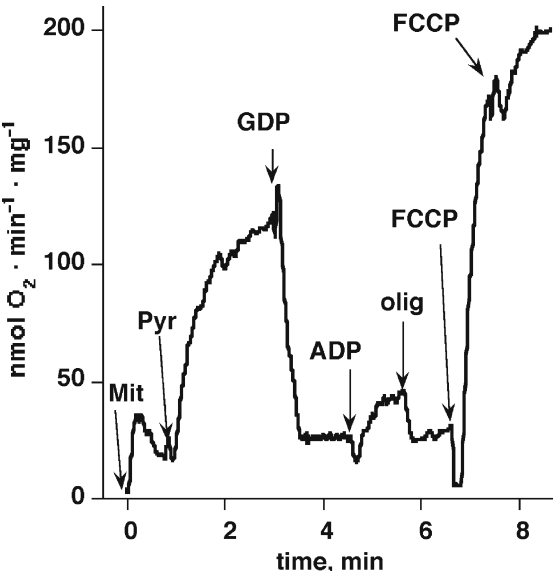

Fig. 8.2 Typical oxygen electrode trace obtained with isolated brown-fat mitochondria. The rate of oxygen consumption is shown. When mitochondria are added, only a very low rate of respiration (thermogenesis) is observed. However, if substrate for combustion, here pyruvate, is added, a high rate of respiration is observed. That this respiration can be inhibited by GDP indicates that it is mediated via the uncoupling protein-1 (UCP1). A low basal rate of respiration is left after addition of GDP. Addition of ADP, which leads to initiation of ATP synthesis, leads, in these mitochondria, to only a very small increase in respiration when contrasted to what is observed in practically all non-brown-fat mitochondria (so-called state-3 rate); the low rate is caused by a very low content of the ATP-synthase enzyme. Addition of oligomycin inhibits the low activity of the ATP-synthase, resulting in so-called state-4 rate. Addition of an artificial uncoupler (here FCCP) reveals the total capacity of the mitochondria for oxidation of the substrate

5. The electrode output can be connected to a computer via an analogue-to-digital converter and the data acquired in a Chart PowerLab application program. (Alternatively, a regular chart recorder can be used.) *See* **Figs. 8.2** and **8.3** for representative results of such determinations.

3.4 Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential can be determined by a number of methods that rely upon distribution of a cationic dye within or across the mitochondrial inner membrane in accordance with the membrane potential. Several compounds can be used for this purpose, such as triphenylmethylphosphonium (i.e., TPMP),

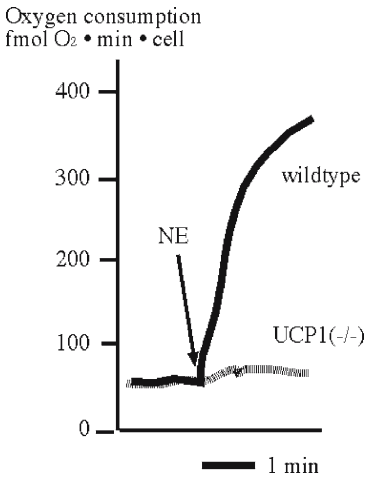

Fig. 8.3 Typical oxygen electrode trace obtained with isolated mature brown-fat cells. Two types of brown-fat cells are compared. In brown-fat cells isolated from mice in which UCP1 has been genetically ablated (UCP1(–/–)), there is a low basal rate of respiration (thermogenesis). In these cells, the addition of norepinephrine (NE) does not lead to an increase in thermogenesis. In brown-fat cells isolated from wild-type mice (WT), the unstimulated rate of respiration is similar to that in brown-fat cells without UCP1, i.e., the mere presence of UCP1 in the mitochondria within the cells does not lead to uncoupling (thermogenesis). However, when the UCP1-containing cells are stimulated with norepinephrine, the high thermogenic potential of the brown-fat cells is demonstrated. Principal sketch based on observations in Matthias et al. ***(20)***

tetraphenylphosphonium (TPP, which can also be used in ion-selective electrodes), Rhodamine 123, and safranine O. The method described here is for safranine, because we have found it to function satisfactorily.

The conditions and media should be identical to those described previously for oxygen consumption measurements in mitochondria, so that the results between the two types of determinations can be compared. The changes in absorbance of safranine O (5 µ*M*) at 511 – 533 nm are followed. Increased quenching of the color gives an upward deflection.

