Mitochondrial function in skeletal muscle in type 2 diabetes

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This PhD thesis is based on the following four manuscripts:
I:
R. Rabøl, P. M. V. Højberg, T. Almdal, R. Boushel, S. B. Haugaard, S. Madsbad, F. Dela
Effect of Hyperglycemia on Mitochondrial Respiration in Type 2 Diabetes. JCEM 2009;94:1372-8.

II:
R. Rabøl, S. Larsen, P. M. V. Højberg, T. Almdal, R. Boushel, S. B. Haugaard, J. Løvind, S. Madsbad, F. Dela
Regional Differences in Skeletal Muscle Mitochondrial Respiration in Type 2 Diabetes and Obesity. JCEM, 2010;95:857-63.

III:
R. Rabøl, R. Boushel, T. Almdal, T. Ploug, S. B. Haugaard, S. Madsbad, F. Dela
Rosiglitazone treatment decreases skeletal muscle mitochondrial content and function in type 2 diabetes. Manuscript prepared for submission.

IV:
R. Rabøl, P. M. V. Højberg, T. Almdal, R. Boushel, S. B. Haugaard, S. Madsbad, F. Dela
Improved Glycaemic Control Decreases Inner Mitochondrial Membrane Leak in Type 2 Diabetes. Diabetes, Obesity and Metabolism 2009;11:355-60.

INTRODUCTION

Mitochondria are the main producers of energy in most cells. Their primary role is to generate ATP from the energy released by the breakdown of nutrients. Mitochondria are essential for the function and survival of cells, and play a central role in the breakdown of carbohydrates, fat and proteins. Mitochondrial function has been a major focus of research attention in recent years due to their involvement in essential parts of cellular metabolism, especially the breakdown of nutrients. Defects in mitochondrial function have been linked to insulin resistance. The primary cause of insulin resistance is unknown, but intramyocellular lipid accumulation and insulin resistance have been shown to be associated in both healthy adults (1) and offspring of type 2 diabetic parents (2). A molecular mechanism explaining how increased amounts of intracellular fatty acid metabolites, especially diacylglycerol and fatty acyl CoA (3), can interfere with insulin signaling has been demonstrated (4;5). Since this discovery the study of skeletal muscle oxidative capacity has been a major focus of attention, but the discovery of decreased oxidative capacity of skeletal muscle in patients with type 2 diabetes is not novel.

EVIDENCE OF REDUCED OXIDATIVE ENZYME ACTIVITY IN TYPE 2 DIABETES.

The first evidence of defects in the oxidative capacity in skeletal muscle of patients with type 2 diabetes came in 1977. Seven enzymes of energy metabolism were examined, and decreased activity was found in six of these enzymes in patients with type 2 diabetes compared to obese, non-diabetic subjects. Four cytosolic (triosephosphat dehydrogenase, glycerol-3-phosphat dehydrogenase, lactate dehydrogenase and hexokinase) and three mitochondrial enzymes (malate dehydrogenase, citrate synthase and hydroxyacyl-CoA-dehydrogenase (HAD)) were examined. Only the activity of HAD was unchanged between the groups. Hexokinase activity was reduced by 34 % and citrate synthase activity was reduced by 30 % in the diabetic subjects compared to the obese controls (6).
Simoneau and Kelley repeated and extended the studies of Vondra et al in 1997. Muscle biopsies from eight obese diabetic subjects were compared to biopsies from eight lean and eight obese non-diabetic subjects. Phosphofructokinase, glyceraldehydes phosphate dehydrogenase and hexokinase activity were measured as estimates of glycolytic capacity whereas the activity of cytochrome-c oxidase (COX) and citrate synthase were used as estimates of the oxidative capacity. The authors found trends

towards a higher glycolytic activity and a lower oxidative activity in the diabetic subjects compared to lean and obese non-diabetics. Simoneau found a comparable reduction in citrate synthase activity of 16 % in type 2 diabetics compared to obese controls and a 20 % reduction compared to lean. These differences did not reach statistical significance, but could reflect the fact that mitochondrial content was reduced in the type 2 diabetic subjects.

SKELETAL MUSCLE FIBER TYPE COMPOSITION IN TYPE 2 DIABETES

The metabolic capacity of skeletal muscle depends on the fiber type distribution. Human skeletal muscle consists mainly of three different fiber types, namely type I, IIa and IIx. Type I is slow twitch oxidative, type IIa fast twitch oxidative and type IIx fast twitch glycolytic. Type I fibers have the highest oxidative activity, whereas type IIx have the highest glycolytic activity (7). Studies on fiber type distribution in skeletal muscle of type 2 diabetic patients show conflicting results. Some have found differences in fiber type composition in type 2 diabetic and insulin resistant states (8-11), while others have not (12-15). Generally, there is a trend of reduced type I fiber content in type 2 diabetic subjects, and considering the possibility of alterations in fiber type distribution in type 2 diabetes, it is important to consider enzyme activity per fiber type.

The above mentioned differences in oxidative enzyme activity could thus be explained by a different fiber type composition in type 2 diabetes compared to lean and obese controls. He et al. further investigated this concept in 2001 where single-fiber analyses were performed on 22 lean, 20 obese and 20 type 2 diabetic subjects. Fiber type composition and glycolytic (α-glycerol phosphate dehydrogenase) and oxidative (succinate dehydrogenase) enzyme activities for each fiber type were determined (15). The authors found no significant group differences in fiber type proportions although there was a trend for a lower proportion of type I fibers in obese and diabetic subjects compared to lean. Using succinate dehydrogenase (SDH) activity as an index of oxidative capacity, He et al. found significantly lower activity in patients with type 2 diabetes compared to lean controls. This was observed for all three fiber types. The authors concluded that patients with type 2 diabetes have a reduction in the oxidative activity of skeletal muscle which is independent of the effect of fiber type. However, this conclusion was proposed without measures of mitochondrial content.

ELECTRON TRANSPORT CHAIN ACTIVITY IN TYPE 2 DIABETES

Kelley et al. conducted more detailed studies of the mitochondrial respiratory chain, by measuring the specific activity of rotenone-sensitive NADH:O2 oxidoreductase (16). Rotenone is a specific inhibitor of complex I in the respiratory chain, and the authors argued that the activity of the NADH:O2 oxidoreductase reflects the overall activity of the electron-transport chain. The authors used citrate synthase activity to normalize for mitochondrial content, and performed electron microscopy of skeletal muscle of 10 patients with type 2 diabetes, 10 lean controls and 10 obese controls. They found a reduced activity of the rotenone-sensitive NADH:O2 oxidoreductase in the diabetic subjects compared to both obese and lean controls. The activity of citrate synthase was also significantly reduced in the type 2 diabetic subjects. Thus, when the authors corrected for the reduction in citrate synthase activity, and hence the reduction in mitochondrial content, they deduced that no differences exist in the activity of the electron transport chain between type 2 diabetics, lean and obese controls. Electron microscopy revealed that mitochondria in skeletal muscle of patients with type 2 diabetes and obese controls were smaller than in the muscle of lean controls. This finding could be explained by the sedentary lifestyle of the type 2 diabetic subjects, but the level of physical activity of the subjects was not reported. The authors concluded that there is an impaired bioenergetic capacity of skeletal muscle mitochondria in type 2 diabetes and their results imply that this impairment could be due to a reduced mitochondrial content and/or volume in the skeletal muscle of type 2 diabetics.

Ritov et al. (17) published a paper in 2005 on deficiencies of subsarcolemmal muscle mitochondria in obesity and type 2 diabetes. In this study muscle biopsies were obtained from 11 patients with type 2 diabetes, 12 obese individuals and eight lean controls. Using differential centrifugation and digestion techniques the authors separated subsarcolemmal and intermyofibrillar mitochondria and measured electron transport activity in both fractions. Electron transport activity was measured using “succinate oxidase” (succinate: O2 oxidoreductase) which is comparable to the activity of complex II in the electron transport chain. The electron transport activity of subsarcolemmal mitochondria was significantly reduced in patients with type 2 diabetes compared with obese and lean controls. The authors quantified mitochondrial DNA (mtDNA) as a measure of mitochondrial content and found lower amounts in the muscle of type 2 diabetics than in lean controls. The decrement in mtDNA was, however, smaller than the decrement in the activity of the electron transport in the subsarcolemmal mitochondria, leading to the conclusion that these mitochondria have a functional impairment in type 2 diabetics. Subsarcolemmal mitochondria are thought to be involved in membrane related processes relevant to insulin action (18), whereas intermyofibrillar mitochondria are thought to support muscle contraction (17). The disproportionate deficiency in subsarcolemmal mitochondria in type 2 diabetes could be related to insulin resistance. Since the subsarcolemmal fraction of mitochondria is most responsive to physical activity (19), a link between insulin resistance, subsarcolemmal mitochondrial function and the positive effect of physical activity seemed to be established. However, an intervention study by Menshikova et al (20) could not find a strong correlation between improvements in insulin sensitivity and activity of subsarcolemmal mitochondria following a program of physical activity and weight loss in 14 obese sedentary men and women. The activity of succinate oxidase in the subsarcolemmal mitochondria increased significantly with training, but the proportionality between intramyofibrillar and subsarcolemmal activity remained unchanged (20).

