Mitochondrial form and function in pancreatic β-cells and brown adipocytes

Jakob D Wikström, M.D.

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Picture on cover: Brown adipocyte stained with the red mitochondrial membrane potential sensitive dye TMRE. In addition a subset of the mitochondrial population is also labeled with photo-convertible GFP. The image was acquired with a Zeiss LSM 710 confocal microscope.
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"If I could remember the names of all these particles I'd be a botanist"

Albert Einstein

"Nothing shocks me. I'm a scientist."

Indiana Jones

"Art is I; Science is we"

Claude Bernard

To my parents

Abstract

Mitochondria stand in the center of metabolism and compromised mitochondrial function has been shown in metabolic diseases such as diabetes and obesity. To develop mitochondrial targeted therapeutics, an improved understanding of the regulation of mitochondrial function is needed. This thesis is focused on the role of mitochondria in two tissues highly dependent on mitochondria: pancreatic β-cells and brown adipose tissue (BAT). The role of mitochondria in these tissues is opposite. In β-cells, mitochondrial ATP production is necessary for insulin secretion while in BAT mitochondria produce heat by disconnecting the respiratory chain from ATP synthesis.

Two main aspects of mitochondria were explored; mitochondrial functional efficiency and the interrelationship between mitochondrial shape and function.

Mitochondria within dispersed individual β-cells were found to exhibit heterogeneity in mitochondrial membrane potential. This functional diversity decreased when cells were challenged with glucose stimuli, suggesting that at higher fuel levels low-activity mitochondria are recruited into a pool of high-activity mitochondria. The BCL-2 family member BAD was identified as a contributor to mitochondrial membrane potential heterogeneity. Glucolipotoxic conditions designed to mimic diabetes in vitro increased the functional diversity suggesting that this may be of importance for diabetes pathophysiology.

To examine mitochondrial efficiency in intact islets a high throughput islet respirometry method was developed. It was found that due to increased uncoupling, islets from a diabetic animal model exhibit lower respiratory efficiency as compared to animals fed control chow. Glucose, free fatty acids and amino acids all decreased respiratory efficiency. A large portion of the respiratory efficiency appeared mediated by reactive oxygen species and the adenine nucleotide translocase. Human islets showed higher respiratory efficiency as compared to mouse islets. However, as in the mouse islets glucose decreased respiratory efficiency. In islets from obese donors there was a trend towards decreased respiratory rates.

The interrelationship between mitochondrial shape and function was examined both in β-cells and BAT. In β-cells mitochondria were found to undergo cycles of fusion and fission. The key mitochondrial dynamics proteins Opa1, Drp1, and Fis1 were shown to regulate β-cell mitochondrial morphology. During glucolipotoxicity mitochondria fragmented and lost their fusion ability. Knock down of the fission protein Fis1 rescued the β-cells from glucolipotoxic induced cell death and maintained β-cell insulin secretion capacity. Similarly, BAT mitochondria also showed fusion and fission. The mitochondrial dynamics proteins Mfn2 and Drp1 were shown to strongly affect BAT mitochondrial morphology. In response to a combination of adrenergic and free fatty acid stimuli mitochondria drastically changed from long filamentous structures to fragmented spheres. This occurred as a wave passing through the cell. The mitochondrial fragmentation was dependent on the β-adrenergic pathway and reactive oxygen species. Inhibiting fission by the negative form of Drp1 decreased BAT response to adrenergic stimuli by half. Thus, mitochondrial fission appeared essential for proper BAT function.

In conclusion, mitochondrial efficiency may be of importance for normal as well as compromised β-cell and islet function. Mitochondrial morphology appears critical for mitochondrial function in β-cells and BAT.

This thesis is based on the following papers, which are referred to in the text by their Roman numerals, respectively.

I. **Wikstrom JD**, Katzman SM, Mohamed H, Twig G, Graf SG, Heart E, Corkey BE, de Vargas LM, Danial NN, Collins S, Shirihai OS. Mitochondrial functional heterogeneity in pancreatic beta cells. *Diabetes*: 56(10):2569-78, 2007.

II. Molina AJ, **Wikstrom JD**, Stiles L, Las G, Mohamed H, Elorza A, Walzer G, Twig G, Katz S, Corkey BE, Shirihai OS. Mitochondrial Networking Protects Beta Cells from Nutrient Induced Apoptosis. *Diabetes*. 58(10):2303-15, 2009.

III. **Wikstrom JD**, Elorza A, Allister EM, Sereda SB, Stiles L, Neilson A, Neilson A, Ferrick DA, Corkey BE, Deng S, Wheeler MB, Shirihai OS. Islet bioenergetic efficiency is regulated by nutrients.
Manuscript

IV. **Wikstrom JD**, Si Y, Twig G, Liesa M, Sereda SB, Las G, Cannon B, Nedergaard J, Shirihai OS. Brown adipocyte activation is characterized by a wave of mitochondrial fission and depolarization that is dependent on β_3 receptor stimulation and Drp1, and is characterized by complete, but reversible, arrest of fusion
Manuscript

It also refers to the following papers.

Wikstrom JD, Twig G, Shirihai OS. What can mitochondrial heterogeneity tell us about mitochondrial dynamics and autophagy? *Intern J. Biochem Cell Biol*. 41(10):1914-27, 2009.

Danial NN, Walensky LD, Zhang CY, Choi CS, Fisher JK, Molina AJ, Datta SR, Pitter KL, Bird GH, **Wikstrom JD**, Deeney JT, Robertson K, Morash J, Kulkarni A, Neschen S, Kim S, Greenberg ME, Corkey BE, Shirihai OS, Shulman GI, Lowell BB, Korsmeyer SJ. Dual role of pro-apoptotic BAD in insulin secretion and beta cell survival. *Nat. Med.* 14(2):144-53, 2008.

Katzman SM, Messerli MA, Barry DT, Grossman A, Harel T, **Wikstrom JD**, Corkey BE, Smith PJ, Shirihai OS. Mitochondrial metabolism reveals a functional architecture in intact islets of Langerhans from normal and diabetic Psammomys obesus. *Am J Physiol Endocrinol Metab* 287(6):1090-1099, 2004.

Twig G, Graf SA, **Wikstrom JD**, Mohamed H, Haigh SE, Elorza A, Deutsch M, Zurgil N, Reynolds N, and Shirihai OS. Tagging and tracking individual networks within a complex mitochondrial web with photoactivatable GFP. *Am J Physiol Cell Physiol* 291, C176-C184, 2006.

Twig G; Liu X; Liesa M; **Wikstrom JD**; Molina AJA; Las G; Yaniv G; Hajnoczky G; Shirihai OS. Biophysical properties of mitochondrial fusion events in pancreatic β-cells and cardiomyocytes unravel potential control mechanisms of its selectivity. *Am J Physiol Cell Physiol*, May 8 2010. [Epub ahead of print]

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Abbreviations

Body mass index (BMI)
Brown adipocytes (BA)
Brown adipose tissue (BAT)
Dynamin-related protein 1 (Drp1)
Free fatty acids (FFA)
Green fluorescent protein (GFP)
Inner membrane PAGFP($_{IM}$PAGFP)
Inner mitochondrial membrane (IM)
Insulin growth factor 1 receptor (IGF1R)
Matrix Red flurescent protein ($_{MT}$RFP)
Matrix targeted PA-GFP ($_{MT}$PAGFP)
Mitochondrial DNA (mtDNA)
Mitochondrial membrane potential ($\Delta\Psi_m$)
Mitofusin (Mfn)
Outer membrane GFP ($_{OM}$GFP)
Photo-activatable GFP (PA-GFP)
Polyethylene glycol (PEG)
Rat insulinoma derived beta cell line (INS1)
Reactive oxygen species (ROS)
Short interference RNA interference (siRNAi)
Tetrachloro-1,1,3,3-tetraethylbenzimidazol-carbocyanineiodide (JC-1)
Tetrakis (4-benzoic acid) porphyrin manganese(III) (TBAP)
Type 2 diabetes (T2D)
Uncoupling protein 2 (UCP2)

1. Introduction

Type 2 diabetes and obesity are metabolic diseases that plague the world in the 21st century. In the United States alone, it is now estimated that 10% of adults fcare diabetic (Centers for Disease Control and Prevention, 2007) and 35% are obese (Catenacci, 2009). In Sweden, 292,000 people were diabetic in 2000 (World health organization, 2010). According to the World Health Organization it affected 171 million people in 2001 and by the year 2030 is expected to grow to a staggering 366 million. Diabetes is the leading cause of blindness, end-stage renal disease and neuropathy in the US (Brownlee, 2003). The direct and indirect costs related to diabetes in the U.S. are estimated at $132 billion in medical expenditures and lost productivity (Hogan, 2003). Although significant research effort has been invested, there has been a poor delivery in terms of therapeutics. Thus, more research is needed.

Normoglycemia is maintained by several tissues including pancreatic islets and skeletal muscle. Diabetes is defined as elevated blood glucose; hyperglycemia. In type 2 diabetes tissues are considered to malfunction. Skeletal muscle display insulin resistance, i.e. low uptake of blood glucose. Pancreatic β-cell insulin secretion is insufficient or dysregulated. Of great importance for normal function of these tissues are mitochondria (Maechler, 2006). There is a growing body of data indicating that β-cell mitochondria malfunction in type 2 diabetes (Mulder, 2009). This is perhaps not surprising considering that mitochondria have a central role in metabolism. The thermogenic brown adipose tissue (BAT) is possibly the organ that is most characterized by its mitochondria. This because the heat produced is a direct product of mitochondrial activity (Cannon, 2004). It has been suggested that BAT may play an important role in metabolic disease as it transforms stored energy into heat when active. A recent study indicates that BAT may be dysfunctional in this category of patients (Cypess, 2009).

Mitochondria take up a substantial portion of the cytoplasmic volume of eucaryotic cells, and they have been essential for the evolution of complex animals. Without mitochondria, present-day animal cells would be dependent on anaerobic glycolysis for all of their ATP. When glucose is converted to pyruvate by glycolysis, only a very small fraction of the total free energy potentially available from the glucose is released. In mitochondria, the metabolism of sugars is completed: the pyruvate is imported into the mitochondrion and oxidized by O_2 to CO_2 and H_2O. This allows 15 times more ATP to be made than that produced by glycolysis alone (Alberts, 2002).

The discovery of the mitochondrion was gradual, however the term was derived from the greek words *mitos* (thread) and *khondrion* in 1898 by Carl Benda (Benda, 1898 544 /id); thus describing the structural double nature of this organelle. Decades ago, influential electron microscopy studies led to the dogmatic view of mitochondria as bean-shaped organelles. These studies revealed the ultrastructural hallmarks of mitochondria, which include double lipid membranes and unusual inner membrane folds termed cristae. Recent studies have led to renewed appreciation for the fact that the mitochondrial structure is highly dynamic. Imaging studies have revealed that mitochondria constantly move and undergo structural transitions; fusion and fission (Detmer and Chan, 2007b).

These processes are important as a number of diseases are directly caused by their dysfunction (Delettre, 2000; Alexander, 2000; Zuchner, 2004; Waterham, 2007).

In this thesis mitochondrial form and function of healthy and diseased β-cells and intact islets as well as brown adipocytes is characterized. Furthermore, we test the hypothesis that mitochondrial form is of importance for function in these cell types. The results are presented in the context of the relevant literature.

2. Mitochondrial physiology

2.1. Mitochondria in a nut shell

Mitochondria have multiple functions within the cell that include ATP-production, calcium signaling, and apoptosis. Each mitochondrion is bound by two lipid membranes. Together they create two separate mitochondrial compartments: the internal matrix space and the intermembrane space (Figure 1). The matrix contains hundreds of enzymes including those required for the citric acid cycle and β-oxidation. The inner membrane is folded into numerous cristae, which increase the surface area similar to intestinal villi. The cytochromes of the respiratory chain are arranged within the cristae. When cristae junctions open upon apoptotic stimuli cytochrome C may be released into the cytosol to cleave caspases (Alberts, 2002). The outer membrane is permeable to all molecules less than 5 kDa, however most can not pass through the inner membrane because of its high selectivity.

The matrix also contains several copies of mitochondrial DNA (mtDNA), and various enzymes required for mitochondrial gene expression. Since each cell has hundreds of mitochondria, the mtDNA copy number is often above 1000/cell (Shay, 1990). The mitochondrial genome is essential for the respiratory function, however the majority of mitochondrial proteins are encoded by the nuclear genome and are then imported into the mitochondria (Wallace, 2005). The 16 kilobase circular mtDNA genome contains 37 genes. Thirteen of these genes encode protein subunits of respiratory complexes I, III, IV, and V; only complex II is composed of proteins encoded solely by nuclear genes (Wallace, 2005). The remaining genes encode transfer RNA (tRNA) and ribosomal RNA (rRNA) necessary for intramitochondrial protein synthesis (Anderson, 1981).

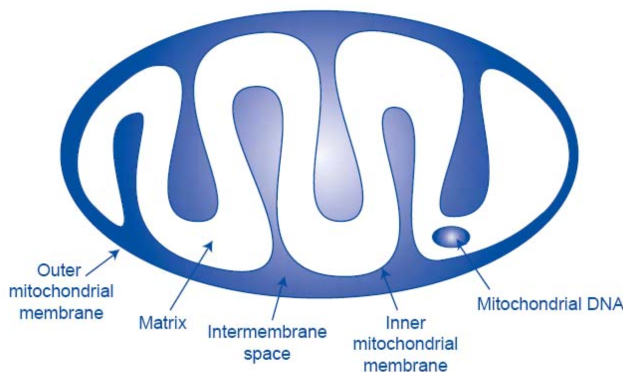


Figure 1. Mitochondrial components: *Image adapted from (Lamson and Plaza, 2002b).*

2.2. Principles of bioenergetics

Mitochondria are often referred to as "the powerhouse of the cell"; this because mitochondria produce approximately 90% of the ATP. ATP is produced by the respiratory chain complexes (I-V) (Figure 2). Complex I (ubiquinone NADH dehydrogenase) is responsible for the oxidation of NADH and pumps protons (H^+) into the intermembrane space while reducing ubiquinone. Complex II (succinate dehydrogenase) oxidizes succinate into malate, thus liberating reducing equivalents (electrons) that are shuttled to complex III via ubiquinone. Complex III (ubiqinol-cytochrome-*c* reductase) receives electrons, liberating H^+ in the process. Complex IV (cytochrome-*c* oxidase) reduces O_2 to H_2O, producing H^+ in the process. As each complex moves electrons along the chain, protons are pumped out of the matrix into the intermembrane space. The proton gradient generated is used to drive ATP synthesis by Complex V (F1F0ATPsynthase), which phosphorylates ADP to ATP. This proton gradient is commonly referred to as the mitochondrial membrane potential ($\Delta\Psi_m$) and represents the energy available to drive changes in ATP/ADP ratio, and reactive oxygen species as well as controls mitochondrial calcium sequestration (Lowell and Shulman, 2005). The $\Delta\Psi_m$ potential is considerably higher than the plasma membrane potential; ~150-180mV (Valdez, 2006) vs. ~60-90mV respectively (Wright, 2004).

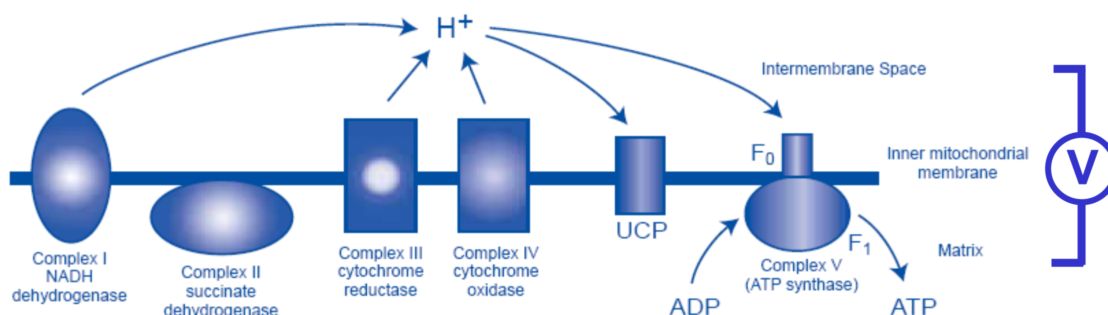


Figure 2. The mitochondrial respiratory chain: The mitochondrial membrane potential ($\Delta\Psi_m$) is the electrochemical gradient that is present across the inner mitochondrial membrane and is generated by pumping H^+ by complexes I,III,IV of the respiratory chain. This potential is used to drive ATP synthesis via complex V (F1F0ATPsynthase). Protons may also re-enter the matrix via other routes, e.g. via uncoupling proteins, without production of ATP. Complex I is considered as the major source of reactive oxygen species (ROS). *Image adapted from (Lamson and Plaza, 2002a).*

During the transfer of electrons along the respiratory complexes, single electrons sometimes escape and result in a single electron reduction of molecular oxygen to form a superoxide anion (O_2^-) (Fariss, 2005). It is estimated that as much as 1% of all oxygen consumed may result in the formation of reactive oxygen species (ROS) such as superoxide anions. The main sites of O_2^- generation are at Complex I and the interface between ubiquinone and complex III (Nishikawa, 2000a). Although previously viewed as toxic byproducts it now appears that ROS may act as intracellular signaling molecules (Pi, 2007).

