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Chapter 12

Skeletal Muscle Mitochondrial Function/Dysfunction and Type 2 Diabetes

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Additional information is available at the end of the chapter

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1. Introduction

"Let food be your medicine and medicine be your food" stated Hippocrates, the father of Western medicine, in 400 B.C. This statement was based on the belief that food was able to influence disease, a concept that was revived several times in later years by painters, writers, scientists, and philosophers. One such philosopher, Ludwig Feuerbach, famously wrote in his 1863-4 essay "man is what he eats" introducing the idea that if we want to improve the spiritual conditions of people we must first improve their material conditions (Feuerbach, 2003). However, for years his warnings remained unheeded, at least in Western countries, in contrast to the teachings of Indian and Chinese medicine which for millennia have argued that a living organism has to assume a healthy diet. Like diet, physical activity has been also considered an important starting point for people's health. Hippocrates wrote in his book *Regimen* "if we could give every individual the right amount of nourishment and exercise, not too little and not too much, we would have found the safest way to health" (Hippocrates, 1955). Our knowledge about the links between diet, exercise, and disease has vastly increased since Hippocrates time. A healthy lifestyle based on diet and physical activity is now considered the keystone of disease prevention and the basis for a healthy aging. However, modern society has created conditions with virtually unrestricted access to food resources and reduced physical activity, resulting in a positive overall energy balance. This is far from the environment of our "hunter-gathered ancestros" whose genes were modulated over thousands of years adapting our metabolism to survive when food was scarce and maximizing energy storage when food became available. In terms of evolution, this radical and sudden lifestyle change in modern society has led to a dramatic increase in the incidence of metabolic diseases including obesity and type 2 diabetes mellitus (T2DM). It seems clear that the development of T2DM has a genetic component that becomes obvious when individuals are exposed to western lifestyle. However, environment

plays a critical role in the incidence of the disease being obesity the main etiological cause of T2DM. Thus, modest weight loss is enough for obese glucose intolerant subjects to prevent the development of T2DM (National Task Force on the Prevention and Treatment of Obesity, 2000).

T2DM also known as "non-insulin-dependent diabetes mellitus" or "adult-onset diabetes", is a metabolic disorder characterized by high blood glucose, insulin resistance, and relative insulin deficiency. T2DM is diagnosed when fasting blood glucose levels are higher than 126 mg/dL (7.0 mM) or the two-hour blood glucose levels higher than 200 mg/dL (11.1 mM) after a glucose tolerance test. T2DM is now considered to be a global epidemic with significant social and economic consequences both at the individual and population level. The International Diabetes Federation estimates that 366 million people suffered from this disease in 2011 and predicts that these numbers will increase to 552 million people by 2030. Risk factors for T2DM include genetic predisposition as well as environmental factors, including adverse intrauterine environment, inactivity, diet, obesity, and aging (International Diabetes Federation, http://www.idf.org/diabetesatlas/news/fifth-edition-release).

The term "prediabetes" is used to describe a condition characterized by impaired glucose tolerance or impaired fasting glucose (Pour and Dagogo-Jack, 2011). The pathophysiology of prediabetes is characterized by alterations in insulin sensitivity and pancreatic beta-cell function, usually associated with increased adiposity (Dagogo-Jack et al., 2009). According to the World Health Organization (WHO) and the American Diabetes Association (ADA), impaired glucose tolerance is defined as a two-hour plasma glucose level between 140 and 199 mg/dL (7.8 to 11.0 mmol/L) after an oral glucose tolerance test. In this condition, fasting glucose levels may be either normal or mildly elevated. The ADA defines a state of impaired fasting glucose when fasting plasma glucose levels are over 100 mg/dL (5.6 mmol/L) but less than 125 mg/dL (6.9 mmol/L). Importantly, subjects with prediabetes are at higher risk for progressing to diabetes. Fortunately, such progression is not inevitable and can be delayed or prevented through pharmacological and lifestyle interventions based on diet and exercise (Knowler et al., 2002, Knowler et al., 2009, Tuomilehto et al., 2001). While these data are encouraging, these interventions are costly, require a very high degree of commitment of the subjects, and are not always successful. Although the progress in understanding the metabolic derangements of T2DM has led to significant advances in the treatment of this disease, it remains unclear whether current therapeutic approaches can really improve the underlying metabolic defects. Therefore, there is an urgent need to characterize the complex pathophysiology of the disease, to identify and target specific mechanisms in order to slow down the worldwide diabetes epidemic.

2. Insulin action and insulin resistance

Insulin essentially provides an integrated set of signals that allow for the balancing of nutrient availability and caloric demands (Samuel et al., 2010). In collaboration with the opposing hormone glucagon, it is responsible for maintaining glucose homeostasis, which is necessary to ensure proper function and survival of all organs. The regulation of plasma glucose concentrations is vital for the entire body and both hypoglycemia and hyperglycemia can impair whole-body physiology, ultimately leading to cellular death. This

is why it is critical to regulate and maintain plasma glucose levels around 5mM, the physiological set point in mammals (Saltiel, 2001).

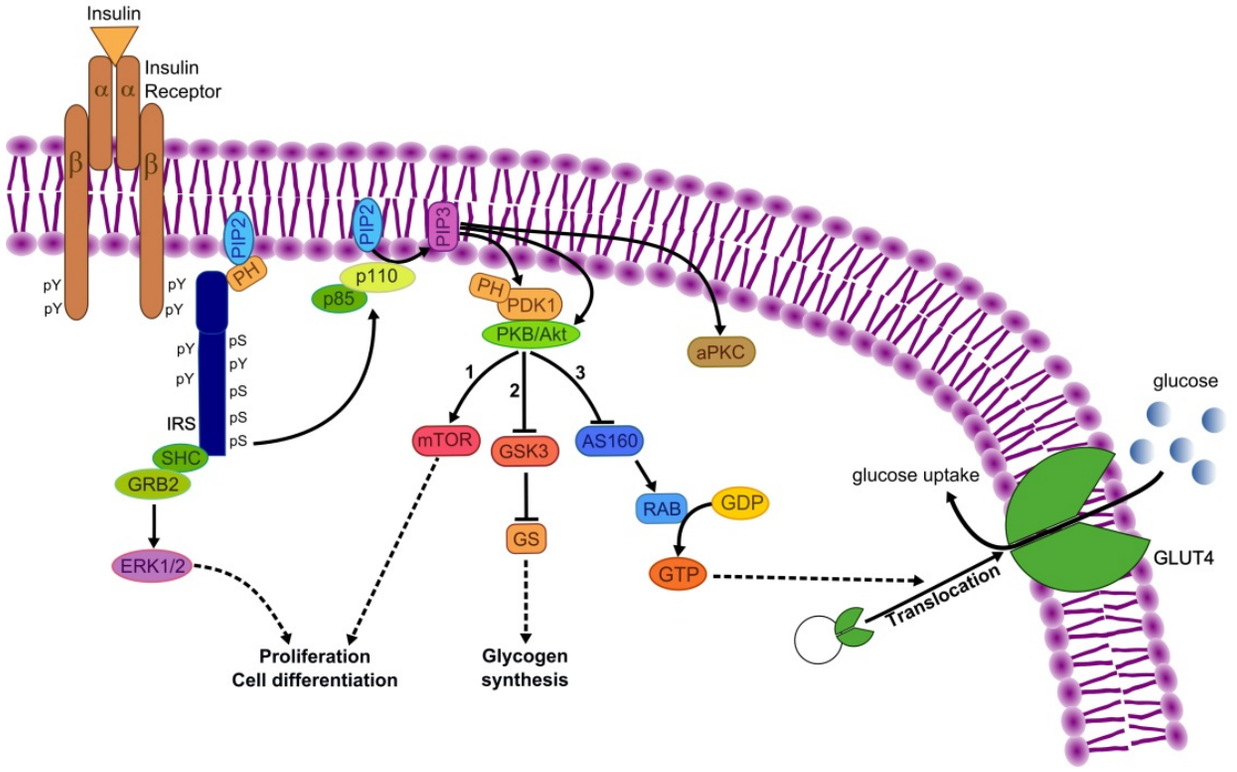
The primary targets of insulin action to maintain glucose homeostasis are skeletal muscle, liver, and adipose tissue. Under physiological conditions, carbohydrates provided by the diet increase plasma glucose levels and promote insulin secretion from pancreatic β cells of the islets of Langerhans. Once secreted, insulin binds to its receptor, triggering a cascade of downstream phosphorylation events that expand the initial signal (*Figure 1*). Insulin binds to its receptor and activates its intrinsic protein tyrosine kinase activity, resulting in the phosphorylation of tyrosine residues located in the cytoplasmic face. The activated receptor, in turn, recruits and phosphorylates a group of substrate molecules. They have the role of docking proteins and are known as "insulin receptor substrates" (IRS). Among these, IRS1 and IRS2 appear to be the major adapter molecules that play a role in insulin cascade. IRS1/2 can activate different intracellular processes, above all glucose metabolism and mitogenesis. Once phosphorylated, these docking proteins recruit the heterodimeric p85/p110-PI3K at the plasma membrane: the regulatory subunit p85 binds to IRS1/2 and this event allows the activation of the catalytic subunit p110, which produces the lipid second messenger PIP3 from PIP2. PIP3 activates a serine/threonine phosphorylation cascade of PH-domain containing proteins: PDK1, the serine/threonine protein kinase B (PKB)/Akt and the atypical protein kinases C ζ and λ isoforms (aPKC ζ - λ). Specifically, PKB phosphorylation causes:

1. activation of the mammalian target of rapamycin (mTOR), an intracellular protein as well as a critical component of the PI3K/AKT pathway, that acts as a central regulator of multiple signaling pathways that mediate growth, proliferation and cell differentiation;
2. glycogen synthase kinase-3 (GSK3) inactivation. This event relieves the inhibitory phosphorylation of glycogen synthase (GS), which becomes activated and promotes glycogen synthesis;
3. insulin-stimulated translocation of the glucose transporter GLUT4 at the plasma membrane, resulting in increased glucose uptake. This pathway involves the protein AS160/TBC1D4. AS160 normally inhibits translocation of GLUT4 through its interaction with RabGTPase protein. The inhibitory phosphorylation of AS160 favors the GTP-loaded state of Rab and relieves the inhibitory effect on GLUT4, stimulating its translocation to the plasma membrane. In this way, insulin can promote the docking and fusion of GLUT4-containing vesicles to the plasma membrane and finally stimulate glucose uptake.

