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Quantification, Localization, and Tissue Specificities of Mouse Mitochondrial Reactive Oxygen Species Production

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Abstract

Mitochondria play a critical role in many different pathologic conditions. Increasing evidence has shown that mitochondrial reactive oxygen species (ROS) production may provide an etiologic link between mitochondria and pathologies. The widespread use of laboratory mice as models for a host of human diseases makes the quantification and localization of ROS production from mice an important endeavor. This chapter presents approaches to the quantification and localization of ROS from mouse brain, liver, and beta cell mitochondria. Techniques for the isolation of mitochondria and mitochondrial fractions and the subsequent quantification of ROS with Amplex Red or a FACS-based method on intact cells are described.

1. Introduction

The laboratory mouse plays an indispensable role in biomedical research. Mouse models have become the choice for basic and applied research because of both adaptability and the presence of highly genetically standardized inbred strains. In addition, the ability to genetically manipulate mice through transgenic, knock-out, knock-in, and tissue-specific deletion adds significant flexibility and depth to experimental design. Yet, most experiments studying mitochondrial physiology have been performed with isolated organelles from rat models. This is likely to change because of the presence of hundreds of spontaneous or genetically manipulated mouse strains with traits that are relevant to human disease.

Mitochondria have a central role in diabetes (Anunciado-Koza *et al.*, 2008; Mathews *et al.*, 2005), aging (Coussens *et al.*, 2008; Katic *et al.*, 2007), apoptosis (Katoh *et al.*, 2008; Kujoth *et al.*, 2005), cancer (Maximo *et al.*, 2008), and neurodegenerative disorders (Fukui and Moraes, 2008; Junn *et al.*, 2008; Ohsawa *et al.*, 2008). A common link shared by the aforementioned pathologic conditions is reactive oxygen species (ROS) and specifically ROS produced by the mitochondria. Although the source of mitochondrial ROS production may differ for each disease, it is likely that the mitochondrial electron transport chain (ETC) is a major contributor. Mutations or sequence variation in the mitochondrial DNA (mtDNA) can modify both the site and quantity of ROS produced by the ETC (Gusdon *et al.*, 2007, 2008; Moreno-Loshuertos *et al.*, 2006).

Complexes I and III have been identified as sites of ROS production by the ETC. Inhibitors of the ETC can be effective in determining the site of ROS production by isolated

mitochondria. Rotenone, an inhibitor of the quinone binding site of complex I (Fendel *et al.*, 2008; Lambert and Brand, 2004), is useful in assessing complex I–derived ROS production. In intact mitochondria, rotenone has contrasting effects comparing liver mitochondria to brain and beta cell mitochondria. Rotenone slightly inhibits complex I–derived ROS in liver (Gusdon *et al.*, 2008) yet increases ROS production in brain (Muller *et al.*, 2008; Votyakova and Reynolds, 2001). Complex I produces superoxide primarily into the mitochondrial matrix (Miwa and Brand, 2003; Muller *et al.*, 2004). Permeabilization of the inner membrane with the antibiotic alamethicin (Qian *et al.*, 2008) allows for a more direct measurement of complex I ROS production when assessed with Amplex Red. The production of submitochondrial particles further simplifies the system by eliminating soluble antioxidants. Complex III, the other main site of ROS production, can be readily assessed by the inhibitors antimycin A and myxothiazol (Chen *et al.*, 2003; Tretter *et al.*, 2007). Several reports have shown that antimycin A and myxothiazol increase brain mitochondrial ROS production (Chen *et al.*, 2003; Young *et al.*, 2002). However, myxothiazol and antimycin A decrease complex III ROS production from liver mitochondria from several mouse strains (Gusdon *et al.*, 2008). Complex III produces ROS on both sides of the inner mitochondrial membrane (Muller *et al.*, 2004); therefore, analysis of complex III ROS production with alamethicin permeabilized is also useful. In nonphosphorylating submitochondrial particles, electron flow from complex I to complex III is drastically inhibited (Hoppel and Cooper, 1969; Malviya *et al.*, 1968; Racker and Horstman, 1967); therefore, the use of submitochondrial particles in the analysis of complex III ROS production is not informative (Gusdon *et al.*, 2008).

