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Integration of cellular bioenergetics with mitochondrial quality control and autophagy

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Abstract

Bioenergetic dysfunction is emerging as a cornerstone for establishing a framework for understanding the pathophysiology of cardiovascular disease, diabetes, cancer and neurodegeneration. Recent advances in cellular bioenergetics have shown that many cells maintain a substantial bioenergetic reserve capacity, which is a prospective index of "healthy" mitochondrial populations. The bioenergetics of the cell are likely regulated by energy requirements and substrate availability. Additionally, the overall quality of the mitochondrial population and the relative abundance of mitochondria in cells and tissues also impinge on overall bioenergetic capacity and resistance to stress. Because mitochondria are susceptible to damage mediated by reactive oxygen/nitrogen and lipid species, maintaining a "healthy" population of mitochondria through quality control mechanisms appears to be essential for cell survival under conditions of pathological stress. Accumulating evidence suggest that mitophagy is particularly important for preventing amplification of initial oxidative insults, which otherwise would further impair the respiratory chain or promote mutations in mitochondrial DNA (mtDNA). The processes underlying the regulation of mitophagy depend on several factors including the integrity of mtDNA, electron transport chain activity, and the interaction and regulation of the autophagic machinery. The integration and interpretation of cellular bioenergetics in the context of mitochondrial quality control and genetics is the theme of this review.

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Introduction

It has long been known that mitochondria show diminished functional activity when isolated from a broad range of tissues subject to pathological stress. Well-established examples are alcohol-dependent hepatotoxicity, cardiac ischemia-reperfusion and neurodegenerative diseases (Bailey and Cunningham, 2002; Brookes et al., 2004; Carreira et al., 2011; Coskun et al., 2011; Jenner, 2003; Krzywanski et al., 2011; Lemasters et al., 2009; Pilsl and Winklhofer, 2012). In all of these cases a role for reactive oxygen or nitrogen species (ROS/RNS) has been proposed in causing mitochondrial dysfunction. Typically, mitochondrial damage is defined as a relative decrease in respiratory parameters such as a change in oxygen consumption in the presence of substrates and ADP (state 3 respiration), in the presence of substrates alone (state 4), or the ratio of these parameters (i.e., respiratory control ratio; RCR). Other parameters include decreased activity of specific enzymes, deletions in mtDNA and increases in oxidative markers such as protein oxidation. The challenge with these data is in integrating them into an overall model of cellular and tissue bioenergetic function. It is becoming apparent that mitochondria under pathological stress exhibit multiple defects which overall can be viewed as a decrease in mitochondrial quality (Karbowski and Neutzner, 2012; Piantadosi and Suliman, 2012; Pilsl and Winklhofer, 2012). Importantly, it has recently been shown that the cell possesses a complex machinery capable of surveying mitochondrial quality and scheduling sections of defective organelles for demolition through a specialized category of autophagy called mitophagy (Kim et al., 2007; Lee et al., 2012; Lemasters, 2005; Youle and Narendra, 2011). This method of mitochondrial surveillance is useful because it allows changes in mitochondrial parameters such as membrane potential, activity of oxidative phosphorylation, and mitochondrial damage to be placed in the dynamic context of cell function.

The controlled regulation of mitochondrial abundance through biogenesis or degradation is also critical for maintaining proper cell function. The formation of “new” mitochondria is a tightly controlled process involving several factors, including *de novo* synthesis of organelle components from cellular precursors, formation of mitochondrial membranes from pre-formed membranous structures, and division of pre-existing mitochondria (Michel et al., 2012). For maintenance and modulation of energy production, the cell then must coordinate the biogenic process: the transcription, import, and assembly of nuclear-encoded genes must match with the replication and transcription of the mitochondrial genome and with biogenesis of mitochondrial membranes. External stimuli have been shown to initiate this dynamic process. For example, caloric restriction, exercise, hypothermic conditions, and energy deprivation result in the activation of signaling pathways responsible for increasing mitochondrial abundance (Bordicchia et al., 2012; Cao et al., 2001; Nisoli et al., 2003; Wu et al., 2002). The PGC1 family, especially PGC1α, along with other multiple factors coordinate the biogenesis of mitochondria (Michel et al., 2012). Interesting examples of the pathological effects of a mismatch between energy demand and mitochondrial number was shown by studies where expression of PGC1α was manipulated in the heart. PGC-1α $^{-/-}$ mice develop signatures of heart failure, and constitutive, cardiac-specific overexpression of PGC-1α results in excessive mitochondrial biogenesis, leading ultimately to death from heart failure (Finck and Kelly, 2007). It appears then that mitochondrial abundance is finely tuned to the energetic needs of the cell—too few or too many mitochondria can result in similar pathological outcomes.

The obligation to match mitochondrial abundance and quality with energy demand is outlined in Figure 1. Once a stable population of mitochondria is established in a cell it must perform a series of integrated biological functions. These include the conversion of caloric energy into metabolic intermediates for cell differentiation, molecular energy (ATP), thermal energy (heat), and oxidants. The generation of ROS from mitochondria appears to serve a cell signaling function when controlled at low levels but can contribute to pathology if it leads to damage to electron transport proteins and "electron leak" to oxygen at a non-physiological locations (Gutierrez et al., 2006; Murphy, 2009). Under normal conditions mitochondrial populations in cells appear highly stable. For example, it has been shown that mitochondria have a half life of ~9 days in the liver, ~10 days in the kidney, ~16 days in the heart, and ~26 days in the brain (Menzies and Gold, 1971). Quality control of individual mitochondrial proteins, is mediated by intra-mitochondrial proteases (Bota and Davies, 2001; Ugarte et al., 2010). The ATP-stimulated mitochondrial Lon protease plays an important role in the degradation of oxidized proteins (Bayot et al., 2008). This process is important in the maintenance of proteins that are highly susceptible to damage and therefore need to be turned over more frequently to sustain normal mitochondrial function. For example, oxidatively damaged aconitase has been shown to be selectively degraded by Lon protease (Bota and Davies, 2002). In response to hypoxia, Lon protease degrades cytochrome c oxidase subunit IV isoform 1 to allow isoform 2 to replace its position (Fukuda et al., 2007). The mitochondrial AAA-proteases have been shown to be important for preventing mtDNA escape from mitochondria to the nucleus possibly through ensuring appropriate fission and fusion, as well as rapid proteolysis of non-assembled inner membrane proteins (Hori et al., 2002; Thorsness and Fox, 1990; Thorsness et al., 1993). In response to more extensive mitochondrial damage, increased mitochondrial fragmentation and decreased fusion can separate damaged mitochondrial proteins, lipids and DNA from functional components, and the entire damaged organelle is processed by mitophagy (Kim et al., 2007; Lee et al., 2012). It has recently been shown that the sub-populations of mitochondria destined for turnover are segregated through a dynamic process involving fission of areas of low functioning mitochondria and fusion of healthy populations (Kim and Lemasters, 2011; Twig et al., 2008b). This turnover of mitochondria by lysosomes is executed in a process now called autophagy of mitochondria, or mitophagy (Green et al., 2011; Kim et al., 2007; Lemasters, 2005; Youle and Narendra, 2011).

Because of their importance in providing cellular energy, modulating ROS and controlling apoptosis, mitochondrial quality control is critical for normal cell function. Mitochondrial morphology and function are perturbed in genetically engineered autophagy-deficient animals, further supporting the hypothesis that autophagy of mitochondria (mitophagy) is essential for mitochondrial quality control (Kim et al., 2007). It therefore follows that damaged mitochondria arise during normal metabolism and are potentially hazardous because of their ability to amplify ROS-dependent damage (Figure 1). Under pathological stressors several interrelated mechanisms can contribute to decreased mitochondrial quality. These include mitochondrial calcium overload, increased levels of ROS/RNS, endoplasmic reticulum stress and accumulation of aggregated proteins. Where extra-mitochondrial sources of ROS/RNS are present, the quality of the mitochondrial population declines as evidenced by increased mtDNA damage, decreased membrane potential, and a diminished bioenergetic reserve capacity (Dranka et al., 2010; Higdon et al., 2012; Hill et al., 2009; Zelickson et al., 2011). Overwhelming the mitophagy pathway exacerbates the problem since the low quality mitochondria now impact on the remaining healthy mitochondrial population through release of ROS and calcium from the organelle. Thus, it is clear that the dynamics, turnover and biogenesis collectively maintain quality control. These processes are regulated by intracellular and extracellular signals that coordinate the biogenesis transcriptional program, cellular bioenergetics, redox signaling and mitophagy. In this review we will discuss emerging concepts in assessing cellular bioenergetic function, and

how mitochondrial quality is maintained in a cellular setting. In particular, we address the following questions:

1. How can bioenergetics be measured and placed in context with disease?
2. What is the role of the mitochondrial genetic landscape in diseases based in bioenergetic dysfunction?; and
3. How does mitophagy contribute to mitochondrial quality control?

The cellular bioenergetic profile and its interpretation

Typically experiments with isolated mitochondria have been performed in the context of established protocols performed with oxygen electrodes. The outputs from such experiments are the rates of state 3 and 4 respiration and the ratio of these two rates—the RCR (Chance and Baltscheffsky, 1958a; Chance and Baltscheffsky, 1958b). Mitochondrial dysfunction has often been defined as a deviation of these parameters in a treatment or disease group relative to control. Classic experimentation with isolated mitochondria cannot effectively address this issue since cellular regulation of mitochondrial function is removed during isolation. To bridge this gap, methods have been developed to determine the behavior of mitochondria in a cellular setting using extracellular flux analysis (Brand and Nicholls, 2011; Dranka et al., 2010; Ferrick et al., 2008; Gerencser et al., 2009; Nicholls et al., 2010; Wu et al., 2007). Recent advances in this technology have allowed for real-time measurements of O_2 consumption and acidification in cell culture with the capacity to inject respiratory inhibitors. This has quickly allowed for the establishment of methodical assays to interrogate bioenergetic function in cells under a broad range of conditions (Dranka et al., 2011; Ferrick et al.; Wu et al.). How the rates of O_2 consumption (OCR) and extracellular acidification (ECAR), which are global properties of the cell preparations, can be ascribed to specific biological processes will be discussed next (Hill et al., 2009; Sridharan et al.).

Fundamentals of the bioenergetic profile assay

Information on how mitochondria function in intact cells has largely been derived from assays similar to that shown in Figure 2. Here, intracellular mitochondrial function can be determined by sequentially adding pharmacological inhibitors of oxidative phosphorylation (Brand and Nicholls, 2011; Dranka et al., 2011). In the first series of measurements, the basal oxygen consumption of the cells is established. The basal OCR represents the net sum of all processes in the cell capable of consuming O_2, including mitochondria and other oxidases. Next, oligomycin, an inhibitor of mitochondrial ATP synthase, is injected. In support of the assumption that the basal respiration represents the demand on the proton motive force (for making either ATP or moving other ions across the inner membrane), the pharmacological inhibition of energy-requiring processes results in a decrease in basal respiration. The decrease in OCR in response to oligomycin is then related to the proportion of mitochondrial activity used to generate ATP.

In contrast to experiments with isolated mitochondria, respiration is sustained in these conditions by respiratory substrates provided by cellular metabolism. To estimate the maximal potential respiration sustainable by the cells, a proton ionophore (uncoupler) such as FCCP is used. Immediately upon exposure to the uncoupler, oxygen consumption increases as the mitochondrial inner membrane becomes permeable to protons, and electron transfer is no longer controlled by the proton gradient. An important feature emerging from such assays is the mitochondrial reserve capacity, which is calculated by subtracting the FCCP-stimulated rate from the basal OCR.