Insulin action in normal conditions differs depending on the target tissue:

- in skeletal muscle, it increases glucose transport, increasing glucose uptake and activating glycogen synthesis;
- in liver, it promotes glycogen synthesis and *de novo* lipogenesis, while inhibiting gluconeogenesis;
- in adipose tissue, it suppresses lipolysis and promotes lipogenesis, leading to a net increase in lipid accumulation.

Insulin secretion from pancreatic β cells is suppressed during fasting. During this state, there is an increase in hepatic glucose production and glycogenolysis. Lipid synthesis diminishes in liver while lipolysis increases in adipose tissue.



IRS, insulin receptor substrate; S, serine; Y, tyrosine; PH, pleckstrin homology domain of the IRS-1; SHC, Src Homology 2 domain; GRB2, growth factor receptor-bound protein 2; ERK, extracellular-signal-regulated kinases or classical MAP kinases; PIP2, phosphatidylinositol 4,5-bisdiphosphate; PIP3, phosphatidylinositol 3,4,5-tridiphosphate; PDK1, phosphoinositide-dependent protein kinase 1; PKB/Akt, protein kinase B; mTOR, mammalian target of rapamycin; GSK3, glycogen synthase kinase 3; GS, glycogen synthase; AS160, 160 kDa Akt substrate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; aPKC, atypical protein kinase C.

Figure 1. Insulin signaling pathway

3. Pathogenesis of Type 2 Diabetes Mellitus

An important early phenotype associated with increased T2DM risk is insulin resistance. Insulin resistance, defined as reduced responsiveness to the effects of insulin to promote glucose disposal into muscle, liver and adipose tissue, is present in high-risk individuals years before the onset of T2DM, and can predict the development of the disease (Martin et al., 1992, Tabak et al., 2009). Given these data, it is alarming that the high prevalence of insulin resistance in the population predicts further dramatic increases in the worldwide epidemic of T2DM. Individuals with established T2DM show several physiological abnormalities, including elevation in fasting glucose levels, elevation in postprandial glucose levels, or both. Insulin resistance in adipose tissue, skeletal muscle, and liver, together with pancreatic beta-cell dysfunction represent the core pathophysiologic defects of T2DM (DeFronzo, 1988).

In the initial stages of development of T2DM, insulin is not able to correctly stimulate skeletal muscle glucose uptake after carbohydrate intake, leading to postprandial hyperglycemia. In adipose tissue, the major fat storage tissue in mammals, insulin resistance results in increased lipolysis and fatty acid release. Increased circulating fatty acids decrease

the ability of insulin to suppress hepatic glucose production and allow a constant increase in fatty acid synthesis. This dysregulation of carbohydrate and lipid metabolism accelerates the progression of insulin resistance. During the first stages of the development of the disease, pancreatic beta-cells have the ability to compensate for insulin resistance by increasing basal and postprandial insulin secretion to correct hyperglycemia. When pancreatic beta-cells can no longer compensate they become unable to respond appropriately to glucose levels. This pancreatic beta-cell failure leads to the deterioration of glucose homeostasis and the development of T2DM. This pattern of physiological abnormalities in skeletal muscle, adipose tissue, liver, and pancreas presents itself in the late stages of the disease (Saltiel, 2001). Additionally, abnormal secretion and regulation of incretins in the gastrointestinal tract, hyperglucagonemia due to alterations in pancreatic alpha-cells, increased glucose reabsorption in kidney, and altered balance of central nervous system pathways involved in food intake and energy expenditure play an important role in the development of T2DM (Defronzo, 2009). This complex pathophysiology makes difficult to identify the primary events responsible for the development of T2DM.

4. Skeletal muscle insulin resistance and T2DM

As mentioned above, insulin resistance is a key component for the development of T2DM. However, the underlying molecular mechanisms are still unclear. Himsworth and Kerr, using a combined oral glucose and intravenous tolerance test, were the first to demonstrate that tissue-specific insulin sensitivity was lower in T2DM individuals (Himsworth, 1940). Ginsberg and colleagues provided another important evidence related to the decreased ability of insulin to promote glucose uptake in subjects with T2DM (Ginsberg et al., 1975). Later on, clear evidences about skeletal muscle insulin resistance in T2DM subjects were provided by DeFronzo and colleagues, who used the euglycemic-hyperinsulinemic clamp technique to quantify insulin-stimulated glucose uptake. With this technique, in a series of studies DeFronzo and colleagues demonstrated that both lean and obese T2DM subjects have marked decrease in whole body glucose disposal during the insulin clamp (DeFronzo, 1988). Skeletal muscle is the largest insulin-sensitive organ in humans accounting for more than 80% of insulin stimulated glucose disposal (DeFronzo et al., 1985). Therefore, insulin resistance in this tissue has major consequences on whole-body metabolic homeostasis.

Several mechanisms have been proposed as potential contributors to insulin resistance in skeletal muscle, including accumulation of intracellular lipid derivatives (diacylglycerol and ceramides), endoplasmic reticulum stress, impaired gene transcription, and pro-inflammatory signals (Ozcan et al., 2004, Straczkowski et al., 2007, Patti et al., 2003, Timmers et al., 2008, Sell et al., 2006). Moreover, several evidences linked mitochondrial defects to insulin resistance and T2DM (Lowell and Shulman, 2005), suggesting that these organelles are key players in maintaining energy homeostasis.

In this chapter we will discuss the potential role that mitochondrial dysfunction plays in T2DM etiology. In addition, a critical review of the current status of the topic will be

presented. In order to facilitate the reader the understanding of this chapter content we will briefly introduce several aspects of skeletal muscle composition, metabolism, mitochondria biogenesis and regulatory machinery that are necessary to comprehend subsequent information.

4.1. Skeletal muscle fiber types and metabolism

Skeletal muscle is a complex tissue composed of different fiber types, which have distinct mechanical and metabolic properties. Adult mammalian skeletal muscle is organized in motor units. Each of these functional systems is composed of a motor neuron and a group of muscle fibers. There are four major fiber types in mammalian skeletal muscle, categorized based on their myosin heavy chain (MyHC) composition: type 1 (slow oxidative), type 2A (fast-twitch oxidative), type 2X (fast-twitch oxidative-glycolytic), and type 2B (fast-twitch glycolytic). In adult human skeletal muscle type 2B fibers are not detectable and the oxidative capacity of type 2X fibers is lower than that observed in rats and mice (Schiaffino and Reggiani, 2011). For additional reading we recommend a review published in Physiological Reviews written by Stefano Schiaffino and Carlo Reggiani that provides an up to date and detailed understanding of this topic (Schiaffino and Reggiani, 2011). For the purposes of this discussion, it is important to keep in mind skeletal muscle diversity: distinct skeletal muscle fibers differ in their energy requirements for cellular function, including contractile activity. Energy is provided by adenosine triphosphate (ATP) hydrolysis to adenosine diphosphate (ADP) and inorganic phosphate (Pi). ATP can be generated by three main mechanisms that vary in their capacity and velocity to resynthesize ATP. The Phosphocreatine (PCr)-creatine kinase (CK) system corresponds to a high-power and low-capacity ATP production reservoir. Glycolysis is the metabolic process by which glycogen and glucose are metabolized to pyruvate and subsequently to lactate; this process has a lower power but a higher capacity for ATP generation than the PCr-CK system. The other energy production resource is the mitochondrial oxidative phosphorylation system, which can obtain ATP from different substrates: pyruvate, fatty acids, amino acids, and ketone bodies. The oxidative phosphorylation system has a very high capacity for ATP generation but a lower power when compared to the other two ATP production systems. It is also important to highlight that mitochondrial mediated ATP resynthesis is highly dependent on oxygen and substrate availability.

Due to its intrinsic characteristics, slow and fast muscle fibers differ in their relative contribution to energy production from PCr-CK, glycolysis, and oxidative phosphorylation processes. The relative contribution of these metabolic pathways is mostly established during differentiation according to the specific function and energy demands of each fiber type. Moreover, it is important to mention that adult skeletal muscle fibers show what has been termed “metabolic flexibility.” Metabolic flexibility is the systemic capacity to switch between different substrates for ATP production depending of their availability and energy requirements (Kelley et al., 1999, Storlien et al., 2004). Thus, skeletal muscle is able to predominantly utilize both glucose and free fatty acids as fuel sources for energy production. The utilization of these two energy sources depends on the fasting/feeding state of the individual:

- During fasting state, muscle glucose uptake is low and plasma fatty acid concentration is elevated due to lipolysis in adipose tissue. Thus, under fasting conditions, fatty acids represent the main source for energy production in skeletal muscle.
- During feeding state, plasma glucose concentration increases and stimulates insulin secretion that exerts two principal and simultaneous actions: suppression of lipolysis in adipose tissue, leading to a reduction in plasma fatty acid concentration, and stimulation of glucose uptake in skeletal muscle. This event, together with the activation of key enzymes in glucose metabolism, leads to a marked increase in muscle glucose oxidation. After glucose is transported into the myocytes trough the GLUT4 transporter, it is immediately phosphorylated by hexokinase II, and the phosphorylated glucose is stored as glycogen or enters the glycolytic pathway for energy production. Thus, during feeding conditions, the main source for energy production in skeletal muscle is glucose.