2.3. Mitochondrial oxygen consumption in cells and pancreatic islets

2.3.1. Assessing mitochondrial function

A variety of cellular parameters may be used as indicators of mitochondrial function, including: the redox state of mitochondrial cytochromes; cellular ATP levels; ROS production; $\Delta\Psi_m$; and oxygen consumption. Although less widely measured, oxygen consumption is arguably the most informative of these parameters (Will, 2006), in that measurement allows a direct and specific assessment of the flow of the electron transport chain. The vast majority of cellular oxygen consumption is mitochondrial and occurs at complex IV in the respiratory chain as protons are pumped out into the intermembrane space (Mitchell, 1976).

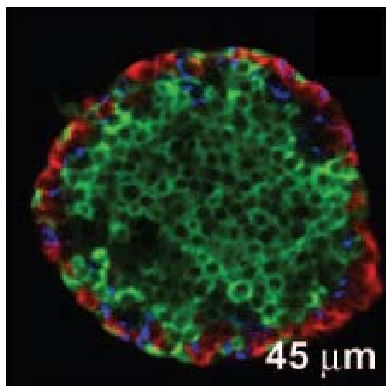
2.3.2. Uncoupled and coupled respiration

The majority of protons reenter the mitochondrial matrix through F1F0ATPsynthase and thereby fuel the conversion of ADP to ATP (Mitchell, 1976). Alternatively protons may reenter through mechanisms not coupled to ATP synthesis, "proton leak", which stimulates "uncoupled respiration". Therefore, oxygen consumption has a dual interpretation. Brand and colleagues have reported extensively on variations in proton leak among different cell types and even different species (Brand, 1991; Hulbert, 2002; Brand, 2003; Else, 2004; Jastroch, 2007; Lambert, 2007; Parker, 2008; Bottje, 2009). In principal, the level of uncoupled respiration is of interest as it reflects the cell's bioenergetic efficiency.

2.3.3. Islet respiration

In cell biology, oxygen consumption (or respiration) is measured either in isolated mitochondria, or in permeabilized or intact cells or tissue. Several assays for measuring oxygen consumption have been used over the years. In principal, assays have utilized either Clark type electrodes or fluorescent probes to report on media oxygen tension. The Clark electrode measures a flow of electrons, i.e. a current, that is dependent on the oxygen tension of the media (Clark, Jr., 1958). The fluorescent probes exhibit quenched fluorescence intensity emission in response to increased oxygen tension (Ji, 2002; Wu, 2007).

Islets of Langerhans consist of several cell types including the β-cells (Figure 3). Mitochondria are essential for proper β-cells or islet function (Maechler, 2006). Mitochondrial metabolism of glucose derivatives is necessary for insulin secretion as described in Figure 3. Several different assays are used to measure islet respiration
(Longo, 1991; Ortsater, 2000; Doliba, 2006; Papas, 2007c; Jung, 2008; Sweet, 2008c). Some have the advantage of having media flow-through and can sample in and outflow, thus e.g. enabling insulin secretion measurements. However, the flow-through methods can only run one sample per experiment and are cumbersome to use, i.e. user-dependent. Alternatively there are multiwell plates coated with oxygen sensitive fluorescent probes (Fraker, 2006; MacGregor, 2006). Though higher throughput, these plates do not provide detailed dynamic data, i.e. limited number of time points. Thus, up to now there has been



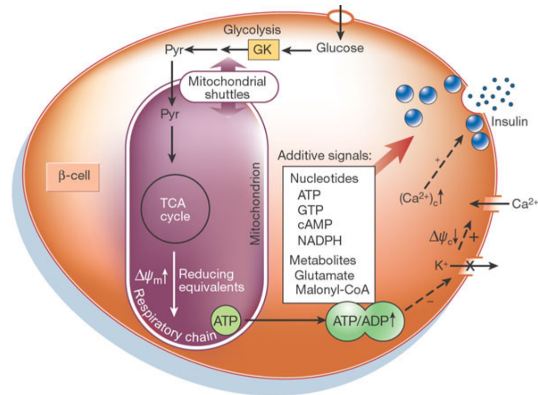


Figure 3. A) Islet of Langerhans from mouse. Islets comprise ~1% of the pancreas and each islet consists of ~1500 cells. Note the different cell types: β-cells (green; secrete insulin; 75% of cells), alpha cells (red; secrete glucagon; 19% of cells), delta cells (blue; 6% of cells). *Adapted from (Brissova, 2005)*. **B)** Model for coupling of glucose metabolism to insulin secretion in the β-cell. Glucose is phosphorylated by glucokinase (GK) and converted to pyruvate (Pyr) by glycolysis. Pyruvate preferentially enters the mitochondria and fuels the citric acid cycle, resulting in the transfer of reducing equivalents to the respiratory chain, leading to hyperpolarization of the mitochondrial membrane ($\Delta\Psi_m$) and generation of ATP. Subsequently, closure of K_{ATP}-channels depolarizes the plasma membrane potential ($\Delta\Psi_c$). This opens voltage-gated Ca^{2+} channels, raising the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), which triggers insulin exocytosis. Several putative messengers, or additive signals, proposed to participate in the metabolism–secretion coupling are indicated. *Adapted from (Maechler and Wollheim, 2001b)*

a lack of high throughput and user friendly assays that at the same time provide high quality data. In paper III the development of a novel approach to islet respirometry based on oxygen sensitive fluorescent probes and multiwell plates especially adapted for islets is presented. This assay can concurrently run 20 islet samples and test multiple conditions over a course of several hours. By applying drugs acting on the respiratory chain levels of basal, fuel-stimulated, uncoupled, maximal as well as non-mitochondrial respiration may be estimated under various conditions.

2.3.4. Significance of measuring islet respiration

As mitochondrial dysfunction is much discussed in β-cell pathophysiology (Mulder and Ling, 2009), islet respiration methods are of importance for basic science. In addition, islet respirometry may also have a clinical use. Islet transplantation is a treatment under development for type 1 and advanced type 2 diabetes (Harlan, 2009). There are currently no reliable methods for assessing islet quality prior to transplantation (Papas, 2009). This is important, since many islet batches have quality issues, foremost because they come from critically ill donors and go through a traumatic treatment during their isolation. It was shown that islets with high oxygen consumption rates are more suitable for transplantation, at least to nude mice (Sweet, 2005; Papas, 2007a; Sweet, 2008b). The respirometry assay presented in paper III may therefore prove useful clinically to evaluate islet quality prior to transplantation. This because of its high troughput capability and its simple design that may make it easy to adapt by different transplantation centers across the world.

2.3.5. Uncoupled respiration of islets

In clonal INS1 β-cells (derived from rat insulinoma) (Asfari, 1992), the proton leak or level of uncoupled respiration was reported to be four times higher than in clonal C2C12 myoblasts (Affourtit and Brand, 2008). However interesting, pancreatic islets function as a functional syncytium (Katzman, 2004) of ~1500 cells and results obtained on cell lines may not readily be extrapolated to the primary tissue. In paper III we present data showing that mouse islet mitochondria are highly uncoupled, ~60% of the basal respiration remains under oligomycin. This is the first characterization of uncoupled respiration in islet. Furthermore, paper III shows that INS1 cells exhibit levels of uncoupled respiration of ~40%. This data is in contrast with a previous INS1 cell study that measured uncoupled respiration to be 75% (Affourtit, 2008). The uncoupled respiration of C2C12 myoblasts (~20%) shown in paper III was similar to the previous study (Affourtit , 2008). Table 1 compares the rates of uncoupled respiration between different tissues and species. Interestingly the uncoupled respiration of islets appears higher than most other cells or tissues.

System	Percentage of total respiration	Reference(s)
Rat β-cells (INS1)	40-75	(Affourtit, 2008), (Paper III)
Mouse islets	60	(Paper III)
Rat hepatocytes	20–26	(Rolfe, 1999),(Nobes, 1990)]
Rat muscle	35–50	(Rolfe, 1996;Rolfe, 1999)
Rat basal metabolic rate	20–25	(Rolfe, 1996;Rolfe, 1999)
Mammal hepatocytes (mouse, ferret, sheep, pig, horse)	~20	(Porter and Brand, 1995)
Avian hepatocytes (finch, sparrow, starling, currawong, pigeon, duck, goose, emu)	Up to 21	(Else, 2004)
Crocodile hepatocytes	Up to 13–30	(Hulbert, 2002)
Lizard hepatocytes	Up to 30	(Brand, 1991)
Frog hepatocytes	Up to 20–25	(Brand, 2000)
Lamprey hepatocytes	Up to 25–50	(Savina, 1997)
Snail hepatopancreas cells	Up to 15–25	(Bishop and Brand, 2000)

Table 1. Comparison of uncoupled respiration across different cell types and species. *Table adapted and modified from (Brand, 1999)*

2.3.6. Significance of uncoupled respiration

The level of uncoupling is an important biological phenomenon as it reflects bioenergetic efficiency. Proton leak contributes to standard metabolic rate, i.e. energy consumption, by converting part of the mitochondrial proton gradient to heat. In fact, 16-31% of standard metabolic rate is caused by proton leak (Rolfe and Brand, 1996). The role of proton leak in human disease is still unknown, however some evidence suggest that it may increase with aging (Serviddio, 2007). Conversely caloric restriction, shown to increase lifespan, was shown to decrease proton leak (Bevilacqua, 2004; Johnson, 2006). Another study however found that long lived mice had higher levels of proton leak (Speakman , 2004).

Theoretically, if the fraction of coupled respiration could be increased, mitochondrial ATP-production may increase as a consequence. In β-cells this could in turn trigger higher insulin secretion as ATP stimulates closure of the ATP sensitive K-channels in the plasma membrane. Thus, the coupling efficiency of the respiratory chain of β-cells within pancreatic islets may represent a therapeutic target. On the other hand, it may be that the uncoupled respiration reflects essential processes for secretion.

2.3.7. Regulation of uncoupled respiration

Proton leak, mirrored by uncoupled respiration, is typically divided into basal and inducible. Basal proton leak is present under resting conditions in all types of mitochondria that have been studied (Table 1) and may make a major contribution to metabolic rate. Inducible proton leak is, as the name implies, not present under resting conditions. A number of different mechanisms may contribute to proton leak.

2.3.7.1. Fuel regulation of uncoupled respiration

In addition to glucose, free fatty acids and amino acids were shown to stimulate insulin secretion, either alone or as augmenters of GSIS (Newsholme, 2005). However, several additional messengers besides ATP are thought to play important roles (Maechler, 2006). In paper III we show that glucose, the free fatty acid palmitate and the amino acids leucine and glutamine in addition to increasing coupled respiration also dramatically increase uncoupled respiration. E.g., the combination of leucine and glutamine increased uncoupled respiration from ~60% to ~ 90%.

The physiological role of fuel induced uncoupling is unclear; from a physiological perspective it appears inefficient. It may be that at the fuel levels tested in paper III mitochondrial ATP production is saturated although the metabolism preceding it is not. E.g., the citric acid cycle with its influxes from glycolysis, β-oxidation and amino acid metabolism may be working at a higher rate than the F1F0ATPsynthase. Uncoupling may act as “release valve”, diverting protons from F1F0ATPsynthase. The purpose of this higher rate may be to maintain a high production of amplifying signals from the preceding metabolism that augments insulin secretion. These may for example be generated from pyruvate shuttle traffic; NADPH, α-ketoglutarate and GTP (Jensen, 2008b) as well as citric acid cycle derived GTP (Kibbey, 2007). Further, it may be that uncoupling serves to protect the β-cell from fuel toxicity. Since the β-cell serve as a fuel sensor, it imports more fuel than is required for maintaining the ATP concentration. These fuels however may render them sensitive to fuel toxicity, as shown by studies on glucolipotoxicity (Poitout, 2008). Increased mitochondrial uncoupling may allow excess fuel to be turned into heat.

2.3.7.2. Lipid composition

Some of the variation in proton leak between tissues and species may be explained by differences in mitochondrial inner membrane surface area. There is a correlation between mitochondrial proton conductance and the fatty acyl composition of inner-membrane phospholipids (Hafner, 1988; Brookes, 1997b; Hulbert, 2002; Brand, 2003). The content of *n*−3 polyunsaturates, particularly docosahexaenoate (C22:6,*n*−3), correlates with high proton conductance, and the content of monounsaturates, particularly oleate (C18:1,*n*−9),

correlates with low proton conductance. However, the proton conductance of phospholipid vesicles prepared from mitochondrial lipids is only 2–25% of the conductance of the mitochondria that they are derived from, and does not change when the composition changes (Brookes, 1997a; Brookes, 1997b). Consequently some other factor than membrane surface area or phospholipid composition must be an important determinant of the basal proton conductance of mitochondria. Naturally, as the mitochondrial membranes have numerous proteins these must be considered.

2.3.7.3. Uncoupling proteins

To date two types of mitochondrial inner membrane proteins have been shown to be involved in proton leak; uncoupling proteins (UCP) and the adenine nucleotide translocase (ANT). UCP1 has long been recognized to mediate noradrenergic stimulated proton leak in brown adipocytes (Nicholls, 2001). The other uncoupling protein homologues, UCP2 and UCP3, are more controversial. UCP2 protein is mostly expressed in pancreatic islets, spleen, stomach, brain and lung while UCP3 is predominantly expressed in skeletal muscle, brown adipose tissue and heart (Chan and Harper, 2006). During the past years, UCP2 in islets have gained much attention. This interest was triggered by studies showing that UCP2 knock-out islets exhibit elevated $\Delta\Psi_m$ and ATP-levels as well as increased insulin secretion (Zhang, 2001a). Furthermore, the UCP2 knock-out animals appeared protected against diet induced diabetes (Joseph, 2002). However, a recent study by Collins and colleagues has indicated that these findings may have been artifacts caused by the genetic background of the knock-out mice (Pi, 2009). UCP2 was knocked out in mice with three different strain backgrounds (C57BL/6J, A/J, 129/SvImJ). In contrast to previous studies, it was found that the insulin secretion was impaired (Pi, 2009). Furthermore, the knock-out islets showed high levels of oxidative stress including elevated levels of antioxidant enzymes and increased nitrotyrosine (Pi, 2009).

Studies on β-cell lines have also showed opposing results. In INS1 β-cells with knock down of UCP2, it was calculated that 20% of the respiration was due to UCP2 (Affourtit, 2008). In addition insulin secretion was increased (Affourtit, 2008). On the other hand, another study where UCP2 was overexpressed found no alteration of uncoupled respiration (Galetti, 2009). Instead, decreased levels of oxidants was shown, thus in line with (Pi, 2009). Thus, the literature is somewhat contradictory. However there is little doubt that islet data is more relevant than clonal β-cell data. In paper III uncoupled respiration of mouse islets with a β-cells specific knock-out of UCP2 is examined. The generation of the knock-out animals was previously described elsewhere (Lee, 2009). We found no difference in uncoupled respiration as compared to control islets (paper III). In fact, UCP2 knock-out islets showed higher levels of basal respiration (paper III). These data add to the literature arguing that UCP2 is not primarily an uncoupling protein like its classic homologue UCP. Instead a primary role of UCP2 may be in regulation of ROS.

2.3.7.4. Adenine nucleotide translocase

The other protein candidate for mediating protein leak, ANT, exchanges ADP for ATP across the mitochondrial inner membrane (Klingenberg, 2008). To examine the role of ANT in proton leak Brand and colleagues examined mice with knock-out of ANT1, an

isoform of ANT, and *Drosophila melanogaster* strains under- or overexpressing ANT (Brand, 2005). Skeletal muscle mitochondria were examined in the mice while whole body mitochondrial isolates were extracted from the flies. It was found that the amount of ANT present in the mitochondrial inner membrane strongly affects the basal proton leak. A major part of the leak appeared to be due to ANT (Brand, 2005). Similar results on the role of ANT were found in liver and brown adipocytes (Shabalina, 2006). It was suggested that ANT2 isoform may mediate fatty acid induced uncoupling while ANT1 may mediate a significant part of the basal proton leak. In paper III the role of the ANT is examined in mouse islets by using its specific inhibitor bongkrekic acid. The contribution of ANT to the basal level of uncoupled respiration was estimated to be ~31% (paper III). This is substantially lower than previously reported on isolated mouse skeletal muscle mitochondria where the ANT contribution was estimated to be between half to two-thirds of the basal proton conductance (Brand, 2005). Under fuel stimulated conditions the contribution of ANT to uncoupled respiration increased to 42%, likely due to increased nucleotide shuttling (paper III).

The results on UCP2 and ANT are not surprising considering the different abundances of these proteins. ANT contributes 1–10% of total mitochondrial protein (Brand, 2005) while only 0.3%, 0.03% and 0.01% is contributed by the pyruvate carrier (Shearman and Halestrap, 1984;Paradies, 1984), UCP2 (Pecqueur, 2001) and UCP3 (Harper, 2002) respectively. In comparison to UCP2, UCP1 comprises 1–5% of mitochondrial protein in brown adipose tissue of mice kept below their thermoneutral temperature of 28°C (Stuart, 2001). Interestingly a recent study has shown that UCP2 content in pancreatic alpha cells is considerably higher than in β-cells (Diao, 2008b). Thus, it may be that UCP2 plays a greater role in alpha cells.

