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MITOCHONDRIAL FUNCTION – ADAPTATIONS TO CHANGED METABOLIC CONDITIONS

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This contribution may seem marginal,
but becomes significant in light of what I have forsaken to get here.

ABSTRACT

The skeletal muscle mitochondria play a decisive role for the metabolic capacity of the body. A capability to adapt to changed metabolic conditions and energy demands is crucial for weight control and physical exercise. The aim of this thesis was to describe how the mitochondria adapt its function to different environmental conditions and changed metabolic demands.

In study I, the aim was to evaluate mitochondrial adaptations to hypoxic exercise. The effect of one-legged cycle training at hypoxia was compared to equivalent normoxic training, performed at the same relative intensity. Eight untrained volunteers performed one-legged cycle training during 4 weeks. Muscle biopsies were taken before and after the exercise period. The leg trained during normoxia increased its mitochondrial population (+20.8%, $P<0.05$) and there was a trend towards increased respiratory capacity (+31.2%, $P<0.08$), while adaptations were absent in the hypoxically trained leg. Altitude training might thus be disadvantageous for mitochondrial adaptations and muscle oxidative function.

In study II, the aim was to investigate the effect of ultra endurance exercise on mitochondrial function. Elite ultra endurance athletes performed running, kayaking, and cycling at 60% of their maximal oxygen consumption for 24 h. Muscle biopsies were taken preexercise, postexercise, and after 28 h of recovery. We found that mitochondrial efficiency was reduced, while the mitochondrial capacity to utilize fat was up regulated (+40%, $P<0.05$) after exercise. This increase in fat oxidation was reflected at whole body level substrate utilization, thus it might benefit performance during prolonged exercise.

In study III and IV, the aims were to study mitochondrial function in obesity and effects of weight loss, respectively. Weight gain varies among individuals despite equal calorie overconsumption. Furthermore, weight loss resulting from low calorie diets is often less than expected and long-term success is low. This suggests differences and changes in metabolic efficiency and basal metabolism. Since mitochondrial uncoupling accounts for a substantial portion of the basal metabolic rate, we compared mitochondrial respiration in obese subjects to normal weight reference groups (study III). In study IV, we studied how mitochondrial capacity was affected by calorie restriction. Muscle biopsies were taken from 11 obese women, with an average BMI of 39 kg/m^2, in conjunction with their gastric bypass surgery and at 6-months of follow-up.

We found that obese subjects had a decreased oxidative capacity (-47%, $P<0.01$) per mitochondrial volume, compared to the to normal weight reference groups. A low capacity for fuel oxidation could play a role in the predisposition for obesity. Six months after the gastric bypass surgery, the subjects had lost on average 25.5 kg of their body weight. Coupled, ADP generating respiration, had increased significantly (+69%, $P\leq0.01$), while the uncoupled respiration was not significantly altered. Mitochondrial efficiency increased significantly. An increased mitochondrial efficiency could partly

explain the reduced basal metabolism and thus the reduced inclination for weight loss at calorie restriction. The reduced capacity among the obese is thus suggested to rather be an effect of the obesity than a casual factor.

LIST OF PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the following text by their roman numerals:

I. BAKKMAN L, Sahlin K, Holmberg HC, Tonkonogi M
Quantitative and qualitative adaptation of human skeletal muscle mitochondria to hypoxic compared with normoxic training at the same relative work rate
Acta Physiol (Oxf). 2007 Jul;190(3):243-51

II. Fernström M, BAKKMAN L, Tonkonogi M, Shabalina IG, Rozhdestvenskaya Z, Mattsson CM, Enqvist JK, Ekblom B, Sahlin K
Reduced efficiency, but increased fat oxidation, in mitochondria from human skeletal muscle after 24-h ultraendurance exercise
J Appl Physiol. 2007 May;102(5):1844-9

III. BAKKMAN L, Fernström M, Loogna P, Rooyackers O, Brandt L, Trolle Lagerros Y
Reduced Respiratory Capacity in Muscle Mitochondria of Obese Subjects
Accepted Obesity facts

IV. BAKKMAN L, Fernström M, Loogna P, Rooyackers O, Svensson M, Jakobsson T, Brandt L, Trolle Lagerros Y
Increased muscle mitochondrial efficiency following calorie restriction in obese subjects
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
COX	Cytochrome *c* oxidase, complexes IV
CPT	Carnitine palmitoyl transferase
CS	Citrate synthase
DNP	Dinitrophenol
EPOC	Excess post-exercise oxygen consumption
ETC	Electron transport chain
FA	Fatty acids
$FADH_2$	Flavin adenine dinucleotid
GBP	Gastric bypass procedures
HDL	High density lipoproteins
IMF	Intermyofibrillar mitochondria
K_m	Michaelis-Menten constant
LCFA	Long-chain fatty acids
LDL	Low density lipoproteins
MCFA	Medium-chain fatty acids
MPTP	Mitochondrial permeability transition pore
NADH	Nicotinamide adenine dinucleotid
PC	Palmitoyl carnitine
P/O-ratio	ATP formed/oxygen consumed
RCI	Respiratory control index
RER	Respiratory exchange ratio
ROS	Reactive oxygen species
RYGBP	Roux-en-Y limb of intestine
SEM	Standard error of the mean
SoReg	Scandinavian obesity surgery register
SOS	Swedish obese subjects
State 3	Respiration coupled to ATP production
State 4	Respiration not coupled to ATP synthesis
UCP	Uncoupling protein
VO_2 peak	Peak oxygen uptake
Wmax	Maximal power output

1 INTRODUCTION

The metabolic capacity of the body plays a decisive role for the development of several diseases and physical ability. An efficient capability to adapt to changed metabolic conditions and energy demands is crucial for weight control and physical exercise. Much of this regulation occurs within the mitochondria, sub cellular organelles known as the "powerhouses" of the cell. In the presence of oxygen, the mitochondria convert metabolites from the food we eat into ATP, a useable form of energy, to support the rest of the cell. The ability to generate energy is thus dependent on mitochondrial content and its functionality, but also of adequate supply of oxygen and energy substrate.

1.1 METABOLIC CHALLENGES FOR THE EXERCISING BODY

The body requires constant energy supply at all times, however, during exercise the energy demand in the skeletal muscle increases dramatically. To sustain repeated muscle contraction the body must generate sufficient amount of ATP. Inability to supply ATP for muscle consumption will result in fatigue.

The exercising body is characterized not only by an elevated energy demand, but also by a dramatic change in prerequisite for energy production, such as the shift in substrate availability and oxygen deficiency (hypoxia). Both hypoxia and physical exercise are potent metabolic stressors [1]. They will both and independently induce adaptations in the mitochondrial supply and utilization of oxygen, with consequences for local and total body metabolism.

1.2 METABOLIC CHALLENGES AT BODY WEIGHT REGULATION

Energy imbalance will affect the metabolism; both at an acute stage and in the long term. A shift in energy homeostasis, where the energy consumed is not balanced by adequate energy utilization results in weight gain and, if prolonged and severe, in obesity. Enhanced mitochondrial activity could improve metabolic homeostasis since even small increases in the energy expenditure have major effects on long-term weight control [2].

Most individuals trying to lose weight find it difficult. At calorie restriction the basal metabolism becomes depressed [3] – an energy economy phenomenon complicating weight loss. Proton leakage over the inner mitochondrial membrane accounts for a substantial portion of the basal metabolic rate [4]. A reduced mitochondrial activity and/or changes in the mitochondrial energy coupling subsequently results in decreased energy expenditure. This may contribute substantially to metabolic dysfunction which in part could explain the weight-plateau appearing for many individuals aiming to lose weight with calorie restriction.