Therefore, muscle energy metabolism has to be capable of switching from predominant oxidation of fatty acids during fasting state, to predominant oxidation of glucose during feeding state. However, obese and type 2 diabetic subjects are unable to shift between substrates (fatty acids or glucose) demonstrating a high degree of metabolic inflexibility (Kelley et al., 1999). This inability to oxidize one substrate or another results in impaired glucose and fatty acid storage as glycogen and triglycerides, respectively. These concepts of metabolic flexibility and inflexibility are documented by studies performed by Dave Kelley and co-workers (Kelley et al., 1999, Kelley et al., 2002a, Kelley et al., 2002b) and summarized by Storlien et al. in the "Proceedings of the Nutrition Society" (Storlien et al., 2004).

4.2. Pathogenesis of Insulin Resistance in Skeletal Muscle

Both obese subjects with or without T2DM have marked skeletal muscle insulin resistance compared to lean non-diabetic subjects. The severity of the insulin resistance positively correlates with BMI (DeFronzo, 1982, Wedick et al., 2009). The mechanism through which obesity causes insulin resistance in skeletal muscle seems to be associated with the accumulation of fatty acids in the myocytes. Among the various types of fatty acids, saturated long-chain ones, including palmitic and stearic acids, are strong inducers of insulin resistant state (Hirabara et al., 2009). Obese subjects with or without T2DM are characterized by an increase in plasma fatty acid concentration, which strongly correlates with reduced insulin-stimulated glucose disposal in skeletal muscle.

In normal conditions, fatty acids are stored in the adipose tissue as triglycerides and released during fasting. During the postprandial state, blood glucose stimulates insulin secretion, which inhibits lipolysis in adipose tissue, therefore limiting the release of fatty acids. In insulin resistant individuals, the ability of insulin to inhibit lipolysis and reduce plasma fatty acid concentration is markedly impaired (Groop et al., 1991). This leads to a chronic activation of lipolysis and higher plasma fatty acid levels. Several studies have demonstrated that chronically elevated plasma fatty acid levels cause insulin resistance in skeletal muscle(Bajaj et al., 2005, Boden, 1997).

One of the proposed mechanisms to explain how fatty acids impair glucose oxidation in skeletal muscle was postulated by Randle and colleagues more than 40 years ago (Randle et al., 1963). They observed that incubation of rat heart with fatty acids was associated with an increase in intracellular concentrations of glucose-6-phosphate (G6P) and glucose. Moreover, incubation of diaphragm muscle with fatty acids led to an increase in glycogen accumulation. According to "Randle's Theory", fatty acid oxidation increases the ratios acetyl coenzyme A/coenzyme A and NADH/NAD+ in the mitochondria, leading to the inactivation of pyruvate dehydrogenase (PDH). Accumulation of citrate inhibits phosphofructokinase and increases intracellular concentrations of G6P, leading to activation of glycogen synthesis, inhibition of hexokinase II, increase in intracellular glucose content and, consequently, reduction in glucose uptake. Thus, this model is based on the inverse relationship between fatty acid availability and glucose utilization. Increase free fatty acid availability inhibits glucose utilization through inhibition of key enzymes involved in glucose metabolism.

In contrast with Randle's hypothesis, Roden and colleagues (Roden et al., 1996) demonstrated that a reduction in muscle glycogen synthesis by elevated fatty acids concentration occurred after a decrease in muscle glucose-6-phosphate levels. Thus, these results demonstrate that free fatty acids induce insulin resistance in humans by initial inhibition of glucose transport/phosphorylation, which is then followed by a reduction in both the rate of muscle glycogen synthesis and glucose oxidation. Therefore, according to Roden et al., insulin resistance induced by fatty acids is primarily associated with impaired glucose uptake rather than glucose accumulation (Roden et al., 1996).

To establish which of these two possible effects takes place, Dresner and colleagues (Dresner et al., 1999) measured intra-myocellular concentrations of free glucose in healthy people under conditions of high and low plasma fatty acids concentrations. If there was a block at the hexokinase step, as proposed by Randle, intra-myocellular glucose concentrations would be expected to increase. Instead, they noted that plasma fatty acid concentrations decreased the accumulation of intra-myocellular glucose, indicating that insulin-stimulated glucose transport activity was reduced. These results, like others carried out by Cline and colleagues (Cline et al., 1999), confirmed Roden's work and suggested that in people with T2DM impairment of insulin action in skeletal muscle is due to reductions in insulin-stimulated glucose transport rather than glucose accumulation.

A second mechanism proposed to explain the pathogenesis of skeletal muscle insulin resistance is related to endoplasmic reticulum (ER) stress (Hotamisligil, 2010). ER is an intracellular membranous network responsible for synthesis, folding, maturation, trafficking and targeting of secreted and transmembrane proteins. It also plays a critical role as a regulator of Ca^{+} homeostasis and lipid biosynthesis. In some diseases, protein synthesis increases in ER-lumen and proteins cannot fold correctly, affecting ER homeostasis. Impairment of ER homeostasis activates an elaborate adaptive stress response, known as "unfolded protein response" (UPR), and results in the phosphorylation and activation of JNK. The link between T2DM, insulin resistance and ER stress in skeletal muscle is still

unclear. It has been demonstrated that ER stress occurs *in vivo* in skeletal muscle when mice are fed a high fat diet (Deldicque et al., 2010a). In another study (Deldicque et al., 2010b), the same authors observed that subjects on high fat diet had increased lipid content and insulin resistance in skeletal muscle with no change in ER stress markers.

Inflammation has also been proposed as a potential mechanism involved in the development of impaired insulin sensitivity. Fatty acids activate inflammatory signals by promoting secretion of pro-inflammatory cytokines including TNFalpha, IL-1beta, and IL-6. Furthermore, fatty acids can directly interact with members of the Toll-like receptor (TLR) family, promoting activation of JNK and IKKbeta. This activation leads to degradation of the inhibitor of kappa beta (IKB) and Nuclear factor-kappa beta (NFKB) activation. This is associated with a decrease in insulin action due to the phosphorylation of IRS-1. A study carried out by Tsukumo and co-workers demonstrated that mice containing a loss of function mutation in the *tlr4* gene (toll-like receptor 4) were partially protected from lipid-induced muscle inflammation (Tsukumo et al., 2007), highlighting the importance of this receptor in skeletal muscle insulin sensitivity.

5. Biology of the mitochondria

Mitochondria are doubled-membrane organelles that constitute the major site for oxidative energy production in the cell. Mitochondria are the only mammalian organelles that contain extra-nuclear DNA (mtDNA), which encodes for 37 genes including 13 subunits of the electron transport chain (Kelly and Scarpulla, 2004). Besides generating the majority of cellular ATP via oxidative phosphorylation (OXPHOS), many other essential cellular functions take place in this organelle. Examples of these include the generation of numerous metabolites via the tricarboxylic acid (TCA) cycle, oxidative catabolism of amino acids and fatty acids, synthesis of ketone bodies, ornithine cycle (also known as the urea cycle), control of cytoplasmic reticulum and calcium signaling (Murgia et al., 2009, Rimessi et al., 2008), synthesis of cellular Fe/S clusters that are essential cofactors for protein translation and DNA repair (Lill and Muhlenhoff, 2008) and generation of reactive oxygen species (ROS) with important signalling functions (Starkov, 2008, Murphy, 2009) and potential damaging consequences.

5.1. Oxidative phosphorylation (OXPHOS)

Mitochondria are able to generate energy by oxido-reduction reactions and proton translocation derived from carbohydrates (TCA cycle), amino acids and fatty acids (β-oxidation). For this purpose, oxygen is consumed to generate water, heat and adenosine triphosphate (ATP). The inner membrane invaginations of the mitochondria, called cristae, contain all transmembrane proteins of the electron transfer system (ETS) and the ATP synthase (Benard and Rossignol, 2008, Vonck and Schafer, 2009). All components of the TCA cycle and β-oxidation pathway are located inside the mitochondrial matrix. Oxidation of substrates generates reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide (FADH2) that will provide electrons to the ETS. Four different complexes –named from complex I to complex IV- form the ETS. Electrons flow from donors

(NADH at complex I and FADH2 at complex II) to an oxygen molecule forming H_2O at complex IV. There is a parallel translocation of protons to the intermembrane space from the matrix that creates an electrochemical gradient used by ATP synthase in a coupled manner to generate ATP. This electrochemical gradient can also dissipate through uncoupling proteins (UCPs) using a non-ATPase-coupled proton leak and generating heat in a process called thermogenesis. The high electronegative potential generated can also drive the entry of calcium into the matrix. Another phenomenon is the loss of electrons during the ETS that can result in generation of reactive oxygen species.

5.2. Mitochondrial biogenesis and dynamics

Mitochondrial biogenesis is defined as the generation of more mitochondrial mass and takes place in response to increased energy demand. These organelles have a heterogeneous morphology due to its dynamic nature. Mitochondrial dynamics is a relatively novel concept that includes movement of mitochondria along the cytoskeleton, regulation of mitochondrial architecture (morphology and distribution), and connectivity mediated by tethering and fusion/fission events (Liesa et al., 2009). Mitochondrial fusion/fission events allow the transcriptional products of mtDNA along with multiple metabolites to be shared within the mitochondrial reticulum. It has been recently established that mitochondrial fission and fusion contribute to multiple essential functions including calcium handling, ROS production and energy output (Chen and Chan, 2005, Parone et al., 2008, Soubannier and McBride, 2009). The relevance of these events in mitochondrial and cell physiology has been partially unraveled and observed that the disruption of such processes results in mitochondrial heterogeneity and dysfunction (Zorzano et al., 2009, Chan, 2006). Therefore, a fine-tune regulation of mitochondrial biogenesis and dynamics is necessary to obtain and maintain functional mitochondria.