2.3.7.5. Permeability transition pore

The permeability transition pore (PTP) is a large channel consisting of multiple subunits that increase mitochondrial inner membrane permeability to various solutes including protons (Rasola and Bernardi, 2007). The PTP classically opens in response to death stimuli and enables release of of cytochrome C that cleaves caspases (Rasola, 2007). However, PTP opening may also be partial and reversible (Liu and Murphy, 2009), and could thus in theory contribute to proton leak and a consequent increase in uncoupled respiration. Furthermore, one of the major components of the PTP complex is the ANT (Tsujimoto and Shimizu, 2007). PTP opening has been extensively examined in myocytes. In rat skeletal muscle exposed to anoxia/reoxygenation an increase in proton leak was demonstrated to be dependent on PTP (Navet, 2006). Interestingly, palmitate appeared to prevent this proton leak, probably because it caused uncoupling by itself. A study on mitochondria isolated from perfused rat hearts that were subjected to ischemia/reperfusion showed somewhat similar results (Nadtochiy, 2006). The increased proton leak after ischemia/reperfusion was inhibited to 50%, by carboxyattractyloside, an inhibitor of ANT, but also by cardioprotective treatments including the PTP inhibitor cyclosporin A. With these data in mind, the role of PTP in regulation of uncoupled respiration in islets was examined in paper IV by using cyclosporin A. No significant effect on the level of uncoupled respiration was found, both under low glucose and leucine/glutamine stimulation, thus pointing to no direct involvement of PTP.

2.3.7.6. Reactive oxygen species

Traditionally, ROS have been thought of as useless by-products of respiratory metabolism in mitochondria and believed to be generally deleterious to biological systems (Finkel, 1998). However, ROS have emerged as physiological mediators of many cellular responses (Rhee, 2006) and some evidence suggests that these molecules may serve a signaling function (Pi, 2007). In β-cells, it was recently suggested that low levels hydrogen peroxide derived from glucose metabolism serves as a signal for insulin secretion, whereas oxidative stress may disturb its signaling function (Pi, 2007; Pi, 2009). Nearly all of ROS are produced in mitochondria because of interaction of oxygen with free electrons released by the respiratory chain.

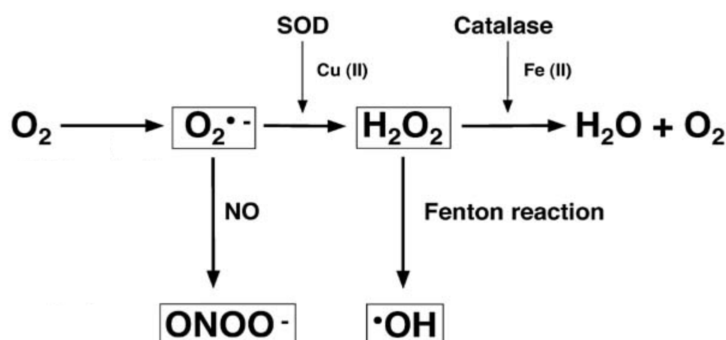


Figure 4. Principles of reactive oxygen species (ROS) generation. ROS are foremost generated by the mitochondrial respiratory chain where the majority of oxygen is consumed. SOD and catalase are important scavengers of superoxide. ($O_2\cdot^{-}$, superoxide anion radical; H_2O_2, hydrogen peroxide; ·OH, hydroxyl radical; ONOO-, peroxynitrite; SOD, superoxide dismutase, NO, nitric oxide). *Adapted from (Kyaw, 2004)*

ROS was shown to induce uncoupling (Echtay, 2002). One mechanism by which ROS does so is by lipid peroxidation that leads to production of reactive aldehydes such as 4-hydroxynonenal (Echtay, 2003). These aldehydic lipid peroxidation products are able to modify proteins such as mitochondrial uncoupling proteins and the ANT, converting them into active proton transporters. Furthermore, in β-cells ROS was described to increase with fuel exposure (Pi., 2007) (paper III). In addition fuels such as palmitate may induce uncoupling in islets (Carlsson, 1999). Considering these data, the effect of scavenging ROS with the superoxide-dismutase mimetic tetrakis (4-benzoic acid) porphyrin manganese(III) (TBAP) on islet respiration was examined in paper III. Interestingly, TBAP totally abolished the fuel stimulated increase in uncoupled respiration. This suggests that ROS may be the molecular link between fuel metabolism and uncoupled respiration in islets. Furthermore, in paper III it is shown that the ANT uncoupling activity increases under fuel stimulated conditions. It was previously shown that in aging ANT is specifically targeted by ROS (Yan, 1998), suggesting that ANT may have a particular sensitivity to ROS. A possible mechanism for how ROS induce uncoupling in the islets may be by stimulating ANT.

2.4. Mitochondrial dynamics

The mitochondrial morphology is a dynamic property that can form a variety of shapes: from long, interconnected tubules to individual small spheres (Frazier, 2006). In

eukaryotes, the overall morphology of the mitochondria is maintained by balancing the opposing processes of mitochondrial fusion and fission, collectively termed mitochondrial dynamics. These processes not only control mitochondrial morphology but also play an important role in mitochondrial function (Detmer and Chan, 2007a; Detmer, 2007b). Without mitochondrial dynamics, the mitochondrial population consists of autonomous organelles that have impaired function that include reduced metabolism, and increased apoptosis (Chan, 2006).

2.4.1. Regulation of mitochondrial fusion

Mitochondrial fusion is defined as the merger of two mitochondria resulting in one larger mitochondrion (Figure 5) (Skulachev, 2001; Detmer, 2007a; Detmer, 2007b). In mammalian cells fusion is regulated by three known transmembrane GTPases: mitofusin-1 (Mfn1), mitofusin-2 (Mfn2) and Optic Atrophy 1 (Opa1). Opa1 is located within the inner mitochondrial membrane (Olichon, 2003). Mfn1 and 2 are located within the outer mitochondrial membrane.

Mfn1 and Mfn2 appear to play similar roles in mitochondrial fusion although Mfn1 requires Opa1 for its function while Mfn2 does not (Zhang and Chan, 2007). Deficiency of either protein results in mitochondrial fragmentation. Mfn1 and 2 can functionally replace each other. Cells lacking Mfn1 can be rescued by overexpression of Mfn2; conversely cells lacking Mfn2 can be rescued by overexpression of Mfn1 (Chen, 2003). Moreover, Mfn-null cells can be fully rescued by overexpression of either mitofusin (Chen, 2003).

2.4.2. Regulation of mitochondrial fission

Mitochondrial fission is the division of a mitochondrion to form two or more separate mitochondrial units (Figure 6) (Yoon, 2004). In eukaryotes mitochondrial fission involves the transmembrane protein Fis1 and GTPase dynamin-related protein (Drp1). Drp1 is a key component of the mitochondrial fission machinery. A minor fraction of Drp1 is localized to punctate spots on mitochondrial tubules, and a subset of these spots

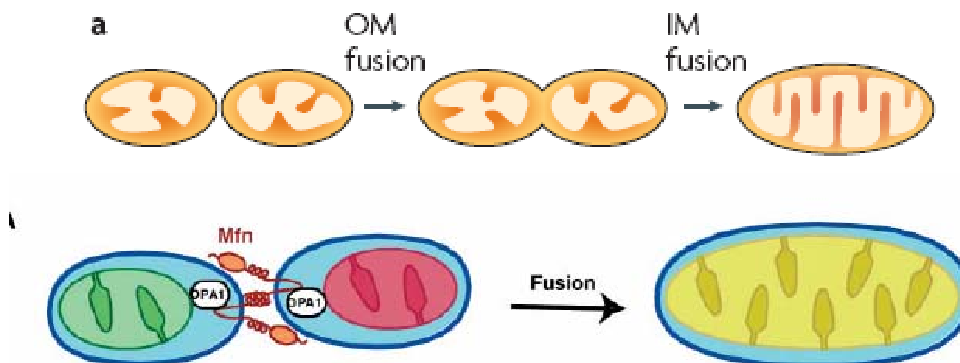


Figure 5. Fusion event: Mitochondrial fusion consists of outer membrane (OM) fusion followed by inner membrane (IM) fusion (top panel). Mfn1 and Mfn2 localized on the outer mitochondrial membrane tether with the inner mitochondrial protein, Opa1 resulting in mitochondrial fusion (bottom panel). *Adapted from (Detmer, 2007).*

mark future sites of fission. The majority of Drp1 is free in the cytosol and is recruited by Fis1 upon induction of fission (Smirnova , 2001). Drp1 activity is inhibited by phosphorylation by protein kinase A which in turn is regulated by cAMP (Cribbs, 2007).Inhibition of Drp1 by expression of a dominant-negative (DN) mutant leads to increased length and interconnectivity of mitochondrial tubules (Lee , 2004).

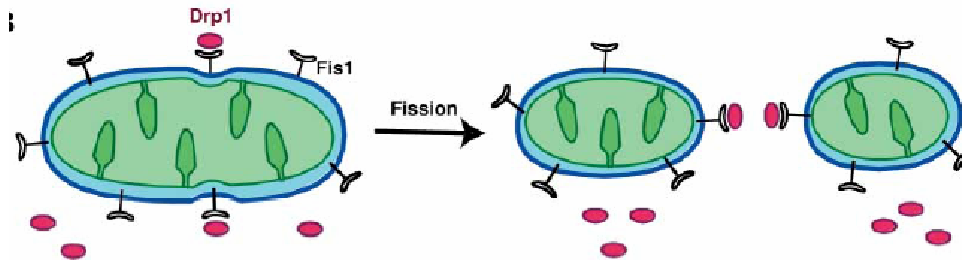


Figure 6. Fission event: Fis1 is localized on the outer mitochondrial (OM) membrane, Drp-1 binds at scission sites upon which fission occurs resulting in two individual mitochondria. *Adapted from (Chen, 2005).*

3. Mitochondrial heterogeneity

Over the past years, it has been shown in numerous studies that mitochondria display functional and structural heterogeneity. Any measured biological parameter varies to some degree. When the magnitude of the variations respond to physiologically and pathologically relevant alterations it may by itself become the parameter of interest. Mitochondrial subcellular heterogeneity is altered by metabolic stress (paper I) and apoptosis (D'Herde , 2000;Krysko , 2001), and therefore deserves attention. Recent years' progress in the understanding of the mitochondrial life cycle, including mitochondrial dynamics and mitochondrial autophagy (mitophagy) suggest that the two processes control and maintain the extent of heterogeneity. Conditions that increase heterogeneity also affect mitochondrial dynamics and autophagy. In cell death, both heterogeneity (Salvioli , 2000;Krysko , 2001) and autophagy (Kroemer and Levine, 2008) increase and mitochondrial dynamics is inhibited (Suen , 2008). In pancreatic β-cells, metabolic stress in the form of high levels of glucose and free fatty acids disrupts mitochondrial dynamics (paper II), increases heterogeneity (paper I) and upregulates autophagy (Choi , 2008).

3.1. Subcellular heterogeneity

Mitochondrial heterogeneity has been reported from a diverse range of primary cells and cell lines including neurons, myocytes, exocrine and endocrine cells, as well as from brown adipocytes (paper IV). A variety of techniques have been used; however, imaging data is dominating the literature. A consensus definition of mitochondrial heterogeneity is lacking in the literature. In principle, heterogeneity is the variance in the measured parameter representing the combined effects of the diversity of the sampled individuals and the noise introduced by the sampling technique. When considering the functional significance of heterogeneity one needs to determine what portion of it is contributed by the noise generated by the methodology of data acquisition and analysis. For example, in the case of confocal microscopy large variance can be generated by the fact that mitochondria are not all in the same focal plane; therefore, the intensity of the

fluorescence signal largely depends on the position of each mitochondrion in the *z*-axis. In addition certain image processing algorithms (such as filters) may widen or narrow the spectrum of the signal. The functional significance of heterogeneity can be determined by demonstrating that heterogeneity is altered by relevant effectors. For example, the span of $\Delta\Psi_m$ heterogeneity is altered in pancreatic β-cells responding to glucose stimulation (paper I). In addition, the temporal characteristic of the heterogeneity is also of importance.

3.1.1. Mitochondrial membrane potential heterogeneity

$\Delta\Psi_m$ is a widely used bioenergetic parameter affecting multiple mitochondrial functions including ATP synthesis, Ca^{++} sequestration, protein import, mitochondrial fusion, mitochondrial autophagy, and the generation of reactive oxygen species (ROS) (Nicholls , 2000). $\Delta\Psi_m$ is regulated by factors contributing to its build up, e.g. fuel input and respiratory chain activity and factors contributing to its dissipation, e.g. F1F0-ATPase activity as well as uncoupling mechanisms and other ion fluxes than protons (Huser, 2000). Within a physiologically relevant $\Delta\Psi_m$ range, the maximum ATP/ADP ratio that can be maintained by mitochondria decreases by up to 10-fold for every 14mV decrease in $\Delta\Psi_m$, thus the level of $\Delta\Psi_m$ reflects a mitochondrion's energetic capacity (Nicholls, 2004). Glucose-induced $\Delta\Psi_m$ hyperpolarization correlates well with induction of insulin secretion (dependent on raises in ATP/ADP ratio) (Heart , 2007) as well as with increased mitochondrial oxygen consumption as shown in paper III. The oxygen consumption data indicates that glucose-induced $\Delta\Psi_m$ hyperpolarization is due to increased proton pumping activity by the electron transport chain. However, in cases where oxygen consumption data is not available, caution should be practiced when interpreting changes in $\Delta\Psi_m$. For example, while depolarization is frequently attributed to mitochondrial respiratory dysfunction, it should be kept in mind that increased ATP-production may lead to depolarization under some circumstances, e.g. in state 3 respiration of isolated mitochondria where ADP, the substrate for oxidative phosphorylation and a possible dissipater of $\Delta\Psi_m$, is in excess. As *in situ* mitochondria cannot be accessed directly, indirect methods which predominantly use membrane-permeate cationic fluorescent dyes have been employed to monitor $\Delta\Psi_m$ in cells by imaging. While these dyes have been widely used they present a number of challenges including unspecific binding, photo-toxicity and interference with cell metabolism. To appropriately interpret data generated using $\Delta\Psi_m$ probes these confounding factors should be controlled for. Unspecific binding can be estimated by adding a mitochondrial uncoupler, which would dissipate the $\Delta\Psi_m$ and lead to loss of mitochondrial staining. Photo-toxicity can be reduced by lowering the intensity of the excitation light during imaging. No matter what intensity is finally being used, the researcher has to address the possibility of photo-toxicity by monitoring the effect of dye excitation on a relevant biological function that can be accepted as a measure of cellular viability and function. By using low dye concentrations metabolic interference can be minimized and controlled for by testing cellular function in the presence of the dye. When imaging mitochondria, it is also important to be aware of the cell's *z*-axis. A difference in dye fluorescence intensity between two different mitochondria in an individual image may arise either from a real difference in dye concentration (and thus $\Delta\Psi_m$) or alternatively from the mitochondria being in different focal planes. This may be corrected for either by using

confocal microscopy *z*-stack imaging, where multiple focal planes are recorded and compiled into a single image, or by combining two fluorescent probes and using their ratio for assessing $\Delta\Psi_m$ as described in paper I. Moreover, ratio imaging can be used to account for artifacts stemming from the limitations of image resolution (200 nm in conventional confocal microscopes). For example, a mitochondrion may cover only part of the factual area that an image pixel "records" from. The light intensity emitted from the mitochondrion will therefore be divided by a larger area than it is actually covering, leading to a false impression of reduced dye concentration. In summary, data generated using $\Delta\Psi_m$ probes should be interpreted with caution and the availability of appropriate controls in which potential artifacts are accounted for should be verified before conclusions are made. Here follows a review of the results and caveats of the main probes used to study heterogeneity in $\Delta\Psi_m$.

3.1.2. JC-1

Tetrachloro-1,1,3,3-tetraethylbenzimidazol-carbocyanineiodide (JC-1) is a fluorescent dye that at low $\Delta\Psi_m$ exists as green-emitting monomers but when $\Delta\Psi_m$ increases, forms red emitting aggregates (Smiley , 1991). The proportion of aggregates to monomers in the inner membrane of the mitochondria is dependent both on the membrane potential as well as on the concentration of the dye in the cytosol. JC-1 has been used to show $\Delta\Psi_m$ heterogeneity in a variety of cells, including intact human fibroblasts (Smiley , 1991); HeLa cells and hepatocytes (Collins , 2002); mouse oocytes and blastocysts (Van , 2003; Van , 2006; Van and Davis, 2006); mouse and human early embryos (Acton , 2004); pancreatic β-cells (paper I); human astrocytes, HEp-2, MDCK and Vero cells (Diaz, 1999); rat cardiomyocytes (Bowser , 1998); as well as in isolated liver mitochondria (Cossarizza , 1996). Although important in revealing heterogeneity, JC-1 possesses a number of drawbacks, of which the most important is that its partition to the inner membrane is not Nernstian and is therefore not reliable for calculating $\Delta\Psi_m$ according to the Nernst equation (Nicholls , 2000). Consequently, it has only been used in qualitative descriptions of $\Delta\Psi_m$ heterogeneity. Moreover, if staining exceeds 30 min, or slightly higher concentrations are used, JC-1 may produce peculiar artifacts (Wikstrom, 2009). In some cells, JC-1 may aggregate into long nail-like structures, very different from mitochondrial architecture. JC-1 appears to load more readily into projections of cells such as neurons and INS1 β-cells, probably reflecting faster dye loading due to the projections' higher plasma membrane to cytosol ratio. Thus, JC-1 also has drawbacks of qualitative nature.

