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Vera B. Schrauwen-Hinderling[a,b], Michael Roden[c], M. Eline Kooi[b], Matthijs K.C. Hesselink[d] and Patrick Schrauwen[a]

Purpose of review

Muscular mitochondrial dysfunction, leading to the accumulation of fat in skeletal muscle, has been proposed to be involved in the development of type 2 diabetes mellitus. Here, we review human studies that investigated various aspects of mitochondrial function in relation to muscular insulin sensitivity and/or diabetes.

Recent findings

In-vivo magnetic resonance spectroscopy allows assessment of mitochondrial functionality from adenosine triphosphate flux in the nonexercising state and from phosphocreatine recovery from (sub)maximal exercising. Application of both approaches revealed reduced mitochondrial oxidative capacity in insulin-resistant (pre)diabetic humans. Reductions in mitochondrial density may contribute to, or even underlie, these findings as well as intrinsic defects in mitochondrial respiration. So far, only two studies reported measurements of mitochondrial respiratory capacity in intact mitochondria in diabetic patients, with inconsistent findings.

Summary

Muscular mitochondrial aberrations in type 2 diabetes mellitus can be detected, but it is so far unclear if these aberrations are causally related to the development of the disease. Alternatively, mitochondrial dysfunction may simply be the consequence of elevated plasma fatty acids or glucose levels.

Keywords

insulin resistance, mitochondrial function, skeletal muscle, type 2 diabetes

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[a]Department of Human Biology, Maastricht University, Maastricht, The Netherlands, [b]Department of Radiology, Maastricht University Hospital, Maastricht, The Netherlands, [c]First Medical Department, Hanusch Hospital (Academic Teaching Hospital - Medical University of Vienna), Vienna, Austria and [d]Movement Sciences, Maastricht University, Maastricht, The Netherlands

Correspondence to Dr P. Schrauwen, Nutrition and Toxicology Research Institute Maastricht (NUTRIM), Department of Human Biology, Maastricht University, P.O. Box 616, 6200 MD, Maastricht, The Netherlands
Tel: +31 43 3881311; fax: +31 43 3670976; e-mail: p.schrauwen@hb.unimaas.nl

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Abbreviations

IMCL	intramyocellular lipids
PCr	phosphocreatine
PGC1α	peroxisome proliferator-activated receptor-coactivator α
T2D	type 2 diabetes

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Introduction

The inability of insulin to stimulate glucose uptake into skeletal muscle is one of the earliest hallmarks in the development of type 2 diabetes mellitus (T2DM), of which the prevalence nowadays is reaching epidemic proportions worldwide [1]. As an excessive fat mass (i.e. obesity) is the major risk factor for the development of type 2 diabetes (T2D), abnormalities in fat metabolism have been linked to the development of the disease. Indeed, there is now compelling evidence that the accumulation of fatty acid metabolites in nonadipose tissues such as heart, liver, pancreas and skeletal muscle has detrimental effects on the functions of these organs. For skeletal muscle, the accumulation of fat inside muscle cells (intramyocellular lipids, IMCL) is inversely correlated with muscular insulin sensitivity in sedentary populations. Moreover, T2D patients and first-degree relatives of T2D patients who are at risk to develop diabetes have increased levels of IMCL [2]. This relationship between IMCL accumulation and insulin resistance can be explained by accumulation of intermediates of lipid metabolism, such as diacylglycerol, fatty acyl-CoA and ceramides, that can interfere with proper insulin signaling (for review see Ref. [3]). It has been suggested that a reduced oxidative capacity (i.e. mitochondrial dysfunction) may limit the complete oxidative degradation of IMCL resulting in increased levels of both IMCL as well as of their intermediates. Here we will review the evidence that abnormal mitochondrial function may be a factor involved in the etiology of muscular insulin resistance and T2DM in humans.