Mitochondrial biogenesis is a complex process that requires the expression of a large number of proteins encoded by both nuclear and mitochondrial genomes. The mitochondrial genome encodes only 13 proteins, which are essential subunits of the respiratory complexes. This genome also provides the 22 tRNAs and 2 rRNAs necessary for the translation of these mitochondrial-encoded proteins. In contrast, transcription of the mitochondrial genome is encoded by the nuclear genome, which is under the control of a single transcription factor named TFAM. Other components needed for the transcription of the mitochondrial genome, including POLRMT, TFB1M, TFB2M, and mTERF are also encoded by nuclear genes. Therefore, fine-tuned coordination is required between the mitochondrial and the nuclear genomes to orchestrate the expression of proteins necessary for a successful mitochondrial biogenesis. This coordination is achieved by complex regulatory mechanisms that involve the action of a relatively small number of nuclear transcription factors, which are discussed in detail below. These transcription factors are in turn regulated by cofactors that integrate physiological signals with the activity of the transcription factors to regulate mitochondrial biogenesis in response to environmental stimuli. Among the most important cofactors are the PGC-1 coactivator family members, which are also discussed in more detail below (Kelly and Scarpulla, 2004).

5.3. Nuclear transcription factors involved in mitochondrial biogenesis

Through their DNA-binding domain, transcription factors bind to specific sequences in the gene promoter region to regulate transcription of a subset of genes. Several transcription factors have been shown to regulate expression of genes involved in the respiratory chain and mitochondrial metabolism, however only a few are considered the major transcription factors crucial for mitochondrial biogenesis.

5.3.1. Nuclear Respiratory Factor 1 (NRF-1)

NRF-1 has a fundamental role in coordinating nuclear and mitochondrial transcription. It induces expression of TFAM, TFB1M and TFB2M (Virbasius and Scarpulla, 1994, Gleyzer et al., 2005), which are essential proteins for the transcription of the mitochondrial genome, and also TOMM20, a key protein required for the transport of nuclear-encoded proteins into the mitochondria. It has also been shown to regulate multiple subunits of the respiratory chain as well as other proteins involved in other mitochondrial functions. Disruption of the NRF-1 gene in mouse models results in mtDNA depletion and impaired mitochondrial membrane potential with an early embryonic lethal phenotype (Huo and Scarpulla, 2001).

5.3.2. Nuclear Respiratory Factor 2 (NRF-2/GABP)

A second nuclear respiratory factor was identified based on its ability to induce expression of a subunit of cytochrome c oxidase, COXIV, and was found to be a complex of the DNA-binding subunit alpha (GABPalpha) and four other subunits (beta1, beta2, gamma1, and gamma2). This respiratory factor was named NRF-2 and was subsequently identified as the human homolog of the mouse GABP (Virbasius et al., 1993). NRF-2 has been shown to regulate expression of key proteins involved in mitochondrial biogenesis and function, including TFAM, TFB and all cytochrome C oxidase isoforms (Gleyzer et al., 2005, Ongwijitwat and Wong-Riley, 2005, Virbasius et al., 1993). Similarly to NRF-1, disruption of the NRF-2 gene also produces a lethal phenotype (Ristevski et al., 2004).

5.3.3. Estrogen-Related Receptor alpha (ERRalpha)

ERRalpha mediates expression of a wide range of genes, including those responsible for fatty acid uptake and oxidation as well as genes for oxidative phosphorylation (Mootha et al., 2004, Huss et al., 2002). Although structurally related to the estrogen receptor, ERRalpha does not bind estrogen. Instead, it is a member of a family of orphan nuclear receptors that also include ERRbeta and ERRgamma. Unlike NRF-1 and NRF-2 where gene knockout proves lethal, disruption of ERRalpha results in a viable phenotype showing decreased body weight and adipose depot size (Luo et al., 2003). This mouse shows normal energy expenditure with no major decrease in mitochondrial proteins. This can be explained by compensation by the other members of the transcription factor family.

5.3.4. Other transcription factors

While not directly involved in transcription of mitochondrial biogenesis or respiratory chain genes, other transcription factors including PPARalpha, PPARdelta, and YY1 are also important for providing other mitochondrial proteins. PPARalpha is responsible for expression of lipid metabolism and mitochondrial fatty acid oxidation genes (Lee et al., 1995, Leone et al., 1999). Through inducible tissue-specific loss of function knockout mouse models, PPARdelta has been shown to regulate mitochondrial biogenesis in skeletal muscle and heart (Schuler et al., 2006, Wang et al., 2010). Finally, YY1 has been shown to activate cytochrome c expression (Seelan and Grossman, 1997, Basu et al., 1997) as well as several key genes for mitochondrial respiration (Cunningham et al., 2007).

5.3.5. PGC-1 coactivator family

While the transcription factors discussed above are part of the transcriptional machinery necessary for mitochondrial biogenesis, the members of the PGC-1 coactivator family provide the integration of physiological stimuli with the transcription factors to adapt mitochondrial biogenesis to changes in the environment. PGC-1 coactivators lack a DNA-binding domain, but they are able to interact with and activate several transcription factors by recruiting other cofactors with chromatin-remodeling activities (Monsalve et al., 2000). PPARgamma coactivator 1alpha (PGC-1alpha), the founding member of the PGC-1 family, was first identified by its ability to activate PPARgamma in brown adipocytes (Puigserver et al., 1998). PGC-1beta and PRC where subsequently identified based on their structural similarity with PGC-1alpha (Lin et al., 2002a, Andersson and Scarpulla, 2001). Interestingly, NRF-1, NRF-2, ERRalpha, YY1 and the PPAR family members are among the transcription factors the PGC-1 family members are able to coactivate (Wu et al., 1999, Cunningham et al., 2007), underlying the importance of these coactivators in the regulation of mitochondrial biogenesis. This role of PGC-1alpha and PGC-1beta in mitochondrial gene expression is well documented in gain of function experiments, where increased expression of these coactivators in skeletal muscle results in an induction of a wide array of genes involved in mitochondrial biogenesis and function (Wu et al., 1999, Lin et al., 2002b, Arany et al., 2007). Furthermore, muscle-specific disruption of PGC-1alpha gene in mice shows decreased expression of mitochondrial genes, resulting in a switch from oxidative fibers to more glycolytic fibers, impairing their endurance capacity (Handschin et al., 2007).

PGC-1alpha is highly regulated at both the transcriptional level and post-translational level, primarily through phosphorylation (Jäger et al., 2007, Li et al., 2007, Rodgers et al., 2010) and acetylation (Lerin et al., 2006, Rodgers et al., 2005). It is this regulatory capacity that allows PGC-1alpha to respond to physiological stimuli and activate the mechanisms leading to increased mitochondrial biogenesis. PGC-1alpha activity is determined by its acetylation status, regulated by the balance between acetylation mainly by the histone acetyltransferase GCN5 (Lerin et al., 2006) and deacetylation largely by the NAD+ dependent deacetylase SIRT1 (Rodgers et al., 2005). This acetylation/deacetylation regulatory mechanism is

involved in the integration of nutrient sensing with transcriptional regulation of mitochondrial genes in skeletal muscle (Gerhart-Hines et al., 2007). In this context, caloric restriction has been proposed to increase mitochondrial biogenesis at least in part through activating SIRT1 and inducing PGC-1alpha deacetylation increasing its transcriptional activity (Baur et al., 2006, Lagouge et al., 2006). Physical exercise has also been recognized as a main activator of mitochondrial biogenesis. In the muscle cell, the AMP-dependent protein kinase (AMPK) responds to low energy levels (increase in AMP content) by inducing a signaling cascade that results in the activation of catabolic pathways and inhibition of anabolic pathways in an attempt to restore energy levels. Therefore, AMPK has been recognized as a key mediator in the physiological and metabolic adaptation to physical exercise. Interestingly, AMPK can directly phosphorylate PGC-1alpha and activate its transcriptional activity regulating expression of mitochondrial genes (Jäger et al., 2007). Furthermore, it has been recently shown that AMPK activation results in a net increase of NAD+ levels with the consequent induction of SIRT1 activity and PGC-1alpha deacetylation (Canto et al., 2010).

6. Mitochondrial dysfunction as a potential mechanism underlying skeletal muscle insulin resistance

Mitochondrial adaptations (biogenesis and dynamics) and function largely affect muscle metabolism and have a significant impact on whole-body metabolism (Patti et al., 2010). As mentioned before, metabolic flexibility is defined as the ability to rapidly modulate substrate oxidation as a function of environmental, hormonal and different energy conditions (Storlien et al., 2004). Defects in pathways controlling glucose and energy homeostasis in skeletal muscle have been shown to impair these adaptations, leading to metabolic inflexibility. What it is important for the role played by mitochondrial dysfunction in T2DM etiology is that this state of metabolic inflexibility is a hallmark of the development of skeletal muscle insulin resistance (Storlien et al., 2004).

Mitochondrial dysfunction is a term that could imply several definitions due to the multiple functions that take place in this organelle. For the purposes of this chapter, we will define mitochondrial dysfunction as both the reduction in mitochondrial oxidative activity and in mitochondrial adenosine triphosphate (ATP) synthesis. Although mitochondrial dysfunction is related to a broad range of diseases, in this chapter we will focus on mitochondrial respiratory dysfunction related to muscle insulin resistance and T2DM etiology.

6.1. Early evidences relating insulin resistance and skeletal muscle mitochondrial dysfunction

Several key studies published between 1999 and 2005 laid the foundation for understanding the underlying mechanisms between mitochondrial dysfunction and subsequent insulin resistance in skeletal muscle and development of T2DM. Significant results from these studies are summarized below.