It has long been considered that diseases resulting from mutations in the mitochondrial DNA (mtDNA) are rare genetic disorders. However, alterations or natural sequence variants in the mitochondrial DNA recently have been recognized to play important roles in the pathogenesis of common diseases. Although some attention has been focused on the accumulation of mitochondrial DNA mutations in somatic cells, inheritance of specific disease associated loci in the nuclear genome in combination with common polymorphisms in the mtDNA may contribute to the development of common pathologic conditions such as cancer, cardiovascular disease, diabetes, and aging. Recently, we have published that a diabetes-associated mtDNA mutation modifies ROS generation from the ETC, but that nuclear genes clearly contribute to the ROS signal. It is likely that mouse models will be exploited to better understand the role of mitochondrial ROS production in specific disease states in the near future. This chapter outlines the methods for isolating mitochondria from mouse brain, liver, and beta cells and provides experimental techniques for the quantification and localization of mitochondrial ROS production with the fluorescent dye Amplex Red and a FACS-based approach.

2. Materials and Procedures for Isolation of Mouse Mitochondria

2.1. Brain mitochondria

Several methods have been used for the isolation of rodent brain mitochondria. Although differential centrifugation can be used to produce enriched mitochondria from many types of cells and tissues (Pallotti and Lenaz, 2007), this method produces a very crude brain

mitochondrial preparation contaminated with synaptosomes and myelin (Sims, 1990; Stahl *et al.*, 1963). Therefore, other methods must be used to obtain a more pure preparation of functional brain mitochondria. Early studies used sucrose gradient centrifugation, yet this method produces mitochondria with poor metabolic parameters given the prolonged exposure to hypertonic conditions (Neidle *et al.*, 1969; Salganicoff and De Robertis, 1965). Improved techniques have used a discontinuous Ficoll gradient (Lai and Clark, 1979; Tanaka and Abhood, 1963). Recently, Sims and Anderson published a protocol that used a discontinuous Percoll gradient resulting in significant enrichment of highly functional mitochondria largely separated from synaptosomes and myelin (Sims and Anderson, 2008). This protocol can also yield distinct myelin and synaptosome bands (Sims and Anderson, 2008). The protocol outlined in the following, based on the work of Sims (Sims, 1991), used a discontinuous Percoll gradient; however, with fewer distinct Percoll concentrations. It allows for the rapid preparation of highly functional and pure mouse mitochondria yet without distinct bands for myelin and synaptosomes.

2.1.1. Materials—Mannitol, sucrose, HEPES potassium salt, fatty acid-free BSA, EDTA, and potassium hydroxide were all purchased from Sigma-Aldrich (St. Louis, MO). Percoll was from Thermo Scientific (Waltham, MA), and the BCA Protein Assay Kits were from Pierce Biotechnology (Rockford, IL).

2.1.2. Procedure for the isolation of mouse brain mitochondria—Percoll was diluted with isolation buffer I (IBI: 225 mM mannitol, 75 mM sucrose, 10 mM HEPES potassium salt, 0.10% fatty acid-free BSA, 1 mM EDTA, pH 7.4 [with KOH]) to produce stocks of 12% v/v, 24% v/v, and 42% v/v. Percoll gradients were prepared in 50 ml Oak Ridge polycarbonate high-speed centrifuge tubes (Nalgene [Thermo Scientific]) by adding 10 ml 24% Percoll, then with a 6-inch long, 18-gauge blunt-ended needle (Popper & Sons, Inc. [New Hyde Park, NY]) fitted to a 10-ml syringe (Becton Dickinson & Co [Franklin Lakes, NJ]) to layer 10 ml 42% Percoll beneath the 24% Percoll. The brain was excised (2 or more for a substantial yield) and placed in 25 ml cold isolation buffer II (IBII: 225 mM mannitol, 75 mM sucrose, 10 mM HEPES potassium salt, 0.1 mM EDTA, pH 7.4 [with KOH]). The brain was washed once with 25 ml cold 12% Percoll. These organs were then homogenized in 15 ml 12% Percoll with a Kontes Dounce homogenizer with pestles A then B (Kimble-Chase [Vineland NJ]). With a 2-inch-long, 18-gauge blunt-ended needle (Popper & Sons, Inc.) fitted to a 10-ml syringe (Becton Dickinson & Co), the brain homogenate was gently layered on top of the Percoll gradient with the centrifuge tube tilted at a 45° angle on ice. The gradient was centrifuged in a Sorvall SS-34 rotor (Thermo Scientific) at 27,000g (15,000 rpm) for 10 min in a Sorvall RC-6 Plus (Thermo Scientific). After centrifugation, the mitochondrial fraction will be located between the 24% and 42% Percoll layers. A 2-inch-long, 18-gauge blunt-ended needle fitted to a 10-ml syringe was used to remove the waste above the mitochondrial fraction. With a clean blunt-ended needle and syringe, the mitochondrial fraction was extracted, placed in a clean 50-ml centrifuge tube, and then the 50-ml tube was filled with IBI. Mitochondria were pelleted at 10,000g (9150 rpm) for 10 min in a SS-34 rotor with a Sorvall RC-6 Plus Centrifuge. Supernatant was removed with care to not disturb the pellet, and then the pellet was suspended in 300 μ l of IBII. The resuspended pellet was then transferred to a 1.5-ml conical bottom microcentrifuge tube and