1.3 THE IMPORTANCE OF STUDYING THE MITOCHONDRIA

In attempts to understand the body's incredible ability to handle with situations of different metabolic conditions and demands, the skeletal muscle mitochondria constitute an obvious target of interest.

Models such as hypoxic exercise, prolonged exercise, obesity and calorie reduction respectively, offer an opportunity to modulate the metabolic stimulus on the muscle. By using a combination of whole body physiological measurements with biochemical analyses of muscle biopsies, we can gain valuable knowledge of the power system of the body´s muscle cells.

2 BACKGROUND

2.1 THE SKELETAL MUSCLE MITOCHONDRIA

Since mitochondria serve as the major source of ATP, the density and intracellular location varies with type of cell and metabolic state [5]. Within the skeletal muscle, mitochondrial abundance and structure differ among fiber types, physiological state, and environment as a reflection of the respiration rates. Inactivity [6,7], aging [8,9] and obesity [10,11] lead to a profound loss of mitochondrial function. Exercise training, on the other hand, enhances mitochondrial functional capacity. In human skeletal muscle mitochondrial volume density ranges from 3.5-10 percent [12].

Skeletal muscle fibers contrast with respect to contraction time, fatigue resistance, capillary density, myoglobin content and mitochondrial availability. Type I, or *slow twitch* muscle, can maintain contraction and sustain aerobic activity during a long period of time. This requires availability of oxygen and metabolic substrate. These fibers are dense in capillaries and myoglobin as well as rich in mitochondria to sustain aerobic activity. The type II, or *fast twitch muscle*, can be divided into different subgroups (IIa and IIx). Type II fibers contract more quickly and with greater force than type I, but since they are less dense in mitochondria and myoglobin they have to rely on anaerobic energy production making them less endure.

Recent research suggests that mitochondria constitutes of an interconnected network rather than individual structures. What was previously thought to be an increase in number and size on a given stimuli, i.e. endurance training, is now described as a proliferation of the reticulum [13]. This might be of importance for facilitation of oxygen and substrate transport to the central region of the muscle fiber [13].

Two populations of mitochondria can be distinguished; the subsarcolemmal mitochondria, located just beneath the cell membrane constituting 10-15 percent of the total mitochondria and the intermyofibrillar mitochondria (IMF) [12]. The IMF mitochondria, located among the contractile machinery, are characterized by a higher respiration rate and higher protein synthesis [14].

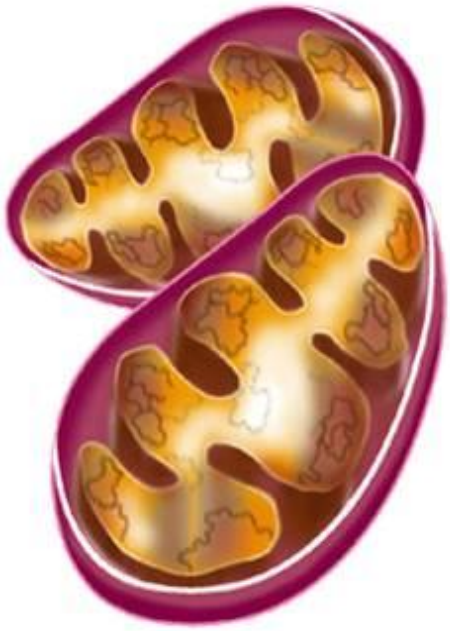

Figure 1. The skeletal muscle mitochondria

The mitochondrial structure is characterized by a double membrane, the intermembrane space and the matrix located within the inner membrane. The outer membrane is quite permeable. The highly folded inner membrane is impermeable to most substances requiring specific transport systems. The respiratory proteins are embedded in multiprotein complexes within the inner mitochondrial membrane.

2.1.1 Mitochondrial energy production

The mitochondria convert metabolites from the food we eat into ATP. Pyruvate and free fatty acids enter the mitochondrial matrix where they are transformed into acetyl coenzyme A and entering the Krebs cycle. This cycle will, besides producing some ATP (Figure 2), generate reduced electron carriers; NADH and $FADH_2$. When these carriers are deoxidized, most of the ATP will be formed. The four enzyme complexes (NADH dehydrogenase, succinate dehydrogenase, ubiquinol:cytochrome *c* oxidoreductase and cytochrome *c* oxidase (COX)) in the inner mitochondrial membrane constitute the Electron Transport Chain, ETC. ETC is the tool of oxidative phosphorylation which is the primary source of energy for aerobic cells. Metabolites such as glucose and Fatty Acids (FA) become oxidized in a process transferring electrons from the fuel molecule to the ETC and to the final electron acceptor oxygen (which becomes reduced to water).

While transferring electrons, protons are being pumped out from the matrix by complex I, III and IV to the intermembrane space, building up a proton gradient across the inner membrane. To restore equilibrium these protons return down their gradient through complex V (ATP synthase), releasing enough free energy to phosphorylate ADP to ATP. The rate at which oxygen is used during prolonged sub maximal exercise is an indirect measure of the rate at which ATP is generated.

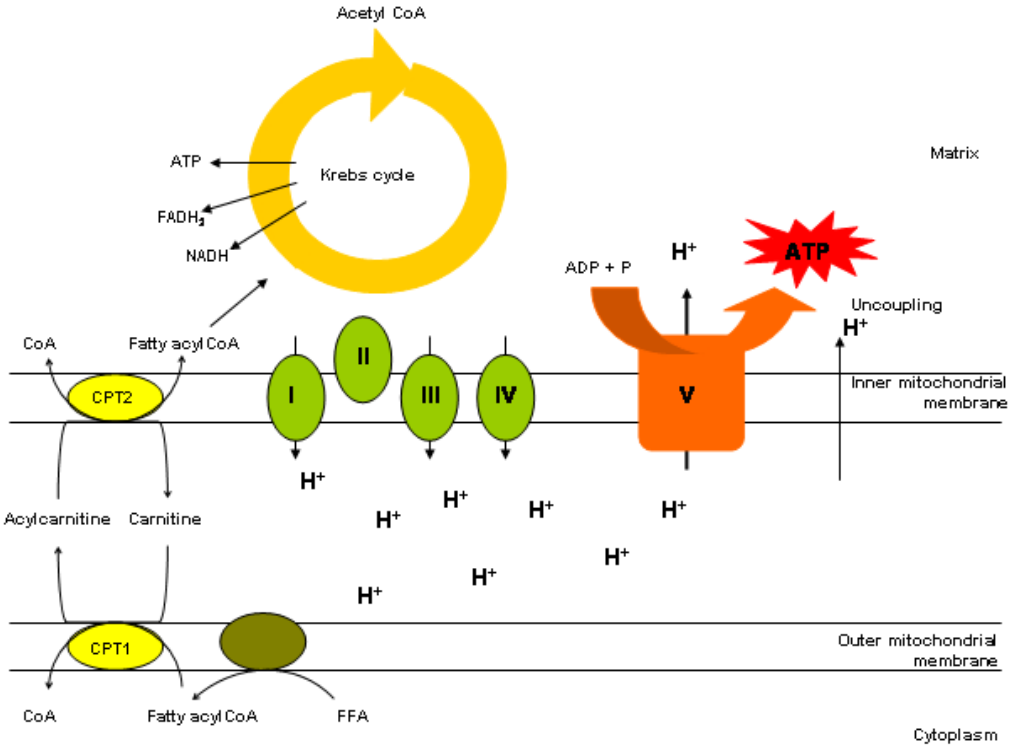

Figure 2. The mitochondrial energy production

Since the mitochondria are responsible for about 95% of the oxygen consumption they also constitute the major potential source for Reactive Oxygen Species (ROS) production. ROS are highly reactive molecules that can participate in unwanted oxidations of lipids, protein and DNA resulting in cell damage. The mitochondrial enzyme complexes, which are in the proximity of ROS production, are prone to be damaged.