6.1.1. Dysregulation of skeletal muscle fat oxidation in obesity

The first studies that identified a relationship between alterations in muscle metabolism and insulin resistance did not mention any link with mitochondrial dysfunction (Kelley et al., 1999). However, research performed by Kelley and co-workers addressed why the pattern of fatty acid utilization in skeletal muscle during fasting conditions might be associated with obesity-related insulin resistance, which is relevant for the scope of this chapter. The study included 16 lean and 40 obese volunteers with leg balance measurements of glucose and free fatty acid uptake. Indirect calorimetry across the leg was also measured in order to determine substrate oxidation during fasting and insulin-stimulated conditions. This study demonstrated that fatty acids were the predominant substrate oxidized by skeletal muscle during fasting conditions in lean subjects. However, rates of fatty acid oxidation during fasting were significantly lower in obese subjects, even though rates of fatty acid uptake were similar to those of lean subjects. Furthermore, the respiratory quotient values across the leg showed a reduced reliance on lipid oxidation in obese subjects. What it is also important is that weight loss only partially improved these patterns; the leg respiratory quotient in obese subjects was unchanged between pre- and post-weight loss, so the reliance of skeletal muscle in fat oxidation during fasting conditions was not improved. The authors suggested that their data pointed to these defects as primary impairments leading to obesity, rather than resulting from obesity. Based on these data and previous observations from the same group (Kelley et al., 1993, Kelley et al., 1999) it could be concluded that the elevated intra-myocellular lipid accumulation in skeletal muscle of obese subjects derives from a reduced capacity for fatty acid oxidation, and this inflexibility in regulating fatty acid oxidation rates, more than fatty acid uptake itself, is related to insulin resistance.

6.1.2. Muscle mitochondria in obesity and type 2 diabetes

In this study, Kelley and co-workers provided early evidence that mitochondrial dysfunction in human skeletal muscle contributes to the development of insulin resistance and progression to T2DM (Kelley et al., 2002b). Previous work by the same group demonstrated that the severity of skeletal muscle insulin resistance in T2DM and obesity is related to diminished activity of oxidative enzymes (Simoneau and Kelley, 1997). Furthermore, triglyceride accumulation in skeletal muscle is also correlated with the severity of insulin resistance and with diminished oxidative enzyme activity. Because it was known that skeletal muscle depends on oxidative phosphorylation to produce energy and that insulin resistance in T2DM and obesity involves altered oxidation of carbohydrates and lipids, the authors attempted to elucidate the potential contribution of mitochondrial dysfunction to skeletal muscle insulin resistance in humans. For this purpose *vastus lateralis* muscle samples from lean controls without T2DM, obese subjects with or without T2DM were obtained. An assessment of the activity of the mitochondrial OXPHOS system and a quantitative study of the mitochondria morphology by transmission electron microscopy was performed in the different muscle biopsies. Creatine kinase and citrate synthase activities were measured as markers of muscle fiber content and mitochondrial content, respectively. Results showed that skeletal muscle mitochondria structure and functional capacity were impaired in T2DM subjects and, to a lesser degree, in obese subjects.

Mitochondrial respiratory complex I activity was reduced by 40% in skeletal muscle from subjects with T2DM when compared to lean controls without diabetes. Moreover, skeletal muscle mitochondrial area and size were smaller in obesity and T2DM and, in some instances, particularly in T2DM, severely damaged. Although age can affect the size of mitochondria, in this case aging did not account for the ~30% reduction in size in obesity and T2DM.

Based on their results, authors proposed a potential mechanism that could explain how impaired mitochondrial function leads to insulin resistance in skeletal muscle, which would be lipid accumulation within myocytes. This was not a new finding, as previous studies (Kelley et al., 2002a) from the same group had shown that increased lipid accumulation in skeletal muscle is associated with insulin resistance and, in turn, lipid accumulation in skeletal muscle in obesity and T2DM is related to a reduced oxidative enzyme activity. Therefore, based on their findings, the authors stated that impaired mitochondrial functional capacity in skeletal muscle can lead to insulin resistance and further T2DM.

6.1.3. Downregulation of oxidative metabolism genes in humans with insulin resistance and diabetes

Patti and colleagues addressed how gene regulation was modulated by T2DM (Patti et al., 2003). High-density oligonucleotide arrays were performed using mRNA samples from skeletal muscle of people with or without T2DM and with prediabetes (insulin-resistant subjects at high risk for T2DM) to identify genes differentially expressed. The results showed that skeletal muscle from subjects with prediabetes and T2DM had decreased expression of oxidative phosphorylation genes, many of which are regulated by nuclear respiratory factor (NRF)-dependent transcription. A decreased expression of the co-activators PGC-1alpha and PGC-1beta, both of which induce NRF-dependent transcription, was also found. Therefore, subjects with insulin resistance and T2DM have a reduced expression of multiple (NRF-1)-dependent genes encoding key enzymes in oxidative metabolism and mitochondrial function. It seems that PGC-1 expression may be responsible for decreased expression of NRF-dependent genes due to alterations in the primary sequence and environmental risk factors for T2DM such as aging, fiber type composition, insulin resistance itself and inactivity, subsequently leading to the metabolic disturbances characteristic of insulin resistance and T2DM.

6.1.4. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes

This study extended the results obtained by Patti and colleagues (Patti et al., 2003). Mootha and co-workers used Gene Set Enrichment Analysis, designed to detect modest but coordinated changes in the expression of groups of functionally related genes to study differential expression among healthy individuals, impaired glucose tolerance subjects, and subjects with T2DM (Mootha et al., 2003). They named OXPHOS-CR to a subset of genes, which include about two-thirds of the OXPHOS genes, strongly expressed in skeletal muscle,

heart and brown adipose tissue. No relationship was found between body mass index (BMI) or waist-to-hip ratio and OXPHOS-CR expression, and neither between quantitative measures of fiber types and OXPHOS-CR. However, expression of OXPHOS-CR correlated positively with the aerobic capacity of the individuals under study and negatively to diabetes. In summary, a set of genes involved in oxidative phosphorylation, whose expression was coordinately decreased in skeletal muscle of T2DM subjects, were identified. Thus, authors hypothesized that the decreased expression of OXPHOS-CR genes might contribute to T2DM. Expression of this gene set was induced by PGC-1alpha, which expression is higher in tissues of insulin-mediated glucose disposal and correlated with total body aerobic capacity.

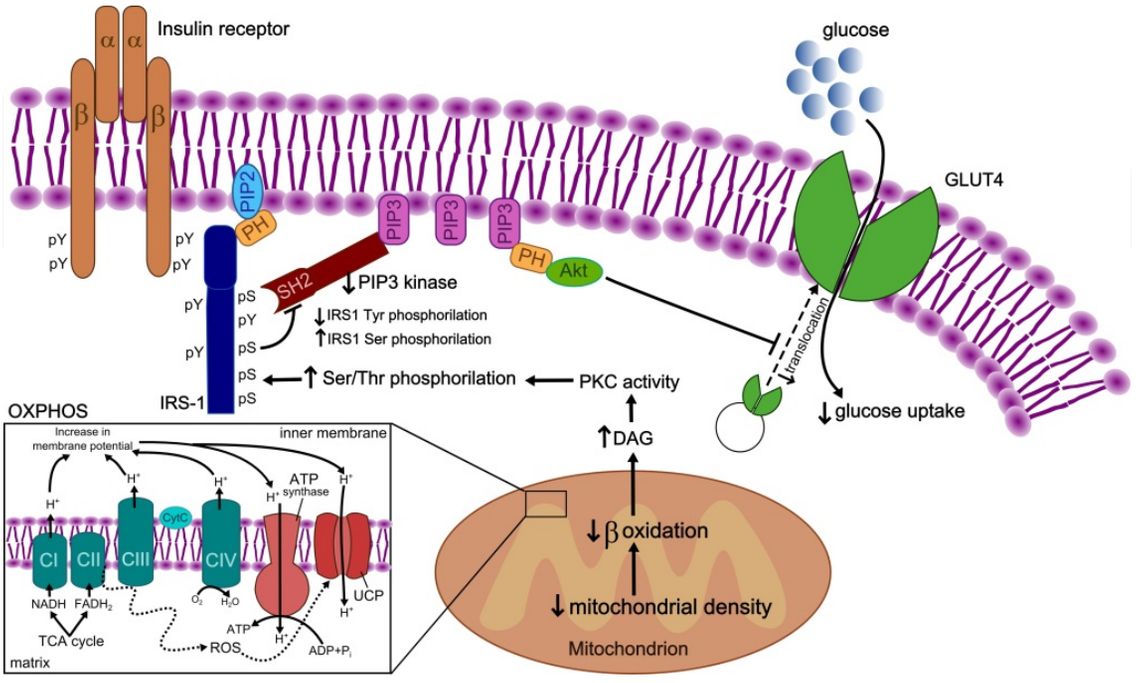
6.1.5. Impaired mitochondrial activity in insulin-resistant offspring of subjects with T2DM

In this study Petersen and co-workers aimed to determine the potential mechanism for the intra-myocellular accumulation of lipids leading to insulin resistance (Petersen et al., 2004). As previously stated, increases in intra-myocellular triglyceride content can occur as a result of increased delivery of fatty acids from lipolysis and/or decreased rates of mitochondrial oxidative phosphorylation. Young and lean insulin-resistant offspring of subjects with T2DM and insulin-sensitive subjects were studied. To test their hypotheses, authors utilized hyperinsulinemic-euglycemic clamps in these subjects to measured intra-myocellular lipid and intrahepatic triglyceride content, assessed whole-body and subcutaneous fat lipolysis rates and determined mitochondrial oxidative-phosphorylation activity in muscle by magnetic resonance spectroscopy. The insulin-stimulated rate of glucose uptake was 60% lower in the insulin-resistant subjects, which could be explained by a 70% reduction in insulin-stimulated non-oxidative muscle glucose metabolism. They also observed an 80% increase in intra-myocellular lipid content and a 30% reduction in mitochondrial oxidative phosphorylation, suggesting that subjects with T2DM have an inherited reduction in mitochondrial content in muscle, which in turn may be responsible for the reduced rates of mitochondrial oxidative phosphorylation. In summary, their data supports the hypothesis that insulin resistance in skeletal muscle of insulin-resistant offspring of subjects with T2DM is linked to an impairment of fatty acid metabolism, potentially due to an inherited defect in mitochondrial oxidative phosphorylation (Petersen et al., 2004).

