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Evaluation of Mitochondrial Functions and Dysfunctions in Muscle Biopsy Samples

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1. Introduction

Within the past decade, the list of publications involving mitochondrial dysfunction in the etiology of metabolic disorders in obesity, insulin resistance and type 2 diabetes, has been growing steadily. Today, large controversies still exist and it is clear that the understanding of the causes and consequences of impairments in mitochondrial functioning are far from being accomplished. In this context, our purpose is to review techniques used in human samples to highlight defects in mitochondrial activity, with a particular focus on skeletal muscle. Several tools are described to assess a large array of mitochondrial functions. In order to facilitate the reading of the review, several details related to methodological concerns are provided in supplementary materials and remain available upon request to the authors.

2. Basic knowledge on mitochondrial functioning

Mitochondria are found in nearly all eukaryotes. They are forming a complex network which shape and functioning is determined by the interaction with the cytoskeleton and by the balance between fusion and fission reactions (Zorzano et al., 2009). They vary in number and location according to cell type. Within skeletal muscle fibers, mitochondrial density and activity mainly varies according to fiber types and physical training (Howald et al., 1985) although type 2 diabetes and genetic inheritance have been also proposed as potential modulators (Petersen et al., 2004; Morio et al., 2005; Befroy et al., 2007). Mitochondria are located either around the nuclei, this subgroup is called subsarcolemmal mitochondria, or nestled between myofibrils. These mitochondria are named intermyofibrillar and have distinct activity from subsarcolemmal ones (Koves et al., 2005; Mollica et al., 2006). Intermyofibrillar mitochondria are mainly dedicated to energy production for muscle fiber contraction. By contrast, subsarcolemmal mitochondria may play a key role in signal transduction and substrate transport. Deficiency in the latter pool has been proposed to contribute to the pathogenesis of muscle insulin resistance in type 2 diabetes (Ritov et al., 2005; Benton et al., 2008).

The most prominent role of mitochondria is to produce the "energy carrier" adenosine triphosphate (ATP) mainly from glucose and fatty acid oxidation, and to a lesser extend from amino acid oxidation. Nutrient oxidation within the cytosol and/or the mitochondrial matrix results in acetyl-CoA production, the main substrate of the tricarboxylic acid (TCA) cycle which takes place in the mitochondrial matrix. The β-oxidation pathway is specific to fatty acid oxidation and results in acetyl-CoA production which feeds TCA cycle. These oxidative pathways are coupled to the mitochondrial electron transport chain (ETC), whose enzyme content and activity together define the mitochondrial oxidative capacity. The latter factor combined with mitochondrial density determines the tissue oxidative capacity. The main rate-limiting oxidative enzymes are citrate synthase and isocitrate dehydrogenase (TCA cycle), β-hydroxyacyl-CoA dehydrogenase (β-oxidation) and cytochrome c oxidase (ETC). Mitochondria are also key organelles for regulation of cell metabolism. Mitochondrial functioning has been proven to control cellular metabolism, redox and calcium signalling, apoptosis-programmed cell death and cellular proliferation. This shows that a perpetual cross talk exists between cell and mitochondria, the result being the cell adaptation to physiological changes or the cell death (Figure 1).

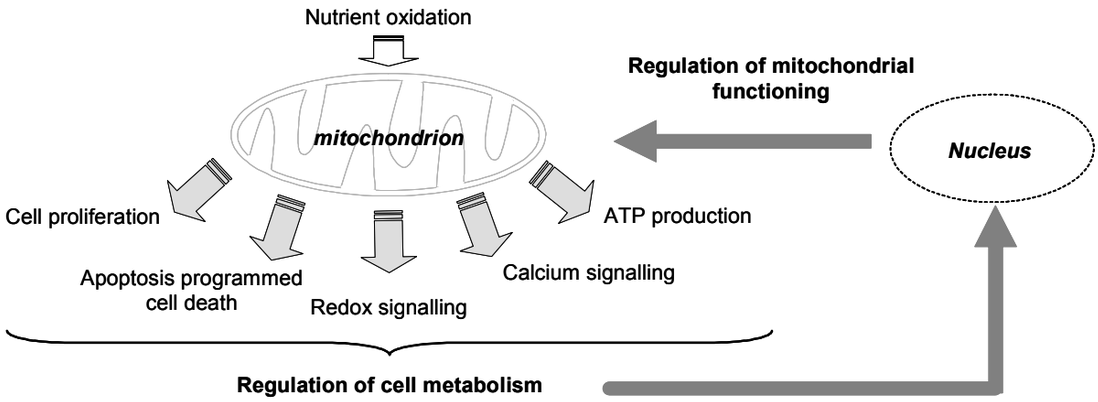

Fig. 1. Involvement of the cross-talk between nucleus and mitochondria in the regulation of cell adaptations.

2.1 Mitochondrial respiratory chain, membrane potential and energy production, and main alterations observed in metabolic disorders

Nutrient oxidation consists in a series of enzyme-catalysed oxidative reactions which aims at transferring energy-rich electrons to the cofactors nicotinamide adenine dinucleotide [NAD+] and flavin mononucleotide [FAD], forming respectively NADH, H^+ and $FADH_2$. The latter reduced carriers provide thereafter the ETC with these energy-rich electrons. The ETC is situated into the mitochondrial inner membrane and consists of four large enzyme complexes (Complex I, NADH-CoQ oxidoreductase; II, succinate dehydrogenase; III, CoQ-cytochrome c oxidoreductase; and IV, cytochrome c oxidase). Mobile electron carriers (e.g. coenzyme Q or ubiquinone, and cytochrome c) transport the electrons from one complex to the next with oxygen acting as the final electron acceptor within cytochrome c oxidase. The transfer of the high-energy electrons along the electron transport chain results in the pumping of H^+ ions across the inner membrane, creating an electrochemical gradient (also named membrane potential, $\Delta\psi m$) that provides the energy required to drive the synthesis

of ATP thanks to a fifth complex called F_0F_1 ATP synthase. The latter consists of a H^+ ion channel (F_0) connected to a catalytic subunit (F_1). The energy provided by the flux of H^+ ions through F_0 is used to drive ATP synthesis from ADP and Pi by F_1 (Figure 2). The electrochemical gradient determines the coupling between the oxidative and the phosphorylative reactions. It is named oxidative phosphorylation (OXPHOS).