Increased oxygen consumption, as for example during strenuous exercise, will increase the oxygen stress and thereby increase the reliance on a well functioning antioxidative defense. In the 1960s, the first report of variation in aerobic capacity on human skeletal muscle was published. The report claimed that oxidative capacity was elevated in people who permanently lived at high-altitude [15].

Since carbohydrate is a relatively limited energy source the body is dependent on a high ability to use FA as fuel during prolonged exercise. The mitochondrial activities are reflected in whole body oxidative capacity. An increased capacity of mitochondria to oxidize FA would therefore be advantageous.

FA cannot simply enter the muscle and cross the mitochondrial membranes to be oxidized. Their transport is facilitated using a number of transporters and enzymes. A carrier (FAT/CD36) is required for FA entrance to the muscle fiber. Inside the muscle cell the FA are prepared for oxidation by conversion to fatty-acyl-CoA (Figure 2), transported through the outer and inner mitochondrial membrane using carnitine, and catalyzed by CPT1 and CPT2.

2.1.2 Proton leakage – a substantial part of the energy expenditure

An intact mitochondrial membrane is a requirement for oxidative phosphorylation. If protons are allowed to cross the membrane without passing the coupling site ATP synthase, the respiration will continue and proton gradient will be equalized without ATP synthesis (Figure 2).

It has long been known that ATP synthesis is not perfectly coupled to oxygen consumption. The proton leakage, or uncoupling, is estimated to constitute a substantial part of the total energy consumption [4]. Although this appears inefficient in an energy perspective, it seems to have important physiological functions.

As the energy is being dissipated as heat instead of ATP formation, an important function is heat production. Other suggested functions are the regulation of body weight, protection against oxidative stress and the prevention of mitochondrial damage induced by fatty acids.

There are different identified ways for the protons to slip through the membrane and uncouple the mitochondria; such as Uncoupling proteins (UCP) or opening of large membrane channels called Mitochondrial Permeability Transition Pores (MPTP) [16].

2.1.2.1 The role of uncoupling protein 3 in human skeletal muscle

In human skeletal muscle UCP3 is found and suggested to function as uncoupler playing a role in reducing ROS formation [17]. UCP3 are also considered as attractive candidates for being involved in energy expenditure, but the results are conflicting [18].

Another hypothesis is that UCP3 could act as a FFA transporter and thereby facilitate lipid oxidation or prevent lipotoxcity [19]. Increased muscle UCP3 expression is associated with elevated levels of circulating FFA in a variety of physiological states (fasting, high-fat feeding, lipid infusion, diabetes, and obesity) independent of changes in energy expenditure [20-22]. Uncoupling and UCP3 expression are also found to be altered with physical training (see further in the section "Metabolic adaptation of the mitochondria").

2.1.2.2 MPTP can uncouple the mitochondria

The MPTP are large membrane channels that are formed in the mitochondrial membrane under certain conditions such as oxidative stress, elevated calcium concentration and low ATP levels [23]. Opened MPTP will depolarize the mitochondria and inhibit oxidative phosphorylation. Induction of the MPTP can also lead to mitochondrial swelling causing cell death and it is important in apoptosis.

2.2 METABOLIC ADAPTATION OF THE MITOCHONDRIA

The skeletal muscle has an incredible capability of adjusting its metabolic capacity and in this way adapt to alterations in conditions and energy demands [24-26]. However, it is not the only determinant of the body's oxidative capacity. Despite their role in oxidative metabolism, the mitochondria may have a minor role in the determination of oxygen consumption rate [27]. Mitochondrial oxidative capacity appears to exceed the capacity of the cardiovascular system to supply oxygen [27] and the latter will be decisive for maximal oxygen consumption [28]. On the other hand there are data showing a good correlation between VO_2 max and whole body mitochondrial content [29]. However, the metabolic adaptations in skeletal muscle are considered critical for improving sub maximal performance [28].

The mitochondrial metabolic adaptation in response to exercise allows a shift of substrate metabolism towards a higher reliance on lipids and decreased accumulation of lactic acid at a given oxygen uptake. Mitochondrial volume density as well as the biochemical properties of the mitochondria within the muscle influences the metabolic capacity [25,26].

In obesity, fat accumulation increases in muscle, this correlates with insulin resistance. The reason for the increased fat accumulation remains to elucidate. Reduced mitochondrial oxidative capacity has been postulated as a contributing factor and has been shown to correlate with systemic insulin resistance [11,30].

2.2.1 Biogenesis – increase in mitochondrial volume

One unique feature of the mitochondria is that they contain their own DNA allowing them to synthesis a number of their own proteins. Of the hundreds genes required for mitochondrial biogenesis, thirteen are encoded by the mitochondrial genome and the remaining of the nuclear genome [31]. Mitochondrial volume is affected by a number of physiological conditions.

2.2.1.1 Exercise induced biogenesis

Repeated bouts of endurance exercise stimulate mitochondrial biogenesis in skeletal muscle to meet the ATP demand via oxidative phosphorylation and thereby resist fatigue. A few weeks of endurance exercise training leads to a 50 percent increase of mitochondrial volume in previously untrained subjects [32,33]. This directly results in improved performance, independent of the training induced change in maximal oxygen consumption (VO_2 peak increased by 24 percent [32,33]) which is of secondary interest [24,34]. This is in line with the changes upon termination of training when the mitochondrial enzymes in muscle decline rapidly, while maximal oxygen consumption remains unchanged [35].

Since adaptation will take place exclusively in the muscle recruited for the exercise, it is probably independent of endocrine influences [5]. Instead adaptation is suggested to arise from combinations of accelerations in ATP turnover, imbalances between synthesis and demand of mitochondrial ATP and cellular calcium fluxes [5]. Hypoxic exercise has been shown to elicit a similar [36] or a greater [37,38] increase in volume densities of mitochondria as equally training load in normoxia.

2.2.1.2 Lower mitochondrial content at obesity

Obese individuals exhibit reduced levels of oxidative enzymes and lower mitochondrial volume compared to normal weight individuals [10,11,39,40]. The mitochondrial area was reduced by approximately 35% in skeletal muscle from obese compared to lean [11]. These findings raise the possibility of impaired mitochondrial content as an additional aspect of the obesity pathogenesis. Furthermore, as shown by intervention in previously obese adults, weight loss is associated with enlargement of mitochondria and an increase in the mitochondrial content in skeletal muscle [41]. Increased mitochondrial volume may provide greater capacity for oxidative phosphorylation [42]. Interestingly, the increases in mitochondrial volume were also shown to correlate with improvements in insulin resistance [41].

2.2.2 Functional improvement decisive for oxidative capacity

The oxidative capacity of skeletal muscles is determined by the total mitochondrial content in the muscle, but also of surface area of mitochondrial inner membranes, and respiratory activities of the single mitochondria. As well as mitochondrial biogenesis can adapt in response to different metabolic conditions, so can the mitochondrial protein composition.

Mitochondrial affinity for oxygen is related to the activity of Cytochrome *c* oxidase (COX) in relation to the capacity of the electron transport chain and Krebs cycle

[43,44]. A greater increase in levels of muscle COX activity than in the activity of Krebs cycle (represented by CS activity) would be an efficient adaptation to hypoxia. Studies on patients suffering from chronic obstructive pulmonary disease, and thereby server chronic hypoxia, reported increased levels of muscle COX activity [45] and decreased activity of CS [46].