1. Add 1 mL of medium (as listed previously for respiratory studies) and 0.5 mg of mitochondrial protein to a suitable cuvette, together with 5 µ*M* safranine.
2. Record the changes in absorbance after the addition of compounds of interest.
3. At the end of the experiment, add 25 µ*M* FCCP, followed by 7 × 10 m*M* NaOH until the mitochondria are solubilized.
4. For each preparation calibrate the membrane potential as follows. Transfer the mitochondria into a state of energy conservation as in **Note 8**. Subsequently, to the mitochondria in the cuvette, add 9 µ*M* valinomycin followed by KCl, at concentrations between 0.1 m*M* and 50 m*M* (choose a range of about 8 concentrations and perform one trace for each concentration). At the end of the experiment, add 25 µ*M* FCCP, followed by 7 × 10 m*M* NaOH, as above.

5. Plot the change in absorbance against log KCl concentration in order to extrapolate to the internal K^+ concentration in the mitochondria and the initial concentration in the medium.
6. Use the Nernst equation and the values obtained in the calibration to calculate the membrane potential under each condition.

4 Notes

1. Some authors use a lightly buffered sucrose solution, containing, e.g., 5 m*M* K-TES. Our experience is that this has no obvious beneficial effect on the preparation. Similarly, for brown fat mitochondria, (in contrast to mitochondria from skeletal muscle or liver), we have not observed any beneficial effect of the use of a chelator, e.g., EDTA (2 m*M*) or EGTA (1 m*M*).
2. Because many studies are now performed on genetically modified mice, the method described here is for such animals. However, mitochondria can also be isolated by the same method from the brown adipose tissue of other mammals (most commonly rats but also Syrian hamsters). They can be isolated from tissue taken from animals kept at colder or warmer environmental temperatures. In these cases, the tissue contains less or more triglyceride and the number of mitochondria per gram tissue is higher in cold-acclimated animals. Triglyceride disturbs the homogenization and a lower relative yield of mitochondria will generally be obtained from animals kept at higher temperatures and similarly higher from animals kept at lower temperatures.
3. When brown adipose tissue mitochondria are isolated, they are uncoupled (***5,6***) and have a collapsed membrane potential. They demonstrate high permeability to many monovalent ions. Presumably as a consequence of this, they have lost the ability to retain osmotic support in the mitochondrial matrix. The matrix is therefore highly condensed after preparation, and oxidation of substrates in the matrix is markedly inhibited ***(7–9)***. To re-expand the matrix, the mitochondria may be incubated in an iso-osmotic medium of permeant ions (such as KCl). Matrix expansion can also be achieved with low osmolarity sucrose (100 m*M*), although this probably gives a less controlled expansion.
4. Brown adipocytes can also be prepared by the same method from rats ***(10,11)*** and mice ***(12)***. It is our experience that the cells from Syrian hamsters are the most robust and for many studies are therefore very suitable. Their robustness also means that they are an appropriate choice for people learning the technique.
5. The details given here for preparation of brown adipocytes are examples of incubation times with collagenase, collagenase concentrations (and types), centrifugation times (or flotation without centrifugation), which can be applied. Different workers tend to develop personal modifications of these, particularly at times and under circumstances when, for unclear reasons, the preparations are less successful. It is difficult to find convincing evidence that these modifications are of major significance, but this idiosyncrasy also indicates that the details specified here are for guidance and need not be adhered to exactly.