Taken together, several studies show reduced activity of oxidative enzymes in skeletal muscle of type 2 diabetics (6;15-17;21), and these reductions in activity are independent of muscle fiber types (15) and are present together with visual evidence of damaged mitochondria (16). The reduced oxidative enzyme activity can in most studies be explained by decreases in mitochondrial content (6;15;16;21), thus evidence of a functional impairment in mitochondria in type 2 diabetes is not convincing. Furthermore, biochemical measurements of oxidative enzyme activity should, in this setting, preferably be compared to functional measurements of mitochondrial function, i.e. measurements of mitochondrial respiration, to verify if changes in enzyme activity influence overall mitochondrial function. None of the studies mentioned above include data on in vitro or in vivo mitochondrial respiration. The study by Ritov et al. (17) is the only study showing functional impairment of skeletal muscle mitochondria in type 2 diabetics, but only in the subsarcolemmal fraction of mitchondria. Altered

capacity of different subcellular mitochondrial fractions in type 2 diabetes cannot be ruled out, but the consequences of such alterations on cellular metabolism are unclear since the physiological role of the different mitochondrial subpopulations is largely unknown (22).

ATP PRODUCTION AND STUDIES ON INSULIN-RESISTANT OFFSPRING OF TYPE 2 DIABETIC PARENTS

Stump et al. applied other methods of measuring mitochondrial function and the effect of insulin on skeletal muscle mitochondrial ATP production, protein synthesis and mRNA transcripts (23). Long term (7-8 hours) insulin-clamps were performed on nine type 2 diabetic patients and nine controls. Subjects were studied at low (39 + 3 pM) and moderate (395 + 12 pM) plasma insulin concentrations and muscle biopsies were obtained at the end of each clamp in order to measure mitochondrial ATP production. Mitochondrial ATP production was measured on isolated mitochondria with a bioluminescent technique using different substrates in combinations with ADP (state 3 respiration). Moderate levels of insulin stimulated ATP production significantly in controls but not in type 2 diabetics. Furthermore, increased activity of citrate synthase (+28 %) and cytochrome-c oxidase (COX) (+29 %) was found in controls, but these enzyme activities were not measured in type 2 diabetic patients. Basal CS activity was similar in type 2 diabetic patients and controls, maybe due to the fact that the type 2 diabetic subjects were not sedentary. The study demonstrates that the capacity for oxidative phosphorylation in skeletal muscle can be enhanced by insulin infusion, and that this insulin-stimulated enhancement is blunted in type 2 diabetes. Questions still remain whether the enhanced oxidative capacity in healthy skeletal muscle is mediated by insulin or simply by an increased substrate flux in the muscle, and whether the decreased ATP production in type 2 diabetics merely reflects muscular insulin resistance. There is evidence, however, that this lack of insulin stimulation of ATP production is an early defect in insulin resistance and type 2 diabetes. Petersen et al. conducted experiments using MR-spectroscopy (MRS) to measure ATP production and phosphate concentration in skeletal muscle of insulin resistant offspring (IR offspring) under insulin stimulated conditions (24). The IR offspring had a 50 % reduction in the insulin-stimulated glucose disposal and a 2-fold higher intramyocellular lipid (IMCL) content, but no significant overall decrease in lipid or glucose oxidation. Insulin stimulation (duration: 150 minutes, plasma insulin concentration: 480 pmol/L) increased skeletal muscle mitochondrial ATP synthesis by 5 % in the insulin resistant offspring compared to 90 % in the control subjects. The change in ATP synthesis rates correlated with the changes in intramyocellular phosphate concentrations (pi), suggesting that insulin regulates mitochondrial ATP production trough control of phosphate transport into the muscle. As in the study of Stump et al., the blunted insulin stimulation of ATP synthesis may well reflect insulin resistance, but the finding of decreased levels of intramyocellular phosphate could be relevant since phosphate is a putative cytosolic signaling molecule regulating oxidative phosphorylation (25).

Petersen et al. have shown earlier that basal ATP production is decreased in offspring of parents with type 2 diabetes (26). Compared to controls, the rate of ATP production was reduced by approximately 30 %. The study was conducted using MRS and no measurements of mitochondrial content were performed. As previously mentioned, these results can be explained by a reduced mitochondrial content in the skeletal muscle of insulin-resistant offspring. A later study by the same group used electron microscopy to measure mitochondrial content (27). A 38 % lower mitochondrial density and a 50 % reduction in mitochondrial encoded protein cytochrome-c oxidase expression were found in the skeletal muscle of insulin-resistant offspring. The expression of nuclear encoded mitochondrial proteins (pyruvate dehydrogenase and succinate dehydrogenase) tended to be reduced as well. Interestingly, mtDNA copy number was not different between the groups, suggesting that the early defect in insulin resistance is manifest in mitochondrial morphology and size, and not number of mitochondria. The reduction in mitochondrial number is not present until later stages of the disease (17). The reduction in mitochondrial density of the young and lean IR offspring is comparable with the 30 % reduction in ATP production previously reported (26).

The studies on insulin resistant offspring of type 2 diabetic parents have provided important insights in the earliest metabolic defects in type 2 diabetes. The offspring are insulin resistant and have increased amounts of IMCL but are all normoglycaemic, implying that hyperglycaemia does not contribute to the detrimental effects on mitochondrial function. This is in contrast to the proposal of Brownlee (28), who argues that excess intracellular glucose leads to ROS production due to a too large voltage gradient across the mitochondrial membrane. In his view, when a critical threshold is reached, electron transport through complex III is impaired and coenzyme Q donates electrons one at a time to molecular oxygen thereby generating superoxide. The superoxide production inhibits glucose degradation and leads to accumulation of glycolytic intermediates which are shunted into different pathways damaging to the cell. However, Brownlee conducted experiments on endothelial cells, and it is possible that the detrimental effects on mitochondria seen in skeletal muscle and endothelial cells in diabetes do not share the same mechanism. We examined this hypothesis in study I of this thesis.

GENETIC AND LIFESTYLE FACTORS INFLUENCING MITOCHONDRIAL FUNCTION

The decreased mitochondrial content found in prediabetic states (27) has led to an intensive search for target genes and proteins regulating mitochondrial biogenesis and function. Decreased expression of peroxisomal proliferator activator receptor γ (PPARγ) coactivator 1α (PGC-1α) has been found in type 2 diabetes (29) and in prediabetic states (30), leading to a decreased expression of genes encoding key enzymes in oxidative metabolism and mitochondrial function (OXPHOS) (including pyruvate dehydrogenase A1, succinate dehydrogenase B, subunits of complex I, II, III, IV and ATP Synthase). More importantly, the activity of mitochondrial proteins Errα and Gabpa/b, transcription factors regulating many OXPHOS genes, are also found to be downregulated (31). A defect in Errα and Gabpa/b could have proven to be a primary cause of diabetes, but Morino et al. did not find differences in mRNA expression between young, lean IR offspring and healthy controls (27).

Environmental factors play a major role in regulating mitochondrial oxidative capacity. Muscle oxidative function improves with exercise (32). After a 16 week aerobic exercise program CS and COX activities increased by 46 % and 87 % respectively, in a large group (n=65) of men and women aged 21-87 years (33). Dela et al found a 42 % increase in COX activity in the trained leg of healthy young volunteers following a 10-week one-leg bicycle training program (34). In other studies on healthy subjects the skeletal muscle content of mitochondrial oxidative enzymes increased by

40-50% following moderate training (35), and activity of the electron transport chain, measured as activity of succinate oxidase, increased significantly as a response to weight loss and physical activity in obese and sedentary adults (20). Mitochondrial ATP production has also been shown to increase following short (10 days) (36) and longer term (6 weeks) aerobic training (37), with increments of 161 % and 170 % respectively. The reduced activity of oxidative enzymes measured in the skeletal muscle of type 2 diabetic patients could therefore simply be a result of a sedentary lifestyle. Of the five studies reporting data on CS activity or mtDNA content in fresh muscle biopsies (6;16;17;21;23) only two comment on the physical activity status of the diabetic subjects, by stating that all subjects were either "active" (23) or sedentary (17), but formal measures of fitness or physical activity were not performed. The view that the reduced CS-activity of type 2 diabetics is simply a result of sedentary lifestyle is supported by the fact that Petersen et al showed reduced mitochondrial function in young, sedentary insulin resistant offspring of type 2 diabetic parents (26). The activity of oxidative enzymes can be induced by physical activity in type 2 diabetes, albeit the results are conflicting. Holten et al. did not find an effect on citrate synthase activity in type 2 diabetics following a six week strength training program, while in the control group citrate synthase activity increased by 7 % (14). As a result of a 10-week aerobic exercise program using one-leg bicycle training Dela et al found a 91 % increase in citrate synthase activity in elderly type 2 diabetic subjects (Dela F, personal communication). The increment in citrate synthase activity was even larger than in groups of young healthy (+ 42 %) and elderly healthy (+ 72 %) subjects.
In spite of parallel increases in oxidative enzyme activities and insulin sensitivity following physical activity, no intervention studies have been able to show a strong correlation between these parameters in healthy and obese sedentary adults (20;33). Toledo and Kelley found a correlation between improvements in insulin sensitivity and increases in intermyofibrillar mitochondrial size (r=0,68, p=0,01) following a 16 week weight loss and physical activity program in obese sedentary adults (38), but this correlation has not been confirmed in type 2 diabetic subjects.

