Assessing mitochondria biogenesis

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Abstract

Mitochondria have their own DNA (mtDNA) and hence biogenesis of mitochondria requires a coordination of nuclear and mtDNA, both of which encode for mitochondria proteins. Our understanding of the molecular control of mitochondria biogenesis has increased in recent years, providing key signatures of the process. To determine whether or not a tissue or an organ of human or animal origin is undergoing mitochondria biogenesis, multiple parameters should be analyzed. First and foremost is visualization and measurement of mitochondria mass/volume in histological sections using fluorescent mitochondria dyes and light microscopy or transmission electron microscopy to yield quantitative results. To confirm or extend these types of analysis, biochemical markers of mitochondria biogenesis should also be included, including assessment of mtDNA copy number, steady-state levels of biogenesis-related transcription factors (e.g. mitochondria transcription factor A, mitochondrial transcription specificity factors, nuclear respiratory factors 1 and 2, and peroxisome proliferator activated receptor gamma coactivator-1-alpha), mtDNA-encoded transcripts and proteins, and rates of mitochondria translation. These techniques are described in isolation and in the context of transgenic and dietary animal models that have been used as tools to study the regulation of mitochondria biogenesis and its role in disease pathology.

1. Introduction

There are genetic, metabolic, and dietary events that result in mitochondria biogenesis and thereby may impact health and disease. Mitochondria diseases may be due to base pair substitutions in the mitochondria genome and/or may involve defects in the nuclear encoded mitochondria proteins. Also, the mechanisms or proteins responsible for ferrying some mitochondria proteins (chaperone proteins) synthesized in the cytoplasm to the mitochondria could be defective and the import of such proteins into the mitochondria could be impaired. All of these factors collectively can lead to mitochondria dysfunction, pathology, and to mitochondria biogenesis.

A number of groups have studied several diseases in humans that affect skeletal and cardiac muscle and peripheral and central nervous system tissue, particularly the brain, the liver, bone marrow, the endocrine and exocrine pancreas, the kidneys and the intestines. (1-5). Fibroblasts isolated from a child afflicted with Leigh's syndrome revealed a disorder involving a nuclear mutation in cytochrome C oxidase, but all subunits were present to lesser degrees (6). Mita et al (7) reported that a quadriceps muscle biopsy of a young patient afflicted with Kearns-Sayre syndrome revealed mitochondria deletion of all of subunit III, parts of NADH-coenzyme Q reductase (subunits III and IV), all of ATP synthase subunit 6 and part of ATP synthase subunit 8. The DNA responsible for encoding cytochrome C oxidase subunit IV was present but not the DNA of mitochondria encoded cytochrome C oxidase subunit II. Another disorder, myoclonus epilepsy with ragged red fibers (MERRF), affects both brain and muscle tissue. Western blot analysis revealed a decrease in cytochrome C oxidase subunit II relative to the other subunits, but Northern analysis failed to show any change in subunits

I, II and III (1). Schwartzkopff et al (8) reported a case of a 30 year old female exhibiting tachycardia in which there was no overt signs of cardiac failure. Subsequent biopsy of the right septal endocardium revealed enlarged and vacuolated mitochondria and increased mitochondria:myofibril values, the appearance of glycogen granules and lipid droplets and a marked decrease in cytochrome c oxidase activity. Muller-Hocker et al (9) and Zeviani et al (10) reported similar observations, particularly lower cardiac cytochrome C oxidase activity in patients suffering from cardiomyopathy. Horvath et al. (11) discovered that the copper chaperone protein, SCO2, is mutated in several forms of fatal infantile cardiomyopathy leading to cytochrome C oxidase deficiency. Salviata et al. (12) demonstrated that copper supplementation of cultured cells (myoblasts, myotubes and fibroblasts) from patients with SCO2 mutations and decreased cytochrome C oxidase activity, could restore the cytochrome C oxidase activity to control levels. Furthermore, a patient with SCO2 mutations and severe hypertrophic cardiomyopathy was reversed with copper-histidine supplementation (13). Mutations in SCO1 or SCO2 results in cell copper deficiency (14).