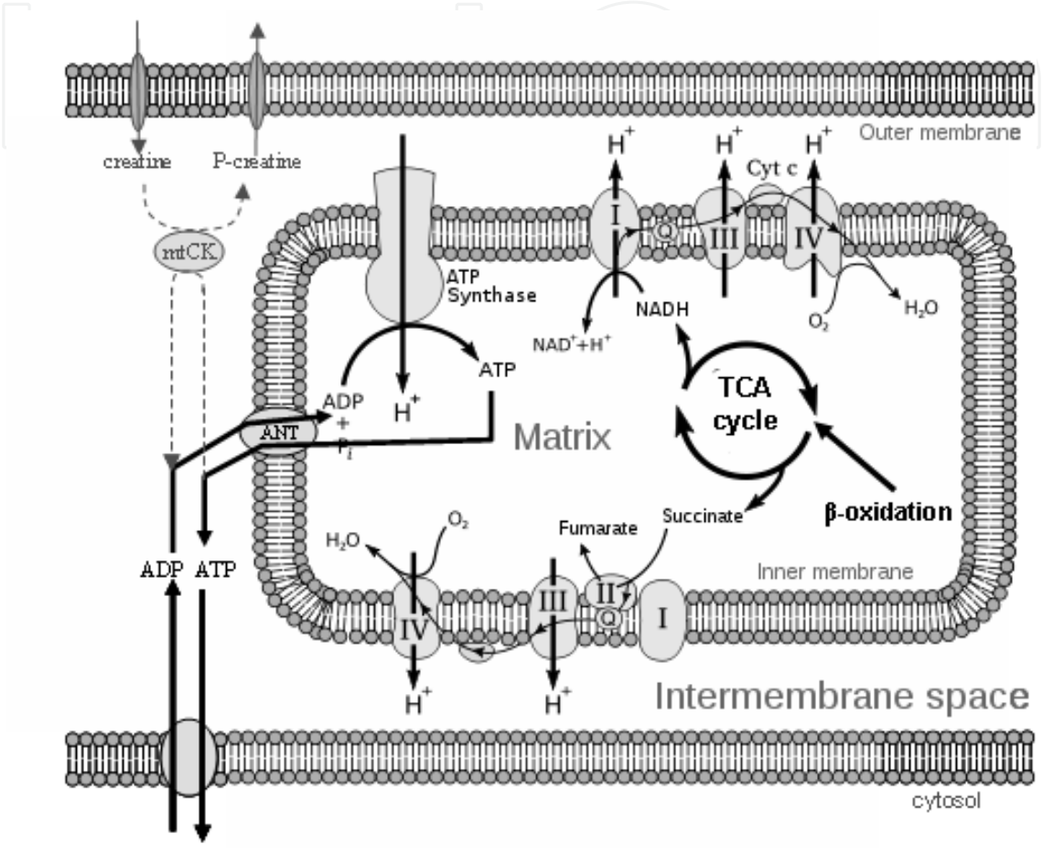

Fig. 2. Main organization of mitochondrial oxidative and phosphorylative pathways. TCA cycle, tricarboxylic cycle; Complex I, NADH-CoQ oxidoreductase; Complex II, succinate dehydrogenase; Complex III, CoQ-cytochrome c oxidoreductase; Complex IV, cytochrome c oxidase; Q, coenzyme Q or ubiquinone; Cyt c, cytochrome c; ANT, adenine nucleotide translocase, ANT; mtCK, creatine kinase. *This figure is derived from Wikipedia.*

Mitochondrial membrane potential is a key indicator of cellular viability. As demonstrated by Peter Mitchell (1961), it is the driving force behind ATP production. It also governs ROS production, mitochondrial calcium storage, opening of mitochondrial permeability transition pores (mPTP) and mitochondrial-apoptotic programmed cell death (reviews in Casteilla et al., 2001; Hand & Menze, 2008). Mitochondrial ATP production has to strictly match cellular needs. It is tightly controlled by different factors. The ATP/ADP-Pi ratio, through a mitochondrial ADP sensing which is still not fully understood (Wilson et al., 1977), the NAD+/NADH-H+ ratio and local oxygen pressure chance. The transfers of ATP, ADP and Pi between the cytosol and the mitochondrial matrix involve ATP/ADP carriers

(adenine nucleotide translocase, ANT). The high-energy phosphate group of ATP may be also transferred to creatine to yield phosphocreatine. This reaction is catalyzed by the mitochondrial isoform of creatine kinase (mtCK), which is located in the mitochondrial intermembrane space. The dynamic of the phosphate group transfer between ATP and creatine and the transport of these metabolites between cytosol and mitochondrial intermembrane space, differs between contractile fiber types. This is due to differential coupling between mtCK, ANT and porin (voltage-dependent anion channel, VDAC) which determines the flux of ADP entrance within the mitochondrial matrix (Saks et al., 1994). Unlike glycolytic fibers (type IIb) for which mitochondrial respiration is regulated by cytosolic ADP, mitochondrial respiration of oxidative fibers (type I) is more controlled by the ratio between creatine and phosphocreatine than by ADP (Zoll et al., 2003). The oxydo-glycolytic fibers (type IIa) present an intermediate situation.

Except in Asian Indians, consistent decrease in muscle mitochondrial ATP production has been reported in insulin resistance in obesity and type 2 diabetes (Petersen et al., 2004, 2005; Szendroedi et al., 2007; Chanseaume et al., 2010). By contrast, the theory linking insulin resistance to decreased mitochondrial oxidative capacity is more challenged. It has been supported by several cross-sectional studies in obese insulin-resistant and type 2 diabetic patients compared to healthy individuals (Howald et al., 1985; Kelley et al., 2002; Pertersen et al., 2004; Ritov et al., 2005, 2010). These observational studies are reviewed in table 1. By contrast, recent studies (Petersen et al., 2005; Chanseaume et al., 2010) and clinical trials that involve interventions (weight loss, physical activity) altering insulin sensitivity (Brons et al., 2008; Toledo et al., 2008) demonstrated a dissociation between insulin resistance and mitochondrial functioning in skeletal muscle. Part of the discrepancy may be due to bias induced by confounding factors, such as physical activity. Indeed when the latter factor is rigorously taken into account, mitochondrial oxidative capacity is similar between lean and obese-insulin resistant volunteers (Chanseaume et al., 2010). However, genetic and/or epigenetic predisposition have also to be taken into account (Petersen et al., 2004, 2005; Morino et al., 2005; Befroy et al., 2007; Nair et al., 2008).

2.2 Mitochondrial fatty acid oxidation and cellular meanings

Mitochondria are the principal site of long-chain fatty acid (LCFA) oxidation for cellular energy production. A defect in this process may trigger the accumulation of toxic lipid metabolites in skeletal muscle. Mitochondrial LCFA oxidation is tightly regulated by the enzyme carnitine palmitoyltransferase 1 (CPT1), which is situated in the outer mitochondrial membrane and regulates the entry of acyl-CoAs into mitochondria. Malonyl-CoA, the first metabolic intermediate of lipogenesis produced by the enzyme acetyl-CoA carboxylase, is the physiological allosteric inhibitor of CPT1 (McGarry & Brown, 1997). This malonyl-CoA/CPT1 partnership is considered as a “fuel sensor” whose role is to regulate the rate of LCFA oxidation according to the relative disposal of LCFA and glucose within the cell. When circulating lipids increase, whereas glucose availability decreases (such as during starvation), lipids are oxidized in mitochondria at the expense of glucose (Rasmussen & Wolfe, 1999). When mitochondrial LCFA oxidative capacity becomes limited, excess LCFAs taken up by the cell are initially redirected towards storage in the form of triacylglycerols (TAG). For these reasons, new concept has emerged pointing out muscle mitochondrial dysfunction as the leading cause for TAG accumulation in obesity and insulin resistance (Petersen & Shulman, 2006).

Measured parameters	Population studied	Results	References
Mitochondrial content	Insulin resistant with family predisposition	**?**	Morino et al., 2005
	Obese	**?**	Holloway et al., 2007
	Obese and insulin resistant*	**=**	Chanseaume et al., 2010
	Obese and type-2 diabetic	**?**	Ritov et al., 2005
	Type-2 diabetic	**?**	Boushel et al., 2007
	Type-2 diabetic	**=**	Nair et al., 2008
	Type-2 diabetic	**=**	Ashmann et al., 2006
Morphology	Obese and type-2 diabetic	? size, area	Kelley et al., 2002
	Type-2 diabetic	? size	Ritov et al., 2005
Biogenesis signalling pathway	Insulin resistant with family predisposition	? PGC-1a, complexes 1-3-4	Heilbronn et al., 2007
	Insulin resistant with family predisposition	= PGC-1a, OXPHOS	Brons et al., 2008
	Obese and insulin resistant*	= PGC-1a/ß, NRF1, OXPHOS	Chanseaume et al., 2010
	Type-2 diabetic and insulin resistant with predisposition	? PGC-1a/ß, NRF1, OXPHOS	Patti et al., 2003
	Type-2 diabetic	? PGC-1a/ß, OXPHOS	Mootha et al., 2003
	Type-2 diabetic	? PGC-1a	Debard et al., 2004
	Type-2 diabetic	= PGC-1a/ß, NRF1, OXPHOS	Nair et al., 2008
Substrates oxidation	Obese	? LCFA oxidation	Kim et al., 2000
	Insulin resistant with family predisposition	? acetyl-CoA oxidation	Befroy et al., 2007
Maximal enzyme activity	Insulin resistant	? Citrate synthase	Heilbronn et al., 2007
	Obese	? CPT1, ßHAD, citrate synthase	Kim et al., 2000
	Obese	? CPT1, ßHAD, COX	Holloway et al., 2007
	Obese and insulin resistant*	= Citrate synthase, COX	Chanseaume et al., 2010
	Obese and type-2 diabetic	? Complexe 2	Ritov et al., 2005
	Obese and type-2 diabetic	? Complexe 2	He et al., 2001
	Obese and type-2 diabetic	? Citrate synthase, complexe 1	Kelley et al., 2002
	Type-2 diabetic	? Citrate synthase	Boushel et al., 2007
	Type-2 diabetic	? Citrate synthase	Ortenblad et al., 2005
	Type-2 diabetic	= Citrate synthase	Nair et al., 2008
ATP synthesis	Insulin resistant with family predisposition	?	Petersen et al., 2004
	Insulin resistant with family predisposition	?	Petersen et al., 2005
	Obese and insulin resistant*	?	Chanseaume et al., 2010
	Obese and type-2 diabetic	?	Abdul-Ghani et al., 2009
	Type-2 diabetic	?	Szendroedi et al., 2007
	Type-2 diabetic	=	Nair et al., 2008
P-creatine synthesis	Insulin resistant with predisposition	=	Brons et al., 2008
	Type-2 diabetic	?	Schrauwen-Hinderling et al., 2007
ROS production	Obese and insulin resistant*	?	Chanseaume et al., 2010
	Obese and type-2 diabetic	? in obese, = in type-2 diabetic	Abdul-Ghani et al., 2009