pelleted again in a tabletop centrifuge at 10,000g for 5 min. The supernatant was then pipetted off and the pellet resuspended in 100 μ l IBII. Mitochondrial protein concentration was determined with the BCA protein assay. Two mouse brains typically yield greater than 3 mg of mitochondrial protein.

2.2. Liver mitochondria

The isolation of mitochondria from mouse liver presents fewer obstacles than brain and can be accomplished effectively by differential centrifugation based on the protocol outlined by Pallade and colleagues (Hogeboom *et al.*, 1948). However, contamination by small particles such as lysosomes and peroxisomes will be present to a varying extent resulting in mitochondrial preparation that is unlikely to exceed 90% purity (Graham, 2001). This mitochondrial preparation exhibits a high respiratory control ratio and functions longer than 4 h after isolation. In differential centrifugation, the initial low-speed spin pellets intact cells, cell debris, and nuclei (Pallotti and Lenaz, 2007). The second, higher speed spin pellets the mitochondria (Pallotti and Lenaz, 2007). The protocol outlined in the following uses the conventional differential centrifugation method with minor modifications. Mannitol is used in this procedure given the previously reported ability of such monosaccharides to increase mitochondrial coupling (Siess, 1983a,b). This protocol can also be applied in principle to skeletal muscle and cultured cells (Frezza *et al.*, 2007).

2.2.1. Reagents—Mannitol, sucrose, HEPES potassium salt, fatty acid-free BSA, EDTA, and potassium hydroxide were all purchased from Sigma-Aldrich (St. Louis, MO). The BCA Protein Assay Kits were from Pierce Biotechnology (Rockford, IL).

2.2.2. Procedure for the isolation of mouse liver mitochondria—The liver was excised and placed in cold IBII (225 mM mannitol, 75 mM sucrose, 10 mM HEPES potassium salt, 0.1 mM EDTA, pH 7.4 [with KOH]) in a 100-ml polypropylene beaker (Thermo Scientific). The organ was washed with 25 ml of IBI (225 mM mannitol, 75 mM sucrose, 10 mM HEPES potassium salt, 0.1% fatty acid-free BSA, 1 mM EDTA, pH 7.4 [with KOH]) and transferred into a beaker with 15-ml IBI. Blunt-ended scissors were then used to cut the liver into small pieces. These pieces were transferred into a Dounce homogenizer and homogenized with pestles A then B, 5 strokes with each pestle. Homogenate was moved to a 50-ml Oak Ridge polycarbonate high-speed centrifuge tube (Nalgene [Thermo Scientific]) and centrifuged at 1300g for 10 min in a Sorvall SS-34 rotor with a Sorvall RC-6 Plus centrifuge. While decanting the supernatant into a 50-ml Oak Ridge polycarbonate high-speed centrifuge tube, care was taken to not disturb the pellet. The supernatant was subjected to centrifugation, 1300g (3300 rpm) in an SS-34 rotor (Sorvall) for 3 min. Afterwards, the supernatant was decanted into a clean 50-ml Oak Ridge polycarbonate high-speed centrifuge tube. This tube was then centrifuged to pellet the mitochondria at 10,000g for 10 min. The supernatant was discarded and the pellet resuspended in IBII and then transferred to a clean centrifuge tube and centrifuged at 10,000g for 5 min. The supernatant was then removed with a pipette and the pellet resuspended in 100 μ l IBII. Mitochondrial protein concentration was determined with the BCA protein assay. One liver typically yields more than 8 mg of mitochondrial protein.