3.1.3. TMRE/TMRM allows for quantitative evaluation of heterogeneity

Tetramethylrhodamine-ethyl-ester (TMRE) and methyl-ester (TMRM) are two similar $\Delta\Psi_m$ probes. They have the advantages of comparatively low mitochondrial toxicity. Their partition to the mitochondria inner membrane is Nernstian, enabling quantitative studies of $\Delta\Psi_m$ heterogeneity (Nicholls , 2000). A number of studies have used these dyes. (Distelmaier , 2008) reported on $\Delta\Psi_m$ heterogeneity in primary human skin fibroblasts. In primary cultures of human fetal astrocytes and adult fibroblasts, longitudinal profiles of single mitochondria were homogenous, while the $\Delta\Psi_m$ between mitochondria differed (Diaz , 2000). Distribution curves of $\Delta\Psi_m$ of mitochondrial populations were shown in several reports (Loew , 1993; Zhang , 2001b) and is also

demonstrated in paper I). The similarity in $\Delta\Psi_m$ heterogeneity measured from neuroblastoma and primary pancreatic β-cells is remarkable, the mean standard deviation in $\Delta\Psi_m$, as expressed in mV, is 11mV in neuroblastoma cells (Loew , 1993) and 9mV in primary pancreatic mouse β-cells (paper I). These subcellular variances in $\Delta\Psi_m$ may appear small but may translate to large differences in ATP production between mitochondria within the same cells (Nicholls, 2004).

3.2. Heterogeneity in compromised cells

While mitochondrial heterogeneity appears to be a universal phenomenon, increased levels of heterogeneity have been associated with cell pathology. In mouse pancreatic β-cells $\Delta\Psi_m$ heterogeneity is increased when cells are metabolically stressed with high levels of glucose and free fatty acids (paper I). The effect of stress on mitochondrial heterogeneity was tested in an ischemia-reperfusion model (Kuznetsov, 2004a; Kuznetsov , 2004b; Kuznetsov , 2004c; Kuznetsov , 2006) It was shown that mitochondria in rat cardiomyocytes are heterogeneous in terms of $\Delta\Psi_m$, Ca^{++}, ROS and flavoproteins and that heterogeneity increased after cold ischemia-reperfusion. This effect may be attributed to heterogeneity in PTP opening and/or cytochrome *C* release (Kuznetsov , 2004b), however this was not directly tested. PTP opening was attributed to ROS, since it was prevented by antioxidants. In mitochondria isolated from ischemic rabbit hearts, it was shown that subsarcolemmal (SS), but not intermyofibrillar (IMF) mitochondria, had a large decrease in oxidative phosphorylation, likely due to decrease in cytochrome C content (Lesnefsky , 1997). It was further shown in quail apoptotic granulosa cells, that cytochrome C release as well as $\Delta\Psi_m$ are heterogeneous, and it was suggested that ATP needed for completion of the apoptotic cascade may be generated in a subset of still respiring mitochondria (D'Herde , 2000; Krysko , 2001). Moreover, by tagging cytochrome C with GFP Heiskanen et al. demonstrated that those mitochondria that were found to be depolarized in staurosporine-treated apoptotic rat pheochromocytoma cells indeed lose their cytochrome C, thus further supporting PTP opening and cytochrome C release as causing $\Delta\Psi_m$ heterogeneity in apoptosis. In HeLa cells stained with calcein-AM and exposed to oxidative stress in the form of peroxide, it was shown that the loss of calcein-AM from mitochondria was heterogeneous, which is likely to reflect heterogeneous PTP opening (Collins , 2002). Furthermore, in unstressed cells PTP may operate in a reversible low conductance mode and not be associated with cell death (Ichas , 1997). This low conductance mode may be implicated in depolarization prior to autophagy (Kim , 2007). Thus it is possible that heterogeneity in healthy cells may also be affected by heterogeneous PTP activity. Finally, in cells that are rapidly dividing, a fraction of the cells examined may be undergoing mitosis. In early mitotic phase in HeLa cells, mitochondria undergo transient fragmentation (Taguchi , 2007). Thus, the proliferation rate may influence mitochondrial heterogeneity measurements to some degree.

3.3. Mechanism of mitochondrial heterogeneity

Heterogeneity in mitochondrial function may be attributed to intrinsic and extrinsic sources. In principle, the metabolic functions of a mitochondrion may be influenced by external signals from the cytosol or other organelles and thus be dependent on its location in the cell. Alternatively, function may depend on intrinsic properties or content of the

mitochondrion itself. In addition, there are several studies indicating that mitochondria, at least in some cell types, may be divided into functionally diverse subpopulations carrying out different tasks in the cell.

3.3.1. Subcellular location

3.3.1.2. Perinuclear vs. peripheral locations

Studies that used the $\Delta\Psi_m$ probe JC-1 have found that $\Delta\Psi_m$ of mitochondria is dependent on the location in the cell (Bereiter-Hahn , 1983; Smiley , 1991; Diaz , 1999). These studies report that $\Delta\Psi_m$ of mitochondria located in the periphery of astrocytes, HEp-2 cells and endothelial cells were relatively hyperpolarized. However studies that have used TMRM or TMRE in astrocytes, fibroblasts and pancreatic β-cells did not find differences on $\Delta\Psi_m$ between perinuclear and peripheral mitochondria (Diaz , 2000)(paper I).

In brown adipocytes, mitochondria close to lipid droplets (peridroplet) exhibit a slightly hyperpolarized $\Delta\Psi_m$ as compared to distant mitochondria (cytoplasmic) (paper IV). Interestingly, this was also accompanied by differences in fusion rates, where peridroplet mitochondria showed slower rates. It was recently reported that the most important parameter influencing fusion probability is mitochondrial motility (Twig, 2010). Thus, it may be that that the peridroplet mitochondria in brown adipocytes are less motile and therefore fuse less. The $\Delta\Psi_m$ result is in contrast with a previous study on clonal INS1 β-cells showing that mitochondria that fuse less are depolarized (Twig , 2008a). This discrepancy may represent different kinds of subpopulations. E.g., the depolarized mitochondria in β-cells were small and destined for degradation by autophagy if they did not repolarize (Twig , 2008a). It may be that this population also exists in BA as most cells had a fraction of minute and depolarized mitochondria. Furthermore, in β-cells the lipid droplets are considerably smaller and scarcer, i.e. the subpopulation of droplet mitochondria found in BA may not exist in the β-cells. In addition, it may be that peridroplet mitochondria are hyperpolarized because of higher fuel access.

3.3.1.3. Calcium

Matrix Ca^{++} regulates the activity of several dehydrogenases of the citric acid cycle and is thereby influencing oxidative phosphorylation (Huser , 2000). In turn, the $\Delta\Psi_m$ generates a diffusion potential that drives Ca^{++} flow from the cytosol into the matrix. As a result, mitochondrial Ca^{++} uptake is highly dependent on cytosolic Ca^{++} concentration which may vary throughout the cell (Filippin , 2003). Thus, it is plausible that subcellular heterogeneity in Ca^{++} availability may influence mitochondrial heterogeneity. Indeed, a number of studies indicate that this may be the case. In mouse pancreatic acinar cells, three distinct groups of mitochondria located in the peripheral basolateral region close to the plasma membrane, around the nucleus and in the periphery of the granular region were described (Park , 2001). These different groups only sequestered Ca^{++} in their immediate vicinity and showed no intergroup connectivity as judged by photo-bleaching (Park, 2001). In conjunction with regions of high Ca^{++}, it has been proposed that there are similar domains exhibiting elevated ROS that may affect mitochondrial activity (Davidson and Duchen, 2006). (Rizzuto, 1998) showed in HeLa cells that some mitochondria have contact sites with endoplasmic reticulum (ER). Release of Ca^{++} from ER at these contact sites results in local increase in Ca^{++} concentrations, approaching the

threshold at which mitochondria may contribute Ca^{++} buffering capacity. Interestingly, this group reported that ER colocalized with only a minority of the mitochondria. In another HeLa cell study it was shown that Ca^{++} released by ER is preferentially sequestered more by peripheral than perinuclear mitochondria. This is in comparison to Ca^{++} from the extracellular sources that is equally sequestered by peripheral and perinuclear mitochondria (Collins, 2002). This finding cannot be attributed to the pattern of ER distribution in the cell as ER is more abundant at the perinuclear region (Collins, 2002). This pattern of heterogeneous Ca^{++} uptake was further shown in a subsequent HeLa cell study where a subset of mitochondria was reported to take up more ER-induced Ca^{++} release (Filippin, 2003). A similar uptake pattern could be repeated after 15min, suggesting that the interactions between mitochondria and ER in these locations remained stable over this period of time (Filippin, 2003). However, since single mitochondria were not tracked, it remains unclear whether mitochondria that responded the second time were the same individual mitochondria or if new mitochondria had moved into close vicinity of the ER. It was suggested that ER induced Ca^{++} release focuses on a few mitochondria allowing a rapid uptake by the low-affinity mitochondrial Ca^{++} uniporter (Filippin, 2003) providing the ER Ca-ATPase with sufficient ATP (Duchen, 2008). Further, it has also been shown that mitochondria may move to regions of high cytoplasmic Ca^{++} a mechanism that may ensure proper Ca^{++} buffering (Yi, 2004;Quintana , 2007).

3.3.1.4. Access to metabolites

Heterogeneous fuel availability and individual differences in metabolism could lead to mitochondrial functional heterogeneity. It has been suggested that diffusion of phosphorous metabolites such as ADP and ATP is uneven throughout the cell (de Graaf, 2000;Saks , 2003), perhaps due to hindrance by intracellular structures (de Graaf, 2000). It may further be that enzymes relevant to fuel metabolism create unevenly distributed microdomains with diverse abundance of cellular fuels. Mitochondrial glucokinase has been reported to be activated by association with the BCL-2 family member, BAD. BAD knock-out rodents exhibit reduced mitochondrial glucokinase activity (Danial, 2003). In pancreatic β-cells of BAD knock-out mice, it was found that $\Delta\Psi_m$ heterogeneity is lower under low glucose conditions, thus indicating that in WT animals mitochondrial glucokinase activity or distribution may be heterogeneous (paper I). Glucokinase generates ADP and its heterogeneous distribution may lead to heterogeneous ADP distribution, translating to a heterogeneous work load, ATP synthesis and $\Delta\Psi_m$. Moreover, increased levels of glucose or methyl succinate decreased the heterogeneity (paper I), suggesting that increased fuel availability may compensate for a diverse ADP distribution. In brown, adipocytes the increased $\Delta\Psi_m$ of peridroplet mitochondria may reflect higher access to acyl-CoA for β-oxidation.

3.3.1.5. F1F0-ATPase

In isolated rat liver mitochondria, $\Delta\Psi_m$ heterogeneity was higher in mitochondria de-energized with ADP (Cossarizza, 1996). This would indicate different levels of F1F0-ATPase or ANT activity among mitochondria. Indeed, in paper I it is described that when exposing mouse pancreatic β-cells to the F1F0-ATPase blocker oligomycin $\Delta\Psi_m$ heterogeneity decreases, which would indicate heterogeneity in F1F0-ATPase activity

under resting conditions. Under some circumstances, e.g. ischemia (Takeda, 2004), F1F0-ATPase has been demonstrated to work in reverse and hydrolyze ATP and thus maintain $\Delta\Psi_m$. However, with oligomycin, only very few mitochondria were observed to depolarize implying that reverse F1F0-ATPase activity is not a major contributor to $\Delta\Psi_m$ heterogeneity in the unstressed pancreatic β-cells (paper I).

3.3.2. Diversity vs. instability

The heterogeneity data described above is based on snapshot images of $\Delta\Psi_m$. In principal, $\Delta\Psi_m$ heterogeneity observed in a snapshot image may represent a state in which the $\Delta\Psi_m$ of each mitochondrion is stable but the population is diverse. Alternatively, it may reflect a state in which each individual mitochondrion's $\Delta\Psi_m$ is unstable over time. To address the two possibilities it is essential to follow individual mitochondria over time. Several studies have tracked $\Delta\Psi_m$ of individual mitochondria. Spontaneous transient depolarizations of $\Delta\Psi_m$, often termed flickers, were first shown by using TMRE (Loew, 1993). Most commonly, flickers have been attributed to transient activation of the PTP (Huser and Blatter, 1999; De, 2000; Diaz , 2000; Collins , 2002; Jacobson and Duchen, 2002).However, at least two types of PTP-independent flickers have also been identified. First, flickers have been linked to reentry of protons into the matrix via the F1F0-ATPase during ATP generation in rat neurons (Buckman and Reynolds, 2001). Second, in rat cardiomyocytes flickers have been attributed to depolarization resulting from focal Ca^{++} influx into a mitochondrion from the sarcoplasmic reticulum (Duchen , 1998). To be able to track an individual mitochondrion, it is essential to first define the boundaries of a single mitochondrion within a complex network, and then follow it over time. (Duchen, 1998) identified individual or groups of mitochondria by their relative decrease in fluorescence intensity (during flickers) compared to the surrounding mitochondria. In theory, a fragment of the mitochondrial web that depolarizes is expected to be electrically coupled and thus share the same matrix space. Recent adoption of the photo-activatable fluorescent proteins enabled for the first time the simultaneous monitoring of individual mitochondrion $\Delta\Psi_m$ as well as fusion and fission events over time. By photoactivation of matrix-targeted photo-activatable GFP ($_{MT}$PAGFP), it was found that adjacent and intertwined mitochondrial structures often consist of several individual mitochondria, as defined by matrix continuity (Twig, 2006). It was further described that the mitochondrial life cycle includes frequent events of fusion and fission (Twig, 2008a). While the $\Delta\Psi_m$ of individual mitochondria was reported to be stable over time (paper I) (Twig, 2008a)), it appeared that following a fission event the two daughter mitochondria often exhibit disparate $\Delta\Psi_m$. Thus, conclusions from $_{MT}$PAGFP studies differ from the previously mentioned studies that found that individual mitochondria's $\Delta\Psi_m$ go through frequent changes. These differences may be due to different definition and identification of individual mitochondria and the portion of the life cycle of the mitochondrion that was captured by each study. Studies that did not monitor fusion and fission events may have considered the changes that occur during fusion and fission as fluctuation of the individual mitochondrion. The different results obtained by the different approaches may also reflect the diverse types of cells studied, as well as different imaging techniques. Finally, when addressing the issue of the single mitochondrion's variation in $\Delta\Psi_m$ over time, it is important to bear in mind that $\Delta\Psi_m$ can fluctuate at the level of the whole cell

or tissue, as exemplified by oscillations in $\Delta\Psi_m$ of cells within rat pancreatic islets (Katzman, 2004).

3.3.3. Organelle content

Several studies indicate that mitochondria within the individual cell may differ by their protein and phospholipids contents which may be accompanied by structural differences. The impact of these differences on mitochondrial function has been shown in isolated mitochondria preparations where the effects of the surrounding cytoplasm and other organelles can be excluded. In cardiomyocytes, it was first reported by (Palmer, 1977) that two populations of mitochondria exist, subsarcolemmal (SS) and intermyofibrillar (IMF). In vitro, the IMF mitochondria displayed higher activities of Complexes I, II, III, and citrate synthase (Palmer, 1977; Palmer, 1985), but had similar protein levels compared to SS mitochondria. Furthermore, Ca^{++} accumulation was found to be higher in IMF mitochondria, as compared to SS mitochondria (Palmer, 1986), perhaps due to higher $\Delta\Psi_m$. In mitochondrial fractions isolated from rat skeletal muscle, IMF mitochondria displayed higher Complex IV activity, higher protein synthesis and content, as well as higher respiration while Complex II activity and cardiolipin content was higher in SS mitochondria (Palmer, 1986). In addition, it was shown that rat skeletal IMF mitochondria have higher ATP content (Takahashi and Hood, 1996).

Since most mitochondrial proteins are imported into mitochondria after being synthesized on cytosolic ribosomes, differences in protein import could potentially explain the diverse content of the IMF and SS mitochondria. IMF mitochondria isolated from rat skeletal muscle were found to exhibit higher rates of import of the precursors of malate dehydrogenase? and ornithine carbamyltransferase (Takahashi, 1996). Differences in the import of uncoupling protein 3 (UCP3), expressed in skeletal muscle mitochondria, were also reported (Nabben and Hoeks, 2008). (Jimenez, 2002) showed that SS mitochondria contain more UCP3 than IMF in the glycolytic muscles tibialis anterior and gastrocnemius, but not in the oxidative soleus muscle of mice. Since the preparation of the IMF and SS fractions differs, it cannot be ruled out that the measured differences are in part due to the different isolation procedures. Using a rigorous protocol to rule out such artifacts, it was shown that IMF mitochondria from piglets have higher respiration and activity of Complex IV and F1F0-ATPase than SS mitochondria, yet with a similar proton leak (Lombardi, 2000). All of the above studies were performed on isolated mitochondria. Studies that have performed subcellular analysis of IMF and SS mitochondria in intact cells are scarce. However, imaging data indicates that SS mitochondria have increased levels of oxidized flavoproteins and higher concentration of matrix Ca^{++} (Kuznetsov, 2006).

In neurons, synaptic mitochondria can be isolated into a heavy and light fraction that differs in the content of glutamate dehydrogenase and aspartate aminotransferase activity, suggesting metabolic compartmentalization (McKenna, 2000). Moreover, in liver and white fat, the heavy mitochondrial subfraction was reported to have a higher content of a wide range of mitochondrial enzymes as compared to the light fraction (Koekemoer and Oelofsen, 2001). By electron microscopy and immunogold labeling, it was demonstrated that the citric acid cycle enzyme alpha-ketoglutarate dehydrogenase is heterogeneously

distributed in mitochondria of mice cortical and cerebellar neurons, and that this could not be explained by random distribution (Waagepetersen, 2006). Finally, organization of mitochondria, i.e. the macro- and ultra-structure, is likely to contribute to the observed functional heterogeneity. Electron microscope tomography has revealed that mitochondrial cristae shape can vary greatly within single cells (Mannella, 2006a). These differences are likely to impact mitochondrial bioenergetics, e.g. by differential access to ADP (Hackenbrock, 1972).