Table 1. Alterations in mitochondrial structure, content and functioning reported in skeletal muscle of insulin resistant, obese and/or type-2 diabetic patients. * all volunteers were similarly sedentary

Cellular TAG buffering capacity in lean tissues such as skeletal muscle, is limited and rapidly flooded, especially for saturated LCFAs such as palmitate which are poorly incorporated into TAG (garcia-Martinez et al., 2005). In these conditions, excess LCFAs may enter alternative non-oxidative pathways that result in the production and accumulation of toxic lipid metabolites, such as diacylglycerols (DAG), acyl-CoAs or ceramides. These LCFA derivatives have been demonstrated to be deleterious for cell functioning since they are able to modulate activity of protein kinases (Shulman, 2000; Hegarty et al., 2003) which ultimately trigger insulin resistance (Shulman, 2000; Chavez et al., 2005), mitochondrial

dysfunction (Coll et al., 2006), oxidative stress (Montuschi et al., 2004) and apoptosis (Slawik & Vidal-Puig, 2006). Whereas mechanisms linking those LCFA metabolites to insulin resistance have raised large consensus, the involvement of mitochondrial LCFA oxidative capacity in the regulation of their synthesis rates is still controversial (Rimbert et al., 2004, 2009). These arguments suggest that more complex interactions exist between LCFA availability and cell metabolism, and determine the intracellular fates of LCFAs (for review Kewalramani et al., 2010).

2.3 Mitochondrial production of reactive oxygen species and cell consequences

During the electron transfer through the respiratory chain, a small percentage of electrons may prematurely reduce oxygen, forming reactive oxygen species (ROS) such as superoxide anion radicals ($O_2^{\bullet -}$). It was demonstrated that the main sites of ROS production are located at complexes I and III, the iron-sulphur centers of complex I being potentially the most important ROS generators (Barja, 1999). ROS are generated either from "normal" electron transfer after oxidation of substrates of complex I and complex II, but also from "reversed" electron transfer from complex II towards complex I (Liu et al., 2002). The latter situation is suggested to be the most physiologically relevant ROS production in mammals (Miwa & Brand, 2003). Recent work from Seifert et al. (2010) showed that LCFA β-oxidation is associated with enhanced ROS production, through a mechanism involving complex III, the electron transfer flavoprotein (ETF) and ETF-oxidoreductase.

As reviewed by Murphy (2009), the rate of ROS production is mainly determined by the membrane potential, the ratio between NADH and NAD+, the ratio between CoQH2 and CoQ and the local O_2 concentration. It is well established that there is a strong positive correlation between membrane potential and ROS production. Small increase in membrane potential has been associated to a large stimulation of ROS production (Korshunov et al., 1997). Similarly, a small decrease in membrane potential deeply reduces ROS production (Votyakova & Reynolds, 2001). Therefore mild uncoupling resulting from a small decrease in membrane potential is considered as a natural antioxidant process (Seifert et al., 2010; Skulachev, 1997). However, not all sites of ROS production are sensitive to membrane potential. Indeed, Seifert et al. (2010) found that the ROS produced during LCFA β-oxidation and involving complex III, ETF and ETF-oxidoreductase, was relatively insensitive to membrane potential changes. In addition, Miwa and Brand reported that the ROS produced at the cytosolic side of complex I through glycerol-3-phosphate dehydrogenase, which donates electrons to the electron carrier Q, is insensitive to membrane potential (2003). By contrast, ROS production from complex I following reverse electron flow is highly sensitive to membrane potential. Mild uncoupling is based on a proton leak across the inner membrane. It is mediated by thyroid hormones, LCFAs, complex IV slipping but most importantly by the activity of the uncoupling proteins UCP2 and UCP3, although molecular mechanism are still not well understood (for review see Murphy, 2009; Schrauwen & Hesselink, 2002; Bezaire et al., 2007). Whereas overexpression of UCP in skeletal muscle has been shown to prevent diet-induced obesity and insulin resistance in mice (Li et al., 2000), their specific involvement in mitochondrial functioning and muscle metabolism in obesity and type 2 diabetes is still under debate in humans (Krook et al., 1998; Bao et al., 1998; Samec et al., 1999).

Spontaneously or by the action of superoxide dismutase (SOD), $O_2^{\bullet-}$ dismutates into hydrogen peroxide H_2O_2, which is more stable and can diffuse through biological membranes. H_2O_2 has been shown to inhibit TCA cycle oxidative enzymes (alpha-ketoglutarate dehydrogenase, succinate dehydrogenase, aconitase) and complex II (Moser et al., 2009; Nulton-Persson & Szweda, 2001). It is an important signal molecule, regulating major redox signalling pathways (for review see Leloup et al., 2011). It can also react to form hydroxyl radical or peroxynitrite, which are both highly damaging. In that context, oxidative stress can occur due to the accumulation of oxidative damages within DNA, protein and lipid components of the organelle. This may contribute to the decline in mitochondrial function and lead in turn to enhanced ROS generation. This vicious circle has been involved in many pathologies and the aging process. It is however questioned in the aetiology of insulin resistance in obesity and type 2 diabetes because reduced mitochondrial ROS production has been reported in those latter situations (Abdul-Ghani et al., 2009; Chanseaume & Morio, 2009). Because mitochondrial dysfunction in type 2 diabetes has been related to oxidative stress (Bonnard et al., 2008), other cellular sites of ROS production are potentially involved.

2.4 Mitochondrial calcium storage and cellular consequences

Mitochondrial calcium uptake has been first described in the early 1960s (Deluca & Engstrom, 1961; Vasington & Murphy, 1962). Since then it has been recognized that mitochondria are able to store amount of calcium bound to phosphate within their matrix large (review in Nicholls & Chalmers, 2004). Differential regulation of mitochondrial Ca^{2+} uptake and release are presented by Hoppe (2010). Calcium taken up by the mitochondria regulates mitochondrial functioning in response to a variety of extracellular stimuli (Jouaville et al., 1999; Territo et al., 2000). It activates three dehydrogenases of the TCA cycle, pyruvate dehydrogenase, isocitrate dehydrogenase, and α-keto glutarate dehydrogenase (63). It may also regulate the ETC, the F_0F_1ATP synthase and ANT (McCormack et al., 1990). This induces an increased substrate uptake by mitochondria, enhanced mitochondrial NADH/NAD^+ ratio and increased ATP production. The high capacity to accumulate calcium confer to mitochondria a key role in the regulation of intracellular calcium signalling (Gunter KK & Gunter TE, 1994; Rizzuto et al., 1998), which includes regulation of gene expression (including those involved in mitochondrial biogenesis and glucose uptake), cell functioning, control of protein trafficking between compartments, and processes linked to the suffering and eventual demise of cells (for review see Lukyanenko et al., 2009).