Fat oxidative capacity in relation to insulin resistance and type 2 diabetes

By definition, lipids and lipid intermediates accumulate in skeletal muscle cells if muscular fatty acid delivery exceeds fat oxidation. Thus, a reduced fat oxidative capacity – combined with elevated plasma FFA levels, as observed in T2D patients [4] – may underlie the muscular fat accumulation observed in the prediabetic

state. Indeed, when comparing T2D patients with BMI-matched healthy controls, a reduced muscular fat oxidation – as determined by measuring rates of oxygen consumption and carbon dioxide production across the leg – was observed in T2D patients [5]. Using tracer technology, a similar conclusion was drawn, as a 50% reduced oxidation of palmitate over the leg, accompanied by decreased activity of carnitine palmitoyl transferase 1 (CPT1) was observed in the obese (insulin resistant) state [6]. A prominent feature of insulin resistance, however, is also an inadequate glucose uptake and oxidation in the postprandial state, in combination with a blunted suppression of fat oxidation. As a result, fat oxidation is, in fact, increased in the postprandial state when compared to insulin sensitive subjects.

The phenomenon of a reduced ability to change fuel selection from predominantly fat oxidation in the fasting state to primarily glucose oxidation in the insulin stimulated condition is referred to as metabolic inflexibility [7]. Interestingly, the metabolic inflexibility observed in type 2 diabetes was preserved in myotubes grown from type 2 diabetic donor satellite cells [8] and could therefore be considered an 'intrinsic' property of type 2 diabetic muscle.

Reduction of PGC1α in type 2 diabetes mellitus

Although several factors that could underlie the reduced fasting fatty acid oxidation and metabolic inflexibility have been reported, no single factor has consistently been shown to be altered in the (pre) diabetic state. With the implementation of the micro array technology in the field of diabetes research, a strong tool became available to screen for multifactor alterations that might underlie skeletal muscle insulin resistance. Using this technology, in muscle biopsies obtained from human T2D patients, two independent studies showed a coordinated reduction of a large cluster of oxidative genes, all under the control of the peroxisome proliferator-activated receptor-coactivator α (PGC1α) and which encode for key enzymes in oxidative metabolism and mitochondrial function [9,10]. Importantly, many of these genes showed only a modest, although consistent reduction, explaining why previous studies with a 'candidate gene' approach may have failed to detect these alterations. The reduction of a cluster of oxidative genes could provide an explanation for the reduced fat oxidative capacity and impeded metabolic flexibility and could underlie the accumulation of IMCL and their intermediates and, ultimately, insulin resistance. With the downregulation of this so-called oxidative phosphorylation (OXPHOS) gene set, the expression of PGC1α itself was also reduced in T2D patients [9,10] and in family history-positive nondiabetic subjects [10]. In line with this, Mensink *et al.* [11•] also reported a ~ 60% reduction in PGC1α gene expression in T2D patients compared to BMI and age matched controls. Interestingly, treating these patients with the antidiabetic insulin sensitizing agent Rosiglitazone restored muscular PGC1α levels along with improved muscular insulin sensitivity and oxidative capacity [11•]. In further agreement with a role for PGC1α as a determinant of insulin resistance, a group of nondiabetic but overweight/obese male subjects showed PGC1α mRNA was reduced by 71% in those subjects that were classified as being insulin resistant, based on the median glucose infusion rate determined using a hyperinsulinemic euglycemic clamp [12•].

In healthy subjects, both the expression and protein content of PGC1α has been reported to be increased with acute exercise [13] and prolonged endurance training [14], conditions that are well known to improve muscular insulin sensitivity. On the other hand, consumption of a high-fat diet for three days reduced PGC1α protein levels by ~20% in human volunteers [15]. Furthermore, the acute elevation of plasma FFA levels for six [16•] or 48h [17] reduced PGC1α expression significantly. These findings may indicate that the reduction of PGC1α in the prediabetic state may be due to environmental factors such as a lack of physical activity and/or the consumption of diets high in fatty acids, although genetic factors cannot be ruled out.