6.2. Putting the pieces together, the link between mitochondrial dysfunction and T2DM

In the year 2005 Drs. Lowell and Shulman wrote a viewpoint where they hypothesized that insulin resistance and hyperglycemia could be caused by a primary mitochondrial dysfunction (Lowell and Shulman, 2005). Insulin resistance occurs due to the accumulation of intracellular fatty acyl CoA and diacylglycerol, which in turn activate critical signal transduction pathways, leading to suppression of the insulin signaling pathway. Among the metabolic perturbations that caused accumulation of fatty acids in liver and/or skeletal muscle and defects in the ability of these organs to metabolize fatty acids, mitochondrial dysfunction seemed to be the reason for inducing insulin resistance. The suggested

hypothesis was that insulin resistance in humans arises from defects in mitochondrial fatty acid oxidation, which in turn lead to increases in intracellular fatty acid metabolites such as fatty acyl CoA and diacylglycerol that disrupt insulin signaling pathway (Figure 2).



OXPHOS, oxidative phosphorylation; CI-CII-CIII-CIV complexes I, II, III and IV within the oxidative phosphorylation system; CytC, cytocrome C; TCA, tricarboxylic acid cycle;NADH, nicotinamide adenine dinucleotide; FADH2, flavin adenine dinucleotide (hydroquinone form); ATP, adenosine triphosphate; ADP, adenosine diphosphate; Pi, inorganic phosphate; DAG, diacylglycerol; Thr, threonine; Ser,S, serine; Tyr,Y, tyrosine; IRS-1, insulin receptor substrate 1; PIP2, phosphatidyil inositol 2; PH, pleckstrin homology domain of the IRS-1; PIP3, phosphatidyl inositol 3; SH2, Src homology 2 domain; Akt, also known as protein kinase B; GLUT4, glucose transported type 4.

Figure 2. Fat-induced insulin resistance hypothesis in skeletal muscle.

T2DM and obesity characterize by chronic nutrient oversupply that can lead to an increase of reducing equivalents (NADH and FADH2) which increases the flux through the oxidative respiratory system (OXPHOS) in the inner membrane in the mitochondria and, in the absence of increased ATP demand, results in ROS generation creating oxidative stress. ROS increased concentration would lead to a diminished mitochondrial density/content or an inherited condition that in turn lead to a mitochondrial dysfunction and consequent decrease in beta-oxidation increasing the intra-myocellular long-chain fatty acids and diacylglycerol (DAG) concentrations. DAG would then activate the phosphorylation of serines and threonines of the insulin receptor susbtrate 1 (IRS-1) through enzymes such as protein kinases C (PKC). PKCs activate the serine kinase cascade and increase the IRS-1 serine (Ser, S) phosphorylation of the the insulin receptor susbtrate 1 (IRS-1). The phosphorylation of serines located in critical sites leads to a blockage of the IRS-1 tyrosines (Tyr, Y) phosphorylation by the insulin receptor, inhibiting insulin-induced phosphatidyl inositol 3-kinase activity (PI3-kinase) resulting in a decreased insulin-stimulated Akt activity. Akt reduced activity fails to activate the translocation of GLUT4 to the membrane, diminishing the insulin-induced glucose uptake and impairing the removing of glucose from blood.

It is still uncertain whether skeletal muscle mitochondrial dysfunction is a cause or rather a consequence of the metabolic derangements that contribute to insulin resistance in T2DM, including lipid accummulation, pro-inflammatory signals or endoplasmic reticulum stress. However, given its complex pathophysiology, establishing causality has proved difficult and the mechanisms leading to insulin resistance remain elusive.

7. Current status of the topic

It is well established that mice and rats with a chronic exposure to a high fat diet (HFD) develop obesity, insulin resistance and, in the long term, T2DM (Oakes et al., 1997, Surwit et al., 1988). Furthermore, it has been shown that skeletal muscle mitochondrial content is diminished in T2DM individuals (Patti et al., 2003, Kelley et al., 2002b). These features, together with impaired energy substrate utilization and the observation that these deleterious effects are not restricted to skeletal muscle, led to the hypothesis that mitochondrial dysfunction plays a major role in T2DM etiology (Lowell and Shulman, 2005). Since the publication of this hypothesis, there has been a growing interest in further assessing the potential implication of mitochondrial function in the etiology of this metabolic disease. One of the first attempts to clearly prove this hypothesis used transgenic mice with defective mitochondria in order to observe whether they would develop T2DM (Pospisilik et al., 2007). Earlier reports from this same group have shown that conditional deletion of apoptosis inducing factor (AIF) provokes OXPHOS dysfunction (Vahsen et al., 2004). Initially, AIF was considered as a mitochondrial protein involved in signaling events leading to cell death. Subsequent studies have demonstrated that the primary physiological role of AIF is the maintenance of an efficient mitochondrial respiratory system. Studies assessing whole body glucose homeostasis and diet-induced obesity and diabetes either in tissue specific (liver and skeletal muscle) AIF knockout mice or in mice with ubiquitous OXPHOS defects showed that these mice were more insulin sensitive and were protected against diet-induced obesity and diabetes, in contrast with previous hypotheses (Pospisilik et al., 2007). Recently, this observation has been confirmed in another study using rats fed with an iron-deficient diet, which provokes a reduction in the iron containing proteins of OXPHOS (Han et al., 2011). Thus, rats under a high fat and iron-deficient diet are protected against high fat diet-induced insulin resistance in skeletal muscle despite a lower fatty acid oxidation capacity (Han et al., 2011).

7.1. Controversy about the effects of high fat diet feeding in skeletal muscle oxidative capacity

In 2007, two different studies were published addressing whether a high fat diet (HFD) decreases or improves skeletal muscle mitochondrial oxidative capacity. In one (Garcia-Roves et al., 2007), rats were fed with a HFD during 4 weeks in order to raise circulating fatty acids and therefore to study the mechanisms that regulate the already reported improved fatty acid oxidation capacity of glycolytic skeletal muscle. Rats fed with the HFD regime showed higher fatty acids content, increased skeletal muscle fatty acid oxidative

capacity in the epitrochlearis (glycolytic muscle), increased expression of enzymes of the fatty acid oxidation pathway and increased protein content of carboxylic acid cycle and OXPHOS system markers. Furthermore, this study showed that this metabolic adaptation occurs through activation of the peroxisome proliferated activated receptor delta (PPARdelta), a nuclear receptor responsible for regulating transcription of enzymes that belong to the fatty acid oxidation pathway and mitochondrial biogenesis process. Fatty acids, mostly unsaturated, are ligands and activators of PPARs, which explain the metabolic regulations observed in this study (Garcia-Roves et al., 2007). Similar results were published, almost simultaneously, by Cooney and colleagues in mice (Turner et al., 2007). C57BL/6J mice were on a HFD either for 5 or 20 weeks. In both periods of time HFD mice showed an increased capacity to oxidize fatty acid in skeletal muscle, concomitantly with an increased enzymatic activity of key proteins in the fatty acid oxidation pathway and higher protein content of different mitochondrial markers. Most importantly, these improvements in fatty acid handling and mitochondrial respiration in fat-fed mice occurred at the time these animals showed skeletal muscle insulin resistance and impaired whole body glucose handling (Turner et al., 2007). These observations were corroborated in the same study using a rat model of obesity (Zucker rats on a HFD) or a mouse model of diabetes and obesity, the db/db mouse (Turner et al., 2007). One year later Holloszy's laboratory, in a follow-up of Garcia-Roves' study, showed that rats fed with two different high fat diets (50% of energy from flax seed/olive oil or lard/corn oil) had skeletal muscle insulin resistance with improved mitochondrial content (Hancock et al., 2008). Therefore, results from these studies led researchers to question whether mitochondrial dysfunction plays a major role in the etiology of T2DM. It seems more probable that skeletal muscle insulin resistance per se or skeletal muscle inactivity could be the origin of the observed decrease in mitochondrial content in subjects with T2DM.

As described over these lines it is uncertain whether skeletal muscle mitochondrial dysfunction plays a critical role in the development of T2DM. We continue the chapter presenting the more recent studies on this topic. First, we will address human based studies and afterwards recent animal studies that provide some relevant information into the field.

7.2. Human studies

It is important to note that in skeletal muscle two different mitochondrial populations exist: subsarcolemmal and intermyofibrillar. Subsarcolemmal mitochondria are located beneath the sarcolemma and have a lower oxidative rate than intermyofibrilar mitochondria. Furthermore, both mitochondrial populations play different roles in skeletal muscle metabolism and, therefore, respond differently to physiological or pathophysiological situations (Palmer et al., 1977). Kelley and coworkers compared both skeletal muscle mitochondrial subpopulations in lean, obese, and T2DM subjects (Ritov et al., 2005). The main observation of this study was that the electron transfer system was dramatically reduced in the subsarcolemmal mitochondrial population in obese subjects, and even to a higher degree in T2DM subjects when compared to lean controls. However, physical activity level was not controlled, therefore it is unknown whether differences in mitochondrial

activity could be related to this parameter. Thus, the authors concluded that an impaired subsarcolemmal mitochondrial activity may have a critical role in the pathogenesis of insulin resistance in T2DM (Ritov et al., 2005).