2.2.2.1 Qualitative changes at exercise

Since the uncoupling constitutes a substantial part of the oxygen consumption in the skeletal muscle in rest, it is important to evaluate uncoupling when studying mitochondrial efficiency. Physiological characteristics for exercise; like elevated levels of circulating FA, increased calcium concentration and high ATP consumption are, as mentioned above, associated with UCP3 expression and MPTP-opening.

However, uncoupling and decreased efficiency would be unfavorable in order to preserve energy. An inverse relationship is found between UCP3 expression and cycling efficiency [47] as well as VO_2 peak [19]. When UCP3 mRNA was found to be increased after exercise [48] it was suggested as a candidate explaining EPOC, the elevated oxygen consumption that remains for several hours after exercise [32]. However, this is not likely since UCP3 protein expression and uncoupled respiration measured in isolated mitochondria were found to remain unchanged after prolonged exercise [32,49].

2.2.2.2 Qualitative changes at obesity

Also, obesity appears to affect mitochondrial function. At least one study has shown a decreased activity of the electron transport chain (ETC) in muscle mitochondria from obese subjects compared to lean control subjects after adjusting for mitochondrial volume [11]. Thus, both the parameters volume and functional capacity prove perturbations in obesity.

2.3 EXERCISE

2.3.1 Hypoxic exercise

Hypoxia, or limited oxygen availability, can be caused by staying/living/training at high altitude. In a clinical setting hypoxia is relevant for ischemia or respiratory difficulties. In a hypoxic environment, capillarity and muscle myoglobin content will increase to compensate for limited oxygen availability [36].

Training during hypoxic conditions is widely used among endurance athletes with the expectation to improve performance. However, the effect of hypoxic training on performance is unclear and the mechanisms remain debated. It is accepted that performance at high altitude will benefit from acclimatization and training in hypoxia, but an improved sea-level performance as a result of hypoxic training is more controversial.

Mainly there are two different strategies used for hypoxic training (1) *live high-train low* when hypoxia is provided at rest or (2) *live low-train high* when hypoxia is provided during exercise.

2.3.2 Prolonged exercise

Prolonged exercise dramatically increases the energy demand of the skeletal muscle and the body, but also considerably changes the prerequisite for energy production. Typical characteristics of prolonged exercise, such as ultra endurance exercise, are sustained elevated metabolic rate, shift in substrate availability (when the carbohydrates are finished we have to rely on fat as energy source) and increased oxidative stress.

Ultra-endurance exercise is defined as events that exceed six hours in duration. Commonly it involves a combination of endurance activities (multi-sport) such as running, cycling, kayaking, skiing or swimming. Both oxygenation and substrate availability become critical components for performance.

2.4 OBESITY

Obesity has become an enormous global health burden in society and, indeed, in the individuals affected. With more than 1 billion adults being overweight – at least 300 million of them clinically obese – obesity has reached epidemic proportions [50]. According to data from 2007, 44 percent of the Swedish population (age 16-84) are overweight or obese. It corresponds to about a 50 percent increase in 25 years [51-53].

Overweight and obesity is accompanied by several metabolic risk factors such as insulin resistance, hypertension and dyslipidemia, which increase the risk of developing cardiovascular disease and diabetes.

Table 1. Reference values of metabolic markers [54]

HbA1c	3.9-5.3% (of totalHb)
HDL	1.0-2.7 mmol/L
LDL	2.0-5,3 mmol/L
Triglycerides	0.45-2.6 mmol/L

So far, progress has been modest in terms of fighting the obesity epidemic. Obesity is a multi-factorial condition affected by genetic and environmental interactions; however, a prolonged positive energy balance is a prerequisite. Even small increases in energy expenditure can have major effects on long-term weight control [2]. Long-term success in weight-loss programs is low [52,53]. In addition, the actual weight loss with low calorie diets is often less than the expected [55-57]. The most obvious explanation for this is compliance with the dietary guidelines and/or lack of regularity in the training program [58]. However, there are increasing evidences supporting the existence of regulatory factors attempting to maintain a stable body weight despite energy imbalance.

2.4.1 Calorie reduction

It is estimated that almost half of adult women and a quarter of adult men are attempting to lose weight at any given moment [59]. One possible explanation for the difficulty to achieve weight loss is reduced basal metabolism following calorie reduction [3]. Even small increases in energy expenditure can have major effects on long-term weight control [2]. Reduction in energy expenditure can be interpreted as a defensive, body weight saving mechanism after food deprivation, aiming at maintaining a relatively stable body weight throughout life [56].

On the other hand, this mechanism becomes a threat to overcome obesity once established [56]. Significant decreases in basal metabolic rate have been reported following calorie restriction [60-62]. It is well documented that this reduction is greater than what could be solely explained by the reduction in body weight and fat-free mass [62-65]. Even a 10 percent reduction in weight leads to a significant (7-8%) reduction in basal metabolism, out of which 40 percent could be explained by adaptive thermogenesis [3]. This may contribute substantially to metabolic dysfunction and partly explain the weight-plateau appearing for many individuals aiming to lose weight with calorie restriction.

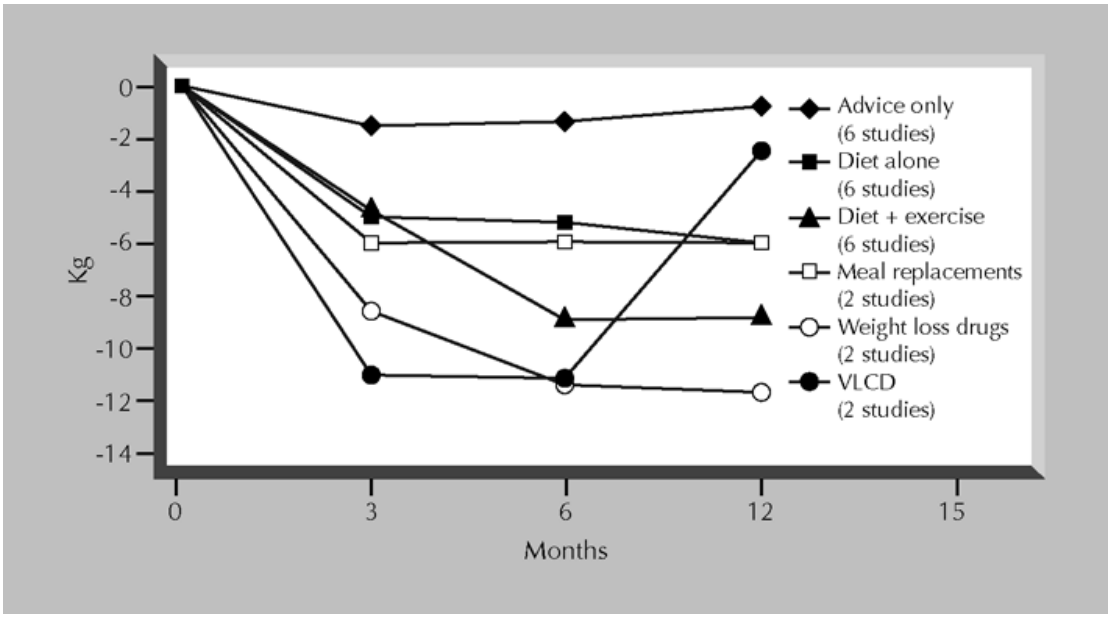

Figure 3. Weight loss plateau following different weight loss interventions. Average weight loss in women completing an intervention. Regardless of the type of intervention, at approximately 6 months a weight loss plateau occurred. VLCD—very low calorie diet. (Franz, Current Diabetes Reports 2004, 4:387-393)

Conversely, it has been possible to demonstrate increased energy losses in overfeeding, which counteracts weight gain to the extent that calorie surplus permits [66]. An adaptive component that can be significant during calorie under- or overconsumtion is suggested [56].