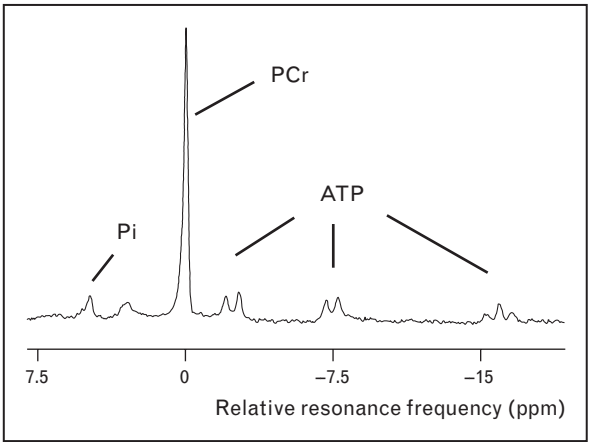
Is mitochondrial function reduced in type 2 diabetes mellitus?

The finding of a reduced expression and protein content of PGC1α in the (pre) diabetic state, resulting in reduced OXPHOS gene expression could explain the muscular fat accumulation that is observed in diabetes, but only if the reduced PGC1α content truly results in a diminished mitochondrial content or oxidative capacity. Since the identification of PGC1α as a putative determinant in the etiology of T2D, many reports have been published that investigated 'mitochondrial function' with a wide range of methodologies and techniques. Unfortunately, many of these investigations only determined, at best, markers of mitochondrial function, such as mitochondrial gene transcripts, mitochondrial protein and DNA content or mitochondrial enzyme activities. Although these markers address different aspects of mitochondrial metabolism, like mitochondrial biogenesis and mitochondrial density, they do not necessarily determine muscular mitochondrial oxidative functionality. Despite this, there are also several reports that did apply *in vivo* or *ex vivo* methodologies to determine muscular mitochondrial oxidative functionality.

In-vivo mitochondrial function assessed by magnetic resonance spectroscopy

An elegant way to noninvasively investigate *in vivo* mitochondrial function is by means of phosphorous magnetic resonance spectroscopy (^{31}P MRS). With this

Figure 1 A ^{31}P-MRS spectrum of the vastus lateralis muscle depicting the peaks originating from inorganic phosphate (Pi), phosphocreatine (PCr) and the three groups of ATP



technique, concentrations of phosphorous-containing metabolites in skeletal muscle can be determined noninvasively and followed over time (Fig. 1). Two different ^{31}P MRS methods have been used to estimate mitochondrial function. With the first technique, the rate of unidirectional flux through ATP synthase (ATP synthetic flux, fATPase) is determined in the nonexercising state by use of the magnetization (saturation) transfer method in which the signal derived from ATP is temporarily suppressed and subsequent changes in the steady state MR signal of free phosphate are quantified to calculate unidirectional ATP synthesis rate. Simultaneously, flux through the tricarboxylic acid (TCA or Krebs) cycle can be determined noninvasively by carbon-13 (^{13}C) MRS. With this technique, concentrations of ^{13}C-containing metabolites in skeletal muscle can be determined. Thereby, the incorporation of ^{13}C label into the muscle glutamate pool is determined after the administration of ^{13}C labeled acetate, which is a direct substrate for the TCA cycle. Using this ^{13}C and ^{31}P MRS method, Petersen *et al.* [18] were the first to report that mitochondrial dysfunction may be a factor in the etiology of insulin resistance. Healthy, younger subjects (average 39 years) without a family history of T2D were compared with BMI-matched healthy elderly volunteers between 61 and 84 years of age. Using a hyperinsulinemic euglycemic clamp combined with labeled glucose, muscular glucose disposal rates were found to be lower in the elderly compared to the young, consistent with age being a major risk factor for the development of insulin resistance and T2D [18]. IMCL content measured with proton MRS was higher in the elderly than in the younger subjects. Muscular fATPase and oxidative metabolism (TCA cycle flux) were decreased by $\sim$ 40% in the elderly, suggesting that an age-associated decline in mitochondrial function might contribute to the development of insulin resistance. Using similar methods, the same research group assessed IMCL and fATPase in lean, but insulin-resistant offspring of T2D patients who were matched for age, BMI and habitual physical activity with insulin sensitive healthy subjects [19]. The offspring of T2D patients were characterized by a $\sim$ 60% lower rate of muscular glucose uptake and an $\sim$ 80% increase in IMCL content when compared to control subjects [19]. Again, rates of muscular ATP synthesis were lower by 30% in the insulin-resistant offspring of T2D patients compared to the insulin-sensitive control subjects. Recently, a similar 30% reduction in TCA cycle flux in these patients was reported [20•]. The authors concluded that mitochondrial dysfunction may be implicated in the aetiology of insulin resistance and T2D. Both studies also suggested that the reduced mitochondrial function may have been responsible for the increased IMCL levels that were observed both in the elderly and the insulin-resistant offspring of T2D patients.