2.3. Submitochondrial particles (SMPs)

Several different techniques exist for the subfractionation of mitochondria into submitochondrial particles (SMPs). The preparation of coupled or uncoupled, as well as inverted or noninverted SMPs, is possible. Typically, inverted SMPs are produced with mild sonication, whereas noninverted SMPs are produced by treatment with digitonin (Hoppel and Cooper, 1969; Malviya *et al.*, 1968; Racker and Horstman, 1967). Malviya *et al.* have published electron micrographs showing that sonicated SMPs have inverted inner membrane subunits, whereas SMPs prepared with digitonin do not, but both types of SMPs seemed to be vesicular (Malviya *et al.*, 1968). Digitonin SMPs are believed to have the same orientation as intact mitochondria because both can establish a pH gradient and lower the pH of incubation media (Mitchell, 1966). It has been reported that a loss of bound divalent cation during the preparation of inverted, sonically prepared SMPs is responsible for some of their properties, including a lack of nucleotide specificity for ADP and ATP (Hoppel and Cooper, 1969). Indeed, it has been shown that Mg^{2+} concentrations are significantly lower in sonic particles than in digitonin particles (Cooper, 1960). However, with more vigorous sonication techniques, it is possible to prepare inverted, uncoupled SMPs. These particles are very useful when attempting to characterize the kinetic aspects of complex I (Estornell *et al.*, 1993; Pallotti and Lenaz, 2007). The submitochondrial preparation that follows is based on the procedure used by Gregg and, more recently, by Lesnefsky and colleagues (Chen *et al.*, 2003; Gregg, 1967). It involves rather drastic sonication and produces SMPs that are inverted and uncoupled. These SMPs are ideal for the purpose of ROS quantification and site localization because of their phenotypic differences from intact mitochondria and facile detection of complex I-generated ROS.

2.3.1. Reagents—4-Morpholinepropanesulfonic acid (MOPS), mannitol, sucrose, HEPES potassium salt, fatty acid-free bovine serum albumin (BSA), EDTA, and potassium hydroxide were all purchased from Sigma-Aldrich (St. Louis, MO). The BCA Protein Assay Kits were from Pierce Biotechnology (Rockford, IL).

2.3.2. Procedure for the production of SMPs—SMPs can be prepared with either fresh or frozen mitochondria. After preparation of intact brain or liver mitochondria, these organelles should be subjected to freeze and thaw. The samples should then be diluted to a concentration between 10 and 20 mg of mitochondrial protein/ml with 10 mM MOPS. In most cases the initial concentration of intact mitochondria will be low, and, therefore, several samples may need to be pooled together.

A salt-ice water bath was prepared in a Fisher Sonic Dismembrator (Thermo Scientific) to make the temperature -4° . The samples were sonicated for 20 sec at 60% maximal output and then allowed to rest for 2 min. This cycle was repeated a total of nine times. Afterwards, the samples were centrifuged at 16,000g for 10 min. Supernatants were decanted into clean centrifuge tubes and centrifuged at 150,000g for 45 min. The pellet was resuspended in IB II and the protein concentration established with the BCA protein assay.

3. Materials and Procedures for Quantification of Reactive Oxygen Species (ROS) Production by Mouse Mitochondria

Several fluorescent dyes have been used to assess the production of ROS from isolated mitochondria. Given its stability, the detection of hydrogen peroxide is very convenient and allows for real-time quantification. Scopoletin has been used for this purpose and fluoresces while reduced but loses its fluorescence after being oxidized by peroxide and horseradish peroxidase (HRP) (Boveris *et al.*, 1977; Corbett, 1989; Loschen *et al.*, 1971). More recently the use of Amplex Red has gained popularity. In contrast to scopoletin, its reduced form is not fluorescent, but after oxidation by peroxide and HRP, it becomes strongly fluorescent. The methods of ROS quantification outlined in the following uses Amplex Red-based model systems. Indeed, Amplex Red has been demonstrated to possess several advantages over scopoletin. These include increased sensitivity and a linear response over a broader range of hydrogen peroxide concentrations (Votyakova and Reynolds, 2004).

3.1. ROS production by intact mitochondria

The production of ROS by intact mitochondria is readily accomplished with Amplex Red and HRP (Votyakova and Reynolds, 2001, 2004). With intact mitochondria, substrates can be chosen that support either complex I- or complex II-mediated ROS production. NADH itself cannot be used as a complex I substrate given that it is not able to pass through the mitochondrial innermembrane. Instead, the mitochondrial glutamate-aspartate shuttle must be used to carry NADH into the matrix (Quagliariello *et al.*, 1965). To this end, glutamate and malate have been widely used.