There are individual differences in the propensity for weight regulation following over- and underfeeding [67]. Some experience large over compensatory responses in energy expenditure, while others low or absent responses [68] in energy expenditure. This

might explain interindividual variability in the susceptibility to weight gain, or loss, between individuals in response to controlled under or overconsumption of calories.

2.4.2 Adaptive thermogenesis alter metabolic efficiency

The component, which in part might explain the limited success in weight regulation, is called adaptive thermogenesis. Adaptive thermogenesis is defined as the regulated production of heat in response to environmental changes in temperature and diet, resulting in alterations in metabolic efficiency. The presence of adaptive thermogenesis, and its underlying mechanism, has been discussed during the past 80 years [56]. In the uncoupled mitochondria, when protons are allowed to re-enter the mitochondria without ATP synthesis taking place, heat is released and thus enable the mitochondria to burn energy without ATP utilization.

Chemical uncouplers have been used to increase the proton permeability in aim at investigating the theory that increased thermogenesis can lead to weight loss [69]. Traditionally, dinitrophenol (DNP) has been used as an uncoupler, affecting all mitochondria of the body [70]. In birds, administration of DNP is associated with weight loss, and a shift in substrate use to more fat oxidation [70,71]. DNP was frequently used as a slimming agent in the USA in the 1930s [69]. The substance was prohibited in 1935 due to reports of lethal overdoses where cause of death was said to be hyperthermia [69].

UCP-1 is a decoupled found in brown adipose tissue of rodents in particular. In mice where UCP-1 is being removed, obesity has been induced even on a normal diet [72]. Until recently this knowledge was considered irrelevant from a human perspective, because it was thought that the brown adipose tissue did not exist in adult humans. Today there are studies showing not only the presence of brown adipose tissue in humans, but also a correlation between the absence of brown fat and susceptibility to obesity [73,74]. Adaptive thermogenesis is today regarded as clinically relevant when it comes to weight loss [3,56,65,67,69,75].

2.4.3 Obesity surgery

Bariatric surgery, or weight loss surgery, was introduced as a treatment method for morbid obesity in the 1950s. Traditionally, obesity surgery has been divided into restrictive and malabsorptive methods. The restrictive method means limitations to consume large amounts of food. Patients are forced to change their eating behavior and to have smaller meals. The malabsorptive principle implies reduction of the length and area of the small intestine, thus decreased energy and nutrient uptake [76].

According to SoReg (Scandinavian Obesity Surgery Register), the number of patients undergoing obesity surgery in Sweden has in principle increased tenfold in ten years, from 500 to nearly 5000, and the volume of operations is anticipated to increase in future. A prediction for year 2010 indicates that the numbers of operations will more than double compared with the year of 2008.

Gastric bypass procedures (GBP) is the most common surgical approach to deal with obesity. During the last several decades, this procedure has been modified into its current form, using a Roux-en-Y limb of intestine (RYGBP), see figure 4. A method that, nowadays, usually is performed laparoscopically [77]. After surgery, food does no longer pass through the excluded stomach or duodenum. However, the metabolic and weight reducing effects might neither mainly be restrictive or malabsorptive but also a result of a dramatic change in the release of gastrointestinal hormone, affecting appetite [77].

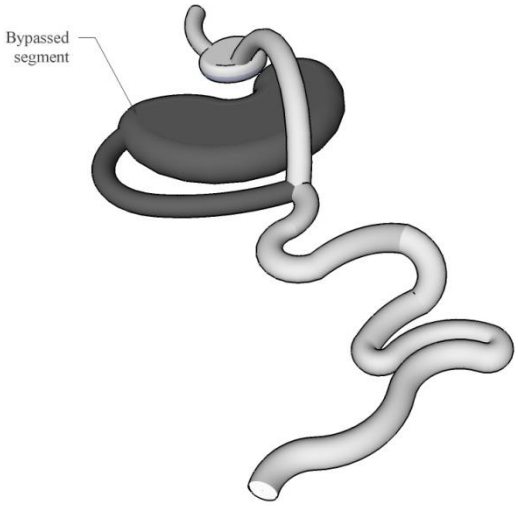

Figure 4. Gastric bypass procedure, drawing by Henrik Bakkman

According to the Swedish Obese Subjects (SOS) study, surgery is the best method of treatment for severe obesity [78]. The SOS study was involving 4047 obese subjects of whom 2010 underwent surgery and 2037 received conventional obesity treatment, weight was recorded repeatedly during the following 15 years. The average weight change in the control subjects was less than ±2%, while those who received gastric bypass lost 32% of their initial body weight in two years. After 10 years their weight was stabilized at -25% of their initial body weight [78].

At the two-year follow-up the control subjects had reduced their energy intake by 2.8%, while those who have undergone surgery had reduced their energy intake by 28.6%. At the ten-year follow-up the decrease in energy intake was -1% and -20.7% respectively [79]. The obesity surgery also lead to improvements in several metabolic markers such as reduction of insulin levels (46% after 2 years and 28% after 10 years), HDL levels (22% after 2 years and 24% after 10 years) and triglycerides (27% after 2 years and 16% after 10 years) [79].

In 1991, the National Institute of Health Consensus Panel on Gastric Surgery for Severe Obesity defined the population who would most likely benefit from bariatric surgery. The criteria suggested are still used to determine the patients that met the criteria for bariatric surgery. The recommendations include those who have a BMI greater than 35 kg/m^2 with significant comorbid conditions (such as diabetes, hypertension, or obstructive sleep apnea), and patients who have a BMI greater than 40 kg/ m^2 with or without significant comorbid conditions, because they have an imminent risk to developing these conditions [80].

Bariatric surgery provides an efficient and in principle guaranteed calorie reduction, which makes it an appropriate intervention model.

3 AIMS

The overall aim of this thesis was to further describe the mitochondrial adaptations to environmental conditions and metabolic demands.

The specific aims were to evaluate how mitochondrial function adapts to:

- limited oxygen conditions, caused by hypoxic exercise.
- sustained elevated metabolic rate and shift in substrate availability typical to ultra endurance exercise.
- obesity, by comparing mitochondrial function in obese subjects with normal weight reference groups.
- calorie restriction and significant weight loss, achieved by obesity surgery.

4 SUBJECTS AND METHODS

In this section, the subjects and protocols are described. Analyses are further discussed, as well as the reasons for choosing different methods. All additional details can be found in respective paper.

4.1 SUBJECTS

An overview of the subjects who participated in the four studies is given in figure 5. The nine trained subjects recruited for study II (prolonged exercise) also served as a reference group in study III (obesity). In Study III, an additional reference group was consisting of nine untrained normal weight subjects. The actual subjects of study III consisted of nine obese, untrained individuals. These nine, in combination with another two, were followed in study IV (calorie reduction), with six months of calorie reduction.