2.5 Mitochondrial involvement in cell apoptosis

Mitochondria are closely involved in the induction of apoptosis. Indeed, the intermembrane space contains several pro-apoptotic proteins which can lead to cell death upon release into the cytosol. Mitochondrial dysfunctions precede and are required for the initiation of the mitochondrial apoptosis pathway (for review see Marzetti et al., 2010). As previously mentioned, subsarcolemmal and intermyofibrillar mitochondria display different susceptibility towards apoptotic stimuli (Adhihetty et al., 2005). Mitochondria may therefore be involved in the pathogenesis of muscle atrophy, in obesity and type 2 diabetes or in

response to short-term immobilisation in ageing individuals (Kim et al., 2010; Magne et al., 2011).

3. Transcriptional regulation of mitochondrial oxidative capacity – interaction with fusion-fission dynamics

A number of transcriptional modulators have been implicated in the regulation of muscle mitochondrial biogenesis and OXPHOS activity (figure 3, see also for review Puigserver & Spiegelman, 2003; Chanseaume & Morio, 2009). They include PPAR gamma coactivator 1 alpha (PGC-1α), in cooperation with several factors such as PGC-1β, the peroxisome proliferator-activated receptors (PPAR), the estrogen-related receptor-α (ERRα), the nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) (Fredenrich & Grimaldi, 2004; Patti et al., 2003; Sparks et al., 2005; Tanaka et al., 2003), or the specificity protein 1 (Sp1), an ubiquitous transcription factor known to regulate the constitutive expression of oxidative OXPHOS genes (Zaid et al., 1999). Sp1 can function as both a positive (e.g. cytochrome c1 and mitochondrial transcription factor A, TFAM) and a negative (e.g. ANT2 and F_1-ATPase beta subunit) regulator of transcription (Yang et al., 2001).

PPARα and PPARβ are involved in the regulation of mitochondrial LCFA oxidative capacity. When bound to their ligands (e.g. LCFA), PPARs form a heterodimeric complex with the retinoid X receptor (RXR) to regulate gene transcription involved in LCFA metabolism. Muscle-specific overexpression of PPARβ in mice was shown to increase oxidative enzyme activities such as citrate synthase or β-hydroxyacyl-CoA dehydrogenase, and to enhance expression of genes implicated in fatty acid catabolism (Luquet et al., 2003).

PGC-1α and PGC-1β, but most importantly PGC-1α, are master modulators of gene expression in skeletal muscle (Moyes, 2003). PGC-1β has been shown to drive the formation of highly oxidative fibers containing type IIX myosin heavy chain (Arany et al., 2007). By contrast, PGC-1α was found to drive the formation of oxidative type I fibres (Lin et al., 2002). In muscle cells, overexpression of PGC-1α was shown to induce the gene expression of NRF-1, NRF-2, TFAM and to activate the expression of genes involved in mitochondrial oxidative capacity (Chabi et al., 2005). PGC-1α gene expression is potently regulated by CREB (cAMP response element-binding protein) binding protein (TORC) 1, a coactivator of CREB (Wu et al., 2006), and by the sirtuin SIRT1 (Amat et al., 2009). Its activity is increased when phosphorylated by p38 stress-activated MAPK (Puigserver et al., 2001) and when deacylated by SIRT1 (Lagouge et al., 2006). Coactivation of ERRα and PPARs by PGC-1α and PGC-1β has been proposed as the major regulatory pathway involved in the control of mitochondrial oxidative capacity (Puigserver & Spiegelman, 2003; Arany et al., 2007; Soriano et al., 2006). For these reasons, alterations in PGC-1α, and to a lesser extent PGC-1β, activity are considered the primary contributors to decreased mitochondrial oxidative capacity in metabolic disorders. Potential intrinsic mechanisms responsible of alterations in mitochondrial biogenesis are reviewed in Chanseaume et al. (2009).

Finally and complementary to mitochondrial biogenesis, recent evidences have demonstrated that the dynamics of mitochondrial network is strongly involved in the control of mitochondrial functioning. It is determined by the balance between fusion and

fission events which are govern by mitochondrial proteins such as mitofusin 1 and 2 (Mnf1, Mnf2) and OPA1 for fusion, and dynamin-related protein (DRP1) for fission. Importantly, Mfn2, which gene expression is regulated by PGC-1α and PGC-1β (Pich et al., 2005; Soriano et al., 2006), stimulates respiration, substrate oxidation and OXPHOS subunits expression (Pich et al., 2005). Zorzano et al. (2009) therefore hypothesized that these mitochondrial dynamics proteins play a key role in mitochondrial dysfunction in obesity or in type 2 diabetes and may participate in the development of insulin resistance. This concept is supported by recent studies in muscle (Zorzano et al., 2009) and neurons (Edwards et al., 2010).

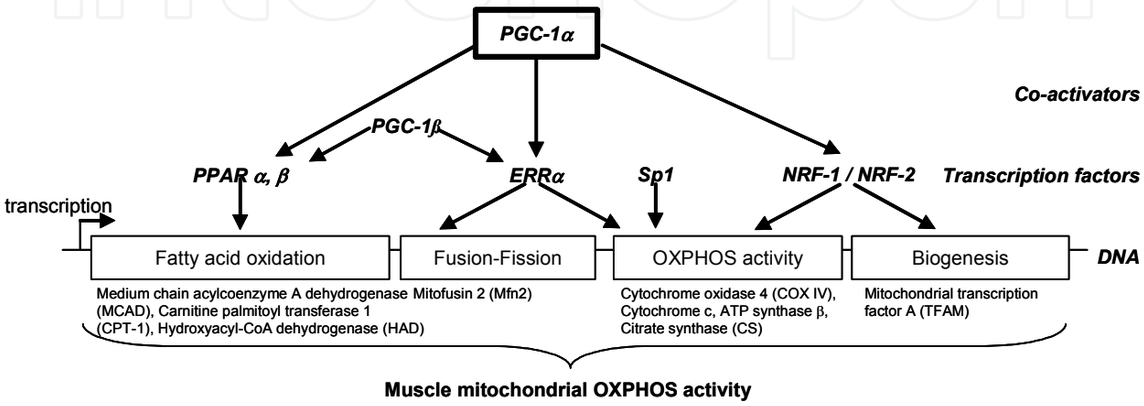

Fig. 3. Major coactivators and transcription factors involved in the regulation of muscle mitochondrial oxidative and phosphorylation (OXPHOS) activity. Non exhaustive key genes, whose expression is regulated by the transcription factors, are given for example.

4. Measurement of muscle mitochondrial OXPHOS activity on muscle biopsy samples

Changes in mitochondrial functioning can be assessed using a battery of biochemical analyses that can often be applied to whole tissue, cells or isolated organelles. These techniques are essential for elucidating intrinsic mechanisms responsible for mitochondrial dysfunctions. However most frequently, they inform about the maximal activity of key enzymes or pathways. In addition, they are limited to the conditions used for the measurements, for example the substrates used to feed the ETC. Therefore, care should be always taken in extrapolating *ex vivo* observations to the *in vivo* situations and generalization should be avoided.

4.1 Samples preparation

Because unfreezing muscle samples brakes mitochondrial structure and alters their functionality, most measurements have to be performed on fresh samples. It is possible to maintain the tissue intact during several hours by using a preservation solution at 4°C until mitochondrial isolation or skinned fiber preparation. Depending on the information required, mitochondrial functioning can be assessed either on isolated organelles or on whole tissue. The latter solution means that one works on tissue homogenates or using the skinned fiber technique. Isolated mitochondria can be obtained by differential centrifugation

from at least 80mg of homogenized muscles (Palmer et al., 1977; Capel et al., 2004; supplementary material). This approach is dedicated to the exploration of the organelle intrinsic functioning. Although contaminated by lysosomes, peroxisomes, tubular Golgi membranes, and small amounts of endoplasmic reticulum, the obtained fraction is suitable for respiratory studies (96). Further purification is possible, for example, with Percoll gradients as described by Mickelson et al. (1980) and Graham (2001). Mitochondrial integrity can be monitored by measuring citrate synthase activity before and after freeze–thaw membrane disruption and Triton X-100 addition (Stump et al., 2003). Similarly, mitochondrial purity can be assessed by assessing marker enzymes for lysosomes (β-galactosidase) and peroxisomes (catalase) as discussed by Graham (2001).