An alternative ^{31}P MRS method to investigate *in vivo* mitochondrial function is by measuring the phosphocreatine (PCr) kinetics during recovery from submaximal exercise [21,22]. During exercise, PCr content decreases transiently and recovers rapidly after exercise. If no substantial acidification occurs during exercise, this recovery follows a mono-exponential time course. In the postexercise state PCr resynthesis is driven almost purely oxidatively [23] and the kinetics of resynthesis (e.g. the half-time of recovery) reflect *in vivo* mitochondrial function [22]. Indeed, postexercise PCr resynthesis was shown to be delayed in various known mitochondrial disorders (for reviews see [24]). A difference of this method compared to the magnetization saturation transfer method is that mitochondrial function is assessed under conditions of increased metabolic demand.

Using this method, we compared *in vivo* mitochondrial function in T2D patients and healthy, obese control subjects matched for BMI [25••]. Consistent with the data from Petersen *et al.* [18,19], the half-times of PCr resynthesis after 5 min of moderate leg exercise were found to be prolonged by $\sim$ 45% (indicating compromised mitochondrial function) in the obese diabetic patients, who were also more insulin resistant compared to the controls. In the T2D patients, PCr resynthesis half-time correlated positively (and hence mitochondrial function negatively) with HbA1c and glucose concentrations. Interestingly, however, IMCL content was similar between the groups, suggesting that impaired mitochondrial function may be a more important determinant of diabetes than IMCL levels *per se*. Consistent with this finding, Szendroedi *et al.* [26••] very recently reported that fATPase was decreased by 27% in nonobese metabolically well controlled T2D patients when compared to young, healthy control subjects, but fATPase was not

different from age-matched healthy controls. Despite these lower fATPase in diabetic patients, IMCL levels were similar in all three groups. Interestingly, fATPase was negatively correlated with plasma FFA levels, suggesting that lipid oversupply may deteriorate mitochondrial function, consistent with the effect of high FFA levels on PGC1α expression [16•,17].

The reports using magnetization transfer mentioned above determined mitochondrial functionality in the fasting state. The same methodology has also been applied to study ATP synthesis under insulin-stimulated conditions, which may mimic the postprandial state. Using this approach, Petersen *et al.* [27] showed that fATPase increased by 90% during a hyperinsulinemic-euglycemic clamp in young, healthy control subjects, whereas this increase was only 5% in age- and BMI-matched lean insulin-resistant offspring of T2D patients. Similar results were obtained by Szendroedi *et al.* [26••], who reported a significant 26% and 11% increase in fATPase during a hyperinsulinemic glucose clamp in healthy young and elderly control subjects respectively, but no such increase in T2D patients. In that study, variance in insulin-stimulated ATP synthesis was largely explained by differences in muscular insulin sensitivity, suggesting that the reduced insulin-stimulated fATPase as observed in the (pre) diabetic state mainly reflects muscular insulin resistance. In line with this, Brehm *et al.* [28•] previously reported that the acute elevation of plasma FFA levels during a hyperinsulinemic euglycemic clamp impaired insulin-stimulated fATPase in healthy, lean young control subjects. Lipid infusion during a glucose clamp is known to result in the acute induction of muscular insulin resistance, due to the accumulation of lipid intermediates in skeletal muscle cells. Thus, this study tends to indicate that the induction of insulin resistance explains the reduced insulin-stimulated ATP synthesis, although an additional direct effect of fatty acids on fATPase cannot be excluded.