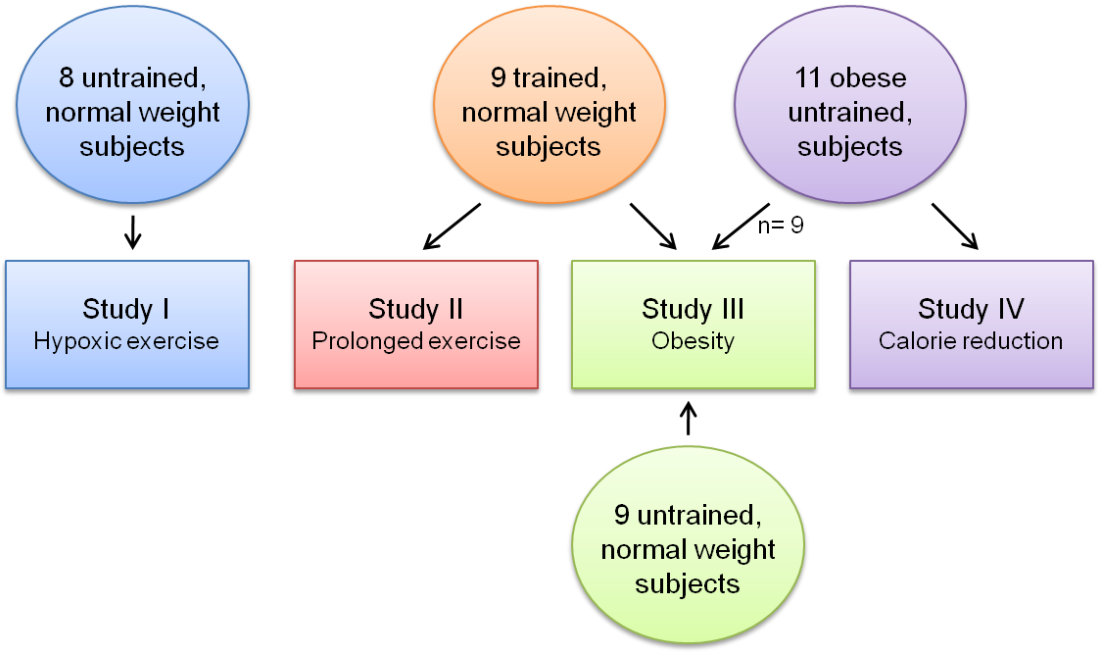

Figure 5. An overview of the subjects participating in study I-IV.

4.1.1 Study I

Subjects volunteering to participate in study I were fairly untrained men and women. Candidates were excluded from the study if they had a record of being engaged in any regular endurance training at least two times per week. All subjects were well-adapted to regular bicycling. Before accepted to the study, all subjects filled out a medical history questionnaire and completed a test of VO_2max. Non-smokers without

contraindications and with VO_2max 40-50 (males) 35-42 (females) were accepted as subjects. Ten subjects (five men and five women) were originally recruited for the study but two women dropped out in the beginning of the training program due to lack of motivation and back injury, resulting in five men and three women.

4.1.2 Study II

In study II, the nine subjects were elite ultra-endurance performance athletes, all men. They had an impressive exercise background with 3-9 years of regular extreme endurance exercise and recent merits from national and international championships. In addition to the characteristics listed in table 2, body composition was measured using air displacement methodology [81] (BodPod S/T. Life measuring inc. USA). The group had a body fat content of an average 16.9% (range 10.8-26.1) of body weight, which is typical for males of their age.

4.1.3 Study III

For study III, eleven consecutive healthy obese subjects, who were referred to obesity surgery at the Bariatric Center, Sophiahemmet, Stockholm during spring 2009, were recruited. Exclusion criteria were smoking and any type of medication. Since the men who were on the waiting list for surgery had pathological values for several metabolic parameters (diabetes, high blood pressure etc.) the restriction resulted in a selection of only women. All women reported an inactive or low active lifestyle, which was confirmed by electronic pedometers.

As reference groups we used two groups of normal weight subjects studied and described in previously published articles. Reference group A consisted of nine normal-weight individuals (5 men and 4 women) not exercising regularly [82] and reference group B consisted of the nine ultra-endurance performance athletes from study II. Since the reference groups consisted of nine subjects each, we chose to limit the number of study participants in study III to cover the first nine overweight patients enrolled in study IV.

4.1.4 Study IV

In Study IV, eleven obese women who underwent bariatric surgery six months earlier were followed up. They all had normal levels of conventional obesity-related metabolic risk factors and were non-smokers. All women reported an inactive or low active lifestyle, which was confirmed by electronic pedometers.

Table 2. Characteristics of subjects from all studies, presented as means (ranges).

Study	Sex	n	Age (years)	Height (cm)	Weight (kg)	BMI (kg/m^2)	Training level
I	♀/♂	8(3/5)	27 (21-33)	175 (165-196)	75 (65-92)	23 (21-25)	Untrained VO_2 – peak ($mL \cdot kg^{-1} \cdot min^{-1}$) = (40.3-49.3)
II/III_B	♂	9	27 (24-32)	182 (175-186)	81 (73-85)	24 (22-28)	Trained VO_2 – peak ($mL \cdot kg^{-1} \cdot min^{-1}$) = (52.7-69.8)
$III_{subjects}$	♀	9	36 (25-42)	167 (159-173)	111 (100-134)	40 (36-45)	Untrained Steps/day 6,600 (5,100-11,300)
III_A	♀/♂	9(4/5)	25 (19-34)	174 (166-191)	72 (54-88)	24 (19-27)	Untrained VO_2 – peak ($mL \cdot kg^{-1} \cdot min^{-1}$) = (43.3-56.9)
IV	♀	11	36 (25-42)	167 (159-173)	110 (100-134)	40 (36-45)	Untrained Steps/day 6,800 (5,100-11,300)

4.2 THE PROCEDURE OF THE RESPIRATION ANALYSIS

The recording of respirations starts with mitochondrial suspension being added to a glass chamber connected to an oxygen electrode. The chamber contains an isotonic medium with substrate and phosphate. Due to that ADP is absent, the initial rate of oxygen consumption (the respiration of the mitochondria) is low. Addition of ADP causes a consistent burst of oxygen uptake when the ADP is converted into ATP. This respiration is referred to as **"state 3"**. When all the ADP has been phosphorylated to form ATP, the respiration rate subsides, representing **"state 4"**.

Here "state 4" is referred to as **uncoupled respiration ,** since oxygen is consumed without any ATP being formed. However, this is a simplification. Some ATP might still be formed without ADP addition, due to possible contamination of ATPase. ATPase contamination might result in breakdown of ATP to ADP, followed by an oxygen consuming re-phosphorylation. Possible contamination is reflected as the respiration in state 4 by the intact mitochondria usually is slightly faster than the original rate, before the first addition of ADP. The oxygen consumption due to non-mitochondrial electrode reactions (drift) is subtracted from the steeper slope in the graph to get state 3 and state 4. To verify that the respiration is non-coupled, an inhibitor, such as oligomycin (which blocks ATP synthase) must be added.

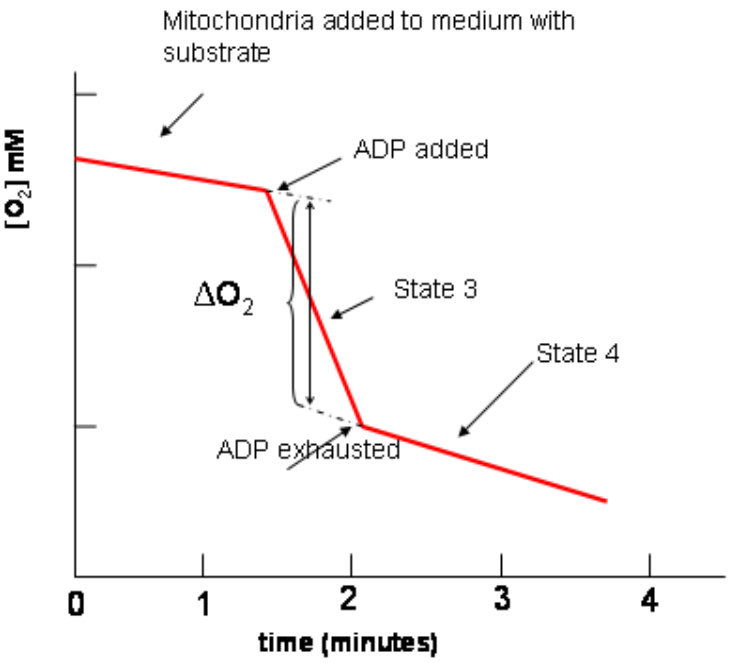

Figure 6. Recordings of mitochondrial respiration with an oxygen electrode (oxygraph).