By contrast, although evaluated ex-vivo, permeabilized fibers take into account both the intrinsic functioning of mitochondria and the cellular content in mitochondria. Of note, skinned fibers require less muscle sample than the isolation procedure. Fifteen to 20 mg of fresh muscle sample is sufficient for one preparation of saponin-skinned muscle fibers. Briefly as described by Saks et al. (1998), fiber bundles are mechanically separated with tongs and permeabilized with saponin on ice. Bundles are then washed to remove ADP, phosphocreatine, soluble enzymes and metabolites. All steps are critical for obtaining clean skinned fibers for accurately measuring mitochondrial OXPHOS activity and coupling. The time of incubation with saponin depends on the cell type and the animal model. This technique requires therefore a well-trained investigator to assure accurate and repeatable measurements. Novices should follow at the beginning the association between the degree of mechanical separation, time of incubation with saponin and percentage of cells permeabilized and/or respirometric responses. Overpermeabilization should be avoided. The degree of permeabilization can be checked using toluidine blue under optic microscopy. Mitochondrial outer membrane integrity can be also verified during respirometry measurements by checking that addition of cytochrome c had no effect on oxygen consumption.

4.2 Evaluation of mitochondrial density

Mitochondrial density, as well as location, shape and structural integrity, can be reliably assessed using transmission electron microscopy. For that purpose, muscle samples are dissected free of adipose and connective tissue, cut into small pieces (1x1x2mm) and fixed. Transverse sections of 8μm are cut using a cryostat and mounted on slides. Images of 10 random and independent transverse sections of muscle fibers at 36,000 X must be considered. Mitochondrial cross-sectional area (size) and mitochondrial volume density (the fraction of cell volume occupied by mitochondria) are measured by digital imaging morphometry and stereological principles of point sampling, in a blind fashion (Weibel, 1979; Gundersen et al., 1988).

Maximal activity of citrate synthase has been often used as an index of mitochondrial density. This measure is performed on whole tissue homogenates. However, citrate synthase activity can be altered in some physiological situations independently of changes in mitochondrial density. For example, it is well known that aging is associated to a decrease in citrate synthase activity whereas other mitochondrial oxidative enzyme activities are unaltered (Rimbert et al., 2004).

Recently the number of mitochondrial DNA (mtDNA) copy has been considered to reflect mitochondrial content (Wiesner et al., 1992; Barrientos, 2002), the ratio between mtDNA and nuclear DNA has been proposed as a reliable index of mitochondrial density. For that purpose, mitochondrial and nuclear DNAs are extracted during the standard procedure of RNA extraction as described in Chanseaume et al. (2010) and in supplementary material. Purified DNA is ready for quantitative real-time PCR analysis of nuclear and mitochondrial DNA content using primer pairs specific for nuclear (β-actin or myogenin promoter) and mitochondrial (NADH dehydrogenase 1 (ND1), ND2, cytochrome B or cytochrome c oxidase 1 (COX1)) genes (Stump et al., 2003; Petersen et al., 2005; Chanseaume et al., 2010).

4.3 Assessment of maximal activity of key oxidative enzymes

This technique allows the use of frozen samples, either whole tissues, homogenates or isolated organelles. Frozen samples should be kept at -80°C for better preservation of the enzymes integrity. It assesses the maximal activity of the enzyme catalytic site. For that purpose, optimal concentrations in substrates are used to stimulate the enzyme activity. Catalytic activity is determined mostly using spectrophotometry on sample homogenates prepared in sucrose, EDTA Tris-HCl buffer (supplementary material)). For example, complex I activity is assessed measuring NADH oxidation at 340nm. Similarly, complex IV activity is performed at 550nm following the oxidation of reduced cytochrome c. Enzyme activities are often expressed per mg of mitochondrial protein but a most relevant normalisation should be citrate synthase activity or, because of the reason exposed in the previous paragraph, mtDNA content. Methods for measuring the separate activity of all ETC complexes as well as citrate synthase activity are extensively described by Barrientos (2002).

Finally, one can also consider the maximal activity of β-hydroxyacyl-CoA dehydrogenase as a fair index of the beta-oxidation pathway. The assay has been established by Bass et al. (1969). Its principle is based on the disappearance of NADH following reduction of acetoacetyl-CoA to ß-Hydroxybutyryl-CoA (supplementary material).

4.4 Measurement of fatty acid oxidative capacity

Whole muscle LCFA oxidative capacity can be easily determined on fresh muscle homogenates. This technique described by Veerkamp et al. (1983), informs on the maximal ability of muscle tissue to oxidize a specific LCFA. Using two distinct preparations, one can separate peroxisomal from mitochondrial beta-oxidation activities. Of note, our *personnal data* repeatedly showed that the peroxisomal contribution to whole tissue LCFA oxidative capacity averages 10-14%, in humans as well as in rodents.

This technique uses a radiolabelled [1 or U-^{14}C] fatty acid (such as oleate or palmitate for the most commons) bound to albumin at the ratio 4.5:1 (Morio et al., 2001; Rimbert et al., 2004; Tardy et al., 2008). A detailed procedure is provided in supplementary materials. Briefly, total LCFA oxidation is measured using sealed vials containing an aliquot of muscle homogenate in the presence of ATP, NAD+, coenzyme A, L-carnitine, L-malate and cytochrome c. Peroxisomal LCFA oxidation is determined in the presence of mitochondrial oxidation inhibitors (rotenone and antimycin A), but in the absence of L-carnitine and L-malate .

CPT-1 is the key enzyme regulating muscle fatty acid oxidative capacity. It is allosterically inhibited by malonyl-CoA (Rasmussen & Wolfe, 1999). The method measures the amount of

palmitoyl-carnitine produced from palmitoyl-CoA and carnitine as described by Kim et al. (2000) and is presented in supplementary materials. CPT-1 activity is measured on freshly isolated mitochondria. It allows assessment of maximal CPT-1 activity as well as its sensitivity to malonyl-CoA inhibition (IC50). Although rarely used, CPT-1 maximal activity can be also assessed from fresh whole muscle homogenates using modification of the above mentioned method (Rimbert et al., 2004). Alternatively, the affinity of mitochondria for palmitoylcarnitine could be assessed using isolated muscle fibers (Ponsot et al., 2005).

4.5 Measurement of mitochondrial respiration

Table 2 summarizes the main substrates used to assess mitochondrial oxidative capacity on isolated organelles or skinned fibers. It also presents the inhibitors required for additional investigation of mitochondrial intrinsic functioning and for measuring state 4 respiration rate. Isolated mitochondria or skinned fiber respiration rates are usually measured at 25°C using an oxygraph system. Higher temperatures, e.g. 30 or 37°C, can be used. This enhances the respiratory rate. Respiration is assayed on 0.25 mg/mL of mitochondrial proteins or 0.5-1.5 mg dried fibers in a specific buffer provided in supplementary materials.

	substrates	**inhibitors**
TCA cycle	Propionyl-L-carnitine Pyruvate/Palmitoyl-L-carnitine/α-ketoglutarate/L-malate (1mM/5µM/10mM/1mM)	
ß-oxidation	Octanoyl-L-carnitine/L-Malate (100µM/1mM) Palmitoyl-L-carnitine/L-Malate (55µM/1mM)	
Complex I	Glutamate/L-Malate (5mM/2mM) Pyruvate/L-Malate (5mM/2mM) α-ketoglutarate (10mM)	Rotenone (2.4µM) DPI (0.4mM) Amytal (2mM)
Complex II	Succinate* (5mM)	Malonate (10mM) TTFA (1mM)
Complex III	Duroquinol (0.6mM) Glycerol-3Phosphate (5mM)	Antimycin A (10µM) Stigmatellin (6.6 µM)
Complex IV	Ascorbate/TMPD (5mM/1mM)	KCN (0.3mM) Azide (10 mM)
Complex V		Oligomycin B (10µM)
ANT		Atractyloside (60µM) Carboxyatractyloside (250µM) Bongkrekate (1.5µM)
Uncouplers	FCCP (1µM) Valinomycin (3µM)	