These disease conditions have lead to a focus on the gene program that drives mitochondria biogenesis. Mitochondria transcription factor A (mtTFA) is a major transcription factor governing mitochondria mitochondria DNA replication and transcription during mitochondria biogenesis (15). Low levels of mtTFA transcript and protein are associated with overall decreased mitochondria gene transcription in HeLa cells. On-the-other-hand, expression of human mtTFA in *Saccharomyces cerevisiae* devoid of mtTFA, restores mitochondria DNA transcription and function (16). Functional human mtTFA is a 25 kD protein, (17, 18) whereby its transcriptional

activation initiates the synthesis of mitochondria RNAs by mitochondria RNA polymerase (17). On-the-other-hand mitochondrial specificity factors, TFB1M and TFB2M, may have even more significant roles than mtTFA (19, 20).

The investigation of nuclear control of mitochondria gene expression has lead to the discovery of several other important transcription factors. Nuclear Respiratory Factor-1 (NRF-1) coordinates nuclear encoded respiratory chain expression with mitochondria gene transcription and replication (21). NRF-1 recognition sites have been found in many genes encoding respiratory functional subunits, such as rat cytochrome c oxidase subunit VIc and the bovine ATP synthase γ subunit. Therefore, NRF-1 activates mitochondria gene expression by upregulating mtTFA (22).

Another nuclear gene product, NRF-2, has also been implicated in the coordination between nuclear and mitochondria gene expression. Although the majority of genes encoding proteins in respiratory functions have a NRF-1 recognition site, some genes such as cytochrome c oxidase subunit IV and ATP synthase β subunit, lack a NRF-mitochondria recognition site but contain a NRF-2 recognition site indicating that these respiratory chain genes may be differentially regulated (23). In some genes, both NRF-1 and NRF-2 recognition sites have been identified (15, 24). It is apparent that NRF-1 and NRF-2 may convey nuclear regulatory events to the mitochondria via mtTFA, and coordinate the gene expression between the nuclear and mitochondria genomes.

Peroxisomal proliferating activating receptor- γ coactivator (PGC-1), is thought to be a master regulator of mitochondria biogenesis and its interaction with mtTFA, NRF-1 and NRF-2 is the subject of investigation. This transcription factor has the ability to induce the production of mitochondria in brown adipose tissue (25). There are various

isoforms of PGC-1 which constitutes a family: PGC-1α, PGC-1β, and PGC-1-related coactivators. Both PGC-1α and PGC1-β have high expression in tissues rich in mitochondria. Unlike some other transcription factors, PGC-1α does not have any response elements, meaning it does not bind to a DNA promoter directly. Rather it acts via a protein-protein interaction but it does not have enzymatic activity (26). Transfection of PGC1-α into C_2C_{12} cells by Wu et al. (27) and into myocytes by Lehman et al. (28) all resulted in indices of mitochondria biogenesis in terms of mitochondria protein, transcripts and mitochondria volume densities of the cells. PGC1-α may act as a coactivator of NRF-1 (27), which then is thought to bind to the promoter of mtTFA to initiate the concomitant upregulation of both mitochondria and nuclear encoded proteins in a coordinated fashion. Another set of transcription factors needed to initiate mitochondria biogenesis are termed Transcription Specificity factors (TFB1M and TFB2M). There are recognition sites within the promoters for NRF1 and NRF2 for these two transcription factors. It is also reported that PGC1-α will upregulate these two transcription factors. Upregulation of mtTFA augments mitochondria biogenesis with these other transcription factors (20).

2 Description of methods To study mitochondria biogenesis

Because of the complex nature of mitochondria, multiple parameters need to be analyzed in order to ascertain whether a mitochondria biogenesis program is occurring in cells, tissues, and/or organs (Table 1). Below, I will describe methods for assessment of mitochondria volume/number by microscopy, mtDNA copy number by PCR, typical

molecular markers of biogenesis by western blot, and mitochondria translation rates by in vivo labeling.