EVIDENCE OF NON-CAUSALITY BETWEEN DECREASED MITOCHONDRIAL FUNCTION AND INSULIN RESISTANCE
Even though the proposed connection between decreased mitochondrial function, intramyocellular lipid accumulation and insulin resistance is compelling and has received a lot of attention, several lines of evidence point to the fact that a direct relationship between these parameters was non-existing. Firstly, mitochondrial content increases with training and decreases with detraining with a half-life of about 10 days. The accompanying increase in insulin sensitivity occurs after the first bout of exercise but disappears 2-3 days after the last bout of exercise. This implies that mitochondrial content and insulin sensitivity might respond to training in parallel but are not causally linked. Secondly, the connection between the amount of intramyocellular lipid and insulin sensitivity can not be confirmed when elite endurance athletes are studied (13;39). Their skeletal muscle contains high amounts of lipid but yet they are very insulin sensitive. The explanation for this "athlete's paradox" has been that skeletal muscle can tolerate high amounts of lipid as long as the oxidative capacity is similarly high. Insulin signaling is only affected

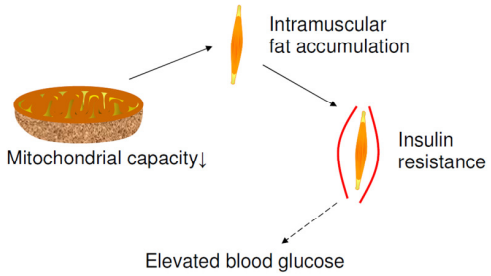

Figure 1. The theory connecting decreased mitochondrial function with insulin resistance and type 2 diabetes. This theory was the basis of the work presented in this PhD thesis.

when an imbalance between lipid content and oxidative capacity is present, and the degradation of lipid is not complete (3;40).

CONCLUSIONS
The above mentioned results formed the basis of the experimental work presented in this PhD thesis. Our aim was to further characterize the association between decreased mitochondrial function, accumulation of intramyocellular lipid and insulin resistance (Figure 1). Furthermore we aimed to investigate whether the observed decrease in mitochodrial function was a consequence of hyperglycaemia, and whether unwanted changes in mitochondrial function in type 2 diabetes could contribute to weight gain.

AIMS AND HYPOTHESIS

STUDY I: EFFECT OF HYPERGLYCAEMIA ON MITOCHONDRIAL FUNCTION IN TYPE 2 DIABETES
Mitochondrial function has consistently been found to be reduced in type 2 diabetes compared to age- and BMI- matched control subjects (6;16;17;21;27;41-44). The occurrence of hyperglycaemia is one of the most prominent pathological features distinguishing the obese insulin resistant subject from an obese patient with type 2 diabetes. Our aim was to test whether hyperglycaemia affected mitochondrial function by decreasing oxidative capacity causing accumulation of intramuscular lipid thus creating a vicious circle. Hyperglycaemia-induced mitochondrial damage had been shown in endothelial cells (28), but had not been investigated in skeletal muscle. However, two weeks insulin treatment partly corrected mRNA transcipt levels of oxidative genes downregulated in poorly controlled type 2 diabetes (45). We therefore treated 11 patients with type 2 diabetes intensively with insulin for seven weeks in order to improve the glycaemic control and measured markers of mitochondrial content and mitochondrial respiration in permeabilised muscle fibers before and after treatment.

STUDY II: REGIONAL DIFFERENCES IN SKELETAL MUSCLE MITOCHONDRIAL RESPIRATION IN TYPE 2 DIABETES AND OBESITY
The analysis of mitochondrial function has been performed on the leg musculature of patients with type 2 diabetes in all the studies so far. It is not known whether the reduction in mitochondrial function is uniformly distributed to other muscle groups of the body, and a uniform decrease could support evidence for a pri-

mary genetic mitochondrial defect in type 2 diabetes. Previous studies have found a relatively better preserved glucose clearance (46) and fatty acid metabolism (47) in arm muscles compared to leg muscles in type 2 diabetes, so we hypothesized that arm muscle mitochondrial function would be comparable between patients with type 2 diabetes and obese controls.
To test this hypothesis we performed measurements of mitochondrial respiration in skeletal muscle of the arm (m. deltoideus) and the leg (vastus lateralis) of patients with type 2 diabetes and compared them to control subjects matched for age, sex and BMI.

STUDY III: EFFECT OF ROSIGLITAZONE TREATMENT ON MUSCLE MITOCHONDRIAL CONTENT AND FUNCTION IN TYPE 2 DIABETES
Insulin sensitizing without exercise is possible with pharmacological treatment. The thiazolidinediones (TZD's) are the only available drugs known to increase muscular insulin sensitivity (48). They act on peroxisomal proliferator-activated receptor (PPAR)-gamma and stimulate the expression of genes that increase fat oxidation, mitochondrial biogenesis and promote fat cell maturation and the formation of more and smaller fat cells (49). Their primary target is adipose tissue, but increases in mRNA levels of peroxisome proliferator-activated receptor-γ coactivator-1-α (PGC1-α), PPAR-β and PPAR-δ have been demonstrated in human skeletal muscle after rosiglitazone treatment (50). Furthermore, muscle specific deletion of PPAR-gamma in mice causes insulin resistance which is not restored by thiazolidinedione treatment (51), so evidence for a direct effect of TZD's on skeletal muscle exists.
Our aim was to measure how improvements in muscular insulin resistance from 12 weeks TZD treatment would affect the activity of mitochondrial enzymes and mitochondrial respiration in 12 patients with type 2 diabetes. Our hypothesis was that the TZD-induced upregulation of genes involved in oxidative phosphorylation would result in improvements in mitochondrial function.

STUDY IV: IMPROVED GLYCAEMIC CONTROL DECREASES INNER MITOCHONDRIAL MEMBRANE LEAK IN TYPE 2 DIABETES
Mitochondrial uncoupling is caused mainly by inner mitochondrial membrane leak and is responsible for a significant proportion (18 % to 22 %) of total oxygen consumption and hence energy expenditure (52).
Most pharmacological treatments aimed at reducing hyperglycaemia in type 2 diabetes cause weight gain (53). This side effect of treatment is particularly undesireable in type 2 diabetes since insulin resistance and obesity are tightly intertwined (54), and obesity further increases some of the risk factors for cardiovascular disease in type 2 diabetes (55).
Several mechanisms have been proposed for this increase in body mass. These include 1) fear of hypoglycaemia, which seems to encourage patients to increase their caloric intake (56), 2) reductions in glucosuria from increased glycaemic control, which can result in less energy loss in urine and lead to a positive energy balance (57-59) and 3) the anabolic effects of insulin on muscle (60) and adipose tissue (61). Treatment of hyperglycaemia with insulin or oral antidiabetic agents also leads to a positive energy balance through a reduction in energy expenditure in both type 1 (58) and type 2 diabetes (62;63). The exact mechanism for this reduction in energy expenditure has not been determined (63), but could be due to suppression of hepatic gluconeogenesis (64), or increases in mitochondrial efficiency which has not been examined to date.

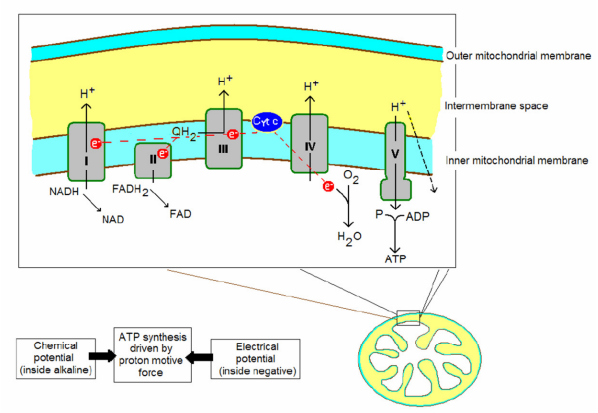

Figure 2. Oxidative phosphorylation at the electron transport chain (65;66). The coupling between oxygen consumption and ATP production is variable (52;67-69). Protons can travel across the inner mitochondrial membrane without generating ATP (inner mitochondrial membrane leak (dotted arrow)) thus reducing proton motive force and mitochondrial efficiency.