Complex II, succinate dehydrogenase, can be assayed for ROS production with the substrate succinate. Succinate dehydrogenase is a member of the electron transport chain and also the only membrane-bound enzyme in the tricarboxylic acid cycle. Succinate dehydrogenase delivers electrons directly to the quinone pool after succinate oxidation (Cecchini, 2003; Sun *et al.*, 2005). Therefore, complex II can be directly assayed by the addition of succinate given its unique dual function. Without addition of the complex I inhibitor rotenone, reverse electron flow from complex II to complex I stimulates high levels of ROS production (Capel *et al.*, 2005; Votyakova and Reynolds, 2001). Therefore, when assessing complex II respiration, rotenone should be added to prevent a 30 to 40% underestimate of respiratory flux because of the accumulation of oxaloacetate (Capel *et al.*, 2005; Ernster and Nordenbrand, 1967).

3.1.1. Reagents—Stocks of Amplex Red (40 mM, Fluka), rotenone (25 mM [Calbiochem]), diphenyliodonium chloride (DPI) (15 mM [Sigma]), myxothiazol [20 mM [Sigma]] were all prepared in dimethyl sulfoxide (DMSO) (Sigma). A 10 mM stock of 4-(hydroxymercuri)benzoic acid (CMB) (Sigma) was prepared in 0.1 M KOH, pH 8.0. Horseradish peroxidase (HRP) (Cayman) was diluted to 2500 U/ml with H₂O, and antimycin A (Sigma) was diluted to 5 mg/ml in ethanol. The incubation media for these experiments was: 125 mM KCl, 2 mM K₂HPO₄, 5 mM MgCl₂, 10 mM HEPES, 10 μ M EGTA, pH 7.4, all from Sigma-Aldrich Co. Glutamate, malate, and succinate were all purchased from Sigma-Aldrich Co.

3.1.2. Procedure for assessing ROS production from intact mitochondria—

Measurements of ROS production by intact mitochondria were made with a RF-5301 spectrofluorometer (Shimadzu) in a quartz cuvette with the reaction chamber heated to 37° and constantly stirred. To study electron transport from complex I or complex II the incubation media were supplemented with either 5 mM L-glutamate and 5 mM L-malate or 5 mM succinate, respectively. Amplex Red (2 µg/ml) and HRP (1 U/ml) were added to the cuvette containing incubation media and the substrates of choice. Each reaction was initiated with the addition of 0.2 mg/ml freshly isolated mitochondria. Fluorescence was measured kinetically with an excitation wavelength of 560 nm (slit 1.5 nm) and an emission wavelength of 590 nm (slit 3 nm). After the data were acquired, the slopes of the linear portions of each trace were determined. These rates were converted into pmol H₂O₂/min/mg by constructing a H₂O₂ standard curve as described (Votyakova and Reynolds, 2004).

3.2. ROS production by alamethicin-permeabilized mitochondria

Alamethicin is an antibiotic isolated from *Trichoderma viride* and consists of 20 amino acids (Mueller and Rudin, 1968) and is unusually rich in α-aminoisobutyric acid, which greatly reduces its backbone flexibility (Marshall, 1972). The membrane-altering properties of alamethicin have been widely studied in both model lipid membrane systems and physiologic systems. The effect of alamethicin on isolated mitochondria has been often studied in the context of providing a positive control for permeability transition pore experiments. Indeed, it has been demonstrated that alamethicin increases ROS production from isolated mitochondria as does the formation of the permeability transition pore (Hansson *et al.*, 2008).

The sites and directionality of mitochondrial superoxide production have been studied in detail. It has been demonstrated that mitochondrial complex I produces superoxide exclusively into the mitochondrial matrix, whereas complex III produces superoxide at two distinct sites—generating ROS into the matrix and into the intermembrane space (St-Pierre *et al.*, 2002). The redox-sensitive dye Amplex Red does not pass through the inner membrane and enter the mitochondrial matrix (Kristian and Fiskum, 2004). Therefore, superoxide produced into the matrix must be converted into hydrogen peroxide by manganese superoxide dismutase and diffused into the intermembrane space before being detected by Amplex Red. Total ROS production detected in intact isolated mitochondria can thus be altered by the matrix concentrations of antioxidant enzymes.

Alamethicin can be used to obtain a direct reading of total mitochondrial ROS production. Treating mitochondrial with alamethicin allows for the rapid detection of complex I–derived ROS, as well as the ROS produced into the matrix by complex III (Gusdon *et al.*, 2008). Furthermore, permeabilizing mitochondria with alamethicin allows NADH to have access to its binding site within complex I. Thus, the use of substrate combinations such as glutamate and malate or pyruvate to produce NADH can be bypassed. This allows for a more direct assessment of electron transport chain–generated ROS in the context of whole mitochondria rather than isolated membrane fractions. However, NADH itself has been reported interact with HRP and produce enough peroxide by means of a free radical–mediated mechanism to substantially oxidize Amplex Red (Votyakova and Reynolds, 2004). However, the addition

of superoxide dismutase (SOD) completely eliminates this reaction, perhaps because of the inhibition of a superoxide-dependent propagation step in a free radical chain reaction (Votyakova and Reynolds, 2004).