2.1 Microscopy.

2.1.1 Fluorescent microscopy using dyes

The most common method to first determine if there is an apparent case for mitochondria biogenesis is using a histological approach. There are dye markers that can enter mitochondria that fluoresce and can be viewed under a fluorescent microscope. One such product is Mito-tracker (Catalogue Number M7513) from Molecular Probes of Eugene, Oregon (Part of Invitrogen Inc, Carlsbad, CA). Lehman et al. (28) used this method to determine if cardiac myocytes transfected with PGC-1α had more mitochondria. The dye fluoresces orange/red. The limitation of this method is an observer must be trained on the intensity of orange/red as well as potential area occupied by the cell that is composed of the orange/red color. This makes this somewhat subjective. However, the use of flow cytometry can help overcome this limitation. Molecular Probes does have a number of mitochondria selective probes that can be used for quantification. The fluorescent dye 10-n-Nonyl-Acridine Orange binds to cardiolipin of the inner mitochondria membrane and at one time was thought to be independent of membrane potential. However, work by Gohil et al. (29) suggests that this is not the case in yeast. This brings up the issue of whether some of these mitochondria dyes may be membrane potential. Rhodamine 123 dye for instance is sequestered in the mitochondria but has also been used as an indication of mitochondria membrane potential. Similarly,

JC-1 dye is very sensitive to membrane potential. Both dyes fluoresces red with high membrane potential. With reduce membrane potential, JC-1 dye fluoresces green and rhodamine 123 fluoresces (30). In essence these dyes are washed out of the mitochondria when they are not respiring and thus suing them to quantify mitochondria is not appropriate.

2.1.2 Transmission electron microscopy

The preferred method to determine evidence of mitochondria biogenesis is transmission electron microscopy. While the fixation, dehydration, sectioning, and staining of sections is rather laborious, the results are worthwhile. However, it is important that any use of microscopy be coupled with an accurate method of assessing the amount of mitochondria. In doing so we should focus on volume density of organelles. Most laboratories are not equipped to do full scale transmission electron microscopy. The assistance of an expert either at a core facility within the institution or elsewhere should be sought out.

2.1.2.1 Fixation.

The one aspect that members of the primary laboratory should try and do is proper fixation of the tissue under investigation. The first few minutes of obtaining tissue from live animals or cell culture are critical to the final quality outcome of the prints or images obtained. Fixation simply means trying to preserve a specimen in an *in vivo* state as much as possible. Whenever possible, in vivo fixation is suggested. This requires the animal be cannulated and the organ of interest be perfused with a buffer to rinse.

Normally oxygenated Dulbecco's phosphate buffered saline supplemented with 14 mmol/L glucose is recommended. This solution should be warmed to 37 ^{o} C. If the biological material you are working is muscle it is important to know the orientation of the muscle fibers. Normally our laboratory will sample the muscle so that the "cut" is tangential to the outer layer of muscle so that muscle fibers may be viewed in a longitudinal plane. Excision of the sample and dicing into 1 mm cubes followed by fixation in 2% glutaraldehyde in 0.1 mol/L Sorenson phosphate-buffered saline and 0.1 mol/L sucrose and placement on a shaker for 90 min assures proper fixation. This is normally followed by rinsing the samples twice in a 0.1 mol/L Sorenson phosphate-buffered saline for 5min each and placed in a 1% osmium tetroxide in Sorenson phosphate-buffered saline fixative (pH 7.4, 4°C)for 150 min. This is usually followed again by two 5-min rinses in Sorenson phosphate-buffered saline and en bloc staining with 0.5% uranyl acetate in acetate veronal buffer (pH 5) for 30 min (31).