3.4. How does mitochondrial heterogeneity coexist with mitochondrial dynamics?

With the exception of specific conditions (mitosis and stress), mitochondria in all cell types examined thus far have been found to go through continuous cycles of fusion and fission (Detmer, 2007b). Laser photo-conversion of $_{MT}$PAGFP in a subset of mitochondria, was found to be followed by its spread throughout the cell's mitochondrial population within 30–45 min in INS1 β-cells (Twig, 2008a; Twig, 2008b). Further, disruption of fusion results in an increase in heterogeneity (Chen, 2005). Hence, it appears that mitochondrial dynamics acts as a content homogenizer. Given the rapid mixing of mitochondrial contents, mitochondria are expected to be homogenous. However, mitochondrial functional heterogeneity exists in healthy cells with presumably normal mitochondrial dynamics and likely, at least to a degree, depends on diverse mitochondrial content. *This presents a paradox*, *how can content remain heterogeneous if frequent mitochondrial fusion events mix mitochondrial components*? A number of observations presented below aim to resolve this apparent paradox. These include that mitochondrial dynamics appears to be an exclusive process, designed to maintain heterogeneity, and that mitochondrial components do not mix equally during fusion.

3.4.1. Fusion—not for everyone

Fusion appears to homogenize the mitochondrial population (Detmer, 2007b); however, it is likely not to involve all mitochondria (Twig, 2008a). In addition fusion/fission events tend to appear in a "kiss and run" like pattern where fusion is brief (1–2 min), with the effect that mitochondria spend the majority of their time in a solitary state. This behavior was reported in COS7 monkey kidney fibroblasts and rat INS1 β-cells (Twig, 2008a), rat myoblast H9c2 and human skin fibroblasts (Liu, 2009), as well as in plants (Arimura, 2004). Disruption of the pro fusion proteins Mfn1 and Mfn2 in fibroblasts leads to mitochondrial fragmentation and widespread heterogeneity of $\Delta\Psi_m$ as well as severe cellular defects, including poor cell growth and decreased cellular respiration (Chen, 2005). This suggests that fusion serves to blend mitochondrial content and perhaps also provide a rescue mechanism for compromised organelles. With $_{MT}$PAGFP, groups of mitochondria in clonal INS1 β-cells were labeled and tracked over time (Twig, 2008a). It was found that some mitochondria did not share their $_{MT}$PAGFP over the course of 1h and that these were depolarized. Following a fission event, depolarized mitochondria had a reduced chance for a subsequent fusion event. Further, a subpopulation of non-fusing mitochondria was identified by its inability to share photo-converted $_{MT}$PAGFP. This subpopulation had depolarized $\Delta\Psi_m$ as compared to the fusing population (Twig, 2008a). Hence, fusion appears to be an exclusive process that excludes some mitochondria, and therefore it does not eliminate mitochondrial heterogeneity on its own.

3.4.2. Mitochondrial "kiss and run" generates heterogeneity

Fission events commonly generate uneven daughter mitochondria: one daughter exhibits relatively higher $\Delta\Psi_m$ and a high probability of a subsequent fusion event within the duration of the experiment (10min); the other daughter mitochondrion is likely to have lower $\Delta\Psi_m$ and a reduced probability for a subsequent fusion event (Twig, 2008a). The mechanism generating uneven daughter mitochondria is unclear. It may be caused by an active or passive reorganization process. Alternatively, it may be that mitochondrial fusion events often are too brief to allow full mixing (Twig, 2006; Busch, 2006). Therefore the resultant dissimilar $\Delta\Psi_m$ of the daughters, at least in part, may reflect difference in the content of the two mitochondria prior to fusion. In support of the latter concept, after either pair of fusion–fission the engaging mitochondria often retain their morphology prior to fusion. Thus, it appears that fusion and fission events occur in a rapid kiss and run pattern. The length of these events is sufficient for matrix exchange, which is achieved in less than a second (Partikian, 1998), but may be slower for membrane components. A novel study recently reported that small vesicles, of 100 nm diameter, bud from mitochondria in mammalian cells (Neuspiel, 2008). The budding of vesicles appeared independent of the fission protein Drp1 and different types of vesicles were described. Vesicles that contained the novel outer membrane protein mitochondria-anchored protein ligase (MAPL) were shown to be targeted to peroxisomes while those that contained TOM20 were not. Moreover, it appeared that vesicles were heterogeneous in $\Delta\Psi_m$, thus the process of vesicle budding may contribute to the level of subcellular heterogeneity. These results are certainly intriguing and future research will cast light on potential interplay between mitochondrial vesicles, dynamics and autophagy.

3.4.3. Do fusion events result in complete equilibration of the fusion mates?

A number of studies have tracked the spread of proteins located either in the OM, IM or matrix, and found that fusion enables their dissemination throughout the mitochondrial network. However, it appears that IM protein equilibration through fusion is less complete than for OM and matrix, which may explain the uneven daughters generated by fission (Twig, 2008a). In this section the evidence for redistribution of components of the different mitochondrial compartments during fusion is summarized.

3.4.3.1. Matrix

The matrix compartment is considered to have a very low viscosity and molecules may diffuse very rapidly within it, comparable to the speed of diffusion in water (Partikian, 1998;Verkman, 2002). Several reports show that matrix contents readily spread throughout the mitochondrial network upon fusion. By polyethylene glycol (PEG) induced fusion of HeLa or osteosarcoma cells transfected with either matrix targeted green ($_{MT}$GFP) or red fluorescent proteins ($_{MT}$RFP), (Legros, 2002) showed a complete mixing of matrix contents within 12 h. By imaging at hourly intervals, a linear rate of increase in colocalization was shown, reaching more than 80% after 8 h (Legros, 2002). Using similar technique, Scorrano and Chan groups showed similar speed of matrix diffusion in mouse embryonic fibroblasts (Chen, 2005; Cipolat, 2004; Koshiba, 2004). A less invasive type of assay used for estimating fusion in intact cells is based on $_{MT}$PAGFP (Karbowski, 2004; Twig, 2008a). PAGFP is non-fluorescent in its native form and emits green fluorescence after photo-conversion. During time-lapse imaging, the spread of

photo-converted $_{MT}$PAGFP from a group of photolabeled mitochondria to the rest of the mitochondria within the cell can be used to quantify fusion activity. By this approach, the time required to reach a steady state in which $_{MT}$PAGFP has spread throughout the mitochondria of a variety of mammalian cells is less than 1h (Karbowski, 2004; Twig , 2008a). This approximate rate of fusion may be evolutionary conserved. In plants with 50% of their mitochondria tagged with the photo-convertible fluorescent protein kaede, the time to a full spread was 2 h (Arimura, 2004). It is not clear why the PEG cell fusion assays are considerably slower, although the significant levels of cell death and the alterations in cell morphology associated with the PEG treatment suggest possible interference with cytoskeleton, and thereby, with mitochondrial dynamics.

3.4.3.2. Membranes

The inner mitochondrial membrane (IM) delineates the matrix space and has a complex architecture with numerous cristae (Mannella, 2006b). It is also considered as the most viscous and protein-rich lipid membrane in the cell (Ardail, 1990; Simbeni, 1991). The mitochondrial outer membrane (OM) has higher lipid content and is topologically simpler (Mannella, 2006b). Precise quantifications are not available in the literature for IM and OM dynamics as for matrix. There are, however, a number of dynamics studies indicating that IM components' mixing proceeds neither as rapidly nor as efficiently as mixing of matrix and OM components. (Malka, 2005) fused cells with $_{MT}$RFP, with cells expressing $_{MT}$GFP or $_{OM}$GFP by exposure to PEG. After 16 h both $_{MT}$RFP, $_{MT}$GFP and $_{OM}$GFP had spread throughout the cells and colocalized. However, after 4 h a significant portion of mitochondria in the fused polykaryons had more $_{OM}$GFP than $_{MT}$RFP (Malka, 2005). This indicates that mixing of IM and OM may occur at different rates and/or may to some extent exist as separate events.

(Legros, 2004) studied the spread of the IM protein subunit 2 of Complex IV (COX2). Osteosarcoma cells that were either wildtype (WT) or ρ0 (devoid of mitochondrial DNA) were tagged with either $_{MT}$RFP or $_{MT}$GFP and fused by PEG. It was found that after 12h a subset of polykaryons did not exhibit full spread of COX2, as identified by immunostaining. In contrast, when fusing WT osteosarcoma with WT HeLa cells, the spread of $_{OM}$GFP became homogenous after 12 h (Legros, 2004). In another study where an IM protein was examined, Complex I in HeLa cells was tagged with RFP or GFP and tracked after cell fusion (Busch, 2006). In order to inhibit de novo protein synthesis, cyclohexamide was added after the PEG-induced cell fusion. After 100 min Complex I had spread to most regions of the mitochondrial network, however it displayed a patchy appearance of colocalization of RFP and GFP (Busch, 2006). Even after 24h, the patchy appearance was maintained as compared to the control fusion polykaryon where no cyclohexamide was added and subsequently de novo synthesis of both RFP and GFP tagged to Complex I could occur. These studies further strengthen the argument that IM mixing through fusion is less complete than for OM and matrix proteins. Moreover, the IM may be divided into inner boundary membrane (IBM), that is closer to the OM and the cristae membrane (CM). By quantitative immunoelectron microscopy, it has been shown that different proteins are distributed in an uneven manner between IBM and CM (Vogel, 2006), indicating content heterogeneity and incomplete mixing even within single mitochondria.

3.4.3.3. mtDNA

Similar to protein components of the IM, it appears that mitochondrial DNA (mtDNA) transfer during fusion may be slower and less complete than that of matrix and OM proteins. This is perhaps not surprising considering the attachment of mtDNA to the IM (Holt, 2007). mtDNA is organized in nucleoids and associated with the mitochondrial IM (Kang and Hamasaki, 2005; Holt, 2007). The majority of discrete mitochondrial structures contain mtDNA even when fragmentation is acutely induced by the use of uncoupling drugs (Legros, 2002). On the other hand, when mitochondrial fusion is abolished by molecular techniques, a large fraction of the mitochondrial population looses mtDNA nucleoids (Chen, 2007). Functional complementation of mtDNA was first shown indirectly within heteroplasmic cells containing varying mutant proportions (Oliver and Wallace, 1982; Hayashi, 1991). More recently, it was reported that following PEG-induced fusion of cells carrying different mutations in respiratory chain components, mitochondrial genomes can complement each other after 10–14 days leading to restoration of respiratory functions (Hayashi, 1994; Takai, 1997; Ono, 2001). This extended duration to recovery is surprising given the rate at which fusion events occur (Ono, 2001). Furthermore, using cell lines with other mtDNA-mutations the Attardi group reported that intermitochondrial complementation was a very rare event; only 0.3–1.6% of fused cells exhibited complementation as judged by recovery of respiratory function (Yoneda, 1994; Enriquez, 2000). The reasons for these divergences have not been resolved (Attardi, 2002), but the observation that mice with high proportions of mutant mtDNA retain functional mitochondria and has no Complex IV negative mitochondria, argues for at least some exchange during fusion of functional mtDNA or RNA or proteins (Hayashi, 1994; Inoue, 2000; Nakada, 2001). Imaging studies confirm that mtDNA spread throughout the mitochondrial population during fusion events. Delivery of mtDNA from ρ+ mitochondria to mitochondria of ρ0 cells has been shown to occur in cybrids (cells created by fusion of enucleated cells with nucleated ρ0 cells). Studies that traced mtDNA using DAPI report that complete repopulation of ρ0 mitochondria with the ρ+mtDNA occurred within 6 h of the PEG fusion procedure (Hayashi, 1994). Using DNA immunohistochemistry and bromodeoxyuridine DNA incorporation, (Legros, 2004) observed that upon fusion of ρ+ and ρ0 cells, mtDNA diffused readily across the mitochondrial network within 12 h. However, when fusing ρ+ and ρ+ cells, the spread appeared slower and mtDNA derived from one cell population was absent from some regions of the polykaryons after 12h.

3.4.3.4. Inner membrane vs. matrix mixing

To investigate whether mixing of IM differs from matrix mixing during fusion spread of an IM protein vs. a matrix protein was compared (Wikstrom, 2009). INS1 cells were transfected separately with PAGFP fused to the carboxyl terminus of ABC-B10 (Graf, 2004), an inner membrane ATP-binding cassette transporter ($_{IM}$PAGFP) or with $_{MT}$PAGFP. In each cell an area comprising approximately 10% of the cell was exposed to the 2 photon laser, resulting in the labeling of 10–20% of the cell's total mitochondrial volume. As a result of fusion, activated PAGFP (IM or matrix) molecules spread to unlabeled mitochondria, resulting in a decrease in the GFP fluorescence of the mitochondria containing activated PAGFP (Figure 7). The spread of $_{IM}$PAGFP was

considerably slower than for $_{MT}$PAGFP. Besides viscosity of the IM, the high complexity of cristae architecture with numerous folds and narrow tubular junctions, may be contributing to the slower diffusion rates of $_{IM}$PAGFP (Mannella, 2006b).

3.5. Autophagy, a mechanism that removes depolarized mitochondria

Macroautophagy is the process by which organelles and fragments of cytoplasm are sequestered by an isolation membrane, and subsequently delivered into lysosomes for hydrolytic digestion and recycling (Levine and Klionsky, 2004). An autophagosome, the intermediary vesicular structure that is formed by the isolation membrane, engulfs the target and is fused with the lysosome. Mitochondrial autophagy (mitophagy) is thought to target damaged mitochondria that are beyond intraorganelle repair (Kim, 2007). Mitophagy may reduce, or contribute to mitochondrial heterogeneity.

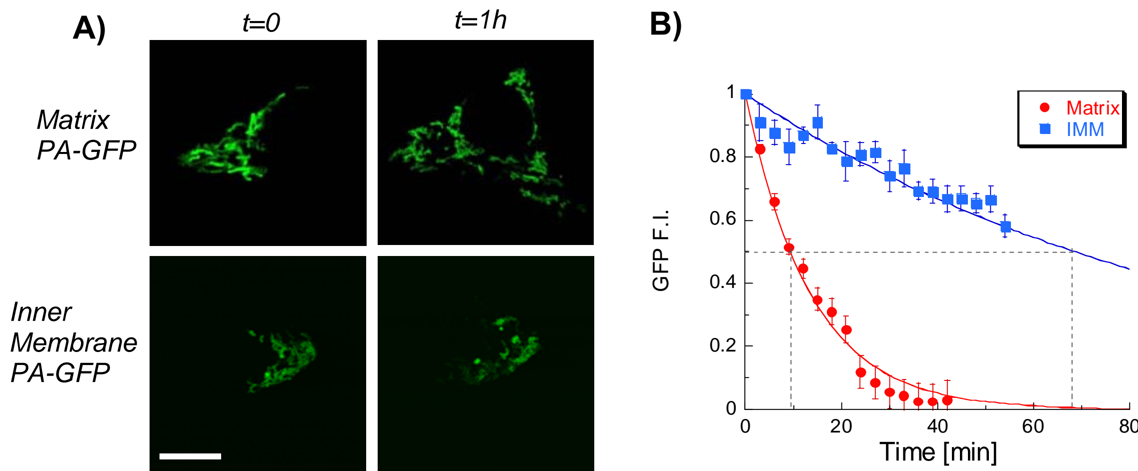


Figure 7. Spread of inner membrane (IM) vs. matrix (MT) tagged PAGFP. INS1 cells were transfected separately with $_{IM}$PAGFP (PA-GFP conjugated to the inner mitochondrial membrane protein ABCB10) or $_{MT}$PAGFP (PA-GFP conjugated to a mitochondrial targeted sequence). **(A)** In each cell an area comprising approximately 10-20% of the cell cross-section area was exposed to 2-photon laser, resulting in the photoconversion of PAGFP. Activated PAGFP molecules spread to unlabeled mitochondria through fusion events, resulting in decay in the GFP fluorescence intensity. **(B)** The kinetics of PAGFP spread for IM (n = 5) and MT (n = 7). Each series of data was fitted to a single exponential decay curve ($y = e^{-\tau x}$) to derive the time constant, τ ($R^2 > 0.98$). For clarity, the decay of each group was normalized to the range between GFP fluorescence intensity after photo-activation and the steady-state level after at least 60min (0). The time constant for $_{IM}$PAGFP was 8.1-fold smaller than that of $_{MT}$PAGFP (τ matrix = 0.074 vs. τ IMM = 0.009). Scale bar 10μm. *Adapted from (Wikstrom, 2009)*

3.5.1. Mitophagy reduces mitochondrial heterogeneity

Mitophagy has been reported to target depolarized mitochondria for digestion and elimination (Elmore, 2001; Priault , 2005; Twig, 2008a). In rat hepatocytes, serum deprivation and glucagon treatment increased the generation of depolarized mitochondria and their targeting by mitophagy (Elmore, 2001). In cells deficient of autophagy,mitochondria are dysfunctional and appear swollen in electron micrographs (Zhang, 2007; Jung, 2008). Further, inhibiting autophagy by the deletion of ATG5 in murine embryonic fibroblasts, for Beclin1 in H4 rat hepatoma cells or pharmacologically in INS1 cells, was found to increase $\Delta\Psi_m$ heterogeneity (Twig, 2008a). A similar result

was also predicted in models testing the long-term combined effect of mitochondrial dynamics' rate and autophagy on mitochondrial activity (Mouli, 2009). Thus, mitophagy decreases mitochondrial heterogeneity by recycling damaged mitochondria that otherwise would add to the level of diversity.