3.2.1. Reagents—Amplex Red (40 mM, Fluka), rotenone (25 mM [Calbiochem]), diphenyl-leneiodonium chloride (DPI) (15 mM [Sigma]), myxothiazol (20 mM [Sigma]) were all prepared in dimethyl sulfoxide (DMSO) (Sigma). A 10 mM stock of 4-(hydroxymercuri)benzoic acid (CMB) (Sigma) was prepared in 0.1 M KOH, pH 8.0. Horseradish peroxidase (HRP) (Cayman) was diluted to 2500 U/ml with H₂O. Antimycin A (5 mg/mL [Sigma]) and alamethicin (25 mg/ml [Sigma]) stocks were diluted to in ethanol. A stock of NADH (10 mM [Sigma]) was prepared in H₂O fresh before each experiment. Superoxide dismutase (SOD) (10 kU/ml [Sigma]) was prepared in H₂O. The incubation media for these experiments was: 125 mM KCl, 2 mM K₂HPO₄, 5 mM MgCl₂, 10 mM HEPES, 10 mM EGTA, pH 7.4, all from Sigma-Aldrich Co.

3.2.2. Procedure for quantifying ROS production by alamethicin-permeabilized mitochondria—Measurements of ROS production by alamethicin-permeated mitochondria were made with a RF-5301 spectrofluorometer (Shimadzu) in a quartz cuvette with the reaction chamber heated to 37° and constantly stirred. Amplex Red (2 µg/ml), HRP (1 U/ml), and SOD (40 U/ml) were added to the cuvette containing incubation media. Each reaction was initiated with the addition of 0.2 mg/ml freshly isolated mitochondria. For control reactions, ROS production was measured for at least 2 min without added substrates followed by the addition of 80 µM NADH. Afterwards, ROS production was measured for an additional 2 min. For experimental traces, mitochondrial ROS production was first assessed in the presence of alamethicin (30 µg/ml) with no added substrates for 2 min. NADH (80 µM) was then added, and ROS production was assessed for an additional 2 min. ROS production was also measured after the addition of mitochondrial electron transport complex inhibitors to study sites of ROS production. In each case, the fluorescence was measured for 2 min after the addition of each inhibitor or combination of inhibitors. For these studies each inhibitor (rotenone [10 µM], rotenone and CMB [10 µM, each], or myxothiazol [10 µM]) was added individually or in specified combinations. Fluorescence was measured kinetically with an excitation wavelength of 560 nm (slit 1.5 nm) and an emission wavelength of 590 nm (slit 3 nm), slopes determined over the linear portions of each trace, and the rate converted into pmol H₂O₂/min/mg by constructing a H₂O₂ standard curve as described (Votyakova and Reynolds, 2004).

3.3. ROS production by SMP

The use of SMPs for the study of ROS production offers a slightly different experimental system than either intact mitochondria or alamethicin-permeabilized mitochondria. SMPs prepared by sonication have been shown to be vesicular; however, electron micrographs have indicated that the inner membrane subunits are oriented outward, whereas digitonin-treated SMPs seem to retain inward-oriented inner membrane subunits (Malviya *et al.*, 1968). Also, SMPs have been shown to be largely devoid of matrix antioxidant enzymes such as MnSOD (Raha *et al.*, 2000). Therefore, the complex I ROS signal, which is directed into the matrix, can be much more readily detected in SMPs (Chen *et al.*, 2003).

Hence, SMPs offer a similar advantage as alamethicin-permeabilized mitochondria but differ by their lack of antioxidant enzymes.

3.3.1. Reagents—Amplex Red (40 mM, Fluka), rotenone (25 mM [Calbiochem]), diphenyliodonium chloride (DPI) (15 mM [Sigma]), myxothiazol (20 mM [Sigma]) were all prepared in dimethyl sulfoxide (DMSO) (Sigma). A 10 mM stock of 4-(hydroxymercuri)benzoic acid (CMB) (Sigma) was prepared in 0.1 M KOH, pH 8.0. Horseradish peroxidase (HRP) (Cayman) was diluted to 2500 U/ml with H₂O. Antimycin A (5 mg/ml [Sigma]) was diluted in ethanol. A stock of NADH (10 mM [Sigma]) was prepared in H₂O fresh before each experiment. Superoxide dismutase (SOD) (10 kU/ml [Sigma]) was prepared in H₂O. The incubation media for these experiments was: 125 mM KCl, 2 mM K₂HPO₄, 5 mM MgCl₂, 10 mM HEPES, 10 mM EGTA, pH 7.4, all from Sigma-Aldrich Co.