Table 2. Substrates, inhibitors and uncouplers used in the polarographic experiments. Concentrations within brackets are indicative. * *used in the presence of rotenone (2 µM); DPI, diphenyliodonium chloride; TTFA, Thenoyltrifluoacetone; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; KCN, potassium cyanide; FCCP, carbonyl cyanide p-(trifluoromethoxyl)phenylhydrazone.*

State 2 (non-phosphorylating) respiration is measured in the presence of respiratory substrates without ADP. State 3 (phosphorylated) respiration is measured after the addition of of ADP, saturating concentration leading to maximal ADP-stimulated respiration being 1 mM (Gueguen et al., 2005). A replicate experiment can be done to evaluate uncoupled respiratory rate in the presence of an uncoupler such as carbonylcyanide p-(trifluoromethoxy)phenylhydrazone (FCCP). State 4 respiration is assayed after addition of oligomycin B, an inhibitor of ATP synthase, or after addition of atractyloside, a potent inhibitor of ANT. This measure validates the quality of fiber preparation (notably complete removal of free ADP during washing) if respiration returns to state 2. However, inhibiting ANT may improve the OXPHOS coupling since ANT may be responsible for basal and LCFA-induced uncoupling (Di Paola & Lorusso, 2006). Respiratory control rate is evaluated by dividing state 3 by state 4 rates. Respiration rates are expressed as natom (nat) O/min/µg protein for isolated mitochondria or natom (nat) O/min/mg dried (24 h at 110°C) fibers for skinned fibers.

When differential regulation of respiration by ADP and mitochondrial kinases (creatine kinase, AK2) is of interest, titration protocols with increasing concentration in ADP (from 0.1 to 1 mM) should be performed in the presence or not of 20 mM creatine (creatine kinase activation), or 10 mM glucose (hexokinase activation), 1 mM AMP (AK2 activation), 10 µM Ap5A (AK2 inhibition) (Gueguen et al., 2005). The functional coupling of miCK to energy production could be determined, calculating the affinity for ADP using a combined protocol on permeabilized fibers (N'Guessan et al., 2004).

4.6 Measurement of mitochondrial ATP production

ATP synthase (ATP_{ase}) activity is assayed on freshly isolated mitochondria. Maximal activity is measured spectrophotometrically by monitoring the increase in absorbance at 340 nm using a NADP-linked ADP-regenerating system. Mitochondria are added to the reaction buffer in the presence of glucose, AMP, NADP, ADP, hexokinase, and glucose-6-P dehydrogenase (see supplementary materials) and the reaction is followed at room temperature (Rustin et al., 1994). Similarly, it can also be assessed using a coupled assay between lactate dehydrogenase and pyruvate kinase, NADH reduction being checked at 340 nm (Rustin et al., 1994).

ATP production can be directly measured using bioluminescence kit assay by incubating isolated mitochondria with various substrates of the ETC (Table 2). Blank tubes are used for measuring background and all reactions for a given sample are monitored simultaneously and calibrated with addition of an ATP standard. Mitochondrial ATP production is calculated from the area under the curve (Wibom & Hultman, 1990).

Finally, mitochondrial ATP production can be measured kinetically in parallel with respiration (on isolated mitochondria or permeabilized fibers) as described by Ouhabi et al. (1998). After ADP is added, five 10µl samples are taken every 15 sec in the oxygraph chamber, and frozen in 100µl DMSO before ATP is assayed using a bioluminescence assay kit. ATP production rate is expressed as nmol/min/mg dried fibers. ATP/O is calculated to evaluate coupling of ATP production to ADP-stimulated oxygen consumption (state 3 in nmol O_2/min/mg) (Chanseaume et al., 2010).

4.7 Measurement of mitochondrial ROS production

Using similar incubation conditions than described for respiration or ATP assays, superoxide anion ($O_2^{\bullet-}$) can be assessed on isolated mitochondria using the chemiluminescent probes 2-methyl-6-p methoxyphenylethynylimidazopyazinone (MPEC) (Nakai et al., 2004) or lucigenin (Bis-N-methylacridinium) (Li et al., 1999). Fluorescent sensitive probes such as hydroethidine, especially MitoSOX™ Red which targets the mitochondrial matrix, can also be used. Isolated permeabilized fibers were recently used for $O_2^{\bullet-}$ generation measurement using the triphenylphosphonium Hydroethidine (TTP-HE) reporter (Xu et al., 2010). Signals were analyzed by micellar electrokinetic capillary chromatography coupled to fluorescence detection.

$O_2^{\bullet-}$ rapidly dismutates into H_2O_2 in the presence of mitochondrial SOD which considerably reduce its matrix level. Then the quantification of the more stable H_2O_2 can be assessed using luminol chemiluminescence (Li et al., 1999). H_2O_2 can be also measured using non-fluorescent dyes that become fluorescent upon enzymatic oxidation by H_2O_2 in the presence of horseradish peroxidase. The most commonly used dyes are homovanilic acid (Ruch et al., 1983), 2',7'-dichlorfluorescein-diacetate (DCFH-DA) or Amplex® Red (Chen et al., 2003). Kinetic or global approaches are both adequate for calculating the rate of mitochondrial ROS production. Standard curve is obtained by adding known amounts of H_2O_2 to assay medium in the presence of the reactants. It is critical to quantify background fluorescence, in the absence of mitochondria. Net fluorescence is then calculated minus background and H_2O_2 production is expressed in pmol/mg of protein/min. The heterogeneity of mitochondrial alteration during metabolic disorders could be studied on permeabilized fibers, characterizing the specificity of H_2O_2 production depending on myofiber type (Anderson et al., 2006). If oxygen consumption and ROS production were determined in similar experimental conditions on isolated mitochondria or permeabilized fibers, the free radical leak can be calculated as an indicator of the fraction of electron reducing oxygen to $O_2^{\bullet-}$ (Sanz et al., 2005). It has been shown that this leakage was higher in glycolytic compared to oxidative fibers (Anderson et al., 2006). More recently, new methods were developed to analyze local change in ROS generation on individual isolated fibers. Hence, ROS production could be analyzed by confocal imaging (Shkryl et al., 2009) or real time fluorescence microscopy (Palomero et al., 2008). Although more reliable than $O_2^{\bullet-}$ quantification, the detection of H_2O_2 as an index of $O_2^{\bullet-}$ production has also its limits, i.e., the reaction of $O_2^{\bullet-}$ with other molecules (such as $NO^{\bullet}$), the removal by matrix peroxidase and contamination by H_2O_2 produce within the intermembrane space (see (Murphy, 2009) as a more detailed review on this topic).

4.8 Additional measurement of mitochondrial functionality

Mitochondrial membrane potential can be determined simultaneously with oxygen consumption using the potential-dependent triphenylmethylphosphonium cation ($TPMP^+$) probe as described by Kamo et al. (1979). Development of fluorescent probes sensitive to mitochondrial membrane potential has facilitated the investigation on isolated organelles as well as cells. The most commonly used dyes are rhodamine 123, its derivatives tetramethylrhodamine methyl and ethyl esters (TMRM and TMRE), 3,3'-dihexyloxacarbocyanine,iodide ($DiOC_6(3)$) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolcarbocyanine iodide (JC-1). The latter has been proposed as the most reliable probe (Salvioli et al., 1997). Results are usually compared with those obtained in the presence of a mitochondrial uncoupler, such as FCCP, to attain

maximum depolarization. Membrane potential could also be determined using isolated permeabilized fibers in a multiwell plate (Xu et al., 2010).