We studied mitochondrial respiration in skeletal muscle before and after seven weeks of intensive insulin treatment of patients with type 2 diabetes initially in poor glycaemic control. Mitochondrial respiration was measured using the technique of permeabilised muscle fibers (42;70-72). The purpose of the study was to test the hypothesis that mitochondrial efficiency is increased as a result of insulin treatment, as determined by increased respiratory coupling of mitochondria or decreased inner mitochondrial membrane leak (Figure 2).

METHODS
In order to ciritically evaluate the results we have obtained, an in depth analysis of the respirometry method will be provided in the following section. We have applied several other methods during our studies, but these are all well established, i.e. hyperinsulinemic euglycaemic clamping, biochemical measurements of intramyocellular triglyceride, citrate synthase etc.
High resolution respirometry has not been used routinely on skinned muscle fibers from patients with type 2 diabetes, and this method will therefore be discussed in this section.

HIGH RESOLUTION RESPIROMETRY
Measuring mitochondrial function with respirometry has been the primary focus of the work presented in this thesis. We have used high resolution respirometry in order to measure oxygen consumption in small pieces of skinned muscle fiber. The method of respirometry is not novel, but the technique has been optimized in order to increase the sensitivity of the oxygen sensors (70-73). To fully understand the strenghts and weaknesses of this method I will describe the work flow from the time a biopsy is taken untill the measurement of oxygen consumption is complete.
1. Biopsy storage. A small piece of the muscle biospy is immidiately placed in ice-cold BIOPS solution. The solution contains 10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl2, 5,77 mM ATP, 15 mM phosphocreatine and has a pH 7.1. The biopsies can survive in this medium for up to 24 hours. Figure 3 shows the

difference in oxygen consumption between a part of a biopsy that was immidiately analysed (blue bars) and one that has been kept in BIOPS solution at 4 °C for 24 hours (white bars).
Even though we have shown that biopsies can survive storage, all our biopsies were examined on the same day of the experiment.

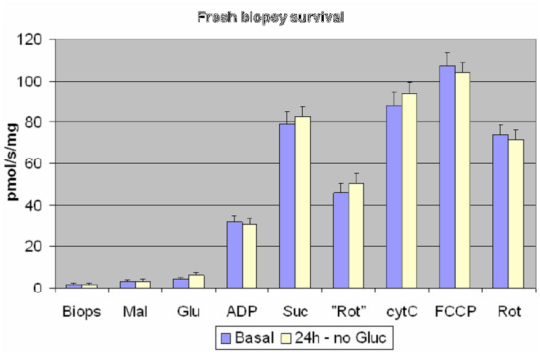

Figure 3. **Storage of biopsies for 24 hours at 4 °C in BIOPS solution does not affect oxygen consumption (N=10). The marks on the x-axis indicate the addition of different substrates and inhibitors.**

2. Dissection and saponin treatment. The muscle biopsies are gently dissected using forceps or needles in order to remove visible fat and connective tissue. Dissection is done under a magnifying glass while the biopsy is still kept in ice-cold BIOPS solution. The muscle fiber membrane is then permeabilised with saponin by adding the biopsy to a vial of BIOPS medium containing saponin (50 µg/mL). Saponin is a mild detergent which dissolves the cholesterol of the sarcolemma. This allows for our substrates and inhibitors to enter the muscle cell during respirometry. The outer mitochondrial membrane does not contain high amounts of cholesterol so the integrity of this membrane is not lost by saponin treatment. It is however crucial that saponin treatment is limited to 30 minutes and that the concentration of saponin is 50 µg/mL, otherwise the outer mitochondrial membrane will become permeabilised. We have not performed methodological studies on the saponin treatment and exposure time since the optimal treatment time is well established (70-72), but we do test the integrity of the outer mitochondrial membrane in each experiment. If the addition of cytochrome c increases respiration then the outer mitochondrial membrane has become permeabilised and cytochrome c can reach the electron transport chain and stimulate electron transport and respiration. If oxygen consumption was increased by more than ten percent after cytochrome c addition the experiment was discarded.
After permeabilisation biopsies were rinsed twice for 10 minutes in MiR05 solution containing 0.5 mM EGTA, 3 mM MgCl2.6H2O, 60 mM K-lactobionate, 20 mM Taurine, 10 mM KH2P04, 20 mM HEPES, 110 mM Sucrose, 1 g l-1 BSA, at pH 7.1. The rinsing ensures that the saponin is removed from the cells.
3. Determining the weight of the biopsies. The most difficult part of our measurements is determining the weight of the biospy sample that will be used for respirometry. We weigh the biospy after blotting off water on a piece of paper. When the biopsy is on the scale water will continuously evaporate, making it difficult to detemine the exact weight. On the other hand, if the biopsy is dried for too long time, the muscle cell will suffer and will not be able to respire as well in the respiration chamber. Figure 4 shows the effect of drying time on respiration. It is crucial that drying time is kept under 5 minutes. Normally samples would be weighed within 1-2 minutes.

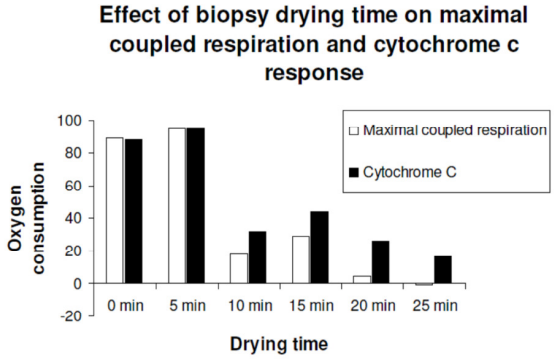

Figure 4. **The effect of biopsy drying time on mitochondrial respiration. If the biopsy is dried for more than five minutes the following measurement of maximal respiration will be significantly decreased. Furthermore, we found evidence of damaged outer mitochondrial membrane since cytochrome c response increases.**

Determining a realistic weight of our sample is crucial for our data since oxygen consumption is measured as pmol O2/s*mg. As will be shown later, imprecise weighing of our samples accounts for a lot of the variation we find in our measurements of mitochondrial oxygen consumption. Other ways of reporting data includes normalizing our respirometry data to mitochondrial content, but this has also proven to be challenging. The issue of normalization will be discussed later in this chapter.
4. Respirometry. All measurements of oxygen consumption are done in duplicate, and the oxygen sensors provide an online reading of the actual oxygen consumption and oxygen concentration in the chambers. Figure 5 shows an example of a trace from the oxygraph. The blue line indicates the oxygen concentration of the chamber, while the red line is the derivative of the blue indicating oxygen consumption. In this protocol substrates are added in series and respiration is stimulated. In the end of the protocol respiration is inhibited by the addition of oligomycin which blocks ATP production in complex V. The addition of oligomycin causes a marked decrease in oxygen consumption.

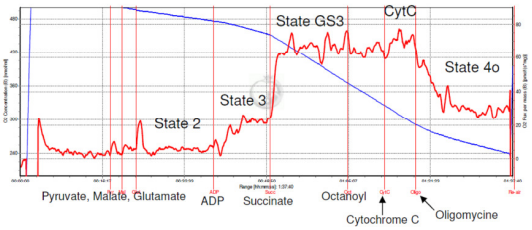

Figure 5. **Example of trace from the oxygraph. The blue line indicates oxygen concentration and the red line indicates oxygen consumption. Substrates are added in series and respiration is stimulated. Data on oxygen consumption are transferred to a PC for further analysis.**

From the results of the individual chambers we can calculate limits of agreement. Figure 6 shows measurements from two chambers analysing a biopsy from the same subjects plotted as a scatter plot. The data are maximal respiration with malate, glu-

tamate succinate and ADP as substrates from 106 subjects studied as part of the experimental work for this thesis.

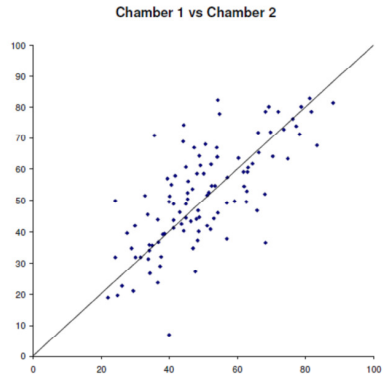

Figure 6. **Agreement between two respiration chambers analysing a biopsy from the same subject. The dataset consists of 106 experimental runs conducted as part of the experimental work for this PhD thesis.**

The mean and standard deviation of the results from chamber 1 is 50.1 + 15.1 pmol O2/s*mg and from chamber 2: 51.5 + 16.5 pmol O2/s*mg. The mean difference and standard deviation between the chambers is 1.4 + 11.9 pmol O2/s*mg. The limits of agreement between the 2 chambers is – 22 pmol O2/s*mg to + 26 pmol O2/s*mg. To examine if the variation increases with increasing oxygen consumption, we can draw a Bland-Altman plot where the difference between chambers is plotted against the average between the chambers (Figure 7).