3.3.2. Procedure to measure ROS production by SMPs—Measurements of ROS production by alamethicin-permeated mitochondria were made with a RF-5301 spectrofluorometer (Shimadzu) in a quartz cuvette with the reaction chamber heated to 37° and constantly stirred. Amplex Red (2 µg/ml), HRP (1 U/ml), and SOD (40 U/ml) were added to the cuvette containing incubation media with either 5 mM succinate or no substrates. Each reaction was started by the addition of 0.05 mg/ml to 0.2 mg/ml SMPs and the rate of ROS production recorded for at least 2 min. For reactions without substrates, 80 µM NADH was added and the rate of ROS production recorded for an additional 2 min. At this time ETS inhibitors were added (rotenone [10 µM], rotenone and CMB [10 µM, each], or myxothiazol [10 µM]) record the rate of ROS production obtained for at least an additional 2 min. The slopes of the linear portions of each trace were determined and converted into pmol H₂O₂/min/mg by constructing a H₂O₂ standard curve as described (Votyakova and Reynolds, 2004).

4. Reagents and Procedures for Evaluating Ros Production by Beta Cells

Beta cell mitochondrial ROS production has been implicated in the pathogenesis of type 1 and 2 diabetes. An increase in mitochondrial ROS production induced by hyperglycemia has been implicated as the link between elevated blood glucose and pathologic damage (Rolo and Palmeira, 2006). Indeed, mitochondrial ROS production has been shown to cause DNA damage leading to poly(ADP-ribose) polymerase activation and subsequently GAPDH inhibition. This leads to increased flux through the hexosamine pathway, advanced glycation end-product formation, polyol pathway flux, and increased activation of PKC isoforms—the main pathways of hyperglycemia-induced pathosis (Du *et al.*, 2003).

Recently, our group has demonstrated that an allele of *mt-Nd2* associated with resistance against type 1 diabetes causes decreased mitochondrial ROS production in mice (Gusdon *et al.*, 2007, 2008). However, isolation of mitochondria from primary islet cells would require the use of far too many mouse islet donors for practical purposes. However, the pancreatic beta cell line (NIT-1) derived from the non-obese diabetic (NOD) mouse has been in use for more than 15 y (Hamaguchi *et al.*, 1991). Use of flow cytometry with the mitochondrial superoxide specific dye MitoSOX red is an effective way to determine ROS production from

beta cell lines. In the following, we outline a protocol based on the work of Mukhopadhyay *et al.* (2007) with minor modifications for use of MitoSOX Red and flow cytometry to detect beta cell ROS production.

4.1. Beta cell mitochondria isolation

Characterizing the physiology of isolated beta cell mitochondria is an important question in type 1 and 2 diabetes. However, considering that the mouse pancreas contains somewhat more than 1000 islets and each islet contains 1000 cells (Takahashi *et al.*, 2007), isolation of a usable quantity of mitochondria from primary islets would require far too many mouse donors for practical purposes. The use of cell lines as a source of mitochondria represents a practical alternative. In the following, a differential centrifugation procedure for the isolation of mitochondria from beta cell lines is outlined.

4.1.1. Reagents—Mannitol, sucrose, HEPES potassium salt, fatty acid-free BSA, EDTA, and potassium hydroxide were all purchased from Sigma-Aldrich (St. Louis, MO). Percoll was from Thermo Scientific (Waltham, MA) and the BCA Protein Assay Kits were from Pierce Biotechnology (Rockford, IL). Hanks' balanced salt solution (HBSS) was purchased from BioWhittaker (Lonza, Basel, Switzerland). For these experiments, as well as those described previously, two isolation buffers were used: isolation buffer I (IBI): 225 mM mannitol, 75 mM sucrose, 10 mM HEPES potassium salt, 0.10% fatty acid-free BSA, 1 mM EDTA, pH 7.4 (with KOH), and isolation buffer II (IBII): 225 mM mannitol, 75 mM sucrose, 10 mM HEPES potassium salt, 0.1 mM EDTA, pH 7.4 (with KOH).