While the great strength of MRS is the possibility to determine whether oxidative metabolism is indeed decreased in the *in vivo* setting, this at the same time limits the mechanistic conclusions that can be drawn from these studies. Using MRS, 'mitochondrial function' of a relatively large muscle volume is assessed, and therefore fATPase or PCr recovery rates not only reflect 'mitochondrial functionality and/or capacity' but may as well reflect the number and the size of mitochondria in the muscle under study. Mitochondrial density, in turn, is related to the muscle fiber type composition, with more glycolytic muscle fibers having a lower mitochondrial density. Considering this, the term 'muscular oxidative functionality' might better reflect what is being measured *in vivo*. Finally, the determination of mitochondrial function/muscular oxidative functionality *in vivo* can also be limited by an impairment in oxygen supply which can occur, for example, when perfusion is disturbed.

Ex-vivo mitochondrial function

To complement *in vivo* findings with more mechanistic data, direct examination of the function of the mitochondria *per se* is warranted. One alternative methodology that has frequently been used is the determination of oxidative enzyme activity in muscle homogenates or isolated mitochondria. Although reduced activity of oxidative enzymes have been reported in muscle of T2D patients (for review, see Ref. [29•]), these assays do not necessarily reflect mitochondrial function, because only part of the mitochondrial oxidative system is studied and – perhaps more importantly – this is done out of its functional context. For example, activity of the single complexes of the electron transport chain are often measured independently of the control of the oxidative phosphorylation system that is present in functional mitochondria. Therefore, true intrinsic mitochondrial function needs to be studied using high-resolution respirometry of isolated mitochondria or permeabilized muscle fibers. So far, only two studies have been published that applied this technique to study mitochondrial function in T2D patients [30••,31••] with inconsistent results. Boushel *et al.* [30••] determined oxygen flux capacity of permeabilized muscle fibers from biopsies of healthy subjects and T2D patients. They found that ADP-stimulated state 3 respiration, both with substrates for complex I and/or complex II, was reduced in T2D patients. Because measurements were performed in permeabilized fibers, however, data was corrected for mitochondrial density. After correction of respiration rates for mitochondrial DNA (mtDNA) content or citrate synthase activity – both markers of mitochondrial density – the differences in respiration rates between T2D patients and healthy controls completely disappeared. In contrast, however, Mogensen *et al.* [31••] compared mitochondrial respiration rates in isolated mitochondria of type 2 diabetic patients and obese nondiabetic controls and reported that maximal ADP-stimulated respiration (state 3) with pyruvate as a substrate and respiration through the electron transport chain (ETC) were reduced in T2D patients. They also reported that the proportion of type 2X fibers were higher in T2D patients compared to obese subjects, suggesting that the differences in mitochondrial respiration may partly be explained by qualitative differences in mitochondria isolated from type 1 versus type 2 muscle fibers.

Why the outcomes of these two studies are different is difficult to explain, but differences in the details of the methodology – isolated mitochondria versus permeabilized fibers – may have affected the results. The differences in state 3 respiration reported by Mogensen *et al.* [31••] were relatively small (~ 10%), and thus less

pronounced than the differences in *in vivo* mitochondrial function that were reported using MRS. Thus, it is well possible that mitochondrial dysfunction that is observed in the prediabetic state is indeed due to a combination of reductions in mitochondrial density and (small) differences in 'real' mitochondrial function. Other factors that only occur in the *in vivo* setting (e.g. impaired perfusion) may contribute to the larger differences found *in vivo*, while they are not taken into account by the *ex vivo* measurement. Clearly, more *in vivo* studies controlling these factors and more studies using high-resolution respirometry are needed to clarify the issue of mitochondrial respiration in T2D patients.