Calcium retention capacity of mitochondria and sensitivity of the permeability transition pore is evaluated on isolated mitochondria using the same incubation buffer and substrates than for respiration, except that 1 µM Calcium Green™-5N is added (Fontaine et al., 1998). Pulses of 10 µM Ca^{2+} are added every minute until pore opening. Specificity of mitochondrial pore opening is assessed by adding cyclosporin A. Comparison between fiber types could be done in a similar approach using permeabilized fibers (Picard et al., 2008).

4.9 Evaluation of mitochondrial biogenesis and fusion-fission dynamics

Supported by steadily increasing literature data, changes in mitochondrial biogenesis and dynamics are relevant mechanisms potentially responsible for mitochondrial dysfunctions. PCR and western blotting analyses are pertinent techniques to investigate these pathways in human samples with limited size (20 to 30 mg). Major proteins potentially involved in these pathways have been described in the previous chapter "Transcriptional regulation of mitochondrial oxidative capacity – interaction with fusion-fission dynamics" and can be targeted using molecular tools.

4.10 Investigation of the mitoproteome

The recent development of genomic tools has considerably improved or knowledge of metabolic disorders within key metabolic tissues. Recently, a proteomic study of mitochondrial proteins (mitoproteome) reported that more than 80% of ETC proteins can be identified (Lefort et al., 2009). The authors were able to reliably detect proteins implicated in ROS generation, scavenging, FA oxidation and apoptosis. One hundred micrograms of human biopsie are required and allow a more than 7 fold enrichment in mitochondrial proteins with a classical isolation procedure as described for respirometry assay. Of note, 30% of identified proteins from the whole tissue proteome are assigned to mitochondria (Yi et al., 2008). Further studies should aim to relate the metabolic disorders and modification of the mitoproteome in insulin sensitive and resistant subjects. This tool combined to transcriptomic analysis should provide key elements to distinguish between mitochondrial functioning defect and mitochondrial density variation during insulin resistance.

5. Conclusions

Exploring mitochondrial functioning in muscle samples, especially from human muscle biopsies, can be achieved using combination of strategies which require a minimal amount of 20mg of tissue for permeabilized fibers for one experimental condition (e.g. respirometry with one substrate), 30mg for molecular exploration and 30mg for an enzyme activity. The most tissue-consuming techniques are those based on isolated mitochondria, which necessitate at least 80mg of tissue for the extraction procedure. Once mitochondrial pellets are obtained, if further purification is not performed, combined and miniaturized methods allow assessing several parameters characterizing mitochondrial functioning (for example respiration, ATP and ROS production and enzyme activities). Hence, 150 to 200mg of tissue sample could be considered as a sufficient condition to build a complete investigation as presented in figure 4. These explorations are however limited in the number of substrates used for testing the ETC.

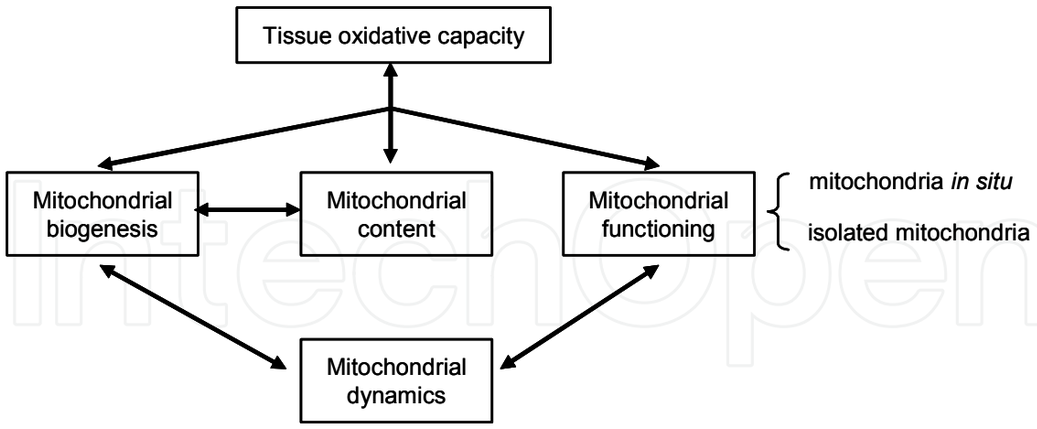

Fig. 4. Main guidelines for exploring mitochondrial functioning in human muscle biopsies.

6. Supplementary materials: buffers composition and protocols

6.1 Preservative solution for isolated fibers

CaK_2EGTA 1.9mM, K_2EGTA 8.1mM, imidazole 20mM, DTT 0.5mM, $MgCl_2$ 9.5mM, MES 53.3mM, taurine 20mM, ATP 2.5mM, phosphocreatine 19mM, pH 7.1.

6.2 Mitochondria isolation buffers and procedure

Tissue grinding: 100mM KCl, 50mM Tris, 5mM MgSO4, 2mM EDTA, 1mM ATP, 0.2% BSA, pH 7.4. To improve the extraction rate of intermyofibrillar mitochondria, treatment with subtilisine type VIII (1.5mg/ml, 5ml/g tissue, incubation 2 to 5 min on ice) is required prior to homogenization.

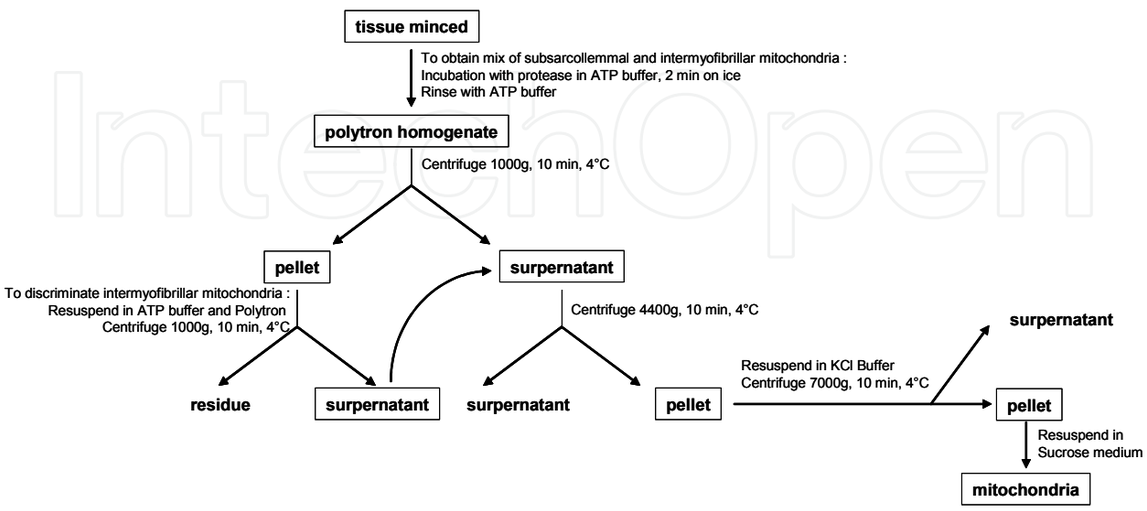

Fig. 5. Procedure for isolating subcellular fractions enriched in mitochondria using differential centrifugation.

KCl buffer: KCl 100mM, Tris 50mM, $MgSO_4$5mM, EDTA 2mM, pH 7.4.

ATP buffer: KCl buffer with ATP 1mM and BSA 0.2%, pH 7.4.

Protease buffer: ATP buffer with subtilisine type VIII (1.5 mg/ml, 5 ml/g tissue).

Sucrose medium: sucrose 0.25M, EGTA 0.1mM, Tris-HCl 10mM, pH 7.4.

6.3 Respiration buffer

CaK_2EGTA 1.9mM, K_2EGTA 8.1mM, imidazole 20mM, DTT 0.5mM, KH_2PO_4 3mM, $MgCl_2$ 4mM, MES 100mM, taurine 20mM, EDTA 20µM and 0.2% BSA.