To determine the extent of non-mitochondrial oxygen-consuming processes, the respiratory chain is inhibited with antimycin A alone or in combination with rotenone. Such

pharmacological inhibition of electron transport typically inhibits the majority of oxygen consumption in the cell, and we attribute the remaining O_2 consumption to non-mitochondrial oxidases present in the cell. This value is subtracted from other OCR measurements to determine the mitochondrial-based parameters. It should be noted that for meaningful comparisons between different conditions or treatments, the basal OCR should be normalized to a factor to correct for different numbers of cells in the assay or changes in cell size, e.g., cell atrophy or hypertrophy. Insights into the specific activity of the mitochondria can be obtained by normalizing to a mitochondrial protein measured in the cell lysates after the assay. Taken together, these data have interesting implications for understanding mitochondrial function in a cellular context.

Interpretation of the bioenergetic profile

In Figure 3 we have selected examples with diverse energy requirements *in vivo* to illustrate how the bioenergetic profile differs between cell type. Aortic endothelial cells are typically regarded as having (low energetic demand), primary hepatocytes (moderately energetic), and adult cardiomyocytes (high energy demand). Differences in ATP-linked oxygen consumption and proton leak are clearly evident between the cell types, with endothelial cells having a much higher proportion of the basal OCR ascribed to ATP turnover. Both hepatocytes and cardiomyocytes show little change when treated with oligomycin. The FCCP-stimulated rate is highest in the more energetic hepatocytes and cardiomyocytes, which leads to a high reserve capacity in both cell. At first glance the profiles look remarkably similar but on closer examination differences are apparent. For example, the lack of sensitivity of cardiomyocytes to oligomycin is likely due to the fact that under these conditions the cells are not contracting. This means that the activity of ATP synthase required to supply the ATP demand is likely very low and therefore the effect of oligomycin is minimal. The pie chart in panel B represents Basal OCR distributed between ATP-linked OCR, proton leak and non-mitochondrial functions. It is interesting to note that proton leak (or ion movements requiring proton motive force) and non-mitochondrial consumption of oxygen is quite different between the different cell types. Data in Panel C are expressed as a % of the maximal respiration, and these data reinforce the conclusion that the contribution of mitochondria to oxygen consumption in cells is both diverse and distinct. This finding assumes an additional importance when we consider the potential role and extent of ROS generation in mitochondria both in relation to mitochondrial genetics and maintenance of organelle quality as will be discussed later.

To assist the investigator Figure 4 shows a typical basis for interpretation of the most common changes in bioenergetic profile observed in routine experiments. Below we describe in detail these interpretations of cellular bioenergetic profiles and some of the principles that underlie changes in the key parameters of basal respiration, ATP-linked oxygen consumption, proton leak and reserve capacity (sometimes also called spare respiratory capacity).

Basal oxygen consumption rate—Taken in isolation, a change in the value of basal OCR may be due to either a physiological adaptation or response to stress. An increase in basal OCR could be due to an increase in ATP turnover, an increase in proton leak, and/or an increase in non-mitochondrial oxygen consumption (e.g., ROS formation). Oligomycin treatment gives insight into the former two possibilities (Figure 4).

ATP-linked OCR and proton leak—The addition of oligomycin is assumed to eliminate completely control of respiration by oxidative phosphorylation, and the remaining rate of mitochondrial respiration is then taken to represent the cumulative proton leak that was present prior to addition of oligomycin. An increase in the ATP-linked rate of O_2

consumption would indicate an increase in ATP demand, and a decrease would indicate either low ATP demand, a lack of substrate availability, or severe damage to the ETC, which would impede the flow of electrons and result in a lower OCR (Figure 4); of note, this would also decrease the maximal capacity as well (see below).

An increase in apparent proton leak could be due to a number of factors: uncoupling protein (UCP) activity could be increased (Divakaruni and Brand; Echtay et al.); the inner mitochondrial membrane and/or ETC complexes could be damaged, allowing H^+ to leak into the matrix; or there could be electron slippage, which would result in oxygen consumption in the absence of proton translocation (Brown, 1992). Treatment of cardiomyocytes with 4-hydroxynonenal (HNE), for example, results in an increase in both ATP-linked oxygen consumption and proton leak (Hill et al.; Sansbury et al., 2011a; Sansbury et al., 2011b).

In such a result, it is important to consider that the pharmacological interrogation of the metabolic profile can bias data output and interpretation. For example, oligomycin increases the mitochondrial membrane potential ($\Delta_{\psi m}$) and induces a State 4-like respiratory condition (Brown et al.), which will also increase proton leak through the mitochondrial membrane (Brand; Brown). The value for ATP-linked respiration is then modestly under-estimated and proton leak over-estimated. It is important to consider also that the rate of apparent proton leak could represent the passage of any ion, e.g., calcium, across the inner membrane which can dissipate the proton gradient. Technologies that allow the simultaneous measurement of mitochondrial membrane potential in conjunction with cellular respiration would need to be developed to test this experimentally.

Maximal OCR—Experimentally, the use of FCCP to estimate the maximal respiratory rate has several limitations. In each cell type the FCCP concentration has to be titrated since at high concentrations it can be inhibitory (Dranka et al., 2011). A lower maximal capacity could indicate decreased substrate availability or that mitochondrial mass or integrity is compromised. A high FCCP-stimulated rate compared with the basal rate suggests that the cell has a substantial mitochondrial reserve capacity, which could be important for responding to acute insults.

Non-mitochondrial OCR—Very few measurements have been performed in cells to determine the contribution of non-mitochondrial sources of oxygen consumption. Using isolated mitochondria and respiratory chain inhibitors it was calculated in the early 1970's that mitochondrial superoxide production was 1–2% of oxygen consumption (Boveris and Chance, 1973; Turrens, 2003). Using cell fractionation and the measurement of hydrogen peroxide generation in hepatocytes, it was calculated that hydrogen peroxide production was 10% of the total oxygen consumption by the cell (Boveris et al., 1972). In Figure 3B we show the distribution of oxygen consumption in the cells calculated assuming basal respiration is 100%; in Figure 3C this is calculated assuming maximal respiration is 100%. In the hepatocytes and cardiomyocytes, non-mitochondrial oxygen consumption constitutes approximately 40% of basal OCR, and in endothelial cells approximately 15%. A more detailed investigation of the potential sources of these oxygen consuming pathways needs to be performed since not all of these pathways will generate hydrogen peroxide. However, it is intriguing that this value is different between cell types. The widely held view that mitochondria consume over 95% of the oxygen in the body should be reevaluated.

Cellular respiratory state$_{apparent}$

It is becoming clear that in most cells mitochondria function at an intermediate respiratory state between State 3 and State 4 and have RCR values far in excess of those found in

isolated mitochondria (Brand and Nicholls, 2011; Dranka et al., 2011; Dranka et al., 2010; Sansbury et al., 2011a). The fact that cells have measurable respiratory state values that vary with insult or injury suggests that the redox status of individual components of the respiratory chain populate a dynamic range of intermediate metabolic conformations. When comparing the state 4 condition (oligomycin) to the state 3/uncoupled condition (FCCP-stimulated), cytochrome *c* oxidase appears to exhibit a potential range of activity of approximately 5–10 fold. This is interesting since experiments with the isolated enzyme have shown the existence of intermediate active forms of cytochrome *c* oxidase that depend on electron flux, and these forms of the enzyme are differentially reactive with respiratory modulators such as CO (carbon monoxide), NO (nitric oxide) and NO_2^- (nitrite) (Cooper and Giulivi, 2007; Sarti et al., 2003).

We have proposed that the cellular $State_{apparent}$ can be calculated as follows:

$$State_{apparent} = 4 - \frac{(Basal - Oligo)}{(FCCP - Oligo)}$$

The $State_{apparent}$ therefore can be modulated by changes in the basal OCR, the coupling status, and/or the maximal respiratory rate.

It is important to note that the calculation of $State_{apparent}$ simplifies the underlying and highly interactive phenomena that act together to determine overall flux through the respiratory chain. Since the factors that determine respiration are multiparametric then it is clear that several different conditions could lead to similar values for these parameters. The underlying molecular mechanisms for changes in $State_{apparent}$ could involve changes in activation or levels of expression of the metabolic proteins controlling these processes which then must then be determined in independent experiments.

Cellular RCR

The respiratory control ratio was originally defined for isolated rat heart muscle sarcosomes and referred to the extent to which respiration is slowed after added ADP is phosphorylated completely (State 3 to State 4 transition) (Chance and Baltscheffsky, 1958b). This ratio, in its various forms, is generally regarded as the “gold standard” for measuring mitochondrial coupling, and the concept has been extended to cells. In the original paradigm, respiratory control can be viewed as a result of the buildup of a high-energy intermediate that links respiration to ATP synthesis, which occurs because all ADP is phosphorylated.

In reality, the phenomena that are responsible for respiratory control are much more complex. Metabolic control analysis (MCA) has undoubtedly been the most useful and quantitative framework for examining mitochondrial respiratory control in cells. The overall approach involves determining the relative extent to which one individual step in a pathway, such as oxidative phosphorylation, exerts control of overall flux through the entire pathway. The parameter that quantitates this control is the control coefficient and can assume a value between 0 and 1. Importantly, if the overall flux is at steady-state the sum of control coefficients for all steps of a pathway is equal to 1. Functionally, the closer the value of a control coefficient for an individual step is to 1 the more control this single step has over the flux through the entire pathway. Metabolic control analysis has been applied to both isolated mitochondria and also to mitochondrial respiration in intact cells and has revealed information which can be used for the quantitative and mechanistic interpretation of the reserve (Pan-Zhou et al., 2000) or spare respiratory (Choi et al., 2009) capacity in cells under various conditions (Brand and Nicholls, 2011).

In terms of interpreting reserve capacity, the assignment of each component of OCR to the biological activities depicted in Figure 2 makes specific assumptions regarding the control coefficients for the various components under the different experimental conditions. Specifically, the control coefficient for the proton leak component must dominate respiration after addition of oligomycin and the coefficient for electron transfer (implicating the integrity of respiratory complexes and/or substrate conditions) must dominate after addition of uncoupler. Previous application of MCA to studies with hepatocytes has demonstrated that under baseline conditions (similar to the basal OCR) control of oxidation is distributed among all three components ($\Delta\psi_M$-generating processes, ATP production, and proton leak) (Brown et al., 1990). Comparison with Figure 3 indeed supports the concept that under basal conditions control of respiration is distributed among these components but this differs between different cell types.

Biological regulation of the RCR is also complex, and it is now known that the RCR varies with both the respiratory substrate used and the activation of uncoupling processes in the inner mitochondrial membrane. These can be specifically regulated by channels known as uncoupling proteins, proton slip in respiratory chain complexes, or non-specific damage to the mitochondrial membrane such as would occur with lipid peroxidation.

The bioenergetic profiles shown in Figures 2 and 3 can be used to calculate a respiratory control ratio (RCR) for oxidative phosphorylation in a cellular setting. To do this, we assume that the OCR after the addition of oligomycin represents State 4 respiration and that after FCCP is equivalent to State 3. The cellular RCR without (Basal RCR) and with (maximal RCR) the addition of FCCP is then calculated as the State 3 rate divided by the State 4 rate. The non-cytochrome *c* oxidase OCR (i.e., in the presence of antimycin A; Anti A) is subtracted from all rates.

$$RCR_{basal} = \frac{(Basal - Anti\ A)}{(Oligo - Anti\ A)}$$

$$RCR_{max} = \frac{(FCCP - Anti\ A)}{(Oligo - Anti\ A)}$$

The RCR calculations for the three cell types are shown in Figure 3. The high RCR values calculated using these data are clearly much above that reported for isolated mitochondria. There could be several reasons for this difference.