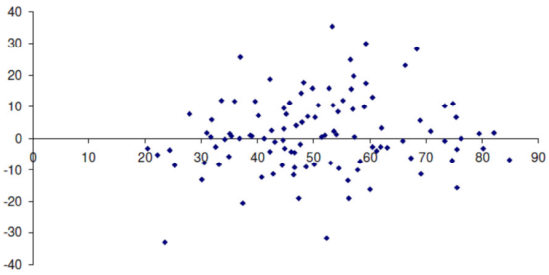

Figure 7. **Bland-Altman plot showing that the difference between the chambers does not increase with increasing oxygen consumption.**

Variation is not increased with increasing oxygen consumption implying that the oxygraph is performing reproducible measurements over a broad range of oxygen fluxes. An alternative explanation for the observed variation in our measurements could be that the small pieces of biopsy contain different amounts of mitochondria. If the mitochondria were heterogenously distributed in the muscle then we would expect to find larger differences between chambers with larger differences in weight of the biopsy. Figure 8 illustrates that this is not the case.

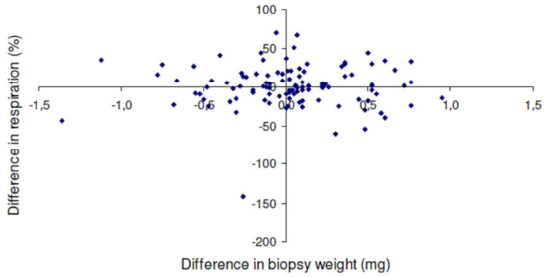

Figure 8. **A large difference between the biopsy in the two chambers does not lead to a large difference in respiration implying that mitochondria are distributed homogenous in our samples.**

In conclusion, the variation between the chambers can not be attributed to variations in sample size or imprecise measurements at higher rates of respiration. The main cause of variation between the chambers is probably introduced by imprecise weighing of the muscle samples.

Knowing the variation in our measurements it is possible to perform a post hoc power calculation. In study I we examined whether tight glycaemic control improved mitochondrial function. If we aim to identify a difference of 25 % in mitochondrial function with a power of 80 % and a significance level of 5 % the calculations would look as follows:

$n > (u+v)2 \times (\sigma 12 + \sigma 22) / (\mu 1 - \mu 2)2$, where u = 0.84, v = 1.96, standard deviation (σ1 and σ2) = 30 % and the minimal relevant difference (μ1 – μ2) = 25 %

$n > (0.84+1.96)2 \times (302 + 302) / (252) \quad \Rightarrow \quad n > 22$ in each group.

If we were looking for smaller improvements the number of subjects required would increase substantially: With a minimal relevant difference of 15 % and the same power and significance level the number of subjects should be over 63.

5. Normalization of mitochondrial respiration to mitochondrial content. Citrate synthase (CS) activity has been established as reflective of nuclear expression of mitochondrial proteins, and hence a marker for mitochondrial content (74). The use of citrate synthase as a marker is not without problems. Maybe not surprisingly, CS activity can, in parallel with mitochondrial content, be increased by endurance training in healthy adults (32;33;35;75) and type 2 diabetic patients (76), but increases in CS activity have also been reported following acute exercise (77;78), whole-body insulin stimulation (23) and incubation of skeletal muscle cells with insulin for four hours in healthy adults (79). These acute effects on CS activity do not reflect increases in mitochondrial content, and it stresses the point, that CS can be used as marker only in the basal state where physical activity is controlled for. Another marker for mitochondrial content is mitochondrial DNA (mtDNA) which is correlated to CS activity in the basal state (74), but is not sensitive to acute changes in muscle metabolism. The level of mtDNA reflects the level of mtDNA genome expression and therefore mitochondrial content (80), and this measure has been applied to address the question of how mitochondrial content and gene expression is altered in diabetes (17) and other dieased states (74), as well as with interventions such as exercise training (18).

In addition to reporting the data on oxygen consumption per mg of tissue we have calculated and reported respiration per CS activity in some of the work presented in this thesis. Mitochon-

drial respiration per CS activity could give information on the capacity of each mitochondrium in a sample. A problem with this method is that CS is not measured in the sample that we performed the respirometry on, and CS activity is measured in another piece of biopsy sample which, in principle, could contain more or less mitochondria than the sample we performed respirometry on. This problem is not solved by perfoming biochemical measurements of other mitochondrial markers (i.e. mitochondrial DNA, mtDNA) and it remains a topic for discussion how to normalize respiration to mitochondrial content. In my opinion the raw data on oxygen consumption per mg tissue is the most appropriate value to present, since it gives an indication of the overall capacity of the biopsy to perform oxidative phosphorylation. If specific information on mitochondrial physiology is needed then it is possible to calculate ratios between the different respiratory states. These ratios are not affected by differences in mitochondrial content.

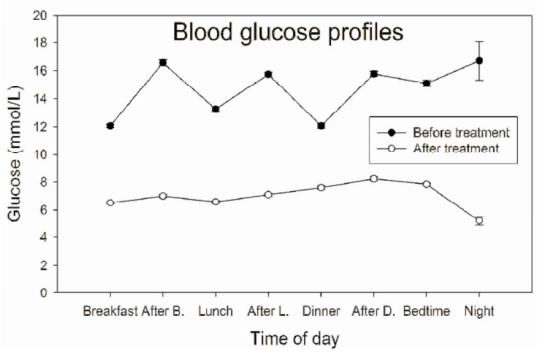

Figure 9. **The result of seven weeks of intensive insulin treatment of the ten patients with type 2 diabetes in study I. The average blood glucose fell significantly at all time-points during the day. Blood glucose profiles are averages of 8-point blood glucose measurements performed on the last three days before insulin treatment and on the last three days of treatment.**

SUMMARY OF RESULTS

STUDY I:
EFFECT OF HYPERGLYCAEMIA ON MITOCHONDRIAL FUNCTION IN TYPE 2 DIABETES

The subjects for this study were recruited at the outpatient clinics of Hvidovre and Gentofte University Hospitals. All subjects were treated with oral antihyperglycaemic agents, either metformin or a sulfonylurea, at inclusion. To avoid any effect of their current medication on our measurements, we took all patients off medication for two weeks before the initial examination day. After the initial examination we began an intesive insulin treatment regimen aiming for fasting blood glucose below 5.5 mmol/L and postprandial values below 7.5 mmol/L. Treatment resulted in significant decreases in fasting blood glucose, average blood glucose (including both pre- and postprandial), HbA1c and fruktosamin. We also included an age- and BMI-matched control group and performed similar measurements at baseline. Baseline characteristics of the control group and the group of patients with type 2 diabetes only differed with regards to glycaemic control.
At baseline mitochondrial respiration was reduced in the subjects with type 2 diabetes compared to control subjects. We measured mitochondrial respiration per milligram muscle and found that respiration was decreased by 17 to 24 % percent depending on the substrates used, $p<0.05$. While the intensive insulin treatment improved the glycaemic control significantly (Figure 9), we did not detect any changes in mitochondrial respiration. Figure 10 shows the individual data from the group of patients before and after treatment and the control group at baseline. Substrates for complex I are malate, pyruvate, glutamate and ADP in saturating concentrations, while substrates for complex I and II includes the addition of succinate in a saturating concentration.

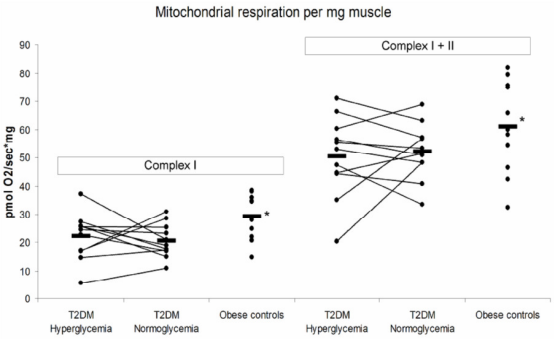

Figure 10. **Mitochondrial respiration with substrates for complex I (malate, pyruvate, glutamate) and complex II (malate, pyruvate, glutamate and succinate). Mitochondrial respiration is significantly reduced in type 2 diabetes when compared to the respiration in the control group. Optimizing glycaemic control had no effect on mitochondrial respiration in type 2 diabetes. Mitochondrial respiration is measured per mg muscle.**
*** p<0.05 compared to patients with type 2 diabetes.**

Using substrates in saturating concentrations ensures that that we obtain the maximal mitochondrial respiration possible. Maximal mitochondrial respiration is correlated to the total number of mitochondria, and our measurements could be interpreted as another way of measuring mitochondrial content. In order to test if we could find intrinsic defects in the mitochondria of the patients we controlled our measurements of respiration by the number of mitochondria present in the muscles of the subjects. Citrate synthase activity is an established marker of mitochondrial content, and the values of maximal mitochondrial respiration and citrate synthase activity correlated significantly in our study ($r2=0.84$, $n=11$, $p<0.01$). When we divided the individual measurements of respiration with the corresponding value of citrate synthase activity, we did not find a difference between the subjects with type 2 diabetes and the control subjects (Figure 11.) This implied that mitochondrial content is reduced in type 2 diabetes, but the intrinsic function of the mitochondria is not affected.