3.5.2. "Death row" mitochondria contribute to heterogeneity

In addition to reducing mitochondrial heterogeneity, it appears that autophagic targeting of mitochondria in itself adds to heterogeneity. Fission events often give rise to one depolarized daughter mitochondrion (Twig, 2008a). If the depolarized daughter did not repolarize swiftly its chance of a second fusion event was found to be six times lower compared to the hyperpolarized daughter. Also, overexpression of the pro-fusion protein Opa1 decreased mitophagy, suggesting that fewer mitochondria were "allowed" to leave the active networking pool. Further, it was found that mitochondria depolarize hours prior to autophagy (Twig, 2008a). Thus, it appears that the time lag between mitochondrial depolarization and the engulfment by the autophagic membrane creates a pool of depolarized mitochondria that are not involved in fusion events. Additionally, one may consider that the depolarized mitochondria inside autophagosomes, that have not yet been digested, also add to heterogeneity. However, since the digestion time is rather short, on average 7min (Kim, 2007), in unstressed cells this contribution to heterogeneity is likely to be minor.

3.5.3. Tags for selective mitophagy requires heterogeneity

Several studies suggest that mitophagy may be selective, both in terms of organelle specificity as well as in its targeting of a particular subpopulation of mitochondria (Kim, 2007;van, V , 2008). Selective mitophagy has been reported to occur in reticulocytes during erythroid maturation (Takano-Ohmuro, 2000), in the oocyte during fertilization, where the sperm mitochondria are specifically targeted (Shitara, 2000) as well as in yeast (van, V, 2008). The precise mechanism that targets mitochondria for autophagy is still unclear (Levine and Yuan, 2005; van, V, 2008). However it was shown that mitochondria depolarize prior to autophagy (Elmore , 2001; Priault, 2005; Twig, 2008a). This may be evolutionary conserved as depolarized yeast mitochondria exhibit similar behavior (Priault, 2005). Logically, for the autophagic machinery to recognize mitochondria destined for mitophagy it would require some sort of molecular tag. A problem for any tag would be dilution by mitochondrial fusion; it is therefore expected that a mitochondrion targeted for autophagy would need to be excluded from fusion with others. Indeed, prior to being autophagocytozed mitochondria join a non-fusing pre autophagic pool (Twig, 2008a). Since the pre-autophagic pool contributes to heterogeneity, the mechanisms that generate heterogeneity may shed light on the molecular and functional characteristics of the mitochondria in the pre-autophagic pool and perhaps on the molecular tag that destine them for autophagy.

3.5.3.1. Characteristics of mitochondria in the pre-autophagic pool

Small mitochondrial size is a prerequisite for mitophagy as mitochondria inside autophagosomes are minute (Kim, 2007). Hence, it may be that the pre autophagic membrane can only engulf small mitochondria. For example, in aging tissues there is accumulation of swollen large mitochondria that do not undergo autophagy (Coleman,

1987). In order to obtain small units, mitochondria need to undergo fission and fragment. Mitochondrial fragmentation was recently shown to be casually linked with alterations in both long and short isoforms of the fusion protein Opa1 (Duvezin-Caubet, 2006), i.e. both isoforms of Opa1 are necessary for fusion. Depolarization by uncouplers, which causes mitochondrial fragmentation as well as depolarization, destabilizes the long isoforms by proteolytic cleavage (Duvezin-Caubet, 2006; Song, 2007; Guillery, 2008). In conjunction with these data, nonfusing depolarized mitochondria, as well as mitochondria inside autophagosomes,were reported to have lower Opa1 levels (Twig, 2008a). Furthermore, it was shown that during apoptosis Opa1 is released from mitochondria (Arnoult, 2005), which could perhaps contribute to the increase in heterogeneity. Thus, modification or degradation of Opa1 may be involved in targeting mitochondria for autophagy.

In yeast there are a number of candidate proteins that may function as tags for mitophagy. Uth1 is located on the OM and its absence selectively triggers 50% less mitophagy although autophagic activity in general is upregulated (Kissova, 2004). Aup1p, a protein phosphatase, localizes to the mitochondrial intermembrane space and is required for efficient mitophagy in stationary phase cells. However, the precise pathways are not understood and deficiency of these proteins has opposite effects on cell viability (Kissova, 2004; Tal, 2007). Further, it was recently reported that the ubiquitin ligase Parkin may selectively label depolarized mitochondria and target them for autophagy in neurons overexpresssing Parkin and treated with mitochondrial uncouplers (Narendra, 2008). These results are certainly of great interest and future studies will determine the role of Parkin in other cell types as well as in housekeeping mitophagy.

ROS induces autophagy, however since ROS are soluble molecules that diffuse rapidly (van, V, 2008), they may mediate their effect by permanently modifying effectors molecules (Scherz-Shouval and Elazar, 2007). It has been suggested that depolarized mitochondria that do not undergo fusion experience further changes, e.g. oxidative, that tags them for autophagy (Kim, 2007). Indeed, a number of indirect observations suggest that mitochondria in the pre autophagic pool may have increased levels of oxidized protein. In INS1 cells inhibition of mitochondrial fission or autophagy results in an increase in the levels of carbonylated proteins (Twig, 2008a). Further, Lemasters' group pointed to PTP as the mechanism by which mitochondria depolarize prior to

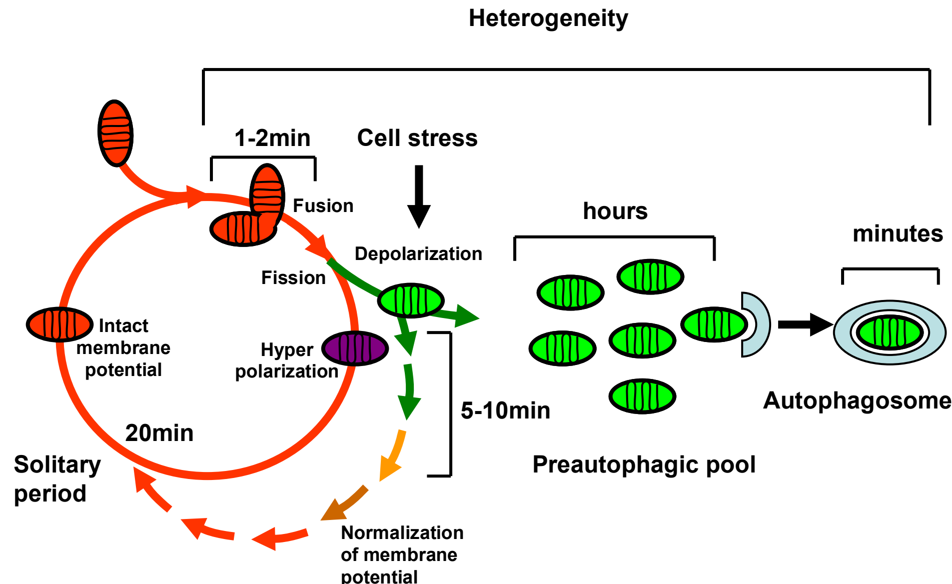


Figure 8. Mitochondrial heterogeneity reflects the mitochondrial life cycle. On average mitochondria undergo fusion/fission events lasting 1–2 min two to three times per hour. After fission one daughter mitochondrion often depolarize, while the other hyperpolarize. Most often these mitochondria return to a normal $\Delta\Psi_m$ within 5–10 min. If the depolarized daughter does not repolarize, it will not be allowed to fuse again. Depolarized mitochondria are targeted for autophagy, however the time lag between depolarization and engulfment by the autophagic membrane is several hours and therefore a pre-autophagic pool exists. Different cellular stresses may increase the size of the pre-autophagic pool. Finally, the mitochondrion is digested within minutes when the autophagosome fuses with the lysosome. *Adapted from (Wikstrom, 2009)*

autophagy in rat hepatocytes (Elmore, 2001); indeed PTP is induced by ROS (He and Lemasters, 2002). However, no effect of the PTP inhibitor cyclosporin A was found on post fission depolarization of mitochondria in INS1 cells (Twig, 2008a). The reason for this discrepancy may be that different types of autophagy were studied; autophagy induced by the combination of nutrient deprivation and glucagon (Elmore, 2001) as compared to housekeeping autophagy in cells in normal culture media with serum (Twig, 2008a). In addition, the difference in cell types studied may also contribute to the disparate results.

4. Mitochondrial pathophysiology in β-cells and islets

Type 2 diabetes (T2D) is a chronic metabolic disease that comprises the vast majority of diabetes onset in adulthood. T2D is characterized by both insulin resistance and β-cell dysfunction.

4.1. Insulin Resistance

Insulin resistance is defined as a diminished biological response to insulin in target tissues, e.g. muscle, liver and adipose tissue. Its progression often parallels that of obesity (Kahn, 2006a). Insulin resistance can be present for many years prior to the development of diabetes due to β-cell compensation (Kahn, 2006b), i.e. higher insulin secretion. However, as β-cell function gradually deteriorates less insulin is secreted, resulting in increased blood glucose levels and progression towards T2D (Steppel and Horton, 2004; Weir and Bonner-Weir, 2004).

4.2. β-cell mitochondrial dysfunction

β-cell dysfunction is a critical component to the development of type 2 diabetes (Poitout and Robertson, 2002a). However, the nature of the primary β-cell dysfunction is not clear. A myriad of studies have found abnormalities in various aspects of the β-cell. It is clear that β-cell mass decrease in diabetics, probably by apoptosis (Butler, 2003) however it is argued that also functional deficits play an important role (Mulder, 2009). A growing body of evidence implies mitochondrial dysfunction in the pathophysiology of β-cell secretory failure (Mulder, 2009). E.g., there have been over 40 different mtDNA mutations reported that increase the predisposition to diabetes (Maechler and Wollheim, 2001a). Transgenic mice with targeted mutations in mitochondria within β-cells become diabetic (Silva, 2000). Moreover, several diabetes animal studies demonstrate alterations in β-cell mitochondrial function as discussed below.

4.3. Glucolipotoxicity

Chronic hyperglycemia and hyperlipidemia can exert deleterious effects on β-cell function, referred to as glucotoxicity and lipotoxicity respectively (Poitout and Robertson, 2002b). Glucotoxicity refers to the slow, progressive and irreversible adverse effects of chronic hyperglycemia on β-cell function (Poitout and Robertson, 2002c). These adverse effects include reduction in glucose-stimulated insulin secretion (GSIS) and loss of β-cell mass. Lipotoxicity refers to the deleterious effects of accumulated fatty acids and their metabolic products on β-cells. Acute exposure to free fatty acids (FFA) potentiates GSIS in β-cells (Warnotte, 1994); whereas chronic exposure to high FFA triggers β-cell apoptosis (El-Assaad, 2003a). Studies by Prentki *et al* (El-Assaad, 2003b) show that saturated FFA are not toxic at low glucose concentrations (5mM) but synergize with elevated glucose (20mM) to cause apoptosis in INS-1 cells and human islets. This synergistic effect on β-cell deterioration is referred to as glucolipotoxicity (GLT) and has been used in several studies (including paper I, II and III) as a diabetes *in vitro* model. Recently, it was also shown in vivo by lipid infusion to rats that lipotoxicity inhibits insulin gene expression (Fontes, 2010).

4.4. Mitochondrial morphology and dynamics in diabetes

Central for mitochondrial function is mitochondrial morphology and dynamics, as discussed above. In muscle, reduction in Mfn2 expression was shown to reduce glucose oxidation and mitochondrial membrane potential (Bach, 2003; Pich, 2005a). PARL, the protease that cleaves the fusion protein Opa1, is positively correlated with insulin sensitivity in human skeletal muscle and was identified as a candidate gene for T2D (Walder, 2005). In β-cells, glucolipotoxic conditions induce increased mitochondrial heterogeneity (paper I) which was also observed to increase in cells deficient of mitochondrial fusion (Chen, 2005). Furthermore, abnormal mitochondrial architecture has been found in β-cells of diabetic animal models. In the Zucker Diabetic Fatty (ZDF) rat mitochondria were fragmented and dysfunctional, as indicated by the increased production of reactive oxygen species (ROS) when compared to the Zucker lean control (Bindokas, 2003). Similar findings were reported from the insulin resistant MKR mouse model (Lu, 2010) and in C57BL6/J mice on high fat diet were β-cell mitochondria were swollen (Fex, 2007). However, mitochondrial dynamics was not described in either of

these studies. In paper II mitochondrial dynamics in clonal and primary β-cells is described. Under normal conditions mitochondria continuously undergo fusion events. Mitochondrial morphology is regulated by pro fusion and pro fission proteins including Opa1and Drp1 (paper II). Under glucolipotoxicity mitochondria rapidly fragment and loose their fusion capacity (paper II). The fragmentation appeared dependent on the pro fission protein Drp1. Interestingly, knocking down the other pro fission protein, Fis1, rescued the β-cells from the apoptosis normally induced by the glucolipotoxic treatment (paper II). The antiapoptotic effect after inhibition of fission is consistent with findings in neurons (Barsoum*,* 2006). Moreover, similar to the findings in paper II it was reported that Drp1 is induced in β-cells under hyperglycemic conditions (Men*,* 2009). Interestingly, Drp1 dominant negative decreased hyperglycemia induced apoptosis (Men*,* 2009). This suggests a role for mitochondrial fission in β-cell apoptosis. Further, another study examined the effect of overexpressing Fis1 or Mfn1 on β-cell function (Park*,* 2008). Overexpression of Fis1 caused dramatic mitochondrial fragmentation, whereas Mfn1 overexpression evoked hyperfusion and the aggregation of mitochondria (Park*,* 2008). Fis1 overexpression caused cellular and mitochondrial dysfunction, including impaired insulin secretion, while dominant negative of Mfn1 did not, although the mitochondria were fragmented. Thus, mitochondria could be fragmented without being dysfunctional which supports the findings on brown adipocytes in paper IV. In conclusion, mitochondrial structure in β-cells is regulated by the fusion and fission proteins and changes in mitochondrial dynamics occur under *in vitro* diabetes conditions. Whether β-cell mitochondrial dynamics have any pathophysiological relevance *in vivo* remains to be determined.

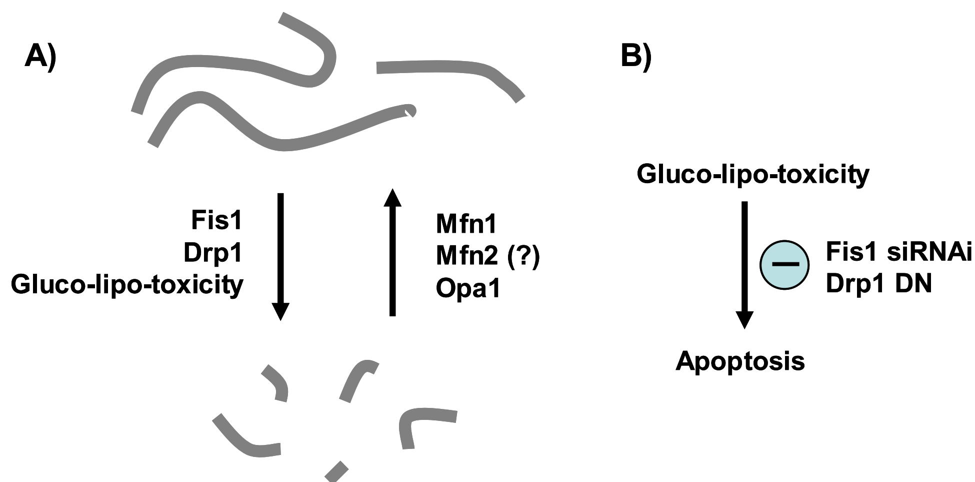


Figure 9. A) Scheme illustrating how mitochondrial morphology in β-cells is regulated by mitochondrial dynamics proteins and glucolipotoxicity. **B)** Inhibiting mitochondrial fission proteins by short interference RNA (siRNAi) or dominant negative (DN) prevents apoptosis.

4.5. Rodent islet mitochondrial function

Only a few studies have examined mitochondrial function of islets from rodent diabetes models. The MKR mouse is a mouse model in which expression of a dominant negative insulin growth factor 1 receptor (IGF1R) in skeletal muscle leads to systemic insulin resistance and diabetes (Asghar*,* 2006). In a study of this model, multiple β-cell mitochondrial abnormalities were described, e.g. anomalous morphology, depolarized

$\Delta\Psi_m$, impaired mitochondrial Ca^{++} uptake as well as reduced oxygen uptake rates, as judged by polarographic methods (Lu, 2010). Another well characterized diabetes animal model is the C57BL6/J mouse that develops hyperglycemia, hyperinsulinemia, and fatty liver when on high fat diet (Baribault, 2010). A study on high fat diet fed C57BL6/J mice found increased islet mitochondrial metabolism and mass (Fex, 2007). However, islet respiration was not tested (Fex, 2007). The study on MKR mice and C57BL6/J are somewhat opposing. Thus, it is still not clear to what extent or how mitochondrial dysfunction occurs in β-cell failure of diabetes animal models. The divergent results are likely, at least some degree, to be caused by strain variability.

To date no study on islets from diet induced diabetic animals have been directly examined for respiration. Adding to this literature paper III examines islets from high fat diet fed C57BL6/J mice that were hyperglycemic and obese. Interestingly, islet respiration was increased. The obvious question arises whether this should be viewed as a dysfunction or not. The observation that the respiratory difference is present only at low glucose but disappears at high glucose (paper III) may suggest a similar dysfunction to basal hypersecretion in diabetic islets (Porte, Jr. and Kahn, 2001). In theory, in a healthy β-cell at low glucose mitochondria would be expected to have relatively low activity as the need for ATP is low. Thus, an unnecessary high function may be viewed as dysfunction, however whether the cause is primarily mitochondrial or not is unclear. On the other hand, the HFD islets responded well to glucose challenge with increase in OCR which would imply that a great deal of the pre-mitochondrial as well as mitochondrial metabolism is intact. Further, the cause of increased respiration may be islet hyperplasia or increased mitochondrial mass. The first was excluded by comparing islet diameters (paper III). The latter still remains an open question.