2.1.2.2 Dehydration and Embedding

The samples are dehydrated in a series of graded ethanol solutions and transferred to propylene oxide for 30 min. Samples are infiltrated with Araldite resin or some other commercial resin (eg Spurrs, Epon) in three steps: 1) 2 parts propylene oxide:1 plastic Araldite resin for 120 min while shaking, 2) 1 part propylene oxide:1 part Araldite resin for 180 min while shaking, and 3) pure Araldite resin overnight. The following day samples are embedded with Araldite in flat embedding molds, placed in an oven until hard and removed from the molds as blocks. Other molds can be used but for morphometric analysis of mitochondria flat embedding molds allows sectioning in a longitudinal plane much better.

2.1.2.3 Sectioning

The embedded blocks containing the sample are trimmed and sectioned using an ultramicrotome with 0.08 μm thickness and placed on grids and stained with lead citrate and uranyl acetate. Sections are now ready for examination by a transmission electron microscope. Here images of interest are exposed on film and developed as negatives followed by development of prints. It is also here at this point that you learn whether the techniques you used have really worked. The entire process of removal of tissue until print development is about 3-4 days. When viewing a sample under the transmission electron microscope, normally 3 random fields are exposed as an image on film. The final print enlargement may vary, but anywhere from 5,000 X to 15,000 X has been used by our laboratory to quantify mitochondria, however it is not unusual for 20,000 to 30,000 X magnification to be used for counting other cell structures.

2.1.2.4 Morphometry

The first step in interpreting the micrographic prints is to make sure you have identified the mitochondria. These are usually not hard to do as they are distinctively composed of two enclosing membranes, with the inner membrane forming cristae (Figure 1). For a complete treatise on quantification or organelles from histology and transmission electron microscopy data, see Weibel (32) and Steer (33). When using electron microscopy for assessing organelles in terms of numbers, the standard term is referred to as volume density or the area occupied by the organelle of interest. For heart muscle the normal volume density of mitochondria is 0.39 $\mu m^3/ \mu m^3$. For skeletal muscle it is around 0.12 $\mu m^3/ \mu m^3$ and a tissue such as brain is around 0.01 to 0.05 $\mu m^3/ \mu m^3$. A major problem in interpreting prints from electron micrographs is that you are dealing with a two dimensional view and have lost the 3-dimensional view. As you recall from above, it was critical for muscle to know the orientation of the fibers. Oblique or

diagonal cuts to the plane of the muscle fiber in this case could lead to error in the accuracy of the data. The image should be at right angles to an axis (34, 35).

A powerful method that our laboratory has used to quantify mitochondria consists of a method know as "point counting grids". Simply put, this is done by placing a transparent square lattice grid over the electron micrograph. The grids normally have lines that are 90 intersect each other at 1 cm distances. We use a lattice grid where every 3^{rd} line is a major heavier line and there are 2 lighter lines between each of the heavier. Some have used grids with a heavy line every 5^{th} line. The transparent grid overlays the print and the number of times an organelle of interest intersects either a major line intersect or minor line intersect is recorded. A lattice of 100-400 points is normally useful for an 8 x 10 inch micrograph. If you are interested in determining the volume density of mitochondria from the heart then counting the number of times a mitochondria is located at a major line intersect can be recorded. If however the number of organelles appeared to be sparse, one can count every intersect to determine if that organelle is located at either major or minor line intersects. Figure 2A gives a simple representation of this. By knowing the total amount of intersects that are possible with the lattice grid and the number of times a mitochondria intersected a line intersect, you simply divide this number by the total. For example if an overlay has 100 possible intersects and 35 of these intersects are where a mitochondria is located, simply divide 35 by 100 and this will yield of volume density of 0.35 $\mu m^3/\mu m^3$. An alternative approach is the use of dashed lines or discontinuous line lattices that overlay the micrograph (Figure 2B) in which the cell component that lies beneath the beginning of the line and that at the end of the line is assigned a point. In this case the line length may vary but is normally 1.8 cm in length

and 1.56 of vertical line spacing (33). Example calculations and other parameters that can be measured in a cell using these approaches with examples are found in the work of Steer (33).