The **respiratory control index (RCI),** calculated as the ratio of the respiratory rate in state 3 / state 4, indicates the tightness of the coupling between respiration and phosphorylation. High RCI values (around 10) implies that the mitochondria are carefully prepared with a well-preserved structural integrity. In the disrupted mitochondrion, the oxygen consumption will continue at high rates even in the absence of ADP.

The efficiency of oxidative phosphorylation, meaning the amount of ATP produced for each oxygen atom consumed, is called the **P/O-ratio.** It is estimated by measuring the decrease in oxygen concentration during state 3, after adding a known amount of ADP (=ATP formed). The maximal value of P/O-ratio is slightly lower than 3. Fat-derived substrates give consistently lower values for the P/O-ratio compared to carbohydrates. This is because, compared to carbohydrates, they generate a higher ratio $FADH_2$/NADH. FADH2 will enter in complex II of the respiratory chain, by-passing

the first coupling site in the ETC where energy is captured for ATP synthesis. For the same amount of oxygen consumed, more ATP will thus be generated from carbohydrates than from fat. At the whole body level this will make it possible to estimate which fuel being oxidized, by calculating the Respiratory Exchange Ratio (RER). RER is the ratio of CO_2 production/O_2 consumption. The RER will be lower (0.7 with all fat oxidation) compared to oxidation of carbohydrate (1.0), since more oxygen is required in fat oxidation,.

Generally, available ADP is the limiting factor with substrate and oxygen in excess. When more ADP is added, the oxygen uptake will increase. With ADP added in excess, while the oxygen-concentration in the chamber is decreased, the oxygen will be the limiting factor for the respiration. An increased mitochondrial affinity for oxygen under these circumstances, would make the system less sensitive to decreased oxygen availability (hypoxia).

The $\mathbf{K_m}$ (also known as the Michaelis-Menten constant), reflects the affinity of an enzyme for its substrate. K_m is defined as the substrate concentration needed to produce half the maximal velocity of an enzyme-catalyzed reaction. The smaller value of K_m, the greater the affinity.

4.3 STUDY DESIGNS AND EXPERIMENTAL PROTOCOLS

4.3.1 Study I – Hypoxic exercise

This study examines the molecular and functional adaptation of the muscle to simulated altitude training. Training at altitude is an integral part of the training process for elite athletes in endurance sports. Advantages on performance are not clear and the mechanisms are discussed.

The aim was to survey the muscular response at a mitochondrial level. We assumed that altitude training (hypoxia, simulated in a low-pressure chamber) would lead to enhanced oxidative function compared to training in the same relative load at normal air pressure (normoxi). We hypothesized that these improvements could compensate for the lack of oxygen supply.

The subjects performed one-legged cycle training, four times per week, during four weeks. They were randomly assigned to have one leg trained at normoxic conditions and the other leg under hypobaric conditions. Each leg was trained for 30 minutes. The hypoxic condition was carried out in a low pressure chamber at 526 mmHg (simulated altitude of 3000 m). Work intensity level corresponded to 65% of pre exercise maximal power output (W_{max}) for the relevant leg (tested under the pressure at which the leg should be trained) giving the same relative intensity. Half way through the training period, the subjects performed a second W_{max} test and the training intensity was adjusted to again correspond to 65% of W_{max}.

Muscle biopsies were taken from vastus lateralis before and after the four week training period. The biopsies were analyzed for the activities of oxidative enzymes [citrate synthase (CS) and cytochrome c oxidase (COX)] and mitochondrial respiratory function.

4.3.2 Study II – Prolonged exercise

In this study the muscular adaptation to extreme endurance work was examined. We wanted to test the hypothesis that prolonged exercise would reduce mitochondrial efficiency and increase the capacity of the mitochondria to oxidize fat.

The subjects performed 12 blocks of exercise (4 x running, 4 x kayaking and 4 x cycling) during 24 hours. Each block consisted of 110 minutes of work, corresponding to a workload of 60% of their individual VO_2 peak, followed by 10 minutes of rest. During the breaks they were provided a diet with standardized energy contents and composition (59% carbohydrate, 29% fat and 12% protein), aiming to give each person 50% of their estimated energy expenditure (based on calculations from pre exercise sub maximal VO_2 tests).

Muscle biopsies were taken before and immediately after the 24 hour work, as well as after 28 h of recovery. Respiration was analyzed in the isolated mitochondria; using first fat as single substrate and then in combination with carbohydrate-based substrate. A part of the muscle biopsy was also used for measurements of UCP3.

4.3.3 Study III – Obesity

In this work we took muscle samples from obese subjects referred to the gastric bypass. The aim was to compare mitochondrial respiration in the skeletal muscle of obese individuals with normal weight reference groups with varying physical activity level (trained and untrained).

One week prior to surgery, the obese subjects were asked to wear electronic pedometers during three consecutive days and to keep record of steps taken. The subjects also underwent ordinary pre-surgical examination with anthropometric measurements and analysis of fasting blood lipids and glycaemic control. Muscle biopsies were taken in conjunction with gastric bypass surgery. Mitochondria were isolated and analyzed for coupled and uncoupled respiration as well as mitochondrial efficiency. All data were compared with the normal weight reference groups.

4.3.4 Study IV – Calorie reduction

In this work we tested the hypothesis that a depressed basal metabolism, caused by reduced proton leakage and increase mitochondrial efficiency, are parts of the explanation for limited success in weight loss following low calorie diets. A reduced basal metabolism could explain why long-term success is low. We evaluated the effects of long term calorie restriction, induced by gastric bypass surgery.

Eleven obese, but otherwise healthy women, referred to gastric bypass surgery participated in the study. In conjunction with the surgery, muscle biopsies were taken from the vastus lateralis muscle. Six months after gastric surgery, in the context of post-operative monitoring, patients were followed up with a new muscle biopsy. Mitochondria were isolated from pre-and postoperative biopsies and analyzed for coupled (state 3) and uncoupled (state 4) respirations, as well as for mitochondrial efficiency (P/O-ratio) to see how calorie restriction effect mitochondrial function.

In addition to ordinary pre- and post surgical examination with anthropometric measurements and analysis of fasting blood lipids and glycaemic control, the subjects wore electronic pedometers during three consecutive days the week before surgery and before the post-surgical examination, to get an estimate of physical activity level.

4.3.5 Estimation of training level

For studies I and II, tests of VO_2 peak were performed to determine aerobic training status and (in study II) to decide oxygen consumption at a given workload. An incremental sub maximal exercise test was performed using ergometer cycle to estimate the VO_2 peak. Following a brief rest, the workload was raised until exhaustion. Expired air was collected in Douglas bags (study I) with its volume determined using a spirometer and analyzed for O_2 and CO_2 using a Beckman analyzer. In study II an online system was used. Heart rate was monitored continuously and the tests were completed within a few minutes and a leveling off in VO_2 was observed. In addition, for study II, sub-maximal tests were also performed on kayak ergometer and on a treadmill.

In study III and IV electronic pedometers (Silva, Ex1 Distance) were used to estimate training level. The subjects wore the pedometers during three consecutive days the week before surgery and post-surgical examination to estimate level of physical activity.