1. The isolation process damages mitochondria and detaches them from the cellular matrix, which results in higher proton leak.
2. The mitochondria respond dynamically to substrate demand in a cellular setting through metabolic regulation which is lost in the isolated mitochondria.

Both factors likely contribute. In any event, it is clear that mitochondria in many cells are normally functioning at a sub-maximal capacity under basal conditions.

Bioenergetic reserve capacity

In addition to the RCR, calculation of the mitochondrial reserve capacity ($FCCP_{rate} - Basal_{rate}$) is a useful qualitative indicator of mitochondrial energetic status in cells. The factors controlling the mitochondrial reserve capacity are complex. Substrate supply, coupling, ATP demand and allosteric regulation of key metabolic enzymes, and the integrity

or level of expression of respiratory chain components are critical features that regulate oxygen consumption.

Substrate supply appears to be extremely important in regulating the bioenergetic reserve in the cellular context. In contrast to experiments using isolated mitochondria, substrate delivery in intact cells is subject to more complex regulation. Allosterism of key metabolic enzymes and the cadence of metabolic cell signaling are two fundamental ways in which respiration is controlled. For example, pyruvate dehydrogenase (PDH), which is the rate limiting step in glucose oxidation, is regulated allosterically by NAD^+/NADH, ATP and AMP, CoA/Acetyl CoA, and calcium as well as by both PDH phosphatase and kinase (Patel and Korotchkina). Furthermore, the TCA cycle is regulated allosterically at several points including isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and malate dehydrogenase (Williamson and Cooper). This is even more important since anaplerotic metabolism, for instance through metabolism of the amino acids glutamine and aspartate entering at the levels of α-ketoglutarate and oxaloacetate, respectively, would influence basal metabolism. Fatty acids can also enter the TCA cycle through anaplerotic metabolism to succinyl CoA (Brunengraber and Roe). These selected examples illustrate how basal metabolism could be altered by different substrates. The use of alternative pathways may also differentially modulate the control coefficients for mitochondrial respiration.

Substrate utilization is cell- and context-specific. Experimentally, this has been validated by simply providing different concentrations, types, or combinations of substrate(s) in the extracellular medium and examining their effects on respiration. For example, hepatocyte respiration is increased by fatty acids, pyruvate and lactate (Korzeniewski et al.; Nobes et al.; Nobes et al.), while insulinoma basal respiration depends primarily on glucose concentration (Affourtit and Brand). In synaptosomes, inhibition of pyruvate uptake leads to a decrease in the reserve capacity by inhibiting the maximal respiratory rate (Brand and Nicholls, 2011; Kauppinen and Nicholls, 1986). Such a result would indicate that glycolysis, in some cells, can exert considerable control over respiration rate. In cardiomyocytes, it appears that glycolysis can *negatively* regulate mitochondrial respiration and that pyruvate is the substrate of choice (Sansbury et al., 2011a). Interestingly, neonatal myocytes in the presence of pyruvate alone have a 20% higher reserve capacity compared with cells having both pyruvate and glucose as substrate, and this difference is due to a suppression of the maximal respiratory rate when glucose is present. The reasons for such inhibition are unclear but may be due to allosteric regulation of the electron transport chain by glycolytic intermediates (Diaz-Ruiz et al.). Recent data suggests that bioenergetic reserve capacity may be particularly important in the cell cycle and related to mitochondrial fission/fusion (Mitra and Lippincott-Schwartz, 2010; Mitra et al., 2009). In addition, it has recently been shown that the regulation of glycolysis is central to controlling the cell cycle suggesting a complex interaction between mitochondrial dynamics and metabolism (Garedew et al., 2012). Collectively, these results exemplify the importance of substrate availability in regulating the reserve capacity, which, as discussed below, translate to the cellular response to differentiation, division and stress.

The bioenergetic reserve capacity is also influenced by overall mitochondrial mass, the activity of individual mitochondrial complexes and the mitochondrial network. It is important to note that an underlying bioenergetic dysfunction can counter-intuitively increase the apparent reserve capacity. For example, conditions which act to inhibit the ATP synthase (or any of its associated functions) would decrease the basal OCR, and, assuming no change in the maximally uncoupled rate, this would produce a “false-positive” increase in the reserve capacity. However, this effect would in fact compromise the production of ATP and actually result in a decreased bioenergetic capacity. This can be identified and tested for as described in Figure 4. The ability of a cell to meet a sustained but high ATP

demand may be a less vulnerable metabolic state than a transient but severe ATP demand in which reserve capacity is exceeded (Brand and Nicholls, 2011). It is then important to consider not just the magnitude of the reserve capacity but the physiological context in which the mitochondria are functioning.

The challenge as we move forward is to develop methods to compare different conditions quantitatively and draw mechanistic conclusions. A limitation in the analysis is the assumption that addition of inhibitors/uncouplers can be used to selectively eliminate certain "blocks or metabolic units," leaving intact and *unchanged* the functions of the other "components." This leads to some error in assignment of metabolic functions such as ATP-linked respiration and proton leak as discussed above. Similarly, the presence of respiratory substrates capable of increasing NADH may increase the control coefficient for chain control of respiration, perhaps increasing the respiration rate. This effect could decrease the apparent reserve capacity when actually proton flux (and thus ATP synthesis) increases, resulting in a true increase in bioenergetic capacity. Lastly, it has been shown that under conditions of increased bioenergetic stress (ATP consumption) a substantial amount of ATP production is via glycolysis in addition to mitochondrial phosphorylation, and this can be estimated by measurement of ECAR under basal or stressed conditions (Dranka et al., 2011).

Role for a glycolytic reserve in the response to stress

It is well known that glucose metabolism plays a fundamental role in maintaining metabolic homeostasis. Branching off of the primary glycolysis conduit are pathways regulating multiple cellular processes and functions, including: (1) the biosynthesis of nucleotides and NADPH (pentose phosphate pathway); (2) cellular osmolarity (polyol pathway); (3) the synthesis of amino sugars (hexosamine biosynthetic pathway); and (4) the synthesis of glycerophospholipids (dihydroxyacetone phosphate pathway). Metabolism of glucose to pyruvate results in two primary possibilities: the metabolism of pyruvate to lactate or the oxidative decarboxylation of pyruvate to acetyl CoA for entry into the TCA cycle. It is the former of these two possibilities that can be measured as an index of glycolytic flux. While lactate assays can give a snapshot view of whether glycolytic flux is increased or decreased over a period of time, XF ECAR analysis gives a time-resolved view of changes in glycolysis (Ferrick et al.). This has allowed for new concepts to develop concerning how glycolysis could regulate cell function.

Interestingly, in hypertrophied neonatal myocytes, it appears that the glycolytic reserve rather than the mitochondrial reserve capacity helps the cell survive HNE-induced injury and cell death. Cellular hypertrophy driven by adrenergic stimuli resulted in a *decrease* in the mitochondrial reserve capacity concomitant with an increase in the glycolytic reserve, and this reserve appeared to help the cell through the oxidative insult. Extant evidence suggests that the hypertrophy of the heart *in vivo* also leads to an increase in glycolytic capacity. For example, in a rodent model of pressure-overload left ventricular hypertrophy (LVH), insulin-independent glucose uptake was increased 3-fold and activators of phosphofructokinase were increased up to 10-fold, leading to a 2-fold increase in glycolysis (Nascimben et al.).

In extracellular flux assays, the glycolytic reserve capacity can be examined through an assay analogous to the mitochondrial function assay where oligomycin and the GAPDH inhibitor koningic acid (KA) are used to examine glycolysis and non-glycolytic ECAR. An example of how this assay could be used is shown in Figure 5. Oligomycin is used in this assay to examine how the glycolytic pathway responds to loss of mitochondrial ATP production. To further inhibit mitochondrial electron flux and remove proton leak, which could act a small sink for reducing equivalents derived from glycolysis (e.g., pyruvate),

antimycin-A and rotenone can be added. This can give a maximal rate of glycolysis that could possibly occur if all mitochondrial electron flux is inhibited. Inhibitors of glycolysis such as KA or 2-deoxyglucose (2-DG) can then be used to measure non-glycolytic ECAR, which is comparable to the use of antimycin-A and rotenone in the mitochondrial function assay.

The impact of mitochondrial DNA haplotype and damage on mitochondrial quality

A unique feature of the molecular machinery controlling cellular bioenergetics is that the proteins necessary for electron transport and oxidative phosphorylation are encoded by both nuclear and mitochondrial genomes. Each cell contains hundreds to thousands of mitochondria and each mitochondrion contains 5 – 10 copies of maternally inherited mitochondrial DNA (mtDNA). The mammalian mtDNA encodes for the 13 key structural subunits required for the catalytic activity of four of the five OXPHOS enzyme complexes (I, III, IV and V). Because of the pivotal role of these proteins in bioenergetics it has been proposed that mutations in the mtDNA-encoded subunits modulate the economics of oxidative phosphorylation in controlling the relative distribution of electrons to ATP generation, heat and the formation of ROS. We and others are now exploring the hypothesis that these features of mitochondrial economy, which have evolved over thousands of years, can ultimately affect mitochondrial quality and the susceptibility to diseases in which the interface between bioenergetics and inflammation are playing a key role (Figure 6) (Krzywanski et al., 2011; Wallace, 2010, 2011).

As the ancestors for contemporary humans migrated out of sub-Saharan Africa, they accumulated missense mutations in their mtDNAs. It is now becoming clear that some of these mutations may have had a selective advantage through their impact on mitochondrial economy. For example, if these mutations altered mitochondrial economy such that caloric utilization for ATP generation was decreased while that used for generating heat increased then this would have been a metabolic advantage for our ancestors as they migrated into northern climates (Ruiz-Pesini et al., 2004; Wallace, 2005). Although these mutations redistributed the economics of electron transfer with a decrease in efficiency in ATP generation, these changes could be accommodated in our migratory ancestors due to changes in their diet (increased caloric intake associated with animal fats) (Cordain et al., 2000). In contrast, the mtDNA haplotypes associated with an economic distribution of electrons to ATP synthesis (e.g., those having sub-Saharan origins) are better adapted to low calorie warmer climates. In the modern era with massive and rapid migration of populations over decades, the mitochondrial haplotype can frequently be maladapted an excessive caloric availability and low energetic demand lifestyle. Figure 6 illustrates this concept by showing that under conditions of high ATP demands (e.g., adequate diet and exercise), electron flow energetics predominately manifests in ATP generation with subtle differences existing in mitochondrial oxidant and heat generation between individuals harboring mtDNA haplotypes originally associated with northern or sub-Saharan latitudes. In contrast, under conditions of low ATP demand (e.g., excess calorie intake and physical inactivity) mitochondrial oxidant production will be differentially increased between individuals with distinct patterns of electron distribution between ATP/ROS and heat (associated with mtDNA haplotype). Specifically, under conditions of excess caloric intake and sedentary lifestyle individuals with sub-Saharan maternal origins will have greater mitochondrial oxidant production relative to those having origins associated with more northern latitudes since excess electron flow in those individuals (sub-Saharan origins) are less likely to be utilized for heat generation and will therefore be diverted to ROS formation.