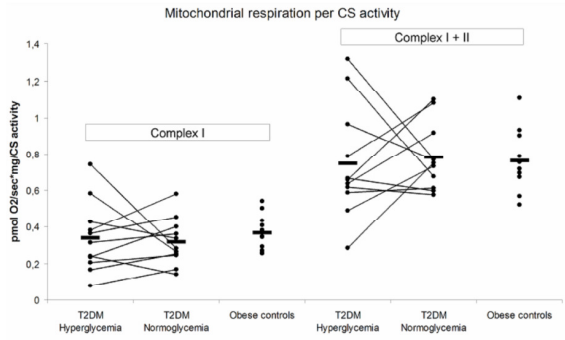

Figure 11. **Mitochondrial respiration with substrates for complex I (malate, pyruvate, glutamate) and complex II (malate, pyruvate, glutamate and succinate) measured as respiration per citrate synthase activity as a marker of mitochondrial content. No differences in mitochondrial respiration are observed between the groups.**

The reduction in mitochondrial function did not translate into increased levels of intramyocellular triglyceride in the patients with type 2 diabetes.

STUDY II:
REGIONAL DIFFERENCES IN SKELETAL MUSCLE MITOCHONDRIAL RESPIRATION IN TYPE 2 DIABETES AND OBESITY
We obtained skeletal muscle biopsies from vastus lateralis and m. deltoideus in ten out of the eleven subjects in study I, and in ten of eleven control subjects. The biopsies were analysed for mitochondrial respiration, citrate syntyhase activity and intramyocellular levels of triglyceride and glycogen. We found that a decrease in mitochondrial respiration could only be found in the leg muscle of patients with type 2 diabetes. This was independent of the substrates used. The combinations of malate, pyruvate, glutamate and ADP (Figure 12) and malate, pyruvate, glutamate, succinate and ADP gave the same results (data not shown). When we controlled for mitochondrial content by dividing mitochondrial respiration with citrate synthase activity, we could not detect differences between the groups. Intramyocellular levels of glykogen and triglyceride did not differ between any of the groups and muscle groups.

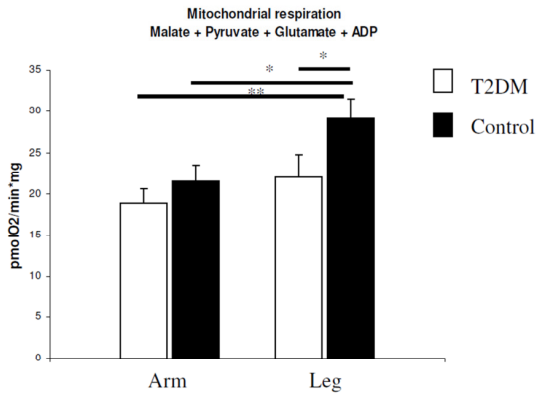

Figure 12. **Mitochondrial respiration per mg muscle using malate, pyruvate, glutamate and ADP in arm and leg muscle of patients with type 2 diabetes and control subjects. Mitochondrial respiration in the leg muscle of the control subjects was significantly higher than in the leg muscle of the patients with type 2 diabetes) and the respiration in the arm muscle of both patients and controls.**

STUDY III:
EFFECT OF ROSIGLITAZONE TREATMENT ON MUSCLE MITOCHONDRIAL CONTENT AND FUNCTION IN TYPE 2 DIABETES
Twelve patients with type 2 diabetes completed 12 weeks rosiglitazone treatment, 4 mg per day. Measurements of insulin sensitivity, body composition, indirect calorimetry, adponectin, skeletal muscle mitochondrial respiration, citrate synthase, β-hydroxyacyl-CoA-dehydrogenase (HAD) activity, electron transport chain protein content, glykogen and triglyceride content were carried out before and after treatment. A group of eight age- and weight-matched healthy subjects were included as controls.
As previously shown, mitochondrial respiration per mg muscle was reduced in type 2 diabetes. This was in accordance with our observations in study I. At baseline the subjects with type 2 diabetes were more insulin resistant and had significantly increased levels of fasting blood glucose and HbA1c compared to controls. Age, BMI, fat free mass and body fat percent were all comparable between groups.
Rosiglitazone treatment significantly improved insulin sensitivity and increased adiponectin levels. The levels of free fatty acids were reduced non-significantly by 18 % ($p<0.09$). Surprisingly mitochondrial respiration decreased from treatment, and markers of mitochondrial content (citrate synthase activity and protein content of complexes in the respiratory chain) tended to decrease as well. We found a relatively larger reduction in respiration with substrates for complex I of the respiratory chain (-31 %) compared to the reduction using substrates for complex I and II (- 10 %), suggesting that rosiglitazone could be directly inhibiting the function of complex I. However, the evidence of an overall decrease in mitochondrial content was convincing, since citrate synthase activity (- 9 %), average electron transport chain protein content (- 15 %) and β-HAD activity (- 13 %) decreased to the same extent.
Resting and insulin stimulated RQ levels did not change as a result of treatment and were not different from the levels found in the control subjects. Intramyocellular levels of triglyceride were also unchanged from treatment, and did not differ from the levels of the controls.

STUDY IV:
IMPROVED GLYCAEMIC CONTROL DECREASES INNER MITOCHONDRIAL MEMBRANE LEAK IN TYPE 2 DIABETES
We observed that an improvement in glycaemic control lead to increases in mitochondrial efficiency as evidenced by a reduction in mitochondrial membrane leak. HbA1c and fruktosamin improved significantly as a result of treatment, and the patients gained an average of 3.4 + 0.9 kg in body weight ($p<0.01$). The reduction in state 4o respiration lead to increases in respiratory control ratio (RCR), which increased by 24 % in the arm musle ($p=0.07$) and 17 % in the leg muscle ($p=0.14$).The patients with type 2 diabetes had significantly lower levels of RCR before treatment than the control subjects, but RCR increased to the level of the controls after hyperglycaemia had been treated with insulin for seven weeks. The changes in state 4o for arm and leg muscle are shown in figure 13. We did not find any correlation between the changes in body weight and changes in state 4o respiration.

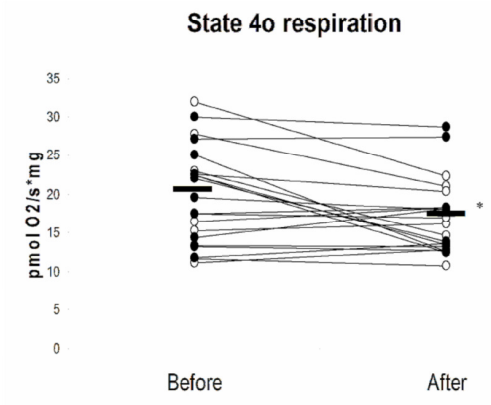

Figure 13. **Individual changes in membrane leak, state 4o, following intensive insulin treatment for seven weeks. Data are shown from arm (open circles) and leg (closed circles). Horizontal bars indicate average value. State 4o decreased by 15% the arm (p=0.07) and 13 % in the leg (p=0.1). Average state 4o decreased by 14 % (p<0.05).**

DISCUSSION

STUDY I: EFFECT OF HYPERGLYCAEMIA ON MITOCHONDRIAL FUNCTION IN TYPE 2 DIABETES

A substantial improvement in glycaemic control did not lead to changes in mitochondrial respiration in skeletal muscle of patients with type 2 diabetes. Mitochondrial respiration was lower in the patients with type 2 diabetes compared to age- and BMI-matched controls. This was due to a lower mitochondrial content. Our results indicate that the reduction in mitochondrial content in type 2 diabetes is not a consequence of hyperglycaemia, at least not within the range of glycaemic control of the present study. Mitochondrial dysfunction following hyperglycaemia has been shown in endothelial cells, where the superoxide damage to mitochondria has been connected to the development of diabetic complications (28). The superoxide production is thought to be increased because of increased substrate oxidation, and the following increase in the voltage gradient over the inner mitochondrial membrane stimulates ROS production. Whether increased ROS production can explain the reduction in mitochondrial content in skeletal muscle in type 2 diabetes remains to be shown (81-88), but oxidative stress has been shown to induce defects in mitochondrial biogenesis, structure and function in high-fat, high-sucrose fed insulin resistant mice (89).

Insulin resistance and decreased insulin action per se could affect mitochondrial biogenesis and function. Ten days of intensive insulin treatment had no effect on muscle mitochondrial protein synthesis and cytochrome c oxidase (45), and caused only a modest increase in citrate synthase activity (90). Insulin infusion studies have confirmed that insulin upregulates genes involved in mitochondrial bioenesis (91), but this effect has been shown to be blunted in type 2 diabetes (92), and the increase in ATP synthesis following insulin infusion is inhibited in type 2 diabetes (23). This has lead to the assumption that the impaired mitochondrial biogenesis and function in type 2 diabetes could be a result of impaired insulin action rather than the cause of insulin resistance, and mice studies have shown that insulin treatment restores expression of electron transport chain proteins down-regulated by streptozotocin treatment (93). Our 7-week insulin treatment did not result in an increase in citrate synthase activity, which is in accordance with one other study of longer term insulin treatment (40 days) (94), but insulin is probably an important regulator of mitochondrial function in human skeletal muscle (95).