Similar conclusions are obtained in a study carried out by Schrauwen-Hinderling and colleagues (Schrauwen-Hinderling et al., 2007) where they attempted to distinguish between the relevance of intra-myocellular lipid content and mitochondrial dysfunction in skeletal muscle insulin resistance. For this purpose, they compared overweight subjects with high intra-myocellular lipid content to weight-matched T2DM subjects. The study showed that T2DM subjects had impaired mitochondrial function and similar levels of intra-myocellular lipid content when compared to weight-matched controls. These data provide more evidence supporting the link between mitochondrial dysfunction and T2DM, although they do not imply causality (Schrauwen-Hinderling et al, 2007). Similar results were found in a subsequent study where Ritov and colleagues compared mitochondrial function in lean, obese and obese-T2DM subjects (Ritov et al., 2010). Along with this idea, Shulman and co-workers performed a study on insulin resistant offspring of T2DM subjects. Offspring subjects with insulin resistance showed a decreased oxidative phosphorylation capacity and increased intra-myocellular lipid content. These results led the authors to conclude that a limited mitochondrial fatty acid oxidative capacity leads to an increased lipid accumulation and subsequently to skeletal muscle insulin resistance. However, the mechanism by which skeletal muscle intralipid accumulation leads to insulin resistance needs still to be elucidated (Befroy et al, 2007). Conversely, in another study performed in subjects with and without T2DM (Asmann et al., 2006), mitochondrial ATP production rate under controlled insulin and glucose levels at the post-absorptive state (low glucose, low insulin levels) and post-pandrial state (high glucose, high insulin levels) was evaluated. The authors found no major differences between subjects with and without T2DM at the post-absortive state. A lower response in the mitochondrial ATP production rate when insulin and glucose rose to achieve post-pandrial state values was observed in T2DM subjects. This study showed how skeletal muscle mitochondrial defects are not intrinsic in subjects with T2DM but are related to impaired insulin action (Asmann et al., 2006). Thus, it is difficult to discern whether mitochondrial dysfunction is a cause or rather a consequence of skeletal muscle insulin resistance due to the cross-sectional nature of most of the human studies carried out.

In 2007 Dela and co-workers added some relevant information to the field using high resolution respirometry to compare skeletal muscle mitochondrial function between T2DM and control subjects (Boushel et al., 2007). T2DM subjects showed a reduced OXPHOS and electron transport capacity when compared to control individuals, as previously observed. However, when the respiratory parameters were normalized by mitochondrial content the authors observed that T2DM subjects did not show mitochondrial dysfunction (Boushel et al., 2007). Therefore, skeletal muscle respiratory capacity could be diminished in T2DM subjects due to a reduction in mitochondrial content and not due to specific defects in the respiratory phosphorylation system. As mentioned before, the observation of a reduced mitochondrial content could be due to a lack of physical activity rather than to a genetic defect in these insulin resistant individuals. These results, showing that T2DM subjects have

similar mitochondrial respiratory capacity when normalized by mitochondrial content, were corroborated by a later study performed in lean and obese insulin resistant subjects by Mandarino and co-workers (Lefort et al., 2010). When mitochondrial function was assessed in isolated skeletal muscle mitochondria from lean and obese-insulin resistant subjects a similar maximal respiration rate was observed in both groups. However, obese-insulin resistant subjects showed a higher reactive oxygen species (ROS) production, which could influence insulin signaling. Additional information was obtained with the comparative analysis of mitochondrial proteins from lean and obese insulin resistant subjects by high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry pointing out to a lower content in obese-insulin resistant subjects for some of the complex I subunits, less carnitine palmitoyltransferase 1B (key enzyme for fatty acid oxidation) and lower content of enzymes involved in branched amino acid metabolism. These differences in skeletal muscle mitochondrial proteins from lean and obese-insulin resistant subjects could explain why there is a higher intra-myocellular lipid accumulation in insulin resistant muscles, a higher ROS production and an improper branched amino acid oxidation (Lefort et al., 2010). Another issue that had not been previously addressed was mitochondrial sensitivity (mitochondrial respiratory performance under submaximal concentrations of substrates). This point was addressed by Dela and co-workers in T2DM subjects, obese controls and age-matched lean individuals. T2DM subjects showed enhanced mitochondrial substrate sensitivity when compared to obese controls. This better mitochondrial substrate handling is limited to non-lipid substrates (malate, glutamate, and succinate) and it is unrelated with maximal respiratory capacity, due to the fact that maximal oxidation rates were the same for the different substrates and experimental groups under assessment (Larsen et al., 2011).

More information, discounting the hypothesis linking mitochondrial dysfunction with the pathogenesis of insulin resistance and subsequently T2DM, arrives from studies of low birth weight (LBW) individuals. It has been previously established that LBW is a risk factor of insulin resistance and T2DM (Phillips, 1998). A recent study by Brons et al. showed that young lean LBW subjects had abnormal glucose metabolism when compared to normal birth weight (NBW) controls, but skeletal muscle mitochondrial ATP production (both in arms and legs) and expression of oxidative phosphorylation genes were similar between LBW and NBW subjects (Brons et al., 2008). More clear evidence against the theory that mitochondrial dysfunction leads to insulin resistance and T2DM was supplied in a follow-up study (Brons et al., 2012). Brons and co-workers assessed skeletal muscle mitochondrial function in LBW and NBW subjects before and after 5 days of high fat diet overfeeding. LBW subjects developed peripheral insulin resistance after high fat diet overfeeding but without any detrimental effect on mitochondrial oxidative capacity (Brons et al., 2012).

7.2.1. Differences in between skeletal muscles, aerobic capacity and aging

The comparison of mitochondrial function in arm and leg skeletal muscles from obese control and T2DM subjects supplies additional and relevant information to determine whether mitochondrial dysfunction is implicated in T2DM etiology. Mitochondrial

respiration, when quantified per milligram of wet tissue or citrate synthase activity, was similar in permeabilized muscle fibers from deltoids muscle biopsies of T2DM and obese-control subjects. However, when mitochondrial respiration was measured from the *vastus lateralis* muscle a significant reduction was observed in T2DM subjects, as previously reported in other studies (Rabol et al., 2010). These results highlight the importance of assessing the physical fitness of the individuals, as this factor could explain the differences found in leg muscles oxidative capacity when healthy individuals and T2DM subjects are compared. The finding that muscle respiratory capacity is not impaired in arm muscles of T2DM subjects goes against the hypothesis of mitochondrial dysfunction playing a role in the pathogenesis of T2DM. More insight into this question was obtained in studies on aging, both in skeletal muscle insulin sensitivity and mitochondrial function. Karakelides and co-workers performed a study assessing insulin sensitivity and skeletal muscle mitochondrial function using a hyperinsulinaemic-euglycemic clamp technique and quantification of mitochondrial ATP production rate, respectively (Karakelides et al., 2010). The subjects were divided in four different groups: young lean, young obese, old lean, and old obese individuals and stratified by sex. The results obtained by this experimental approach indicated that insulin sensitivity is related to the levels of adiposity and not to aging. In contrast, skeletal muscle mitochondrial function is reduced by age and not related to the levels of adiposity. Furthermore, men showed a higher mitochondrial ATP production rate than women, but women were more insulin sensitive than men. Taken together, this study clearly dissociated skeletal muscle mitochondrial function from insulin sensitivity (Karakelides et al., 2010). However, the subjects were not matched by maximal oxygen uptake capacity (VO2max.), which could be a confounding factor due to the fact that aerobic capacity is related to mitochondrial respiratory capacity. A recent study addressed whether aging has an effect in mitochondrial function when VO2max. values are taken into account. Young and middle-aged individuals with matched VO2max. had similar skeletal muscle mitochondrial respiratory capacity per wet tissue weight. However, when mitochondrial respiratory capacity was normalized by mitochondrial content, middle-aged subjects showed a lower capacity per mitochondrial unit (assessed by mtDNA content), implying that with age skeletal muscle keeps mitochondrial respiratory capacity increasing mitochondrial density (Larsen et al., 2012).

7.2.2. Ethnicity and skeletal muscle insulin resistance

It is known that Asian-Indians have one of the highest prevalence of T2DM in the world. In order to understand the metabolic differences that lead to this higher prevalence, a study was performed by Nair and co-workers comparing diabetic Asian-Indians, non-diabetic Asian-Indians, and non-diabetic Northern European-Americans (Nair et al., 2008). A hyperinsulinaemic-euglycemic clamp assessing insulin sensitivity and whole body glucose disposal, along with skeletal muscle maximal mitochondrial ATP production rate, protein content and mRNA expression information of different mitochondrial markers was used to evaluate mitochondrial function in the three experimental groups. Diabetic Asian-Indians and non-diabetic Asian-Indians had similar mitochondrial function even though they had

marked differences in insulin sensitivity, an observation that does not support mitochondrial dysfunction playing a role in T2DM development. Moreover, when the two ethnicities were compared, Asian-Indians had a higher OXPHOS capacity than Northern European-Americans even though Northern European-Americans were more insulin sensitive, providing additional data against a causative role of mitochondrial dysfunction in T2DM etiology (Nair et al., 2008).

7.2.3. Lifestyle interventions in type 2 diabetic individuals

It has been shown that lifestyle interventions that achieve significant body weight loss, reduction of body fat content, and improved aerobic capacity, are one of the most important tools, if not the best, in the prevention or amelioration of T2DM (Knowler et al., 2002). After completing an exercise training regime (cycling on a cycloergometer for 45 minutes, twice a week, for 10 weeks), T2DM subjects showed improved skeletal muscle lipid oxidation and mitochondrial oxidative capacity (Bordenave et al., 2008). However, this study did not address whether this significant improvement in mitochondrial function was related to enhanced glucose homeostasis, skeletal muscle insulin resistance, or both. Meex and co-workers attempted to elucidate whether an exercise training regime (12 weeks of aerobic exercise) could have a positive effect on skeletal muscle mitochondrial dysfunction, elevated intra-myocellular lipid accumulation, and insulin resistance, characteristic features for T2DM (Meex et al., 2010). After the completion of the exercise training intervention both overweight controls and T2DM subjects showed improved insulin-mediated glucose disposal, and a significant increase in mitochondrial respiratory capacity measured by ^{31}P-magnetic resonance spectroscopy (Meex et al., 2010) and in permeabilised muscle fibers by high resolution respirometry (Phielix et al., 2010). T2DM subjects recovered metabolic flexibility and showed an increase in intra-myocellular lipid content, in relation with what had been observed in trained individuals and in disagreement with the hypothesis that increased skeletal muscle lipid storage is related to insulin resistance and mitochondrial dysfunction (Meex et al., 2010). Similar observations were obtained by Hey-Mogensen and co-workers in obese subjects with and without T2DM (Hey-Mogensen et al., 2010). After 10 weeks of aerobic training both experimental groups showed significant improvement in mitochondrial respiratory capacity despite the observation of significant differences in insulin sensitivity between both groups (Hey-Mogensen et al., 2010). Obese subjects with T2DM showed increased reactive oxygen species (ROS) production (Hey-Mogensen et al., 2010), which is in line with the results of Lefort and co-workers in a similar group of subjects (Lefort et al., 2010). Obese T2DM subjects that follow a lifestyle intervention program combining caloric restriction (25% reduction of daily energy intake) with regular physical activity (30-40 min aerobic exercise per session) achieved an approximately 7% weight reduction after four months. These individuals improved skeletal muscle mitochondrial density, citrate synthase activity and insulin sensitivity. Thus, proving that lifestyle interventions have a beneficial effect in the treatment of T2DM (Toledo et al., 2007). A follow-up study from the same research group (Toledo et al., 2008) attempted to discern whether mitochondrial improvement after weight loss due to a lifestyle intervention is

related to the decrease in body weight or requires the improvement in aerobic fitness obtained by regular exercise. Both intervention groups (diet alone and diet plus exercise) achieved the same body weight loss (10%) and similar insulin sensitivity improvements. However, only the subjects under the diet plus exercise intervention program showed a significant enhancement in mitochondrial function (Toledo et al., 2008). This observation is pertinent because it distinguishes between insulin resistance improvements and skeletal muscle mitochondrial performance.