While cybrid cell culture studies have provided conflicting data regarding the role of mtDNA haplotype on *in vitro* bioenergetics, other studies have shown that mtDNA haplotype can influence tumor growth, age-related deafness, cognition, behavior, reproductive behavior, and susceptibility to autoimmune disease in mice (Amo and Brand, 2007; Amo et al., 2008; Gomez-Duran et al., 2010; Moreno-Loshuertos et al., 2006). Similarly, molecular epidemiologic studies have shown that human longevity correlates with mtDNA haplotypes having temperate and arctic origins (yet have increased risk for illnesses related with energetic insufficiency) whereas those of sub-Saharan origins appear to be more frequently associated with certain types of cancer and diseases thought to be due to increased oxidative stress and/or mutagenesis (Bhopal and Rafnsson, 2009; De Benedictis et al., 1999; Petros et al., 2005; Rose et al., 2001; Wallace, 2005).

Mitochondrial quality, mtDNA damage and disease

From the previous discussion it is clear that since mitochondrial function declines with the accumulation of mitochondrial damage, then assessment of overall mitochondrial quality can serve as a prognostic indicator for disease risk. For example, studies on primary vascular endothelial and smooth muscle cells have shown preferential and sustained mtDNA damage, altered mitochondrial transcript levels, and decreased mitochondrial protein synthesis in response to oxidant treatments (Ballinger et al., 2000). High levels of nitric oxide have also been shown to decrease the levels of mitochondrial respiratory proteins in endothelial cells and increase the susceptibility to cell death (Ramachandran et al., 2004). *In vivo* studies have shown increased mtDNA damage in atherosclerotic and aged tissues from both humans and animals, and moreover, that vascular mtDNA damage occurs prior to or coincidental to pathologic changes (atherogenesis) in mice and non-human primates (Ballinger et al., 2002; Westbrook et al., 2010). It is also well established that numerous environmental oxidants, cardiotoxicants and disease risk factors (e.g. hypercholesterolemia, hyperglycemia) cause significant mtDNA damage, and it has been suggested that developmental exposure to these factors can significantly increase the risk for adult disease development by causing early damage to the mtDNA (Cakir et al., 2007; Chuang et al., 2009; Gutierrez et al., 2006; Yang et al., 2007). In addition to the obvious impact of damaged mtDNA on organelle function, oxidant production, and energy generation, studies are now suggesting that the damaged mtDNA can serve as a Damage Associated Molecular Pattern (DAMP) which contributes to a pro-inflammatory cellular environment (Krysko et al., 2011). Finally, certain mtDNA haplotypes may be more prone to mtDNA damage by virtue of mitochondrial oxidant generation associated with conditions of chronic excess energy balance (e.g., mtDNA haplotypes associated with greater oxidant production will sustain more mtDNA damage compared with those that generate less oxidants). Consequently, the mitochondrial bioenergetics and organelle quality are likely dependent upon organelle economy that may be influenced by mtDNA haplotype which in turn may influence the accumulation of mtDNA damage.

Regulation of mitochondrial quality control and the role of mitophagy

Mitochondrial turnover is stimulated by a broad range of conditions including nutrient deprivation. Electron microscopy studies in the early 1960s showed that lysosomes are increased in number in glucagon-treated rat liver and many of the lysosomal structures contained mitochondria or remnants which appeared to be in various stages of breakdown or hydrolysis (Ashford and Porter, 1962). During development, mitophagy plays a key role in eliminating the paternal mitochondria after fertilization in *C. elegans* (Sato and Sato, 2011). As maternal inheritance of mtDNA is shared by many organisms, including humans, mitophagy may play a critical role in the genetics and diversity of mtDNA haplotype and, as discussed previously, disease susceptibility.

Mitophagy and mitochondrial quality

In addition to the developmental process, tissues that are highly dependent on mitochondrial quality, including liver, heart and brain, maintain active mitophagy that appears to be protective against toxins, hypoxia, and age-dependent diseases (Karbowski and Neutzner, 2012; Lee et al., 2012; Okamoto and Kondo-Okamoto, 2012; Osiewacz, 2011). Oxidative stress may be a key mechanism through which mitochondria are damaged and targeted for mitophagy (Apostolova et al., 2011). We have recently shown in heme-dependent toxicity in endothelial cells that one of the earliest events is bioenergetic dysfunction and the engagement of mitophagy (Higdon et al., 2012). In a number of human diseases with mitochondrial deficits, mitochondrial respiratory chain activities and CoQ10 levels are decreased, and oxidative stress and mitochondrial membrane permeabilization are increased (Cotan et al., 2011). Mitophagic turnover in these cells is important for preventing accumulation of additional damage. For example, in MELAS fibroblasts prevention of autophagy resulted in apoptotic cell death, supporting the idea that mitophagy plays a protective role in cell survival (James et al., 1996).

Mitophagy is important for control of mitochondrial quality and ROS. Key factors in the mitophagy pathway include Atg1/Atg13/FIP200 activation, an ubiquitin-like conjugation pathway involving Atg 3, 5, 7 and 22 leading to lipidation of LC3I and its conversion to LC3II, and LC3II insertion into autophagosomal membranes (Figure 7). Autophagosomal expansion is also stimulated by Beclin/VPS34 complex activities. Some of the key molecular mediators are shown in Figure 7 and are important for mitochondrial quality control. Deformed mitochondria have been observed in *Atg3*$^{-/-}$ (Jia and He, 2011), *Atg5*$^{-/-}$, *Atg7*$^{-/-}$ (Pua et al., 2009), and *Beclin* (*Atg6*)$^{+/-}$ cells. These effects also impact on pathology since livers of *VPS34* knockout mice exhibit significant increases in mitochondrial mass and steatosis (Jaber et al., 2012). Significantly increased levels of ROS have been found in various cells in *FIP200*, *Atg3*, *Atg5*, and *Atg7* knockout mice (Jia and He, 2011). LC3B knockout macrophages generate more superoxide and in response to LPS, these macrophages displayed more swollen mitochondria (Xu et al., 2011). It is important to note that inhibiting mitophagy has not always been shown to be detrimental. For instance, a mouse model with a targeted deletion of Atg7 in adipose tissue had increased amounts of brown-like adipose tissue containing higher levels of mitochondria and increased fatty acid oxidation. The mice were resistant to diet-induced obesity and insulin resistance (Singh et al.; Zhang et al.). These results illustrate that, in some organs and under certain pathological conditions, a dysfunctional mitochondrial quality control system may have unexpected advantages.

Molecular control of mitophagy

Because mitophagy impacts mitochondrial homeostasis, it is not surprising that it is a tightly regulated process. Depending on the cell type, genetic makeup and environmental stimuli, mitophagic regulatory mechanisms include: mitochondrial membrane depolarization followed by recruitment of Parkin (Elmore et al., 2001; Jin and Youle, 2012), fission and fusion activities, phosphorylated ERK targeting to the mitochondria, and cell signaling processes that detect nutrient conditions and energy status (Figure 7). Importantly, there exists a module of key proteins that regulate the interaction of mitochondria with autophagosomes, and considerable progress has been made in identifying these proteins and their mechanisms of mitochondrial recognition. BNIP3 and Nix (BNIP3L), both BH3-domain containing Bcl-2 family of proteins, have been shown to play critical roles in mitophagy (Band et al.; Ding et al., 2010; Zhang and Ney). These proteins localize to mitochondria and are important in targeting mitochondria for degradation. The significance of the involvement of Nix in mitochondrial clearance is supported by deficient reticulocyte maturation in *Nix* knockout mice (Zhang and Ney, 2009a). Another important regulator of

mitochondria-autophagosome targeting is FUNDC1, which is an outer mitochondrial membrane protein that binds to LC3, and may be important in mitochondrial quality control in response to hypoxia (Liu et al.).

The rapid pace of investigations concerning mitophagy has led to a detailed understanding of two particularly important components of the mitophagic machinery—PTEN-induced putative kinase 1 (PINK1) and Parkin. It is clear that PINK1 is critical in identifying mitochondria destined for autophagy. In "healthy" mitochondria, PINK1 is rapidly turned over by proteolysis; however, mitochondria that become damaged appear to allow mitochondrial accumulation of PINK, which leads to the recruitment of Parkin (Matsuda et al.; Narendra et al., 2010b). How membrane potential and bioenergetic function regulate this PINK-Parkin mechanism is unknown; nevertheless, it is well-established that the machinery is acutely sensitive to changes in mitochondrial function. Within one hour after administration of uncoupling agents, Parkin translocates to mitochondria; this translocation can also be induced by mitochondrial poisons such as azide (Narendra et al., 2008; Suen et al.). The induction of mitophagy following this recruitment is thought to involve the ubiquitin ligase activity of Parkin, which ubiquitinates substrates such as mitofusin 1 (Mfn1), mitofusin 2 (Mfn 2) (Gegg et al.; Tanaka et al.), and voltage-dependent anion channel (VDAC) (Geisler et al.). The ubiquitin-binding adaptor p62 may then be involved in recruiting the mitochondrial cargo into autophagosomes (Ding et al., 2010; Geisler et al.; Pankiv et al.), although contradictory reports exist concerning whether p62 is indeed essential for mitophagy (Narendra et al.; Okatsu et al.). Parkin also interacts with Ambra1; this is important for Ambra1 to activate class III PI3K which is needed for subsequent mitochondrial clearance (Van Humbeeck et al., 2011).

Role of fission and fusion in mitophagy

The shape and functional attributes of mitochondria appear to be important for determining whether they are targeted for mitophagy (Gomes et al., 2011). In hepatocytes, fission is associated with mitophagy (Kim and Lemasters, 2011). The blocking of mitochondrial fusion results in loss of membrane potential in distinct mitochondrial subpopulations and recruitment of parkin (Youle and Narendra, 2011). Parkin, once localized to mitochondria, develops an increase in its E3 ubiquitin ligase activity (Matsuda et al.), and ubiquitinylates mitofusins (Gegg et al.; Tanaka et al.). In turn, this appears to mediate the proteolytic degradation of mitofusin and to promote mitochondrial fragmentation. Hence, parkin may interact dynamically with the fission/fusion machinery and facilitate the removal of only damaged mitochondria by autophagy.

Other proteins affecting fission and fusion, including Fis1, Drp1, and Opa1, have been shown to be important as well (Chan, 2006). For example, in INS1 β-cells, Fis1 knockdown, overexpression of Opa1 or dominant negative Drp1 resulted in decreased mitophagy as assayed by colocalization of mitochondria with autophagosomes (Twig et al., 2008a). It is important to draw attention to the fact that all mitophagic events appear to be exquisitely sensitive to changes in mitochondrial function. In most cases, mitochondrial membrane depolarization appears to serve as a mechanism to recruit signaling molecules that target damaged mitochondria to be degraded. This may then help prevent injured mitochondria from releasing pro-apoptotic agents and producing undesirable ROS (Figure 8). Additional evidence suggests that the bioenergetic reserve capacity may be sensed by the mitophagic machinery (Higdon et al., 2012). An attractive hypothesis that integrates all of these findings is that loss of the ability to proteolyze initial recruitment proteins such as PINK occurs once the bioenergetic reserve is breached. Such a hypothesis integrates the bioenergetic status of each mitochondrion tightly with targeted mitophagic processes.

Regulation of Mitochondrial quality in disease

The concept that mitochondrial quality control is a critical element in the role the organelle plays in the pathogenesis of disease is gaining support. Some of selected examples are discussed below.