2.2 Biochemical and molecular methods

2.2.1 Mitochondria DNA determination

In addition to histological methods, many times it is common to use one or several biochemical methods to help verify that mitochondria biogenesis is occurring. Since mitochondria have their own DNA that ranges from 16.5 kB in humans and rats, the measure of mitochondria DNA will be proportional to the number of mitochondria, although each mitochondrion may have from 2-10 copies of DNA. The amount of mitochondria DNA has traditionally been accomplished using Southern blot analysis. However, newer and more rapid methods have exploited real time PCR to quantify mtDNA in cells and tissue and comparing it to the total amount of genomic DNA as one marker. If the real time PCR method is used, NADH dehydrogenase subunit I is a mitochondria encoded protein and a measure of the quantity of DNA encoding for this enzyme will indicate the mitochondria number, but this should be corrected for the genomic DNA. The latter can use lipoprotein lipase. For the NADH dehydrogenase subunit 1, a 205 base pair fragment primers can be used (Forward: CCC ATT CGC GTT ATT CTT; Reverse: AAG TTG ATC GTA ACG GAA GC). The lipoprotein lipase can be 225 base pair fragment primers (Forward: GGA TGG ACG GTA AGA GTG ATT C; Reverse: ATC CAA GGG TAG CAG ACA GGT) (36, 37). These are simply examples

of what can be used. The strategy to employ is that any set that amplifies an mtDNA sequence and a nuclear gene sequence will work. An important note is that mtDNA target sequence used should avoid common deletions found in disease and with aging. In the circular mtDNA this would mean that using sequences between the small arc of the oriH and oriL portion is recommended. A multicopy nuclear locus such as the 18S of rRNA is also recommended as a method of correcting for genomic DNA (38, 39).

2.2.2 Western blotting of selected markers of mitochondria biogenesis.

There are several strategies one can use to provide evidence that a tissue is likely to have more mitochondria. Transcripts or proteins of cytochrome c oxidase subunits should be elevated if there are a greater number of mitochondria. Various subunits of ATP synthase should be elevated. Whether one uses northern blots for transcripts or western blots for protein quantification, either one should be done in studies confirming if mitochondria biogenesis has exists. These are indirect measures of mitochondria biogenesis. Another approach we have used is to look at key proteins or transcripts known to be involved with mitochondria biogenesis. Here the candidates are NRF-1, NRF-2, mtTFA, and PGC-1α. In our hands we have used gel shift assays to determine if NRF-1 and/or NRF-2 are upregulated. RNAase protection assays may also be used for NRF-1 and NRF-2 assessment. One problem though is that some of these transcription factors may be only transiently upregulated. These two transcription factor proteins have recognition sites on the promoter of mtTFA. We have used western blots to demonstrate quantify changes in mtTFA. In our lab we have been studying cardiac mitochondria biogenesis as a result of dietary copper deficiency. While we have been able to demonstrate increased mitochondria mass using transmission electron microscopy, we

were able to demonstrate a robust increase in mtTFA in hearts from copper deficient rats compared to controls (40). Moreover we demonstrated that NRF-1 and NRF-2 were elevated, but prior to any change in mtTFA, demonstrating the temporal pattern to some of these proteins (41). Recently we were able to demonstrate elevations in PGC-1α using real time PCR and western blotting (Figure 3) in rats fed a copper-deficient diet where increased mitochondria are known to occur. Taken together, these markers revealed to us that the gene program for mitochondria biogenesis was tuned on and supported the transmission electron microscopy data. Many of these antibodies are commercially available such as from Santa Cruz Biotechnology (Santa Cruz, CA) and MitoSciences (Eugene, OR). These include antibodies for cytochrome c oxidase nuclear and mitochondria encoded subunits, PGC1-α, mtTFA, and ATP synthase subunits.