Moreover, a large portion of the increase in respiration in high fat diet islets appeared to be contributed by uncoupled respiration. This may be a direct effect of free fatty acids in the diet as these may act as weak uncouplers (Rottenberg and Steiner-Mordoch, 1986). In fact we found that 48h palmitate incubation increased the level of uncoupled respiration under high glucose (paper III). Similar findings have previously been reported in rat islets that were cultured under similar conditions, although uncoupling was only measured by indirect methods (Carlsson, 1999). Interestingly, no major difference in mitochondrial mass was observed (Carlsson, 1999).

Further, the results in paper III may have a broader implication for mitochondrial studies. Many studies have examined OCR of various cell lines and tissues; in the diabetes field primarily skeletal muscle and β-cells. One conclusion from our study for future investigations is inevitably that when measuring respiration it should be done with and without inhibition of F1F0ATPsynthase in order to estimate coupled vs. uncoupled respiration. Otherwise, an increase in respiration may be mistakenly interpreted as potentiated mitochondrial function.

4.6. Human islet mitochondrial function

The lack of consistent data on mitochondrial function in diabetes rodent models stresses the importance of studying human islets. As described above, mitochondria are essential

for proper insulin secretion from rodent islets and β-cell lines. In humans a rare form of diabetes is caused by a specific mutation in mtDNA (A3243G mutation) (Maassen, 2002). Thus, mitochondria appear essential also for normal human islet function. To date there are no studies directly examining respiration of diabetic human islets (Mulder, 2009) although somewhat of the opposite was studied. It was shown that human islets with robust oxygen rates to a higher degree reverse diabetes when transplanted to nude diabetic mice (Papas, 2007b; Sweet, 2008a). In perhaps the only study thus far on mitochondria in human diabetic islets, a number of mitochondrial alterations were shown in addition to insulin secretion dysfunction (Anello, 2005). These included decreased $\Delta\Psi_m$ hyperpolarization in response to glucose challenge and increased expression of complex I and V (F1F0ATPsynthase) as well as increased expression of UCP2. The mitochondrial number appeared similar, however their volume was larger (Anello, 2005).

In paper III human islet respiration was plotted against body mass index (BMI) of donors. A trend towards decreased oxygen consumption rates with increased BMI was found. These results may be interpreted in several ways. The findings may suggest that obesity is accompanied by islet mitochondrial dysfunction. However there are a number of possible confounding factors. First, it may be that different handling of the islets at islet centers in combination with variations in shipping conditions affected the islets. Second, diverse donor medical histories may also have contributed to the islets phenotypes. Third, it may be that increased size of the islets from obese donors caused ischemia and cell death in the islet cores.

Furthermore, in comparison with mouse islets human islets showed less uncoupled respiration (37% vs. 60%); however the mechanism of glucose induced uncoupling was reproduced in them (paper III).The reason for this relatively large discrepancy in coupling efficiency between the two species is unclear. One potential mechanism may be architectural differences in alpha cell distribution, which in mouse islets are at the islet periphery but in the human islets are spread throughout the islet (Brissova, 2005). Alpha cells express more UCP2 than β-cells (Diao, 2008a) and their distribution may therefore affect the level of uncoupled respiration measured. Again, it may also be that different isolation and culture conditions affected the mouse and human islet phenotypes.

5. Mitochondria in brown adipose tissue

The majority of type 2 diabetes subjects are also obese. Multiple different factors are considered to be involved in the pathogenesis of obesity. These include among others inflammation and defect hormone signaling, but perhaps foremost increased energy intake and decreased energy expenditure. Thus, too much food and too little exercise. BAT has gained interest in metabolic disease due to its capacity to transfer energy from food into heat. In theory, if BAT activity or mass could be increased it could function as an overweight countermeasure.

BAT and β-cells share several characteristics. Both are highly dependent on mitochondria for their function. β-cells can not secrete insulin without functional mitochondria (Maechler, 2006) while BAT can not produce heat without the mitochondrial protein UCP1 (Matthias, 2000). Moreover both tissues have sympathetic innervation. In addition

recent studies indicate that BAT may similarly to β-cells be dysfunctional in obese individuals (Cypess, 2009; van Marken Lichtenbelt, 2009).

5.1. BAT function and significance

Although BAT is present in all mammals it has been studied mostly in rodents. These studies have identified BAT as the organ providing non-shivering thermogenesis (Cannon, 2004). This heat production is essential at critical periods in life such as birth and also enables animals to function in cold environments. Morphologically brown adipocytes differ from white in that they have numerous mitochondria and multiple lipid droplets (Cannon, 2004). Molecularly, the most prominent difference is that BAT expresses UCP1 which is activated in response to noradrenergic stimuli. Embryonically, at least part of the BAT pool is believed to stem from progenitor cells that are shared with myocytes but not with white adipocytes (Atit, 2006; Timmons, 2007). Another part of the pool however, may share ancestry with white adipocytes (Seale, 2008). White adipocytes have few mitochondria and commonly only one large lipid droplet. However, there appears to be some degree of plasticity in brown and white adipocyte phenotypes. White preadipocytes treated with PPAR gamma activation during differentiation develop brown adipocyte characteristics (Petrovic, 2010). Another study where the cannabinoid type 1 receptor was inhibited showed similar results (Perwitz, 2010).

Several older studies report on BAT in human adults, however the physiological relevance was not clear (Cramer, 1920; Wegener, 1951; English , 1973; Heaton, 1973; Huttunen , 1981; Hany , 2002). E.g., microscopic evidence of BAT (cells with multiple lipid droplets) was described (Heaton, 1973). Recent studies have sparked a renewed interest in BAT in adults (Nedergaard, 2007; Cypess, 2009; van Marken Lichtenbelt, 2009; Virtanen, 2009; Zingaretti, 2009). In brief, these studies utilized positron-emission-tomography (PET) data. When carefully examined spots of high activity (uptake of 18F-fluorodeoxyglucose) was discovered in anatomical regions where BAT is present in other species. Biopsies from these areas proved positive for the BAT marker UCP1 (Cypess, 2009;Virtanen , 2009;Zingaretti , 2009). In addition, 18F-fluorodeoxyglucose uptake increased when human subjects were exposed to cold, thus suggesting a physiological role (van Marken Lichtenbelt, 2009). Interestingly, obese individuals and glucose intolerant individuals appeared to have less BAT (Cypess, 2009).

5.2. UCP1 and BAT signaling

Mitochondria are at the core of BAT function and therefore the mitochondria are numerous. It is well established that the mitochondrial inner membrane protein UCP1 directly mediates the uncoupled respiration that makes BAT a thermogenic organ (Cannon, 2004). UCP1 is a 32kDa size member of the large mitochondrial carrier protein family. Although proteins similar in structure to UCP1 have been described (UCP2, UCP3, UCP4), UCP1 is the only true thermogenic protein thus far (Bouillaud, 2009).

Free fatty acids (FFA) are central to BAT function. UCP1 is directly activated by FFA or its derivatives (Cannon, 2004). In addition FFA serves as the main mitochondrial substrate for UCP1 mediated uncoupled respiration. Fatty acids are derived both from intracellular lipolysis as well as from the circulation. In brief, noradrenergic stimulation

of β-adrenergic receptors stimulates an increase in cyclic AMP which triggers increased protein kinase A activation. This causes increased activity of hormone-sensitive lipase which triggers lipolysis and a rise in intracellular FFA. FFA then directly activates UCP1 and enters β-oxidation as acyl-CoA. As UCP1 is activated, proton conductance over the inner mitochondrial membrane is increased which causes a drastic increase in respiration and production of heat.

BA activity is best assessed by measuring the increase in oxygen consumption in response to stimuli. In intact cells BA mitochondria uncouple both in response to adrenergic stimulation and exogenous FFA (Cannon, 2004). However these have not been used together in the literature. *In vivo* these stimuli would be expected to coexist as FFA's are present in plasma. Furthermore, adrenergic response includes lipolysis in white adipose tissues (Bizzi, 1968) which would further increase the FFA levels that the brown adipose tissue is exposed to. In addition, BA may be found among white adipocytes (Cousin, 1992) and may in such locations be exposed to even higher concentrations of FFA. With this in mind it appears natural to stimulate BA with both NE and FFA. Captivatingly, when doing so in paper IV a *"synergistic effect"* was discovered; i.e. the response to the combination was greater than the sum of their individual responses (paper IV). This was true for both palmitate (saturated FFA) and oleate (mono-unsaturated FFA).

In BAT, β_3- and α_1-receptors are considered the most significant for noradrenergic signaling (Cannon, 2004; Kim, 2008). β_3-receptor signaling is considered the main pathway for thermogenesis. However, α_1-receptor signaling also stimulates significant intracellular events, perhaps foremost being Ca^{++} release from the endoplasmic reticulum (Cannon, 2004). To examine if one pathway dominates the synergistic effect of NE and FFA the β_3 agonist CL-316243 and the α_1 agonist cirazoline was used (paper IV). It was apparent that while CL-316243 showed a similar effect to NE cirazoline did not. To further test the involvement of α_1-receptor signaling, Ca^{++} was chelated however this did not dampen the synergistic effect (paper IV). This finding suggests that the synergistic effect is part of the β_3-receptor stimulated thermogenic signaling pathway.

In a broader physiological context the synergistic effect is certainly intriguing. It may be that plasma FFA's are necessary for maximal thermogenesis from BAT. From a technical perspective, *in vitro* studies that only stimulate BA with NE may therefore underestimate the maximal uncoupled respiration.

5.3. ROS in BAT

In the process of cell respiration, there are at least three stages that are associated with increased superoxide generation. These include increased substrate supply, decreased ADP concentration, and increased intracellular Ca^{2+} concentration (Fridlyand and Philipson, 2004). In stimulated BA substrate supply increase (lipolysis), ADP and AMP increase (Pettersson and Vallin, 1976) and Ca^{2+} increase (Zhang, 2002). In addition, an increase in mitochondrial oxygen consumption is followed by an increase in rate of ROS generation (Barja de, 1992). In addition, it was shown that pharmacological uncoupling can accelerate ROS production (Zamzami, 1995). Thus, the large increase in respiration

in thermogenically active BAT may include a concurrent increase in ROS levels. Indeed, ROS was shown to be produced by BAT mitochondria (Sekhar, 1987) and possible to be inhibited with antioxidants (Rauchova, 2006). However the change in ROS levels in intact BA upon noradrenergic stimuli is unknown. Interestingly though, the rate of hydrogen peroxide generation found in BAT mitochondria was increased in male Wistar rats after cold acclimation (Sekhar, 1987). In that work, *in vitro* hydrogen peroxide generation/mg of mitochondrial protein was tripled after acclimation. Moreover, ROS may affect mitochondrial structure (Liot, 2009; Tian, 2009) and function (Addabbo, 2009).

With the above observations in mind the consequence of scavenging ROS is examined in paper IV. With TBAP, an antioxidant with superoxide-dismutase and catalase activity, the fraction of depolarizing cells decreased and the increase in oxygen consumption due to NE and palmitate was dampened by ~50%. Yet, no increase in ROS was measured with NE and palmitate stimulation. In fact ROS decreased slightly (paper IV). The reasons for this contradiction remain unclear. Further, a recent study on methamphetamine induced mitochondrial fragmentation in neurons show that Drp1 oligomerization and translocation to mitochondria is ROS dependent (Bernardi, 2006; Tian, 2009). Thus, it is possible that ROS may affect BA function through its effect on mitochondrial fission.

5.4. UCP1 content

It is becoming well characterized what pathways trigger transcription and translation of UCP1. The transcription factor PRDM16 appears central as it stimulates a broad program of brown fat determination including expression of the transcription factors PGC-1α/β (Wolf, 2009). However, although UCP1 is considered essential for BAT thermogenesis few studies have examined the correlation between UCP1 content and BAT function. This is not trivial since one therapeutic approach to increase BAT activity may be to increase UCP1 content. In a recent study Feldman and colleagues correlated UCP1 expression in animals on high-fat-diet (which increased UCP1 expression) with whole-animal thermogenesis (Feldmann, 2009). There was an increase in UCP1 expression of ~200-300% with high fat diet. In contrast, the increase in whole animal oxygen consumption in response to norepinephrine injection was only ~20% higher in the high fat diet animals. Thus, there appeared to be somewhat of a disconnection between UCP1 content and BAT function. In paper IV it is shown that in cultured mouse brown adipocytes there is no correlation between $\Delta\Psi_m$ depolarization and UCP1 content. Cells were first imaged live and the $\Delta\Psi_m$ depolarization in response to norepinephrine and FFA was captured with fluorescent dyes. This was followed by immunostaining for UCP1 and imaging. Image analysis showed no correlation between degree of $\Delta\Psi_m$ depolarization (in mV) and UCP1 content, as calculated per cell. Thus, cells that underwent major depolarization and thus would be expected to have massive uncoupling did not have higher UCP1 content (paper IV).

These findings suggest that although UCP1 is essential for BAT thermogenesis (Matthias, 2000) it may play more of a permissive rather than rate-limiting role. The molecular

nature of rate limiting steps is unclear, but FFA could be involved as these induced a synergistic increase in respiration when added together with NE (paper IV).

5.5. Mitochondrial membrane potential

When the respiratory chain is uncoupled by UCP1 the proton gradient across the inner mitochondrial membrane is dissipated; $\Delta\Psi_m$ depolarization. By using cationic dyes and the Nernst equation that describes their partitioning over membranes, changes in $\Delta\Psi_m$ may be quantified. A drop in $\Delta\Psi_m$ of 20mV was theoretically considered needed for the transition from coupled to uncoupled respiration in isolated BAT mitochondria (Nicholls and Bernson, 1977; Locke, 1982). Experimentally the extent of NE induced $\Delta\Psi_m$ depolarization was estimated to be 15mV (Rafael and Nicholls, 1984). Paper IV adds to this literature. The average $\Delta\Psi_m$ depolarization due to NE only and NE and palmitate together was estimated to be 3.6mV and 32.2mV respectively (paper IV). This discrepancy is most certainly reflecting that a larger fraction of cells depolarize with NE and palmitate, i.e. not all cells are recruited just by NE alone. Accordingly, the incongruity to the literature may also rest on a similar ground; however technical differences such as different animals and fluorescent probes used as well as freshly isolated vs. cultured cells may also contribute. Moreover, it is true that $\Delta\Psi_m$ is a static measure that reveals less about mitochondrial function than oxygen consumption. However, it is far easier to measure. In paper IV changes in $\Delta\Psi_m$ most often correlate with oxygen consumption. Thus, $\Delta\Psi_m$ may be a measure suitable for high-through-put screening.

Furthermore, in paper IV a previously unknown feature of $\Delta\Psi_m$ depolarization in BA is described. When studying the depolarization over time it appeared as a migrating wave passing through the cell within minutes (paper IV). It has been suggested that mitochondria may act as "power cables" connecting distant parts of the cell bioenergetically (Skulachev, 2001). To test if this could be true in the case of the depolarization wave cells with fragmented mitochondria due to Mfn2 KO were examined. Nonetheless there were still depolarization waves in the Mfn2 KO cells. This suggests that a cytosolic signal and not mitochondrial is mediating the wave propagation. In addition, as mitochondria are not connected throughout the cell and fusion events only occurs ~3 times/h/mitochondrion (Twig, 2008a), mitochondrial dynamics is probably too slow to mediate the wave.

Several years ago it was shown that cytosolic Ca^{++} spikes in myotubes exposed to apoptotic agents initiate depolarization of mitochondria in discrete subcellular regions (Pacher and Hajnoczky, 2001). These mitochondria then initiate slow waves of $\Delta\Psi_m$ depolarization and Ca^{++} release from the sarcoplasmic reticulum propagating through the cell. Mitochondrial Ca^{++} uptake appeared critical for the wave propagation (Pacher, 2001). With this in mind the Ca^{++} chelator BAPTA-AM was used to indirectly test if Ca^{++} could be involved (paper IV). However, despite Ca^{++} chelation $\Delta\Psi_m$ depolarization waves were still found. The large differences in wave propagation rates, ~45µm/min (Pacher, 2001) vs. 11.7µm/min in paper IV further suggests that a Ca^{++} wave is not present; however Ca^{++} imaging would be necessary to conclusively exclude this.

In cardiomyocytes photo-toxicity induced ROS was shown to trigger waves of mitochondrial depolarization and repolarization passing through the cell (Brady, 2004). This was likely due ROS induced opening of the PTP and subsequent ROS-induced-ROS-release. Interestingly the propagation rate of the depolarization wave in the cardiomyocytes was 5μm/min, in the same order as in the BA. Due to this background we examined the effect of the antioxidant TBAP on the wave. Fewer cells depolarized, however cells that did depolarize still did this in a wave like fasion.

The PTP is a high conductance channel whose opening leads to an increase of mitochondrial inner membrane permeability (Bernardi, 2006). Opening of PTP is commonly associated with cell death. The primary consequence of PTP opening is $\Delta\Psi_m$ depolarization, however it may also lead to morphological changes. To examine if PTP play a role in BA depolarization we used the PTP inhibitor cyclosporin A however it had no effect.