The reserve capacity and its regulation of cell (patho)physiology

An emerging concept in the energetics field is that the reserve capacity can be used as an index of mitochondrial health (Brand and Nicholls, 2011; Dranka et al., 2010; Higdon et al., 2012; Hill et al., 2009; Zelickson et al., 2011). By indicating how close a cell is to operating at its bioenergetic limit, predictions can be made regarding the cellular response to stress or increased energy demand. This has been demonstrated in the response of cells to oxidative stress. In intact myocytes, stressors that are both causes and consequences of oxidative stress can impact the reserve capacity. For example, the lipid peroxidation product, HNE, increases ATP demand and proton leak to a point where the reserve capacity is depleted (Hill et al.). It appears that a higher bioenergetic reserve capacity results in a greater ability to withstand oxidative stress. In cells given only glucose to respire on, the reserve capacity was only 30% of that of cells provided with both glucose and pyruvate, and the cells given only glucose showed a much earlier HNE-induced bioenergetic collapse compared with cells having extracellular pyruvate available (Sansbury et al., 2011a). Another example was shown in neurons, where glutamate excitotoxicity similarly depleted the reserve respiratory capacity, resulting in respiratory failure (Johnson-Cadwell et al.; Yadava and Nicholls). In the development of resistance to chemotherapy, changes to the mitochondrial respiratory chain (predominantly at cytochrome *c* oxidase) were associated with an increase in reserve capacity (Oliva et al., 2010). In endothelial cells, exposure to nontoxic concentrations of NO or low levels of hydrogen peroxide reversibly decreased mitochondrial reserve capacity. However, combined NO and superoxide and hydrogen peroxide treatment resulted in an irreversible loss of reserve capacity and was associated with cell death (Dranka et al.). These findings suggest that the reversibility of deleterious changes in the reserve capacity may also be critical; the mechanisms controlling the recoverability of the reserve capacity are unclear, but likely relate with irreversible oxidative protein modification to metabolic and respiratory chain components or non-repairable damage to proteins or organelles that regulate ion fluxes.

The role of the bioenergetic reserve has been extended beyond its relationship simply with cell death. In particular, emerging evidence implicates the mitochondrial respiratory capacity in immunity. For example, it was shown that CD8+ memory T-cells possessed a substantial reserve capacity that was responsive to interleukin-15 (IL-15), which regulated the stability of memory T-cells after infection (van der Windt et al.). A high reserve capacity may also be linked to proliferation of certain cell types, which could result in pathology. For example, it was shown that cancer cells proliferate more rapidly in the presence of exogenous pyruvate when compared with lactate and that pyruvate supplementation increased the reserve respiratory capacity. Inhibition of cellular uptake of pyruvate led to decreased mitochondrial respiration and cell growth (Diers et al.). Hence, cells that transform to a phenotype with high proliferative potential could use the mitochondrial reserve capacity to increase energy for biosynthesis reactions that drive cell division.

Neurodegenerative Diseases

Some of the most interesting mechanisms implicating mitochondrial quality control in pathologies are associated with bioenergetic dysfunction characteristic of many neurodegenerative diseases.

Mutations in *Parkin, Pink1* and *DJ-1* have been shown to be responsible for a small subset of autosomal-recessive Parkinson's disease cases. The recent finding that these proteins also play an important role for mitochondrial quality control and mitophagy in a variety of cell types have stimulated new research regarding neurodegeneration pathogenesis in Parkinson's disease (Jin and Youle, 2012). As discussed above, studies in HEK294, HeLa cells, cardiomyocytes and primary cortical neurons have shown that Parkin accumulates in depolarized mitochondria (Narendra et al., 2008). PINK1-Parkin interactions lead to ubiquitination of VDAC and involve changes in the activity of fission and fusion proteins such as Drp1 and Mfn (Chu, 2010; Geisler et al., 2010b; Rakovic et al., 2011). In addition, Parkin activates the ubiquitin-proteasome system (UPS) which in turn ubiquitinates a large number of mitochondrial proteins (Chan et al., 2011), which may serve as signals for mitophagy. DJ-1, has been shown to act in a parallel pathway to Parkin and PINK1 to regulate mitochondrial fragmentation and is critical in Parkinson's disease (Schapira and Gegg, 2011). Knockout of DJ-1 in flies decreased the respiratory control ratio (RCR) and mitochondrial DNA/nuclear DNA ratio without changing membrane potential or complex I subunit NDUFS3 level (Hao et al., 2010). DJ-$1^{-/-}$ mice exhibit decreased skeletal muscle ATP (Hao et al., 2010), associated with down regulation of uncoupling protein 4 and 5 (and decreased mitochondrial length and fusion rate. DJ-$1^{-/-}$ MEFs exhibit a profound decrease in mitochondrial quality. This is evident from impaired mitochondrial respiration, increased mitochondrial ROS, decreased mitochondrial membrane potential and characteristic alterations of mitochondrial shape, and depression of autophagy (Krebiehl et al., 2010).

The concepts of reserve capacity and cellular RCR can be applied to mitochondrial quality control *via* fusion/fission in the context of these Parkinson's disease associated proteins. The sensing of mitochondrial depolarization during mitophagy occurs *via* PINK1 whereby depolarization prevents the engagement of the N-terminus mitochondrial targeting sequence (MTS) with the Tim23 pathway and prevents localization to the mitochondrial matrix side of the inner mitochondrial membrane (Narendra and Youle, 2011). This interruption in localization inhibits the normally active proteolytic cleavage of PINK1 and it accumulates at the outer mitochondrial membrane where it recruits and activates Parkin resulting in polyubiquitination of multiple mitochondrial protein targets and subsequent targeting for mitophagy. It is noteworthy that either uncoupler (which will collapse both the pH and $\Delta\psi_M$ component of the proton motive force) or valinomycin plus potassium (which collapses only the $\Delta\psi_M$ component) (Narendra et al., 2010b; Youle and Narendra, 2011) will prevent processing of PINK1, suggesting that it is the transmembrane electrical field component of the proton motive force that is responsible. Studies with isolated rat hepatocytes (Hafner et al., 1990) indicate that under normal conditions there is only modest control of the proton motive force exerted by the respiratory chain, phosphorylating system, or proton leaks, illustrating the high degree of homeostasis of this component of mitochondrial quality. This result supports the idea that under these normal conditions mitochondrial $\Delta\psi_M$ homeostasis is maintained and mitophagy is avoided, even under changing metabolic conditions (*e.g.*, "State3–4"-like transitions).

Alcohol-dependent hepatotoxicity

In the liver, an early event in response to ethanol, endotoxin and cytokines are released, this depresses mitochondrial function, leading to hepatotoxicity (Arteel et al., 2003; Bailey, 2003; Scaglioni et al., 2011). The reserve capacity of the mitochondria after chronic alcohol exposure is decreased and this is exacerbated by the combined exposure of the cells to hypoxia and nitric oxide. Nitric oxide (NO) in combination with reactive oxygen species (ROS) and the associated formation of potent prooxidants such as peroxynitrite contributes to steatosis and decreased mitochondrial quality (Jaeschke et al., 2002; Pacher et al., 2007; Shiva et al., 2005). For example, alcohol consumption is associated with increased mtDNA

damage, enhanced sensitivity to the mitochondrial permeability transition, increased sensitivity to the NO-dependent control of respiration, extensive modification of the mitochondrial proteome, protein nitration and lipid peroxidation (Venkatraman et al., 2004a; Venkatraman et al., 2004b; Venkatraman et al., 2004c; Zelickson et al., 2011). Consistent with these findings, a mitochondrially targeted antioxidant decreases oxidative stress and steatosis (Chacko et al., 2011). It is now becoming clear that repair of damaged mitochondria is a complex process, mediated by the autophagy-lysosomal system, which is defective in EtOH toxicity (Ding et al., 2011; Donohue, 2009; Lemasters, 2007). Interestingly, enhancing autophagy with the therapeutic agent rapamycin decreased acute EtOH dependent hepatotoxicity (Ding et al., 2011). In this context, autophagy is protective since inhibition of the autophagic process resulted in increased mitochondrial-dependent apoptosis. These data also suggest that inhibition of the autophagic process occurs and is associated with increased steatosis and marked deterioration of mitochondrial quality probably through a Cyp2E1 mediated mechanism(Wu et al., 2010). This is consistent with the observation that damaged mitochondria which are not removed by mitophagy are susceptible to uncoupling and may promote cell death. The formation of Mallory-Denk bodies is characteristic of alcoholic steatohepatitis in human patients and are comprised of protein aggregates containing cytokeratins, ubiquitin and p62 (Rautou et al., 2010; Zatloukal et al., 2007). These findings suggest that therapeutic strategies that link mitochondrial oxidative damage and autophagy may be beneficial in alcoholic liver disease.

Cardiovascular disease

Given the high energy demands of the cardiovascular system it is not surprising that defects in bioenergetics are associated with the pathology of ischemia-reperfusion and heart failure. An example of how the $State_{apparent}$ changes based on cell phenotype can be drawn from our previous work, where cardiomyocytes were exposed to phenylephrine (Sansbury et al., 2011b). This resulted in hypertrophy of the cells and changes in cellular bioenergetics. Notably, the hypertrophied cardiomyocytes demonstrated a small movement toward state 3 compared with non-hypertrophied cells. In this instance, the difference in respiratory state was driven by a single factor, i.e., a higher basal OCR due to increased ATP demand. A similar response was shown *in vivo* in hypertrophied and failing hearts, where failure was shown to deplete bioenergetic capacity almost completely (Gong et al.), which would be thought to push the $State_{apparent}$ to near 3.0. These are several examples illustrating that an intermediate respiratory state exists under physiological conditions and that intracellular mitochondria possess the ability to move toward a more energetic condition using the available reserve cellular bioenergetic capacity. Evidence for a reserve capacity in functioning organs and tissues has also been presented using ^{31}P-NMR techniques in tissues under metabolic stress (Sako et al.).

During heart failure, the heart decompensates when there is an imbalance between energy production and use, leading to a decreased energy reserve in the heart (Ingwall, 2009; Liao et al., 1996). For example, with volume overload (VO), there is a twofold increase in myocardial oxygen consumption and increased ATP consumption (Strauer, 1987; Yun et al., 1992). As shown in Figure 9, the increased ATP demand in VO is also associated with the activation of the ROS-generating enzyme xanthine oxidase (XO) (Gladden et al., 2011a; Gladden et al., 2011b). This finding was translated to the clinic in a recent study which showed that left ventricular biopsies taken from patients with isolated mitral regurgitation had significant cardiomyocyte myofibrillar degeneration along with increased protein nitration and lipofuscin accumulation, consistent with increased formation of ROS/RNS (Ahmed et al., 2010). These oxidants are generated from a number of sources including XO. In addition to being a major source of reactive oxygen species, XO is linked to bioenergetic dysfunction since its substrates derive from ATP catabolism (Figure 9). Correspondingly,

there was also evidence of aggregates of small mitochondria in cardiomyocytes, which is generally considered a response to bioenergetic deficit in cells. This increase pro-oxidant environment could decrease mitochondrial quality and underlie the finding that mitochondria isolated from rats with chronic VO have a greater sensitivity to Ca^{2+}-induced PTP opening, and these volume-overloaded hearts have increased ischemia-reperfusion injury (Marcil et al., 2006).