*2.2.3 Radiolabelling***.**

Incorporation of an amino acid into the mitochondria protein using an *in vivo* model and time course is an approach that is used to assess biogenesis. The drawback is the used of radioactivity in that is a large dosage since dilution into a whole body must be taken in account. Leucine is frequently used. In our laboratory we have used 250 μCi L-(4, 5- ^{3}H) leucine per 100 g body weight (42). We normally cannulate the left jugular vein by making a small incision on the ventral surface of the neck of anesthetized rats. This is when we are looking at the heart, but the femoral vein may also be used. We conduct a time course in which the animals are sacrificed 30 min, 60 min, or 90 min. after infusion. Clearly multiple animals need to be used for each time point (at least an n of 3) and the total number depends on the number of treatments. After the time period has

elapsed, rats are exanguinated by opening the thoracic cavity and severing of the great vessels of the heart. The tissue of interest is placed on ice for subsequent analysis.

The hearts are radioactive as is the remainder of the carcass and proper disposal of radioactive bio-materials must be followed. The heart needs to be prepared to have the mitochondria protein separated from the remainder of the tissue. If muscle is used, then you are essentially separating myofibrillar protein from non-myofibrillar protein which is mitochondria and 1% sarcoplasm. 0.3 to 0.6 g of heart is homogenized at high speed in 10X volume of 0.1M KCl and 1.5% Triton X-100 with a Polytron for 30 sec. A 10-µl sample of the crude homogenate is added to 5 mL of a counting cocktail and placed in a scintillation counter. The remaining homogenate is centrifuged at 1100 g for 20 min to separate the detergent soluble nonmyofibrillar supernatant from the insoluble myofibrillar pellet. A 10-µL sample of the nonmyofibrillar fraction is added to 5 mL of cocktail and counted for radioactivity. The myofibrillar fraction is diluted back to the original volume through the addition of KCl-Triton-X mixture and rehomogenized. A 50-µL sample of the myofibrillar fraction is added to 5 mL cocktail and counted for radioactivity. A ratio of the nonmyofibillar to myofibrillar radioactivity is used to determine the mitochondria biogenesis, which would be indicative of greater radioactivity in the non-myofibrillar fraction.

3 Inducing mitochondria biogenesis: over-expression models to nutritional models.

While there are many models that have been and are being produced whereby there is an over-expression of PGC-1α to study mitochondria biogenesis, we will focus on those produced by the Kelly lab at Washington University School of Medicine. In

trial studies using an overexpression model of PGC-1α resulted in 100% mortality in offspring due to over-production of mitochondria. Knowing the lethality of simple over-expression, an inducible system allows the animals to mature and then induce mitochondria proliferation under controlled conditions. Also, one of the most known nutritional models of mitochondria biogenesis is that produced by feeding diet deficient in copper. Here the heart enlarges and is characterized by increased volume density of mitochondria. Deficiencies of other nutrients can lead to similar findings. I will discuss both genetically modified and dietary models of mitochondrial biogenesis.

3.1 Transgenic models.

This murine model was developed by the Kelly group at Washington University School of Medicine (43). It is a double transgenic inducible model for cardiac-specific overexpression of PGC-1α. Constitutive overexpression of the PGC-1α in the heart of transgenic mice using the myosin heavy chain promoter results in uncontrolled proliferation for mitochondria in heart cells and high mortality. The development of an inducible system allowed for more control over mitochondria proliferation as well as the opportunity to study the molecular pathways that are responsible for mitochondria biogenesis.

The model was created from two transgenic strains. One strain expressed a codon-optimzed reverse tetracycline transactivator from the α-myosin heavy chain promoter (MHC-rtTA). The PGC-1α transgene was developed by inserting a PGC-1α cDNA with a C-terminal myc-his tag into a pTRE2 vector from Clontech. The linearized construct was injected into blastocysts to generate the TRE-PGC-1α lines. The two

strains were crossed to generate the double transgenic mice (Tet-on-PGC-1α). The TRE-PGC-1α gene is "turned" on by administering 2 g/L doxycycline in their drinking water supplemented with 5% sucrose.