Mitochondrial density and damage

As mentioned, a reduced mitochondrial density might at least partly underlie the reduced mitochondrial function/oxidative capacity that is observed in the prediabetic state. Indeed, Ritov *et al.* [32] reported a reduced content of mtDNA, which is considered a valid marker of mitochondrial density, in T2D patients compared to age, sex and BMI-matched nondiabetic controls. Using electron microscopy, Morino *et al.* [33] reported a 38% lower mitochondrial density in insulin-resistant offspring of T2D patients compared to insulin-sensitive controls, in which previously a reduced *in vivo* mitochondrial function had been reported [19]. Also using electron microscopic examination, Kelley *et al.* [34] reported qualitative differences in mitochondrial morphology in diabetic patients, too. Mitochondrial area was significantly lower (~35%) in both hyperglycemic obese individuals and obese hyperglycemic T2D patients compared with normoglycemic lean subjects. Mitochondrial area was found to correlate positively with insulin stimulated glucose disposal [34]. Interestingly, in ~ 30–40% of the obese patients, electron microscopy revealed large vacuoles surrounded by mitochondrial membranes, implicating damaged mitochondria. Whether increased mitochondrial damage, in combination with a reduced (PGC1α-driven) mitochondrial biogenesis is responsible for the reduced mitochondrial content in T2D patients is so far unknown.

Conclusion

The topic of mitochondrial dysfunction has gained a lot of attention in the T2D field. If anything, the results obtained so far taught us that the relation between mitochondrial function and the development of T2D is not as simple as originally assumed. Thus, the initial assumption that a reduced mitochondrial function would lead to the accumulation of IMCL and thereby to insulin resistance is not supported by all studies, as several studies showed no differences in IMCL despite reductions in mitochondrial function in insulin-resistant and/or diabetic subjects [25••,26••]. This however may not be surprising, as it is well established that IMCL *per se* is not causally related to muscular insulin resistance, but IMCL may rather be a marker of other fatty acid intermediates such as diacylglycerol, fatty acyl-CoA and ceramides that can directly interfere with muscular insulin signaling. It is tempting to suggest that these fatty acid intermediates specifically accumulate if the balance between oxidative capacity and fatty acid delivery is disturbed. Alternatively, however, it cannot be excluded that a reduced mitochondrial function may, in fact, be the consequence of the diabetic state, for example, be caused by fat accumulation in skeletal muscle cells [35]. In support of this hypothesis are the findings that plasma fatty acid levels reduce the expression of PGC1α [16•,17], are negatively related to fasting muscular ATP synthesis [26••], and impair insulin-stimulated ATP synthesis [28•], as well as the observation of increased mitochondrial damage in T2D patients [34]. Also, insulin resistance and/or hyperglycemia *per se* may negatively affect mitochondrial function, as could be suggested from the findings that mitochondrial function was negatively related to hyperglycemia [25••] and insulin sensitivity [26••]. In this context, it is important to notice that a reduced mitochondrial function in prediabetic subjects is only reported in subjects that were already more insulin-resistant and had higher IMCL levels compared to their control groups. Therefore, more research is clearly needed to elucidate the specific role of mitochondrial function in the etiology of T2D. If mitochondrial function proves to be of relevance in the etiology of T2D, it provides a fundamental explanation for the well documented effects of physical activity and exercise training – major enhancers of mitochondrial function and biogenesis – on glucose homeostasis and T2D risk. If anything, the interest in mitochondrial function has taught us once more that the growing prevalence of physical inactivity in our westernized society may be more important in the etiology of T2D than sometimes appreciated.

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