To gain further insight into changes from oxidative stress and bioenergetic dysfunction in VO, we have shown that cyclical stretch of adult rat cardiomyocytes causes increased oxidative stress, mitochondrial swelling, and cytoskeletal disruption—all of which are prevented by an XO inhibitor or the mitochondrial targeted drug MitoQ (Gladden et al., 2011b). More importantly, MitoQ prevented XO activation, suggesting that the mitochondria themselves represent a more direct target in preventing oxidative stress, XO activation, and cellular remodeling in cardiomyocyte stretch. In a similar fashion, 24 hours of VO *in vivo* also results in increased cardiomyocyte XO activity with decreased state 3 mitochondrial respiration and decreased LV contractility, which are significantly improved with allopurinol (Gladden et al., 2011b; Ulasova et al., 2011). Most recently, ROS production from XO has been linked to myocardial high energy phosphates and ATP flux through creatine kinase in the failing human heart (Hirsch et al., 2012). Treatment of heart failure patients with an XO inhibitor increased phosphocreatine to ATP ratio and creatine kinase flux (measured using *in vivo* ^{31}P magnetic resonance spectroscopy), which is consistent with the high susceptibility of creatine kinase to oxidative modifications resulting in decreased activity and efficiency of high energy phosphate transfer from mitochondria to the contractile apparatus.

Obesity and Diabetes

An excess consumption of high calorie foods appears to be one of the key factors in the epidemic of obesity (Briefel and Johnson; Duffey and Popkin; Kant and Graubard; Nielsen and Popkin; Popkin et al.; Wang et al.), which increases the risk for developing diseases with a bioenergetic component (Haslam and James). As described above, these include cardiovascular disease (Roger et al.), hepatotoxicity (Zakhari and Li), and neurodegenerative diseases (Bruce-Keller et al.). While a high caloric intake can be offset by exercise (for example, the gold medalist Michael Phelps remains lean despite consuming ~12,000 calories per day!), increasing evidence suggests that physical activity, on average, is declining (Hu et al.; Robinson; Roger et al.; Smith et al.; Williamson et al.). This has led to heightened efforts to promote increased energy utilization and to better understand how altered intermediary metabolism, perturbations in cellular bioenergetics, and genetic differences contribute to the risk for becoming obese and insulin resistant.

Mitochondria lie at the heart of systemic metabolic regulation. They act as endpoint regulators of metabolic rate and affect thermogenesis primarily by increasing proton leak. In brown fat, the relatively high expression of uncoupling protein 1 (UCP1) allows re-entry of protons into the mitochondrial matrix without generating ATP. This uncoupling therefore generates an increase in substrate utilization and electron transport chain turnover as well as energy in the form of heat (Tseng et al.). Despite the low amounts of brown fat in humans, as little as 50 g of brown fat has been estimated to be capable of utilizing up to 20% of basal caloric needs (Rothwell and Stock). This suggests that *decreasing* mitochondrial efficiency is an attractive therapeutic option for obesity. Indeed, synthetic uncouplers such as 2,4-dinitrophenol were used in the 1930s as a weight loss drug, and it was capable of increasing metabolic rate by up to 40% (Cutting; Harper et al.). However, DNP has a narrow therapeutic window (similar to the narrow dose-response curves observed in XF experiments), and side effects led to removal from the market (Harper et al.). Nevertheless,

it is clear that decreasing mitochondrial efficiency may be a robust treatment option for obesity.

The obvious drawback for such an approach is its systemic effects. Mitochondria in organs with high energetic needs (e.g., the heart) should likely maintain a high level of mitochondrial coupling, while other organs such as adipose tissue could afford to be less economical. Indeed, overexpression of UCP1 in adipose tissue (Kopecky et al.) and skeletal muscle (Li et al.) prevented diet-induced obesity in mice, suggesting that uncoupling of oxidative phosphorylation in these two organs is beneficial. Interestingly, oxidative phosphorylation is more uncoupled in endurance athletes compared with sedentary subjects (Befroy et al.), and this appears to enhance fatty acid oxidation and diminish oxidative stress. Moreover, genes encoding fatty acid oxidation are increased in athletes compared with sedentary subjects (Schrauwen-Hinderling et al.), which display an increased gene profile favoring fat storage (Schrauwen-Hinderling et al.). However, it is important to note that a higher metabolic capacity does not always equate with a decreased risk for metabolic disease. For example, American subjects of Asian Indian ancestry are predisposed to obesity and type 2 diabetes and have higher mitochondrial genomic content and oxidative phosphorylation capacity compared with Americans of European descent (Nair et al.). The South Asian population does appear to have more coupled mitochondria, which has led to a "mitochondrial efficiency hypothesis" that could explain the propensity for obesity in some populations (Bhopal et al, 2009). Other hypotheses relating to genetic predispositions such as the "thrifty gene" hypothesis and the "predation release" hypothesis have also been postulated (Speakman, 2008). The fact that mtDNA variants relate with the risk of type 2 diabetes does support a role for genetic factors relating to energetics and metabolism (Irwin et al.; Sun et al.; Wallace, 2011). However, the sudden increase in obesity in areas that have genetically dissimilar populations (such as the Southeastern United States) does not appear to support any of these hypotheses exclusively. It appears that a stronger hypothesis integrating physiological, genetic, and environmental frameworks is required to explain the relatively abrupt increase in the prevalence of obesity and diabetes.

The regulation of autophagy also appears to be important in the context of metabolic diseases such as obesity. Interestingly, autophagy appears to be upregulated in adipose tissues of obese humans (Kovsan et al.), suggesting that it may be involved in adipocyte hypertrophy, and inhibiting autophagy appears to have beneficial effects in the context of obesity. Conditional knock-out of *atg7* in adipocytes was shown to prevent diet-induced obesity and to increase insulin sensitivity in mice (Singh et al.; Zhang et al.). Interestingly, deletion of *atg7* was associated with significantly increased levels of mitochondria in white adipocytes, supporting a critical role of autophagy in regulating mitochondrial number in adipose tissue. These changes were also associated with an increase in adipocyte fatty acid oxidation as well as changes in whole body metabolism (Singh et al.; Zhang et al.). Inactivation of other autophagy genes has resulted in similar results *in vitro*. For example, mouse embryonic fibroblasts (MEFs) isolated from $atg5^{-/-}$ mice accumulate less lipid when stimulated to develop into adipocytes, and $atg7^{-/-}$ MEFs similarly cannot undergo adipogenesis to the same extent as their wild-type counterpart cells. This suggests that inhibiting autophagy may be a therapeutic option for treating or preventing obesity or type 2 diabetes although the impact on mitochondrial quality is unclear. In line with this view, a prospective, observational multicenter clinical study showed that hydroxychloroquine, an inhibitor of autophagy, is associated with a reduced risk of diabetes (Wasko et al.).

While the above-mentioned studies suggest that inhibiting autophagy is beneficial in the context of obesity, the role of autophagy and components critical to the autophagic machinery remain unclear in the context of insulin resistance. For example, an interesting recent study demonstrated that autophagy deficits undermine the benefit of exercise in

protection against high-fat-diet-induced glucose intolerance (He et al., 2012). Moreover, systemic knock-out of the *parkin* gene, which as described in the previous sections is involved in mitophagy, has also been shown to prevent diet-induced obesity, steatohepatitis, and insulin resistance. However, the effect of *parkin* deletion did not appear to be due to regulation of mitochondria or intermediary metabolism. A lack of parkin expression prevented high fat diet-induced upregulation of lipid transport proteins such as CD36, Sr-B1, and FABP and the uptake of fat from the diet (Kim et al.). Hence, it appears that the metabolic effects of parkin are complex and regulate much more than autophagy and mitophagy.

Summary

Collectively, these studies illustrate how differences in mitochondrial economy, bioenergetic reserve capacity, and autophagy regulate health and disease. The control of mitochondrial quality in which biogenesis is balanced with mitophagy is likely to serve as a productive framework for the design of mitochondrial therapeutics. As expected, many of the factors controlling metabolism and susceptibility to disease appear to be driven by genetics and the environment. It is now also becoming clear that physiological processes such as cell differentiation, are associated with profound changes in cellular bioenergetics including reserve capacity (Schneider et al., 2011). The challenge for us is to continue to strive to more fully understand how bioenergetics regulates cell and tissue function. Certainly, this would best position us to develop better and more targeted therapies.

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Abbreviations

Ambra1	activating molecule in Beclin 1-regulated autophagy
AMPA	AMP-activated protein kinase
Anti A	antimycin A
Atg	autophagy related
BNIP	Bcl-2/adenovirus E1B 19-kDa interacting protein 3; DJ-1/PARK7 (mutated in Parkinson's disease)
DNP	Dinitrophenol
Drp1	dynamin-related protein 1
FIP200	focal adhesion kinase (FAK) family interacting protein of 200 kDa
Fis1	fission 1
FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
LC3	microtubule-associated protein 1 light chain 3
MEF	mouse embryonic fibrobalsts
Mfn	mitofusin
LIR	LC3-interacting region

Nix	NIP3-like X, BNIP3L
NO	nitric oxide
Opa1	optic atrophy 1
Parkin	PARK2 (mutated in Parkinson's disease)
PARL	presenilin-associated rhomboid-like
PINK1	PTEN-induced kinase 1/PARK6 (mutated in Parkinson's disease)
PTP	permeability transition pore
RCR	Respiratory Control Ratio
ROS/RNS	Reactive Oxygen Species/Reactive Nitrogen Species
UCP	uncoupling proteins
UPS	ubiquitin-proteasome system
VPS	vacuolar protein sorting
XO	xanthine oxidase
PVA	pressure volume area
HX	hypoxanthine
LV	left ventricle

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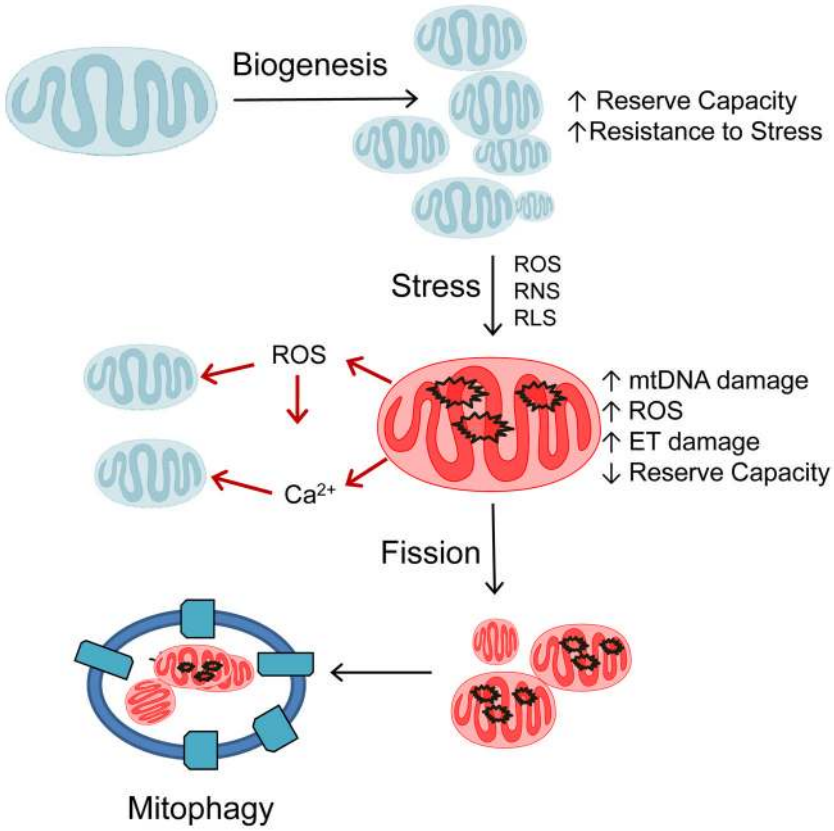

Figure 1. Regulation of mitochondrial quality control and the response to oxidative stress

An existing mitochondrial population is shown subjected to a pathological stress with the formation of reactive oxygen, nitrogen and lipid species (ROS/RNS/RLS). This oxidative stress damages mtDNA impairing the ability of the organelle to replace damaged electron transport proteins and decreasing bioenergetic reserve capacity; the resulting increased mitochondrial ROS then oxidatively damages previously unmodified mitochondria. The damaged mitochondria are turned over by a mitophagic mechanism that then suppresses this vicious cycle. The mitochondrial population is now renewed through mitochondrial biogenesis. The bioenergetic reserve capacity is essential for resistance to oxidative stress and supplying ATP demand. Once the bioenergetic reserve is depleted, bioenergetic failure occurs and the cell is programmed for cell death.