There are neonatal and adult models that have been studied (43). Expression of this transgene in a neonatal model results in a dramatic increase in mitochondria volume density, and is coincident with the upregulation of markers of mitochondria biogenesis. Expression in the adult model leads to an increase volume density of mitochondria, but not to the same extent as the neonatal model. However, the mitochondria ultrastructure appears deranged and there is development of cardiomyopathy. Both models are specific to heart only in that other tissues do not express the transgene or is not leaky. Northern hybridization studies demonstrate that with doxycycline administration, the PGC-1α-myc-his transcripts are up-regulated. Its target gene, NRF-1 is upregulated along with a downstream target, mtTFA. Studies have been conducted on this model where doxycycline has been administered for up to 7 weeks without mortality.

3.2 Mitochondria biogenesis using a copper-deficiency model.

The phenotypes of the two above models display a similar phenotype exhibited by the copper-deficient model. However, mitochondria biogenesis is not limited to the heart. The use of this model is rather simple in both mice and rats. Rats that are recently weaned are housed individually in stainless steel cages. This stainless steel is needed since other cages may contain copper and rodents may tend to bit the cages. Having them housed singly is important since group housing would slow down the copper induction as rodents tend to preen one another and this can lead to unanticipated copper

ingestion. The diets used are purified and most investigators use a diet recommended by the American Institute of Nutrition either called the AIN-76A diet (44) or the AIN-93G diet (45). The biggest difference between these two diets is the types of carbohydrates. Either diet will work. We have used the AIN-76A diet and have copper omitted from the diet for those rats that will be made copper deficient. The control consists of the regular AIN-76A diet which contains 6 μg Cu/kg diet. You can obtain such diets from many commercial diet preparation companies. One company we use in particular is Research Diets of New Brunswick, New Jersey. A quick scan of the internet will reveal other viable commercial sources. Deionized-distilled water must be given to the animals tap water has too much copper that can prevent a copper deficiency from occurring. Feeding these to rats for only 3 to 5 weeks will result in copper deficiency. The hearts will demonstrate marked cardiac hypertrophy and mitochondria biogenesis. Presumably the absence of cytochrome c oxidase activity, a copper-containing enzyme, may help initiate this response. We have demonstrated mitochondria biogenesis from both histological evidence and numerous biochemical studies (34, 35, 40, 41).

4. Concluding remarks

Determination of whether a model system demonstrates the presence of mitochondria biogenesis cannot be relied upon by only one method. Multiple assessments should be done to muster peer review. Clearly histological methods are needed with transmission electron microscopy being the gold standard. However, in addition, some biochemical markers should be presented. In particular the preferred

measures are mtDNA and mtTFA using Northerns or Westerns of mitochondria transcripts or proteins, respectively.

The methods described herein address mitochondria biogenesis, but not functionality. Since increased biogenesis can be associated with gain of function as well as pathological responses, functional studies should be coupled with biogenesis studies to glean a more accurate picture of whether the observed changes are adaptive or potentially maladaptive (46).

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FIGURE LEGENDS

Figure 1. Comparison of mitochondria under "control" conditions (A) versus mitochondria volume density increased as a result of inducing mitochondria biogenesis gene program (B). In this example, a transgenic mouse was induced to turn on the mitochondria biogenesis program by over-expressing PGC-1α.

Figure 2. An example of how mitochondria volume density can be simply determined with a transparent grid overlaying an electron micrograph. In panel A, the major line intersections are used and the cell component corresponding to that intersection is given a point. In panel B, there are many dashed lines or a discontinuous line lattice layered over the micrograph. The organelle corresponding to the beginning and end of each line are assigned points. Either approach will lead valid data for volume density.

Figure 3. PGC1-α levels are increased as a result of increased mitochondria in the copper deficiency model in a typical western blot. Here rats were fed diets either adequate (Cu+) or deficient (Cu-) in copper for 5 weeks. Cardiac hypertrophy occurred in the hearts with increased mitochondria.