Defects in lipid metabolism could also have negative effects on mitochondria. Lipid infusion causes an immidiate increase in insulin resistance (5), and a decrease in gene expression of genes involved in oxidative phosphorylation (96;97). Furthermore, Brehm et al. showed that the increase in muscular ATP production caused by hyperinsulinemic-euglycaemic clamping was inhibited with concommitant lipid infusion. ATP production rate measured with MR-spectroscopy increased by 60 % during clamping under control conditions but was 24 % lower during the lipid infusion (98). More chronic elevation of lipid supply have also been shown to affect mitochondrial gene expression since a high fat diet coordinately down-regulated genes involved in oxidative phosphorylation (99). We did not find a difference in intramyocellular triglyceride levels between type 2 diabetes and obese control subjects, but did not measure fatty acid oxidation intermediates such as diacylglycerol (DAG) and ceramide which are directly involved in the inhibition of insulin signaling (3). It has been proposed that incomplete fatty acid oxidation due to excess lipid supply to muscle could be the link between skeletal muscle insulin resistance and mitochondrial dysfunction (40;100).

In conclusion, we found that hyperglycaemia does not seem to be the major determinant of skeletal muscle mitochondrial content and function in type 2 diabetes. Other factors such as defects in lipid metabolism and decreased insulin action are more likely causes of the obeserved reduction in mitochondrial content in type 2 diabetes.

We have confirmed that mitochondrial content is reduced in type 2 diabetes (6;16;17;21;27;41-44), but we can not demonstrate intrisic defects in mitochondrial function (101).

STUDY II: REGIONAL DIFFERENCES IN MITOCHONDRIAL RESPIRATION IN TYPE 2 DIABETES AND OBESITY

The decrease in mitochondrial function found in type 2 diabetes is only present in the leg musculature. We found no diference in mitochondrial respiration between arm muscle samples from patients with type 2 diabetes and BMI- and weight-matched controls, and arm muscle respiration was significantly decreased compared to leg muscle respiration. This indicates that decreased skeletal muscle oxidative capacity is not a primary genetic defect common for all skeletal muscle in patients with type 2 diabetes, but rather that decreased mitochondrial function is a combination of genetic and lifestyle factors.

Our results support the notion that reduced mitochondrial function is at least in part a consequence of a sedentary lifestyle and decreased muscle use. It has previously been speculated that the upper body musculature has adapted to be less dependent on muscle usage (46). It is possible that the arms have adapted to inactivity as a consequence of the upright posture in which only the legs are used for movement. This is supported by the fact that insulin sensitivity is relatively better preserved in arm muscle than leg muscle in type 2 diabetes compared to control subjects (46), and that fatty acid kinetics are only impaired in the leg muscles but not the arm muscles of patients with type 2 diabetes (47). Following this argumentation, the muscles of the lower extremities “suffers” more from inactivity than the muscles of the upper part of the body since the latter has adapted to inactivity during the course of evolution. This is in accordance with our data showing decreased mitochondrial respiration in the legs but not in the arms of the patients with T2DM.

Another explanation for our findings could be that insulin resistance leads to "exercise resistance". This theory is based on the observation that a bout of exercise increases mRNA and protein levels of peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1) as well as AMPK posphorylation at 30 minutes and 300 minutes post exercise in healthy subjects. This effect is delayed and reduced in obese, insulin resistant individuals (102). According to this obeservation the decreased mitochondrial function that we find in the leg muscle could be a result of decreased stimulation of mitochondrial biogenesis in T2DM even though the activity level is the same in the group of T2DM and controls. The reason for the similar mitochondrial function in the arm muscle could be that m. deltoideus is used to such a low extent that differences in mitochondrial biogenesis can not be detected. In other words, the mitochondrial content of m. deltoideus in obesity and T2DM is close to the minimal level required to meet cellular ATP demands. It should be noted these results are from a very limited group of subjects (102), and that PGC-1α levels have previously been shown not to be reduced in obese T2DM following exercise (103). Furthermore, 6-week exercise program in obese insulin resistant and insulin sensitive subjects produced the same response in markers of mitochondrial biogenesis and content, but the baseline level was reduced in the insulin resistant subjects (104). Allthough controversial, the theory of "exercise resistance" is appealing and warrants futher investigation.
In conclusion, our results demonstrated that the reduction in mitochondrial function found in T2DM is only present in the leg musculature. This supports the notion that the observation of reduced oxidative capacity in skeletal muscle of patients with type 2 diabetes could be a combination of genetic and lifestyle factors.

STUDY III: IMPACT OF ROSIGLITAZONE TREATMENT ON SKELETAL MUSCLE MITOCHONDRIAL CONTENT AND FUNCTION
Rosiglitazone treatment caused a decrease in skeletal muscle mitochondrial enzyme activity and respiration. This decrease occured in parallel to improvements in insulin sensitivity and questions a direct relationship between insulin resistance and mitochondrial function.
It is well established that rosiglitazone improves skeletal muscle insulin sensitivity (48;49;105-108), but the mechanism of action is not completely understood. The primary target is adipose tissue, where fatty acid uptake and oxidation is upregulated and mitochondrial biogenesis is increased (109). Other targets of action have been identified including skeletal muscle AMPK activation (110;111) and upregulation of GLUT4 (48). An increase in adiponectin levels have also been demonstrated (112;113), and adiponectin has been proposed to be an important regulater of skeletal muscle mitochondrial oxidative capacity (114;115) .
We found all the expected effects of rosiglitazone treatment including improvements in insulin sensitivity, increases in adiponectin levels and a tendency to a decrease in FFA levels. Unexpectedly, we demonstrated a decrease in mitochondrial respiration as a result of treatment. We were not able to determine whether the reduction in oxygen consumption was due to decreased mitochondrial biogenesis or an intrinsic down-regulation of mitochondrial activity or both. Cytosolic enzyme activity (citrate synthase, HAD), ETC protein content and maximal oxygen consumption are all decreased to the same degree (down 9 to 13 %) which is indicative of a decrease in mitochondrial content. We found, however, that oxygen consumption using substrates for complex I was reduced by more than 30 % indicating that complex I activity is targeted by rosiglitazone. It has previously been shown that rosiglitazone treatment is connected to a down-regulation of the activity of Complex I of the respiratory chain thereby activating AMP-activated protein kinase (APMK) (110), and AMPK activity has been shown to be restored in skeletal muscle of patients with T2DM after rosiglitazone therapy (116). If AMPK activitation is central to rosiglitazone action it could be expected that the transport of fatty acids into mitochondria and beta-oxidation would be increased. We found no evidence of increased beta-oxidation (HAD activity decreased significantly during treatment) and 26 weeks rosiglitazone treatment did not increase FAT/CD36 mRNA expression in newly diagnosed patients with T2DM (106). The decrease in complex I activity could be a direct toxic effect of rosiglitazone, but this effect cannot explain the decreases in citric acid cycle enzyme activity that we and others (117) have found as a result of treatment.
Several more recent interventional studies using rosiglitazone have demonstrated improvements in insulin action while mitochondrial function and/or content decreased or were unchanged (117;118). Toledo et al. demonstrated that an 18-week diet intervention leading to over 10 % weight-loss in subjects with impaired glucose tolerance, resulted in increased insulin action while mitochondrial density, mtDNA copy number and NADH-oxidase activity was unchanged, and mitochondrial size decreased (119).
In conclusion, 12 weeks of rosiglitazone treatment improved insulin sensitivity, increased adiponectin, lowered FFA concentrations while IMTG levels were unchanged and caused a decrease in skeletal muscle mitochondrial respiration and content. Our data supports the emerging evidence that mitochondrial function and insulin sensitivity are not directly related since we demonstrate that insulin sensitivity can be improved while mitochondrial function is decreased.