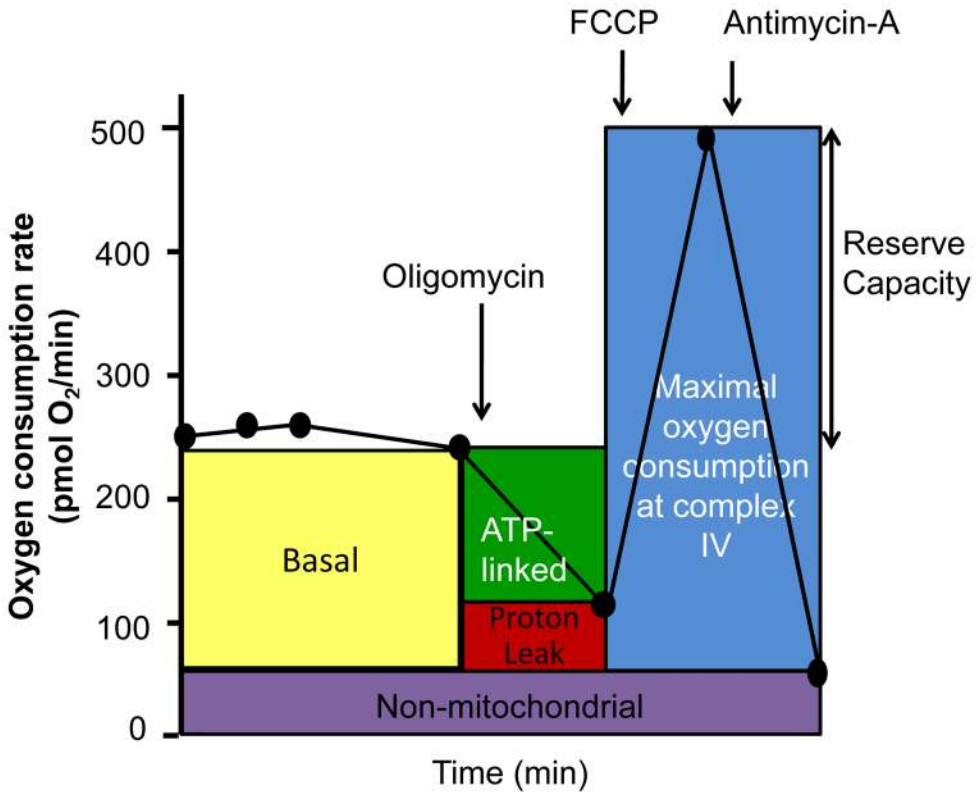

Figure 2. The cellular bioenergetic profile

This mitochondrial stress test can be used to measure several indices of mitochondrial function in intact cells in real time and allows for the identification of critical respiratory defects. A typical experiment is shown in which basal oxygen consumption rate (OCR) is allowed to stabilize before the sequential addition of oligomycin, FCCP, and antimycin A and/or rotenone with a measurement of changes in OCR as indicated. This time course is annotated to show the relative contribution of non-respiratory chain oxygen consumption, ATP-linked oxygen consumption, the maximal OCR after the addition of FCCP, and the reserve capacity of the cells.

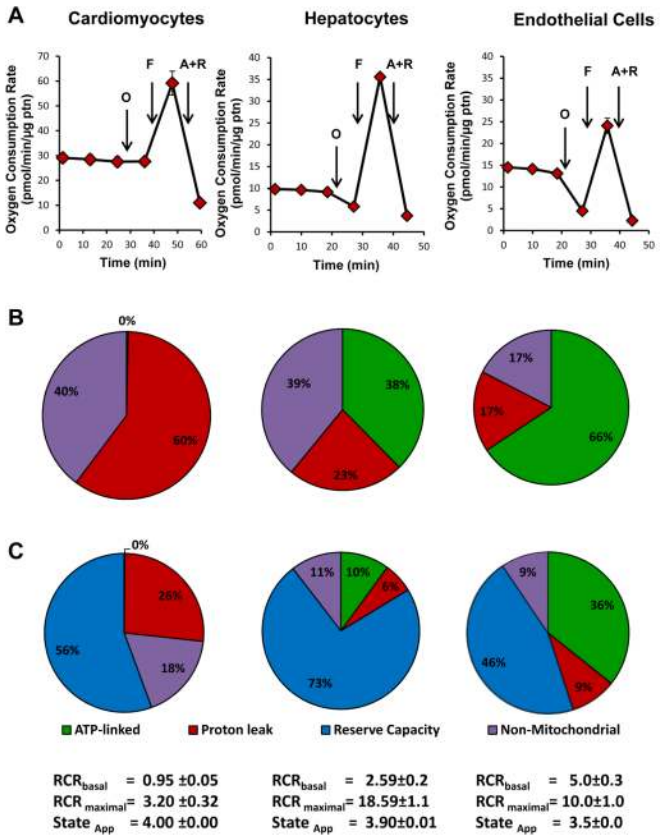

Figure 3. Cellular bioenergetics in different cell types

Extracellular flux analysis using the mitochondrial stress test assay: Panel A: Adult rat cardiomyocytes, primary hepatocytes or bovine aortic endothelial cells were subject to a metabolic stress test as shown, and the OCR rates were normalized to protein. Panel B: in this analysis basal OCR was established as 100% OCR and the proportion of ATP-linked OCR, proton leak and non-mitochondrial oxygen consumption is shown. Panel C: in this analysis maximal OCR is established as 100% and the oxygen consumption is apportioned between ATP-linked OCR, proton leak, reserve capacity and non-mitochondrial O_2 consumption. The RCR for basal and maximal OCR is reported together with the $state_{apparent}$ for each cell type.

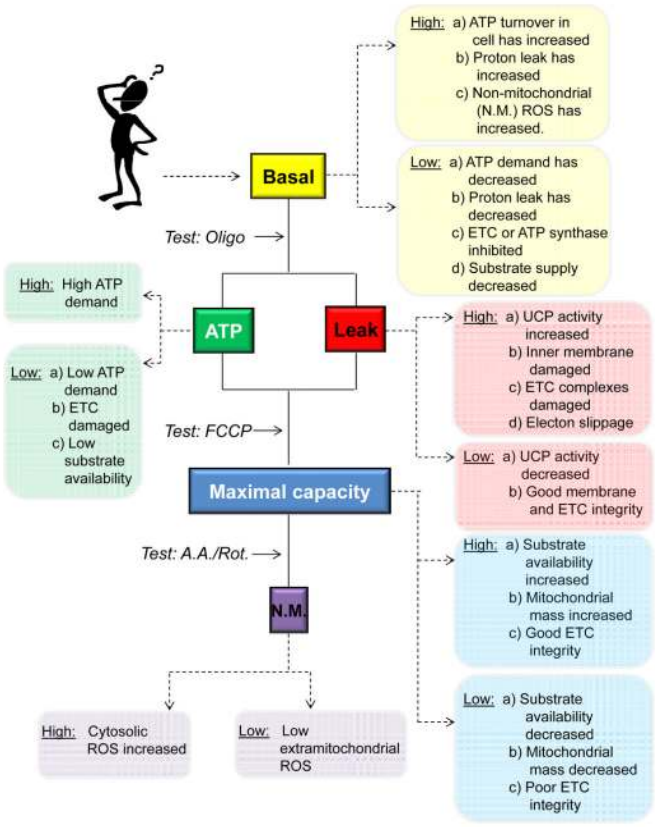

Figure 4. Possible interpretation tree for the mitochondrial bioenergetic profile

In cells, differences in basal oxygen consumption rate (OCR) could be due to several factors including ATP turnover reactions, proton leak, non-mitochondrial (N.M.) oxygen consumption, or damage to the electron transport chain. This can be tested by adding oligomycin, and some potential outcomes are shown. Next, the changes in the maximal capacity (and the reserve capacity) can be identified using the FCCP-stimulated rate. An increase or a decrease in this rate compared with controls could be due to changes in substrate availability, mitochondrial mass, or electron transport chain (ETC) integrity. Lastly, the effects of non-mitochondrial sources on oxygen consumption may be examined.

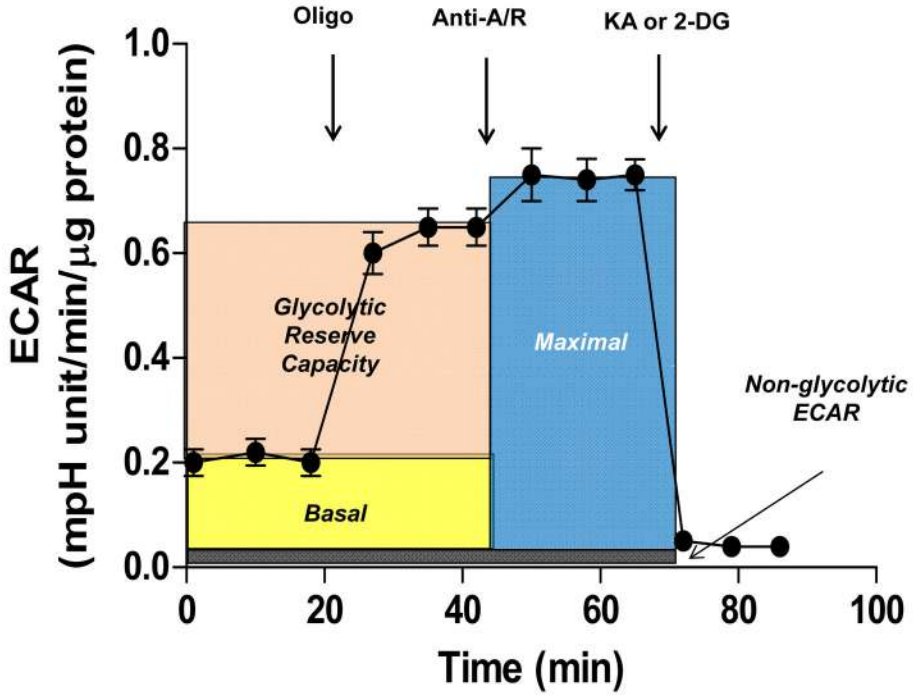

Figure 5. Glycolysis stress test

This extracellular flux assay demonstrates how basal glycolytic rate, maximal glycolytic rate, and the glycolytic reserve capacity can be determined. After measurement of the basal extracellular acidification rate (ECAR), oligomycin can be added, which generally increases glycolytic rate in response to loss of mitochondrial ATP production. The addition antimycin A/rotenone, depending on experimental conditions and cell type, may further increase the ECAR, giving an index of lactate production occurring when all mitochondrial electron transport is inhibited. Non-glycolytic extracellular acidification (i.e., acidification not due to lactate production) can then be measured by introducing koningic acid (KA; an inhibitor of glyceraldehyde-3-dehydrogenase) to inhibit glycolysis; alternatively, 2-deoxyglucose (2-DG) may be used. This assay may be prefaced by addition of oxidants or other stressors.