4.1.2. Procedure for isolating mitochondria from beta cell lines—For the following techniques we have used the NOD insulinoma tumor 1 (NIT-1) cell line. NIT-1 cells were grown to confluence in 15 75-cm² cell culture filter flasks. Media were aspirated and cells washed with 5 ml HBSS. Cells were scraped in 5 ml HBSS until all were dissociated. Cells were then collected in 50-ml conical tubes. Each flask was washed with another 5 ml HBSS and collected in the same conical tubes. Cells were pelleted at 1200 rpm for 10 min in a Sorvall Legend RT. Pellets were saved, resuspended in IBI, and transferred into a Dounce homogenizer. Cells were homogenized with pestles A then B, 5 strokes per pestle. Homogenate was then transferred to 50-ml polycarbonate centrifuge tubes (Nalgene Oak Ridge High-Speed) and centrifuge at 1300g (3300 rpm) in an SS-34 rotor (Sorvall) for 10 min. Supernatant was decanted into a clean centrifuge tube and centrifuged at 10,000g for 10 min. Afterward the supernatant was discarded and the pellet resuspended in IBII. The suspension was then transferred to a 1.5-ml Eppendorf tube and centrifuged at 10,000g for 5 min. Pellets were then resuspended in 100 μ l IBII. Protein concentration was determined with the BCA protein assay. Fifteen confluent flasks typically yield more than 2 mg of mitochondrial protein.

4.2. Reactive oxygen species production (ROS) and quantification from isolated beta cell mitochondria

To assess ROS production from isolated beta cell mitochondria the protocols detailed under section 3 can be used. It is feasible to obtain enough mitochondria to measure ROS from intact and alamethicin-permeated mitochondria. We have never attempted to produce beta

cell SMPs because of the amount of starting material necessary to generate enough beta cell SMPs for experimental purposes.

4.3. Reactive oxygen species production from intact beta cells

4.3.1. Reagents—Hanks' balanced salt solution (HBSS) was purchased from BioWhittaker. MitoSox Red, 2',7'-dichlorodihydrofluorescein diacetate (2',7'-dichlorofluorescein diacetate; H2DCFDA), Sytox Green, and 0.25% trypsin-EDTA were from Invitrogen (Carlsbad, CA), annexin V was obtained from eBioscience (San Diego, CA), and 7-AAD was purchased from BD Bioscience. Annexin V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂, 2% heat-inactivated FBS).

4.3.2. Procedure for determining ROS production by intact beta cells—In 25-cm² cell culture flasks 1.5×10^6 beta cells were plated. Cells were allowed to adhere for 24 to 48 h. Cells were incubated with 5 μ M MitoSOX Red or 5 μ M H2DCFDA in the culture media for 1 h or 15 min, respectively, at 37°, whereas control cells were left untreated. Caution: Avoid exposure to light. After the labeling period, media was aspirated and cells washed with 5 ml HBSS. Cells were then dissociated with 1.5 ml 0.25% trypsin-EDTA for approximately 5 min at room temperature. Caution: Use of enzyme-free cell dissociation buffer results in a larger fraction of dead cells. Afterwards 2 ml of culture buffer was added to stop trypsinization. Cells were collected by centrifuging at 1200 rpm for 5 min in a Sorvall Legend RT and washed twice with 1.5 ml annexin V binding buffer. The pellets were resuspended in 1.5 ml of annexin-V binding buffer, and the cells counted with a Fisher Brand Hemocytometer. Cells, 5×10^5 in 500 μ l, were then placed into sterile 12 $\times$ 75-mm polystyrene tubes (BD Falcon). Cells were stained with annexin V (5 μ l) and either Sytox Green (1 μ l) or 7-AAD (5 μ l) for the detection of dead and apoptotic cells. Results were collected with a FACSCalibur (BD Biosciences). With MitoSOX Red we collected events with FL-2 and -3 to collect MitoSOX Red, FL-1 for Sytox Green, and FL-4 for annexin V. With H₂DCFDA we collected events with FL-1 for H₂DCFDA, FL-3 for 7-AAD, and FL-4 for annexin V. *Note:* We have not observed an increase in beta cell ROS production with antimycin A.

5. Concluding Remarks

Mice are the most widely used models of human disease in biomedical research. The elegant technology available to modify mice genetically coupled with the ability to assemble these genetic modifications with well-described and accepted mouse models presents an effective system to better understand disease mechanisms. Because free radical production has been implicated in a wide variety of pathologic conditions, the attributes of mouse systems make them well suited to better understand the contribution of mitochondrial ROS production in both health and disease. To this end we hope that the techniques and tips described herein will assist researchers to accurately measure the quantity and site of ROS production in their model system.

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