Human studies have produced clear evidence that T2DM is associated with skeletal muscle mitochondrial dysfunction, but have not supported the claim that mitochondrial dysfunction plays a major role in the development of the disease. It seems more likely that mitochondrial dysfunction could be a consequence of the general metabolic disarrangement originated in most cases by nutrient oversupply and insulin resistant status. As detailed before, skeletal muscle mitochondrial function is most probably modulated by the aerobic capacity of the individual, sex, age and ethnicity.

7.3. Animal studies

We have already mentioned that rats (Garcia-Roves et al., 2007, Hancock et al., 2008) and mice (Turner et al., 2007) develop insulin resistance with increased skeletal muscle mitochondrial content and fatty acid oxidative capacity after following a HFD for several weeks. These studies provided one of the first experimental evidences to show a discrepancy between mitochondrial dysfunction and T2DM etiology. However, at the same time a study was published by Lionetti and co-workers (Lionetti et al., 2007) in which rats were fed either with a low-fat diet or HFD for 7 weeks. The results obtained in this research showed how HFD induced hyperglycemia in rats and that it was related to an elevated fat supply, but not to higher energy uptake. Moreover, derangements in glucose handling are associated with a lower functionality of the subsarcolemmal mitochondrial population in skeletal muscle. This observation is in line with mitochondrial dysfunction playing a role in the development of T2DM and is also in agreement with a human study performed by Ritov and co-workers (Ritov et al., 2005).

To decipher the role played by skeletal muscle mitochondria in T2DM etiology, De Feyter and co-workers (De Feyter et al., 2008) compared obese ZDF rats to lean controls. These groups were studied at 6, 12 and 18 weeks of age, going from a pre-diabetic state (week 6) to T2DM (week 12 and 18). Mitochondrial function (measured *in vivo* by magnetic resonance spectroscopy and *in vitro* by enzymatic activities) together with intra-myocellular lipid content, fasting plasma glucose, insulin and free fatty acids data were collected. All these results showed how T2DM develops in line with increased intra-myocellular lipid accumulation but without a decrease in skeletal muscle mitochondrial function, again showing a clear divergence between T2DM and skeletal muscle mitochondrial function (De Feyter et al., 2008). These observations were corroborated in later studies by the same research group where mitochondrial respiratory capacity was measured by high-resolution respirometry (Lenaers et al., 2010). A similar analytical approach was also performed in mice fed with a HFD for 4

weeks (Bonnard et al., 2008) with similar results to those reported by De Feyter and co-workers (De Feyter et al., 2008), highlighting that mitochondrial dysfunction did not precede insulin resistance. However, when mice were fed with HFD for a longer term (16 weeks), mitochondrial defects were evident and mitochondrial respiration rates were lower in permeabilized muscle fibers. Mitochondrial defects were related to a higher ROS production rate and it was shown that a supplementation with antioxidants restored mitochondrial defects (Bonnard et al., 2008). Similar observations were made by Yokota and co-workers (Yokota et al., 2009) in mice fed with a HFD for 8 weeks. In Yokota's study they attempted to assess if insulin resistant mice with higher ROS production and impaired mitochondrial dysfunction would have a reduced exercise performance and whether antioxidant supplementation could reverse the adverse effect on exercise performance. When mice on a HFD were supplemented with apocynin, a compound that reduce free radical formation by inhibition of NAD(P)H oxidase activity (which reduces oxygen to superoxide), mice showed similar mitochondrial function, ROS production and exercise performance that mice under a low fat diet (LFD). However, these mice (HFD+apocynin), despite having better mitochondrial function, still showed impaired glucose tolerance (Yokota et al., 2009).

A subsequent study used Zucker rats to evaluate whether fatty acid transport played a key role in the observed increase in intra-myocellular fatty acid accumulation (Holloway et al., 2009). Additionally, fatty acid oxidation rates, mitochondrial density and intra-muscular triacylglycerol content were also determined. In summary, this study showed how skeletal muscle fatty acid transport was increased, mainly trough an upregulation of the levels of the fatty acid transporter CD36 (Holloway et al., 2009). Skeletal muscle subsarcolemmal mitochondria were increased and had a higher capacity to oxidize fatty acid, in contrast to previous observations (Ritov et al., 2005, Lionetti et al., 2007). However, based on their *in vitro* studies, fatty acid transport exceeded mitochondrial fatty acid oxidative capacity and therefore, intra-myocellular lipid accumulation was increased, which in a long term could explain skeletal muscle metabolic derangements observed in obesity and T2DM (Holloway et al., 2009).

Another aspect of lipid oversupply related to insulin resistance is the saturation of the phospholipids (fatty acid composition), which could alter membrane properties and therefore cellular function. Thus, insulin resistance correlated with the level of membrane phospholipids in skeletal muscle of Pima Indians, a population with the highest T2DM incidence (Pan et al., 1995). On this respect, two recent studies have addressed whether phospholipid fatty acid composition affects mitochondrial function and whether this is the mechanism leading to insulin resistance and further T2DM. For this purpose, C57BL/6J mice were fed with a LFD or a HFD for 3 or 28 days, and fasting glucose, insulin, gene expression profile, mitochondrial proteins and lipid composition were determined at each time point (3 and 28 days). After day 3, mice on a HFD showed increased fasting blood glucose and insulin levels. A marked increase in protein content of the different mitochondrial OXPHOS complexes was also observed at day 28 along with an increase in the degree of phospholipids saturation (de Wilde et al., 2008). Later on, the same research group attempted to understand whether mitochondrial membrane phospholipids composition

could affect mitochondrial function. In this study C57BL/6J mice were fed with a LFD or HFD for 8 or 20 weeks. From week 8, mice on a HFD had a higher level of saturation in their skeletal muscle mitochondrial membrane phospholipids. Mice were insulin resistant although mitochondrial respiratory capacity was not affected. Therefore, these observations were against mitochondrial dysfunction being a key feature in T2DM etiology and also questioning the role played by phospholipids composition in mitochondrial performance and glucose handling (Hoeks et al., 2011).

It seems clear, from the information obtained in the different studies using HFD-induced insulin resistance in mice or rats that at earlier and intermediate stages there are mitochondrial adaptations that improve skeletal muscle oxidative capacity to overcome fatty acid oversupply. To maintain skeletal muscle oxidative capacity, mitochondrial density is increased as showed by Van den Broek and co-workers (van den Broek et al., 2010) and in line with the observation made in humans by Dela and co-workers (Larsen et al., 2012).

7.4. Conclusions

Over these years we have reached a better understanding of how skeletal muscle mitochondria respond to different pathophysiological conditions. The evidence also clearly indicated that, at least at later stages in the etiology of the disease, mitochondrial dysfunction is present in T2DM both in humans and in animals. However, mitochondrial dysfunction does not play a major role in skeletal muscle insulin resistance and the development to T2DM. Most probably mitochondrial dysfunction results from the different disturbances produced by nutrient oversupply and lack of physical activity characteristic of the sedentary lifestyle of modern societies. Skeletal muscle mitochondrial function will be influenced by age, sex, ethnicity, and aerobic capacity. And more importantly, skeletal muscle mitochondrial defects and insulin resistance characteristic of T2DM could be rescue by changes in lifestyle that lead to an improved physical fitness and balanced diet.

7.5. Future perspectives

T2DM is a complex metabolic disease that involves disturbances not only in skeletal muscle, liver and pancreatic beta-cells (Defronzo, 1988) but also implies malfunction of adipose tissue, pancreatic alpha-cells, kidney, gastrointestinal tract and brain (Defronzo, 2009). Therefore, with the aim to find how to successfully prevent or treat T2DM we should address our research taking into account the systemic nature of this metabolic disease.

All cell types present in our body have a lower or higher degree of specialization that will determine their metabolic requirements and ability to adapt to different physiological or pathophysiological stresses. Thus, mitochondrial plasticity to metabolic derangements are cell-type specific. In this regard, Garcia-Roves and co-workers showed how different tissues respond differently to obesity and T2DM in terms of mitochondrial adaptive capacity (Holmstrom et al., 2012). Thus, db/db mice, an animal model of diabetes and obesity, showed clear evidence of mitochondrial dysfunction in liver at 16 weeks of age. However,

glycolytic skeletal muscles from the same mice had a better mitochondrial respiratory capacity and mitochondrial content than the lean control mice. More importantly, oxidative skeletal muscles show earlier evidences of mitochondrial function impairment. This study brings into account cell type plasticity and that time is also very important in the development of T2DM and should be taken into account to better interpret the different results already published (Holmstrom et al., 2012).

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