6.4 Measurement of mitochondrial ATP production

Add freshly isolated mitochondria to the reaction medium containing glucose 400 mM, $MgCl_2$100 mM, KPO_4 100mM, potassium succinate 200mM, AMP 110mM, Hepes 10mM, NADP 7.5mM, ADP 50mM, hexokinase 10µg, and glucose-6-P dehydrogenase 10mg. The total volume in the cuvette is 1 ml. The increase in absorbance at 340nm is followed at room temperature for 3 min (Rustin et al., 1994).

ATP production can also be assessed using a coupled assay between lactate dehydrogenase and pyruvate kinase, NADH reduction being checked at 340 nm. Aliquots of mitochondria (20-40 µg protein) are added to a medium (Tris 50mM, BSA 5mg/ml, $MgCl_2$ 20mM, KCl 50mM, carbonyl cyanide m-chlorophenylhydrazone 15µM, antimycin A 5µM, phosphoenolpyruvate10mM, ATP 2.5mM, 4 units of lactate dehydrogenase and pyruvate kinase, NADH 1mM, pH 8.0) pre-incubated for 5 min at 37°C. The reaction is followed for 3 min before and after oligomycin 3µM is added in order to distinguish the ATPase activity coupled to the ETC (Short et al., 2001).

6.5 DNA purification for mtDNA quantification

Remove the aqueous phase with RNA. Isolate and spun the interphase/organic phase at 12000×g for 5min at 4°C. Remove the remaining aqueous phase to limit contamination with RNA. Add back extraction buffer (4M Guanidine Thiocyanate, 50mM Sodium citrate, 1M Tris; 0.5mL per 1mL of Trizol used for RNA extraction) to each tube and mix for 10 min. Centrifuge at 12000×g for 30min at room temperature. Incubate the upper phase with isopropanol (0.4mL per 1mL of Trizol used for RNA extraction) 5min at room temperature and spun at 12000×g for 15 min at 4°C. Pellets which contain DNA are washed twice with ethanol 70% and spun at 12000×g for 15min at 4°C. Final dissolution is done with sterile water after ethanol removal. DNA concentration is determined spectrophotometrically at 260nm. Total DNA solutions could be stored at -20°C.

6.6 Maximal activity of key oxidative enzymes

Homogeneization buffer for muscle samples: sucrose 0.25mM, EDTA 2mM and Tris-HCl 10mM, pH 7.4.

Buffer for assessing β-hydroxyacyl-CoA dehydrogenase activity: Na_2HPO_4 0.1M, EDTA 2mM, pH 7.5 adjusted with NaH_2PO_4 0.1M, EDTA 2mM. Samples are homogenised in buffer

(Na_2HPO_4 0.1M, EDTA 2mM, pH 7.5 adjusted with NaH_2PO_4 0.1M, EDTA 2mM). 75µl of homogenate is added to 925µl of medium (triethanolamine 100mM, EDTA 5mM, acetoacetyl-CoA 0.1mM, β-NADH 0.45mM, pH 7) and incubated at 30°C during 2 min whilst absorbance is measured at 340nm (Morio et al., 2001).

6.7 Measurement of fatty acid oxidative capacity

At least 50mg of fresh muscle sample is cut into small pieces and crushed in the ice-cold homogeneization buffer (cf 6.6). Total LCFA oxidation is measured using sealed vials in a total volume of 0.5mL containing 75µL of muscle homogenate in a medium (sucrose 25mM, Tris-HCl 75mM, KH_2PO_4 10mM, $MgCl_2$ 5mM, EDTA 1mM, pH 7.4) supplemented with ATP 5mM, NAD^+ 1mM, coenzyme A 0.1mM, L-carnitine 0.5mM, L-malate 0.5mM and cytochrome c 25µM. Peroxisomal LCFA oxidation is determined in the presence of mitochondrial oxidation inhibitors (rotenone and antimycin A) in a medium lacking L-carnitine and L-malate. Blanks are produced by replacing the muscle homogenate with homogeneization buffer.

After 5 min of pre-incubation at 37°C under shaking, the reaction is started by injecting 0.1mL of 600µM of the radiolabelled [^{14}C] fatty acid. Incubation is carried out at 37°C for 30 min and stopped by the addition of 0.2mL of 3M perchloric acid. After 90 min at 4°C, the acid incubation mixture is centrifuged for 5 min at 10,000 g, and the 0.5mL supernatant containing ^{14}C-labelled perchloric acid-soluble products is assayed for radioactivity by liquid scintillation. LCFA oxidation rate is calculated from ^{14}C-labelled perchloric acid-soluble products and expressed in nmoles of fatty acids oxidized per minute per gram wet tissue weight. Mitochondrial oxidation rate is calculated by subtracting the peroxisomal rate from the total oxidation rate. Of note, $^{14}CO_2$ production can be trapped in 0.3mL ethanolamine/ethylene glycol (1:2, v/v) and measured by liquid scintillation.

6.8 Measurement of CPT-1 activity

CPT-1 activity is measured from freshly isolated mitochondria (0.1mg of protein/ml) at 30°C from palmitoyl-L-[methyl-^{3}H]carnitine formed from L-[methyl-^{3}H]carnitine (200µM; 10Ci/mol) and palmitoyl-CoA (80µM) in the presence of 1% (w/v) BSA. Increasing concentration of malonyl-CoA (from 0.01 to 600µM) are used for the estimation of the IC50 value (concentration of malonyl-CoA required to achieve 50% inhibition if CPT-1 activity). This method measures the amount of palmitoyl-carnitine produced from palmitoyl-CoA and carnitine and is originately described by Kim et al. (2000).

CPT-1 maximal activity on fresh whole muscle homogenates: 50mg of muscle is homogenized in ice-cold incubation buffer containing KCl 75mM, Hepes 5mM, KCN 2mM, EGTA 0.2mM, DTT 1mM, ATP 5mM, pH 7.3. Fifty microliters of a 5-fold diluted muscle homogenate are preincubated for 5 min at 30°C with 350µl of incubation buffer and 100µl of 80µM palmitoyl-CoA. Reactions are initiated when 50µl of 10mM L-[^{3}H]carnitine (1µCi) are added at 30°C for 15 min. The reaction is terminated with 1ml of isobutanol. The formed [^{3}H]palmitoyl-carnitine is extracted with saturated $SO_4(NH_4)_2$ and counted by liquid scintillation. CPT I activity is expressed in nanomoles [^{3}H]palmitoyl-carnitine per gram of wet tissue per minute corrected for malonyl-CoA (0.3mM)-insensitive [^{3}H]palmitoyl-carnitine synthesis (Rimbert et al., 2004).

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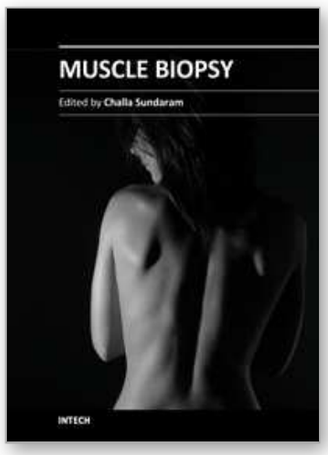

Muscle Biopsy
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Investigation of muscle diseases has changed dramatically with the understanding of genetic basis of a wide range of muscle diseases. Muscle biopsy has become a powerful tool not only to provide diagnosis but to make tissue available for genetic studies and to basic scientists for biomedical research. Accurate interpretation of muscle biopsy to detect cell dysfunction/ damage/death or absence / abnormality of a protein or genetic defect by the sophisticated technologies is important to guide treatment of various muscle diseases. In this book on muscle biopsy various chapters deal with the procedure and interpretation of muscle biopsy, its use in the culture of myotubes and membrane transport studies.Muscle biopsy is an important technique to investigate mitochondrial dysfunction and the mitochondrial DNA integrity in oxidation. Phosphorylation in various metabolic diseases like obesity, type 2 diabetes mellitus and peripheral vascular disease is explored in the other chapters with detailed descriptions on methodology. This book provides the advances in the basic techniques of muscle biopsy for a neuroscientist.

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