5.6. Mitochondrial morphology in BA

Mitochondrial structure is important as mitochondrial function depends on it (Detmer, 2007b). The main studies on BAT mitochondrial structure have been performed on rodent tissue samples examined by electron microscopy decades ago (Desautels, 1980; Suter, 1969; Vallin, 1970). They were performed before the era of detailed live cell imaging; i.e. without the access to fluorescent probes and confocal microscopy. Thus, the literature completely lacks a characterization of mitochondria in intact live BA.

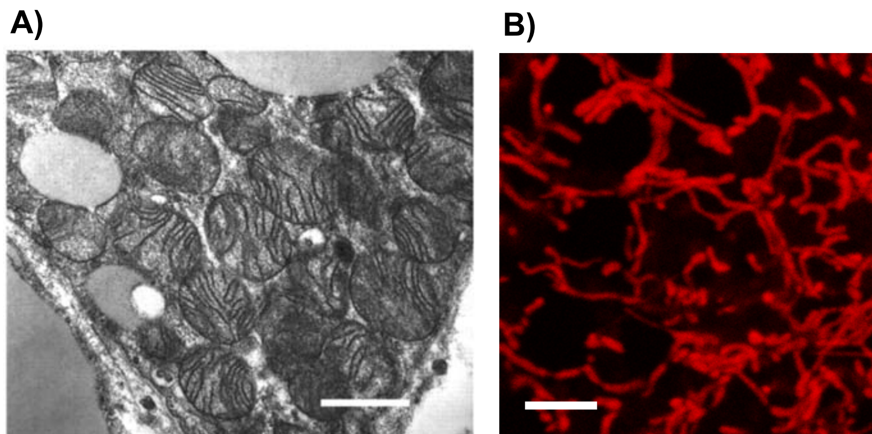


Fig 10. A. Mitochondrial morphology in brown adipocyte; electron microscopy. White bar 1μm. *Adapted from (Rodriguez-Cuenca, 2002)* **B.** Mitochondrial morphology in brown adipocyte; confocal microscopy. Mitochondria stained with fluorescent dye TMRE (unpublished observation). White bar 5μm. Electron microscopy images are highly magnified and made from thin tissue samples. This gives an impression that mitochondria are spherical.

Normal GFP exists as a mix of neutral phenols and anionic phenolates, which produces a major absorbance peak at 397nm and a minor at 475nm respectively (Brejc, 1997). Upon intense illumination of the protein with ultraviolet or 400nm light, the chromophore population shifts predominantly to the anionic form (green emission). This produces an increase in fluorescence of about threefold upon excitation at 488 nm (Yokoe and Meyer, 1996). Photo-activatable GFP (PAGFP) differ from normal GFP in only one amino acid

(Patterson and Lippincott-Schwartz, 2002). This makes the absorbance peak at 475nm initially lower. When illuminated with 400nM light a greater increase in absorbance at the 475nm peak is achieved and thus a more noticeable optical contrast under standard 488-nm excitation (Patterson, 2002). By tagging proteins with PAGFP the precise location, structure and dynamic life of proteins or organelles may be appreciated (Patterson and Lippincott-Schwartz, 2004). In addition to PAGFP there are other photo-convertible proteins, e.g. kaede (Yampolsky, 2008).

By using 2photon-laser excitation of the PAGFP, the laser can be aimed accurately and laser toxicity minimized. The use of PAGFP on mitochondria were previously developed for whole cell assessment of mitochondrial fusion (Karbowski, 2004) and for individual mitochondria (Twig, 2006) by labeling PAGFP with a mitochondrial matrix targeting signal. An alternative approach to PAGFP is fluorescence recovery after photo-bleaching (FRAP) which have been used in many studies (Collins, 2002; Mitra and Lippincott-Schwartz, 2010). However, as the term implies, photobleaching is toxic as it delivers enough photons to bleach fluorophores. By utilizing the techniques of photo-bleaching and PAGFP activation in single mitochondria, it is shown in paper IV that BAT mitochondria are filamentous and form intricate networks (Figure 7). This is in contrast with previous electron microscopy studies where mitochondria appear shorter (Desautels, 1980; Suter, 1969; Vallin, 1970). This discrepancy may partly be caused by the difference in imaging techniques. However a more recent study using tomography did not show filamentous mitochondria, although the study was focused on cristae structure (Perkins, 1998). Furthermore, in other cell types highly dependent on mitochondrial metabolism, such as pancreatic β-cells (paper I and II) and neurons (Knott, 2008), mitochondrial structural alterations have been described in diseased cells. It would certainly be interesting to examine mitochondrial morphology of dysfunctional BAT, e.g. from obese subjects that was reported to have reduced BAT mass (Cypess, 2009).

Mitochondrial fragmentation is typically associated with dysfunction. E.g. in cell death, mitochondria fragment alongside with cytochrome C release and caspase activation (Arnoult, 2007). Under other circumstances mitochondria may become more connected, e.g. during G(1)-S phase in cell division (Perkins, 1998; Mitra, 2009). Inhibition of mitochondrial fusion proteins in HeLa cells results in fragmentation as well as decreased oxygen consumption rates (Chen, 2005). Moreover, mitochondrial fragmentation has been shown in a number of common diseases, e.g. neurodegenerative (Knott, 2008). Moreover, mitochondrial depolarization is usually associated with fragmentation which is the case in cell death but also with pharmacological uncoupling agents such as DNP or FCCP (Kim, 2008). These agents cause both increase in respiration as well as mitochondrial fragmentation and depolarization (Kim, 2008). With these results in mind mitochondrial morphology in stimulated BA was examined in paper IV. Fascinatingly, mitochondria underwent a drastic transformation in morphology alongside with increase in respiration. In BA mitochondrial fragmentation appears associated with increased mitochondrial function, as oxygen consumption rise drastically upon their activation.

Fragmentation may be a slightly inaccurate term since the mitochondria decreased in length but at the same time some of them increased their diameter; i.e. they became

spherical. In fact, older electron microscopy studies report on related findings. E.g., it was shown that mitochondria isolated from cold-acclimated rats are enlarged (Desautels, 1980). This process took 7-14 days and was reversible within a week. Interestingly there was also a transient increase in mitochondrial size during the first 12h of cold-acclimation (Desautels, 1980). A similar finding was reported in another study where BAT was sampled after NE injection to rats and examined by electron microscopy (Vallin, 1970). At 30min after NE injection there was a peak in mitochondrial swelling that had regressed completely at 40min. Further, in comparison to the physiological stimuli NE and palmitate, the pharmacological uncoupler FCCP induced a quite different morphology; mitochondrial rings (paper IV). This disparity suggests that something else besides depolarization is contributing to the spherical morphology. Further, it is compelling to argue that the change in morphology may also involve the ultrastructure. It has been shown that cristae structure can change between state III (excess ADP) and IV (limited ADP) respiration (Hackenbrock, 1966). In addition mitochondrial fusion and fission processes may affect the cristae (Mannella, 2006b).

5.7. Mitochondrial dynamics in brown adipocytes

As described above, mitochondrial fusion is dependent on a set of fusion proteins and in absence of these mitochondria fragment. Fragmented mitochondria have previously been shown to exhibit decreases in mitochondrial fusion rates (Karbowski, 2004; Molina, 2009). In paper IV it is shown that this is also the case in BA. The fragmented mitochondria in NE and palmitate stimulated cells retained their PAGFP to a much higher degree than unstimulated cells. However, also cells only stimulated with NE showed decreased fusion rates. The majority of these cells maintained their filamentous mitochondrial morphology. Thus, the decrease in fusion rates appeared to be independent of morphology. To the authors best knowledge there are no similar findings in the literature.

The drastic change in morphology and dynamics upon BA stimulation led to the hypothesis that mitochondrial shape is of importance for BA function. To test for this two proteins central for fusion and fission, Mfn2 and Drp1 (Detmer, 2007b), were examined. Mfn2 knock-out (KO) cells exhibited fragmented mitochondria as expected from previous studies (Chen, 2003; Chen, 2005). Interestingly, Mfn2 KO cells showed a normal fraction of depolarizing cells in response to NE and palmitate. Similarly, it was found that mouse embryonic fibroblasts deficient of Mfn2 have fragmented mitochondria but lack any major dysfunction (Chen, 2005). This suggests that the fragmented mitochondria in BA do not have any loss of function.

To further examine the role of fragmentation the pro-fission protein Drp1 was inhibited by expression of Drp1 DN. In contrast to the Mfn2 KO cells as well as control cells, Drp1 DN cells showed higher frequency of tubular mitochondria and some of the mitochondria had “lolly-pop” appearance. Grippingly, in contrast to Mfn2 KO the Drp DN cells exhibited functional deficits. Both the fraction of cells depolarizing and respiration in response to NE and palmitate was decreased. These findings have support in the literature. In HeLa cells it was shown that depletion of Drp1 with shRNA, leads to a loss of mitochondrial DNA, decrease in respiration, increase in ROS and a drop in ATP levels

(Parone, 2008). Taken together, the findings in paper IV suggest that mitochondrial morphology in general and mitochondrial fission in particular may play an important physiological role in BA (Figure 11).

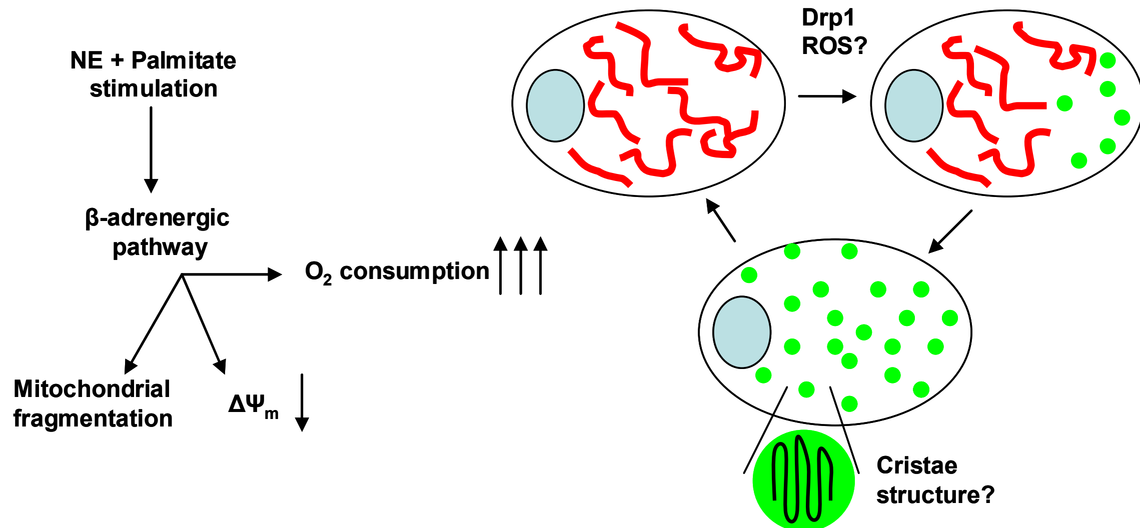


Figure 11. Mitochondrial morphology in brown adipocytes. Mitochondria are highly networked and their structure dependent on fusion and fission proteins. In response to stimulation with norepinephrine (NE) and free fatty acid such as palmitate there is a synergistic increase in oxygen consumption rates and mitochondrial membrane potential depolarization ($\Delta\Psi_m$). The synergistic effect goes through the β-adrenergic pathway. In addition, mitochondria fragment upon stimulation and mitochondrial fusion activity seizes. $\Delta\Psi_m$ depolarization and fragmentation progress as a wave through the cell. The mitochondrial fission protein Drp1 is necessary for the fragmentation and its inhibition dampens response to stimuli. Further, ROS may be involved as inhibition of reactive oxygen species (ROS) with antioxidant dampens oxygen consumption, $\Delta\Psi_m$ as well as fragmentation. Changes in cristae structure was not examined but is likely to occur.

6. Conclusions and summary of thesis

6.1. Thesis summary

There is little doubt that one of the main medical challenges of the 21st century is the pandemic of diabetes and obesity. These are complex metabolic diseases that are caused by a wide range of factors including environmental and genetical. Thus far the therapeutic options are limited and a greater understanding of the pathophysiology is needed in order to develop new approaches. Naturally, as the mitochondrion stands in the center of metabolism this organelle has been examined extensively. In this thesis, focus is on two metabolically important cell types where mitochondria are essential; the insulin secreting β-cell and the energy consuming brown adipocyte. Novel data is presented on the diverse nature of this organelle, its efficiency and the interrelationship between its structure and function.

Paper I characterizes mitochondrial heterogeneity in mouse pancreatic β-cell. It is shown that mitochondria display a wide heterogeneity in $\Delta\Psi_m$ and a millivolt range that is considerably larger than the change in millivolts induced by fuel challenge. Increasing glucose concentration recruits mitochondria into higher levels of homogeneity, while

glucolipotoxicity (an in vitro diabetes model) results in increased $\Delta\Psi_m$ heterogeneity. Exploration of the mechanism behind heterogeneity revealed that temporary changes in $\Delta\Psi_m$ of individual mitochondria, ATP-hydrolyzing mitochondria, and uncoupling protein 2 are not significant contributors to $\Delta\Psi_m$ heterogeneity. BAD, a proapoptotic BCL-2 family member previously implicated in mitochondrial recruitment of glucokinase, was identified as a significant factor influencing the level of heterogeneity.

Paper II examines the regulation of mitochondrial structure in the β-cell and its role in apoptotic resistance. It is shown that β-cell mitochondria are constantly involved in fusion and fission activity that underlies the overall morphology of the organelle. Networking activity among mitochondria is capable of distributing a localized green fluorescent protein signal throughout an isolated β-cell, a β-cell within an islet, and an INS1 cell. Under glucolipotoxic conditions, β-cell mitochondria become fragmented and lose their ability to undergo fusion. Interestingly, manipulations that shift the dynamic balance to favor fusion are able to prevent mitochondrial fragmentation, maintain mitochondrial dynamics, and prevent apoptosis induced by glucolipotoxicity.

Paper III describes the respiratory function of intact pancreatic islets. To enable this study a high-throughput islet respirometry method was developed. Unexpectedly, we found that islets from diabetic HFD fed animals exhibit higher levels of respiration as compared to animals fed chow. Part of the increase in respiration appears to be due to increase in uncoupled respiration. Examining it's regulation we found that fuels that stimulate insulin secretion also increase uncoupled respiration, and that this may be mediated by ROS. Moreover dissecting the molecular mechanism, we show that the ANT contributes to one-third of the uncoupled respiration while UCP2 and PTP appear not to contribute. Finally, we examined a cohort of human islets and found lower levels of uncoupled respiration, however as in the mouse islets glucose challenge induced increase in uncoupled respiration. Interestingly, there was no difference in respiration between healthy and diabetic human islets.

Paper IV is a novel study on mitochondrial morphology and function in brown adipocytes. Mitochondrial morphology was found to be highly networked and dependent on mitochondrial dynamics proteins. When stimulating cells with a combination of norepinephrine and free fatty acids a synergistic response was found. This included a marked increase in oxygen consumption rates and somewhat unexpectedly also a massive $\Delta\Psi_m$ depolarization and mitochondrial fragmentation. The fragmented mitochondria appeared sphere-like and had dampened fusion; however cells regained normal function as well as mitochondrial morphology and $\Delta\Psi_m$ within 24h. Interestingly, $\Delta\Psi_m$ depolarized and mitochondria fragmented in a wave-like fashion where depolarization preceded fragmentation. Furthermore, inhibition of the pro-fission protein Drp1 was found to inhibit the synergistic response, while knock-out of the pro-fusion protein Mfn2 did not. Finally, we found the synergistic response to go through the β-adrenergic pathway and be dependent on ROS but not on Ca^{++}, PTP or UCP1 expression levels.

6.2. Conclusions and future perspectives

In conclusion, mitochondrial form and function are interrelated both in β-cells and brown adipocytes. This knowledge is of importance as mitochondria are essential for proper function of these tissues. Mitochondrial dynamics proteins in β-cells and brown adipocytes may represent a future therapeutic target in both in diabetes and obesity. A challenge will be to selectively target these tissues as mitochondrial structural and functional requirements may vary between cell types. More research is required to expand the understanding of mitochondrial dynamics in these cells.

Proper mitochondrial function appears essential for normal β-cell function. It is unclear however whether and how mitochondrial dysfunction occur in diabetic beta cells. Further studies on human islets are definitely needed. Since human islets are scarce this will require large scale international collaborations. In addition, animal models with inducible alterations of mitochondrial function would be a great asset. E.g., a model where overexpression of the mitochondrial pro fusion protein Mfn2 could be induced at a point in time of choice during development of diabetes would certainly be useful. Furthermore, a drug inhibiting the action of the pro fission protein Drp1 was recently developed; Mdv-1 (Cassidy-Stone, 2008). Examining if Mdv-1 has an anti-diabetic effect in diabetic animal models would definitely be worthwhile. Moreover, perhaps one of the largest drawbacks of the islet literature is that almost all studies are performed ex vivo. Examining islet function in vivo with imaging will certainly lead to a greater understanding of islet function and dysfunction. Some rodent studies have already done this by using MRI for islets transplanted to the kidney capsule (Medarova, 2006) and confocal microscopy for islets implanted in the anterior chamber of the eye (Speier, 2008). It would be a milestone in the diabetes field if islet functional imaging could be performed on islets in human pancreata in vivo.

Likewise in BAT, in vivo manipulations of mitochondrial dynamics proteins would be an appealing avenue to pursue. The main question would be if by manipulating these proteins BAT activity could be boosted. Further, it would certainly be of interest to examine mitochondrial function in diseased BAT. Finally, on a larger scale, by using the high throughput respirometry assay described in paper III and IV, it may now be possibly to screen for compounds that increase BAT activity.

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