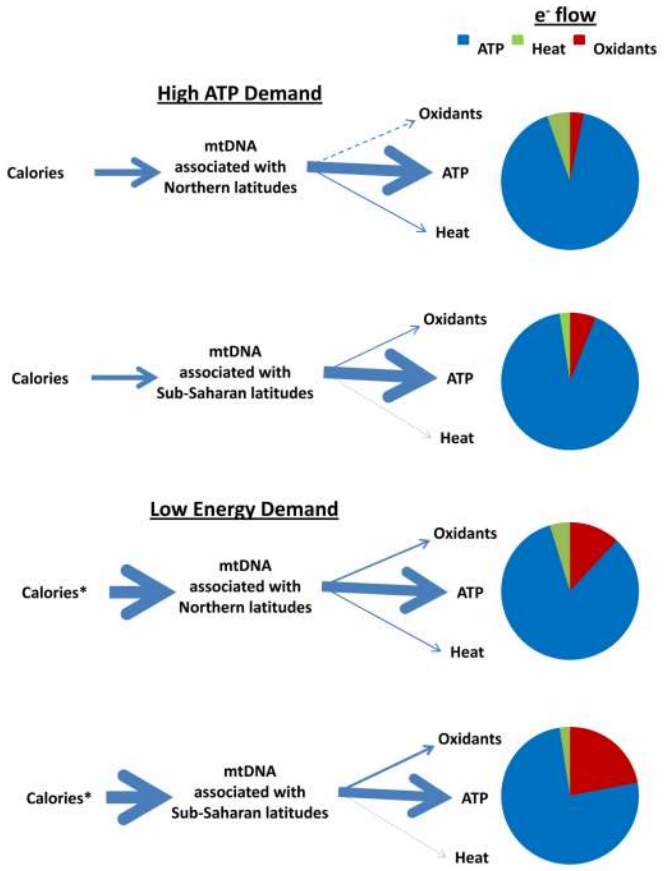

Figure 6. Hypothetical presentation of electronic energy utilization between mitochondria having mtDNA haplotypes representing Northern or Sub-Saharan origins in terms of latitude

Under conditions of high energy (ATP) demand, differences in energy utilization exist between mitochondria having different mtDNAs that manifest in differences in electrons used to generate ATP, oxidants and heat as illustrated by the arrows and pie charts. Under conditions of low energy (ATP) demand and excessive caloric intake (*), these profiles shift to decreased use of electronic energy for ATP generation and greater production of oxidants, with those mitochondria having sub-Saharan latitude mtDNAs generating more oxidants compared with those with Northern latitude mtDNAs as indicated by the arrows and pie charts.

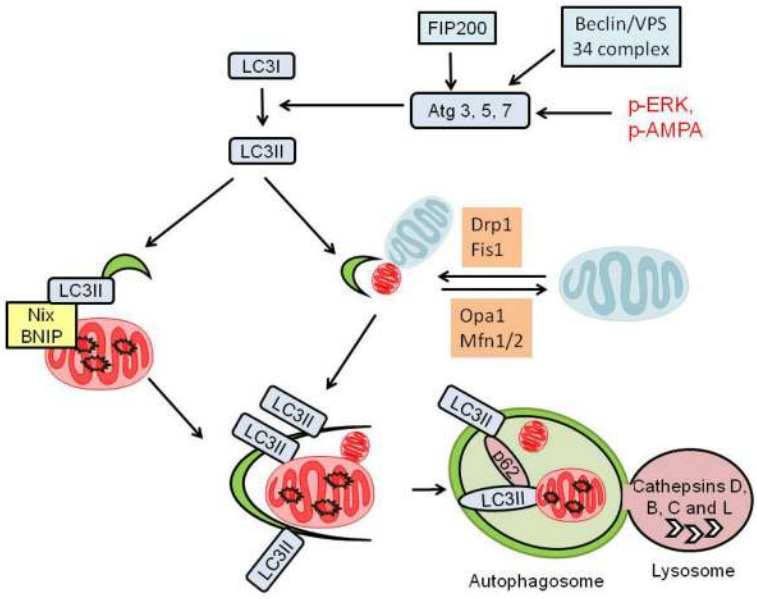

Figure 7. General pathway for activation of autophagy

Autophagy is mediated by the interaction or activation of several components including Atg1/Atg13/FIP200 and a ubiquitin-like conjugation pathway involving Atg 3, 5, 7 and 22. This leads to the lipidation of LC3I, converting it to LC3II. LC3II is inserted into autophagosomal membranes and is essential for autophagosomal formation. Autophagosomal expansion is also stimulated by Beclin/VPS34 complex activities. Autophagosomes then fuse with lysosomes and the content of autophagosomes are degraded in the acidic environment of the lysosomes. Nix and BNIP can target to mitochondria and bind to LC3II via a LIR consensus sequence and are thus important for mitophagy. ERK and AMPA activities have been shown to stimulate mitophagy. Mitochondrial fission and fusion proteins such as Drp1, Fis1, Opa1, and Mfn1/2 are also critical in regulating mitophagy.

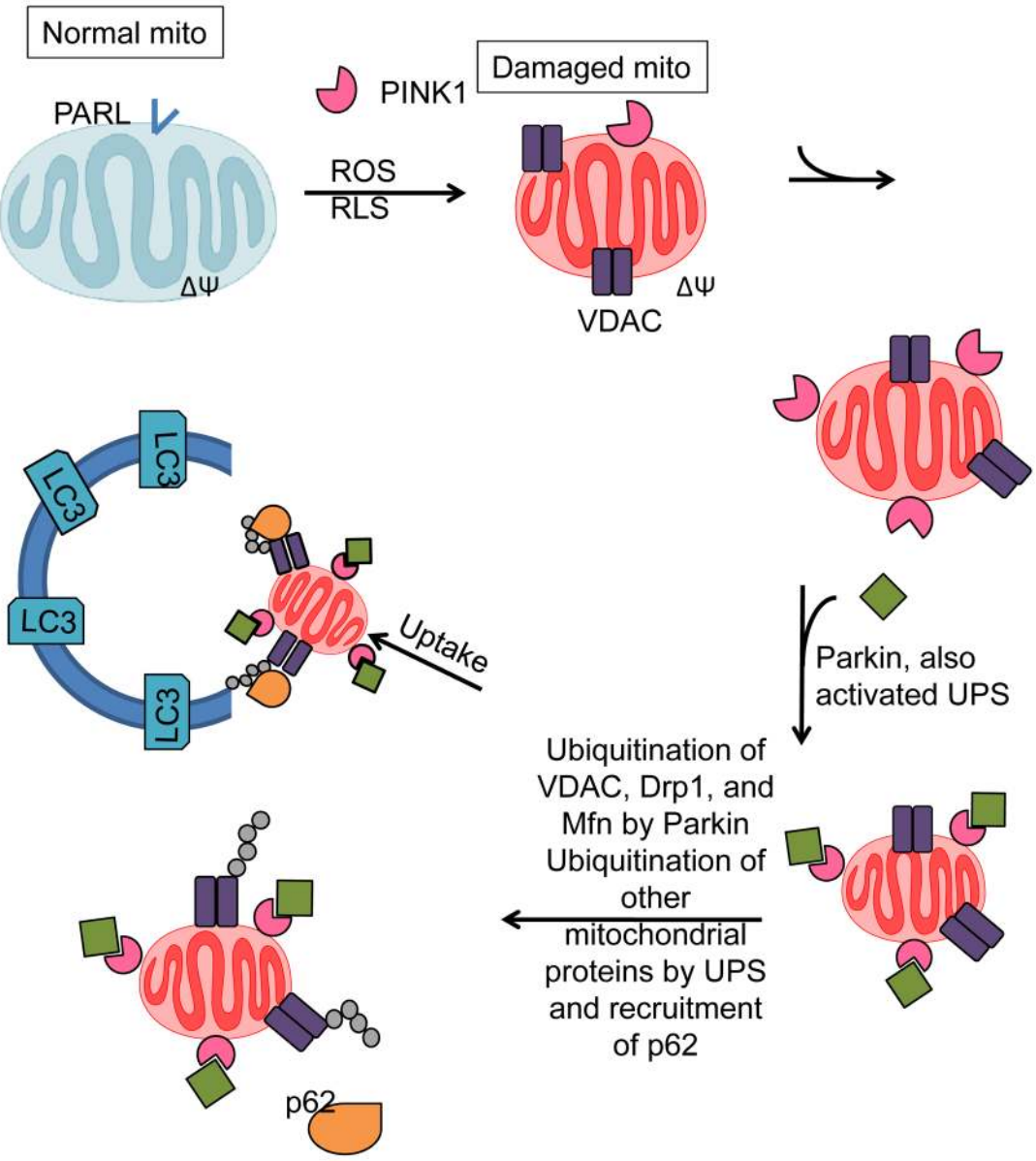

Figure 8. Regulation of mitophagy in response to mitochondrial membrane depolarization

Mitochondria with normal membrane potential and active presenilin-associated rhomboid-like protein (PARL) prevent mitochondrial PINK1 accumulation. When mitochondria become damaged, mitochondrial membrane potential decreases, PARL is inactivated, and mitochondrial PINK1 is stabilized and accumulates in the affected mitochondria. This recruits Parkin, which promotes the ubiquitination of Drp1 and Mfn, as well as a large number of other mitochondrial proteins. Ubiquitinated proteins can be recognized by p62 which binds LC3 and may help traffic damaged mitochondria to autophagosomes for degradation.

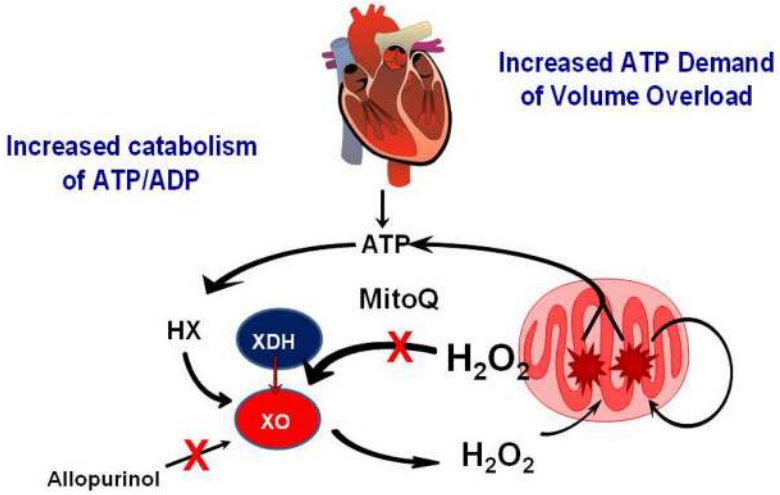

Figure 9. Mitochondrial quality control and xanthine oxidase in the heart

The increase in VO leads to increased usage of ATP, resulting in increased levels of ADP and AMP. ADP and AMP are then degraded to hypoxanthine (HX) via purine catabolism. XO reacts with HX forming superoxide and hydrogen peroxide (H_2O_2) which damage mitochondria, leading to bioenergetic dysfunction and increased ATP catabolism. Collectively, this causes increased electron leak to form more ROS and decreased ATP production. The increase in mitochondrial ROS leads to increased conversion of xanthine dehydrogenase (XDH) to xanthine oxidase. The enhanced ROS generation from mitochondria and XO cause further damage to the mitochondria and left ventricular dysfunction. Mitochondrially targeted ubiquinone (Mito Q) inhibits the conversion of XDH to XO which may depend on mitochondrial H_2O_2.