STUDY IV: IMPROVED GLYCAEMIC CONTROL AND INNER MITOCHONDRIAL MEMBRANE LEAK IN TYPE 2 DIABETES
Mitochondrial function plays a central role in the control of energy metabolism. The efficiency of the mitochondria is highly variable and can influence the ability of a person to loose weight. Some of our data have provided a possible connection between treatment of hyperglycaemia, mitochondrial function and the risk of weight gain. When hyperglycaemia is reduced the level of inner mitochondrial membrane leak decreases approaching the levels seen in obese, non-diabetic control subjects. This leads to increased mitochondrial efficiency as indicated by increases in respiratory control ratios (RCR). In previous studies, improvements in glycaemic control have been accompanied by reductions in resting energy expenditure with concomitant improvements in efficiency of fuel usage (62;63). To our knowledge, the molecular mechanism behind this improvement in efficiency has not been established. We have provided data supporting the view, that mitochondria increase their efficiency with improvements in glycaemic control, and this is likely to be the background for the previous findings of reductions in resting energy expenditure with antihyperglycaemic treatment.
We have found that state 4o respiration and inner mitochondrial membrane permeability is increased with increases in blood glucose. This mechanism is thought to protect the cell from excess ROS production, and evidence for such a mechanism has been demonstrated for endothelial cells (28). By decreasing blood glucose with intensive insulin treatment inner mitochondrial proton leak is decreased to values approaching those of non-

diabetic but obese control persons. Decreases in membrane leak leads to increases in mitochondrial efficiency and hence decreases in resting energy expenditure (63;120).
Our data can possibly explain why it is more difficult for patients with type 2 diabetes to lose weight (121) and sustain their weight loss (122) compared to obese subjects with normal insulin sensitivity. If interventions aiming at reducing blood glucose (including dietary and weight loss programs) lead to more efficient mitochondria, patients with type 2 diabetes are accordingly more prone to regain weight compared to obese non-diabetic subjects. Indeed, inner mitochondrial membrane leak is an important marker for success in weight loss trials, since a high state 4o (high membrane leak and low efficiency) has been shown to result in a larger weight loss than if state 4o is low (120).
In conclusion, we show evidence of increased mitochondrial efficency when glycaemic control is improved in type 2 diabetes. This could be the molecular mechanism explaining the increased efficiency in fuel usage seen in patients with type 2 diabetes upon initiation of antihyperglycaemic treatment. This mechanism, involving decreased inner mitochondrial membrane leak, is a possible contributing cause of the weight gain seen during treatment of hyperglycaemia in type 2 diabetes.

STUDY LIMITIATIONS

Our studies have several limitations. In study I we do not find an improvement in mitochondrial respiration with increased glycaemic control. But data from high resolution respirometry has a standard deviation of approximately 30 %, so small improvements in respiration are difficult to detect with a small sample. A post hoc power calculation reveals that we would need a sample of 23 subjects to find a difference of 25 % in respiration with a power of 80 % and a significance level of 5 % (for calculations see methods section). Furthermore, our subjects did not reach normoglycaemia and the relatively short term treatment of seven weeks could be too short period to detect changes in mitochondrial respiration from changes in glycaemic control. We did not stratify our patients with regard to diabetes duration which ranged from 1 to 15 years. Stricter inclusion criteriae could possibly have improved our ability to draw conclusions from our data, but it must be noted that we have not been able to detect correlations between mitochondrial function and diabetes duration in post hoc analyses.
Generally, all data on skeletal muscle mitochondrial function should be accompanied by a measure of physical activity or VO2max of the subject. Physical activity on the day of the biopsy should be kept to a minimum or should at least be kept constant on all examination days. This has been difficult to control for, and can explain some of the variation in our day to day measurements. Ideally, subjects should be observed overnight in order to make sure that they are fasted and have not performed rigorous physical activity.
In study IV we did not control the diet of the subjects and we did not measure energy expenditure of the subjects. The study can be used to generate a hypothesis concerning the problems of weight gain during antihyperglycaemic treatment, but this hypothesis should be confirmed in a properly designed study where food intake and physical activity is controlled and where energy expenditure is measured.

CONCLUSIONS AND PERSPECTIVES

Skeletal muscle mitochondrial content is reduced in type 2 diabetes, but it is unclear what causes the reduction in mitochondrial content. We are still far from a complete understanding of the links between mitochondrial function, intramyocellular lipid and insulin sensitivity, but our studies have not been able to demonstrate a direct relationship between insulin sensitivity and mitochondrial function.
The results of our studies can be summarized as follows:
- Short term (7 weeks) correction of hyperglycaemia does not improve mitochondrial function in type 2 diabetes.
- Skeletal muscle mitochondrial dysfunction is not uniformly distributed to all parts of the body.
- Insulin sensitivity can be improved while mitochondrial function is decreased.
- Correction of hyperglycaemia leads to improvements in mitochondrial effciency which could lead to weight gain.

With regards to the broader issue of mitochondrial function in type 2 diabetes we believe that mitochondrial content is reduced in type 2 diabetes. This defect is probably not primary even though it has been shown in a highly selected group of subjects who are predisposed to type 2 diabetes (26). Our randomly selected cohort of patients with type 2 diabetes is probably very heterogenous with regards to disease pathology and mitochondrial dysfunction might contribute to some of the pathogenesis in muscular insulin resistance in some patients, but it is not the unifying mechanism leading to the accumulation of intramyocellular lipid and insulin resistance.
Our work has generated new hypotheses that require further investigation. We will study the effect of pioglitazone on mitochondrial function, in order to see if the inhibitory effect on mitochondrial function is specific to rosiglitazone or is a class effect of TZD's. This study is important since new drugs targeting the PPAR system are in development which could have serious adverse events linked to mitochondrial inhibition not detected in premarketing studies.
It is currently not known whether mitochondrial content is reduced in all tissues in patients with type 2 diabetes, and mitochondrial content in other tissues such as heart muscle and adipose tissue is also a focus for continued research.
We are also pursuing the hypothesis of increased mitochondrial efficiency in insulin resistance and type 2 diabetes, and we are planning a study in collaboration with the department of endocrinology at Yale University School of Medicine, where we will study the effect of a hypocaloric diet on mitochondrial efficiency during rest and muscular work in young insulin resistant subjects who are predisposed to type 2 diabetes. Our hypothesis is that these subjects will have stronger defense mechanisms against weight loss than insulin sensitive control subjects due to a more rapid and profound improvement in mitochondrial efficiency.

SUMMARY

Reduced skeletal muscle mitochondrial function has been proposed to lead to insulin resistance and type 2 diabetes. It has been known for several years that oxidative capacity of skeletal muscle is reduced in patients with type 2 diabetes compared to weight matched controls. The reduction in oxidative capacity supposedly leads to the accumulation of intramyocellular lipid which inhibits insulin signalling and causes insulin resistance.

It is not known whether this reduction in mitochondrial capacity is the cause or the effect of type 2 diabetes. This PhD-thesis describes the effect of different pharmacological interventions on mitochondrial function in type 2 diabetes and describe whether mitochondrial function is uniformly distributed to both upper and lower extremities. Furthermore, a hypothesis on the molecular mechanism for weight gain observed with anthyperglycaemic treatment will be presented.

STUDY I

To study the role of hyperglycaemia on mitochondrial function 11 patients with type 2 diabetes were included. All were treated with oral antihyperglycaemic agents at inclusion. The patients were studied after a period of poor glycaemic control (2-week washout of antidiabetic medication) and again after a period of optimal glycaemic control (6-week intensive insulin treatment). The insulin treatment brought the HbA1c and fruktosamin down significantly, but did not change mitochondrial respiration measured on saponin-treated skinned muscle fibers. A control group matched for age- and BMI was also included. The non-diabetic controlas had, on average, a 20% higher respiration than the patients with type 2 diabetes. This was due to a higher mitochondrial content in their muscle measured as a higher citrate synthase activity.
We conclude that the reduction in mitochondrial capacity in type 2 diabetes is not due to hyperglycaemia.

STUDY II

Measurements of skeletal muscle mitochondrial capacity have traditionally been carried out in the leg musculature. We included 10 patients with type 2 diabetes and obtained muscle biopsies from m. deltoideus and m. vastus lateralis. We compared intramuscular triglyceride, mitochondrial respiration and citrate synthase activity between these two muscles. We also included a control group, matched for age- and BMI. The groups had comparable VO2max. M. deltoideus and m. vastus lateralis were selected because of the similar fibertype composition of these muscles.
When comparing mitochondrial respiration in patients with type 2 diabetes and obese control subjects, we could only find a significant difference in the leg muscle. The arm muscle in the patients and the controls had similar levels of respiration, and were comparable to the level of the legs of the patients with type 2 diabetes.
We conclude that reduced mitochondrial capacity is not present in all muscle groups in type 2 diabetes. This finding does not support the assumption that mitochondrial dysfunction is a primary genetic defect in type 2 diabetes, but rather related to physical activity.

STUDY III

In order to demonstrate a direct relationship between insulin sensitivity and mitochondrial function we treated 12 patients with type 2 diabetes with an insulin sensitizer (rosiglitazone). All patients had their insulin sensitivity measured, and muscle biopsies from m. vastus lateralis were obtained at baseline and after three months therapy.
Treatment resulted in a significant improvement in insulin sensitivity, but a paradoxical, significant decrease in mitochondrial respiration. We conclude that insulin sensitivity can be improved without concommitant improvements in mitochondrial function in type 2 diabetes.

STUDY IV

Optimal glyceamic control did not affect mitochondrial respiration in study I. However, we found a decrease in inner mitochondrial proton leak as a result of the improved glyceamic control. This proton leak can be measured when ATP production is blocked, and is a measure of mitochondrial efficiency. The lower the proton leak, the higher the mitochondrial efficiency. Proton leak is directly correlated to energy metabolism, and we have shown that improvements in glyceamic control leads to increases in mitochondrial efficiency and a drecrease in energy metabolism. This could be one of the mechanisms explaining why patients with type 2 diabetes gain weight on antihyperglycaemic treatment.

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