6. The preparation technique is dependent upon the property of the cells to float on top of an aqueous medium. If cells are isolated from animals that are cold-exposed, the triglyceride concentration may be so low that the cells sink in the medium and can therefore not be separated. The yield of cells will thus be lower than normal ***(13,14)***. In general, the yield of mature adipocytes as a percentage of total adipocytes in the tissue is not very high. The representativeness of the cell population can perhaps therefore be discussed. Also, if cells are prepared from animals living at thermoneutrality, the cells are very replete with triglycerides and their diameter larger than of cells from animals at room temperature. This large cell size seems to make the cells more fragile and sensitive to mechanical manipulation.
7. This value is, of course, only valid for normal atmospheric pressure. However, the effects of normal fluctuations in atmospheric pressure are normally ignored.
8. To transfer the mitochondria into a state of energy conservation, incubation should be performed in the presence of fatty-acid-free albumin (0.1–0.5 %), to remove fatty acids and related substances, and of purine nucleotides. The nucleotides bind to the brown-fat specific uncoupling protein UCP1 and in so doing close the proton leak through this protein ***(9)***. The most commonly used nucleotide is GDP, which is used at a concentration of 0.1 – 1 mM. Other di- and triphosphate purine nucleotides are also more or less efficient. The nucleotide binding site is on the outer side of the inner mitochondrial membrane.
9. When respiratory studies are performed on isolated mitochondria, it is of great importance that a suitable substrate is used. The most relevant is a fatty acid or its derivative, such as long-chain acyl-coenzyme A or acyl-carnitine. In all cases, to permit complete fatty acid oxidation, malate (in 5 m*M* concentration) must be added to the buffer to replenish citric acid cycle intermediates which have been lost during isolation ***(15)***. In some species, the reuptake of malate is low and this may even limit fatty acid combustion. When acyl-carnitine esters are used (50 µ*M*), no further additions (except malate) are required. For acyl-CoA derivatives (similar concentrations), a further addition of L-carnitine (2 m*M*) guarantees that availability of this compound does not limit oxidation. If free-fatty acids are used, further additions of ATP (100 µ*M*) and coenzyme A (5 µ*M*), in addition to carnitine, allow unlimited fatty acid oxidation. A further NADH-coupled substrate that demonstrates fairly high rates of respiration is pyruvate, used at 5 m*M* concentration together with malate (5 m*M*). Glutamate is inappropriate. Brown fat mitochondria also demonstrate a high rate of oxidation of glycerol-3-phosphate (used at m*M* concentrations), a flavoprotein-coupled substrate which is oxidised on the external face of the inner membrane and thus does not require transport ***(16)***. In many species, succinate (which is a classical substrate for studies of liver mitochondria) permeates only poorly into the mitochondria and its use may therefore lead to severe underestimates of oxidative capacity. This is also often the case for other potential substrates, mainly the intermediates of the citric acid cycle, which have a low rate of permeation in certain species ***(17)***.

10. Respiration in the brown adipocytes is most notably stimulated by the physiological agent norepinephrine, in which case endogenous lipolysis provides the substrate and also permits uncoupling of respiration from the constraints of a requirement for ATP utilization ***(10,18)***. This uncoupling is entirely dependent upon the presence of UCP1 ***(19,20)***. Free fatty acids can also be added to the cell suspension and provide an adequate substrate ***(18)***. Their combustion is also fully dependent on the presence of UCP1, and this demonstrates that fatty acids can directly or indirectly activate UCP1 ***(19,21)***. If other substrates are utilized, there is a transport requirement into the cells, in addition to which rates of respiration are generally low unless respiration is artificially uncoupled with e.g. FCCP (20 μ*M*). The maximum respiratory rates then seen are usually much lower with exogenous substrates such as pyruvate (5 m*M*) than with the endogenously generated or exogenously added fatty acids.

White adipose tissue:

Mitochondria from white adipose tissue. To obtain mitochondria representative for the mature adipocytes in the tissue, isolated adipocytes, prepared essentially as described for the brown adipocytes , can be used as the starting material. The cells are homogenized and the mitochondria isolated by routine differential centrifugation, as described. The yields are very low. The mitochondria are well-coupled and can be stimulated to respire on citric acid cycle intermediates, in the presence of ADP ***(22)***.

Adipocytes from white adipose tissue: as noted previously, isolated adipocytes can be readily prepared ***(2)***. Because of the appearance of the cells (one unilocular fat droplet filling most of the cell volume), intact cells are not easily distinguished from large fat droplets. Few respiratory studies have been performed on such cells. Their rate of respiration is very low and high cells densities must be used. Basal metabolic/respiration rates can be estimated and hormonal stimulation can be performed, but this is generally evaluated in terms of metabolic changes other than respiration. A number of microcalorimetric studies have been performed on isolated white adipocytes from humans. Basal metabolism has been determined ***(23)*** and comparisons made between tissue taken from obese and lean ***(24)*** or hypo/euthyroid ***(25)*** individuals. Effects of hormone stimulation can be determined.

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