4.3.6 Physiological tests

In study I, progression of W_{max} over the training period and comparisons between training conditions were followed by repeated tests (72 hours before training period, two weeks into the program and after). W_{max} for each leg, determined as the highest workload the subject could maintain for a complete 2-min period, was used to calculate the training intensity. The test was performed for each leg separately, under the condition (normoxic or hypoxic) at which the leg should be trained. Workload was increased (from initial ♀40W/♂75W) every two min by 15 W until subjects could no longer maintain a cadence of 60 rpm, despite verbal encouragement. When the last workload was not maintained for 2 full min, W_{max} was calculated as follows: $W_{com} + 15 \times t \times (120)^{-1}$, where W_{com} is the value of the last complete workload, t is the number of seconds at the final (not completed) workload, and 15 is the power output difference between the last two workloads.

During the test, heart rate was recorded by a heart rate monitor (610I, Polar Electro OY, Kempele, Finland). Ratings of perceived exertion for the legs and the breathing were made according to the Borg scale at 2 min intervals. Values were compared at the highest work rate maintained by each subject during hypoxia or normoxia for 2 min pre-training. This means that within the individual, the comparisons were done at the same absolute work rate and between individuals, at approximately the same relative intensity (90 ± 3% of hypoxic W_{max}-value and 85 ± 2% of normoxic W_{max} -value).

In the ultra-endurance study heart rate was recorded during the 24 hours**.** Before, immediately after and 28 hours post-exercise oxygen consumption (VO_2) and respiratory exchange ratio (RER) were measured while subjects cycled at the same absolute work rate (40-50% of individual VO_2 peak).

4.3.7 Muscle sampling

All four studies involved muscle biopsy sampling from the vastus lateralis muscle. The biopsies were taken about 15-20 cm proximal to the superior border of the patella and from the superficial portion of the vastus lateralis muscle (20–30 mm depth). Muscle biopsies were obtained by the needle biopsy technique of Bergstrom [83] (study I, III and IV) or with a Weil Blackesly conchotome (study II). For study I, suction was added to achieve greater material exchange.

Figure 7. A Bergström biopsy needle

Study I involved a total of three biopsies per subject. One biopsy were taken from a randomly selected leg 48h before the training period and one biopsy from each leg l-2 days after the training period. In study II three biopsies were also taken; pre (5-12 days prior experiment), post (within 30 minutes after completed experiment) and 28 hours post-exercise. In study III one muscle biopsy per subject were obtained from the left leg which was followed up in study IV, with a second biopsy from the same location, approximately six months later.

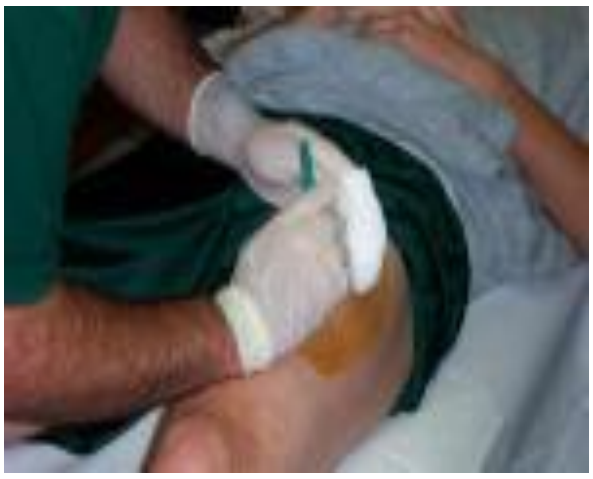

Figure 8. Muscle biopsy sampling

A 5-7 mm long incision was made through the skin and fascia by a scalpel under local anesthesia in all four studies. In study III and IV this was done in combination with general anesthesia in the pre-surgical biopsy sampling. Each muscle sample was cleared from blood, fat, and connective tissue before it was divided in portions and weighed. The portion not used for isolation of mitochondria was rapidly frozen in liquid nitrogen and stored at -80°C until the analysis of enzyme

activity, protein assay, or histological determination. All on-line measurements of respirations need to be completed within three hours after biopsy sampling. The limited time frame is an important concern in the study design.

4.4 ANALYTICAL PROCEDURES

4.4.1 Isolation of mitochondria

Between 10 and 150 mg muscle sample was used for isolation of mitochondria. To isolate the mitochondria we used the technique of Tonkonogi & Sahlin [84]. Muscle specimens were disintegrated mechanically with scissors and treated with protease to degrade myofirillar proteins. This was followed by homogenization and differential centrifugation to separate mitochondria from other cell organelles. The final mitochondria pellet was resuspended in a preserving medium and kept on ice until the analysis of respiratory capacity. In the original protocol, used in study I and II, the resuspension was performed in a cold environment (cold room, 4 °C). For study III and IV the technique was modified and improved so that resuspension could be done in room temperature.

4.4.2 Assessment of mitochondrial density

CS activity, a frequently used marker of Krebs cycle activity and mitochondrial density, was used for the quantification of mitochondrial volume and estimation of muscle oxidative power in study I, III and IV. Activity of CS was established by a spectrophotometer in an aliquot of the mitochondrial fraction with oxaloacetate as a start reagent [85]. In study I, measurements of CS activity in the mitochondrial suspension was combined with CS measurements in freeze-dried muscle tissue [86] to allow calculations of mitochondrial yield in whole muscle.

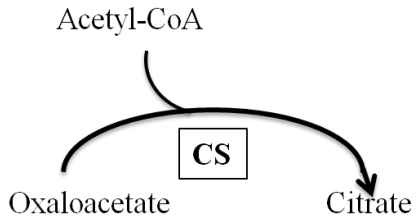

Figure 9. Reaction catalyzed by citrate synthase

4.4.3 Mitochondrial respiratory activity

The respiration rates of isolated mitochondria were measured using a Clark-type electrode in a reaction medium (25°C). In study I, III and IV the carbohydrate sources (pyruvate) were used as substrates. In study II, respiration was measured first with fat derived sources (palmitoyl-carnitin (PC)) representing ATP generation through β-oxidation of fatty acids, followed by a second measurement with a combination of fat and carbohydrate sources (PC and pyruvate).

The mitochondrial suspension was added to the reaction medium with substrate and coupled respiration (state 3) was initiated by the addition of ADP. The respiratory rate returned to the rate measured prior to the addition of ADP, when all ADP was

phosphorylated to ATP (state 4, non-coupled respiration), see figure 6. The Respiratory Control Index (RCI) was calculated as State 3/State 4. The ratio between phosphorylated ADP and consumed oxygen (P/O-ratio) was calculated as the amount of added ADP divided by the oxygen consumed, according to Chance and Williams [87]. All respiration data have been corrected for electrode drift. The relative mitochondrial FA oxidation in study II was calculated as state 3 respiration with PC /state 3 respiration with PC + pyruvate.

4.5 STATISTICS

4.5.1 Study I – Hypoxic exercise

For the analysis examining the differences over time (before and after the training period), such as W_{max}, enzyme activities and mitochondrial functional parameters, we used repeated measures analysis of variance ANOVA with Tukey's post hoc test. Differences between the hypoxically and the normoxically trained leg, such as perceived exertion for breathing and leg, were tested by the non-parametric Wilcoxon–Mann–Whitney test. The significance level was set at $p<0.05$. The results are presented as means ± standard error of the mean (SEM).

4.5.2 Study II – Prolonged exercise

Data were tested for normal distribution before the parametric statistics were performed. Differences between time points were tested with one-way repeated measures ANOVA. If a difference was detected, the location of significance was determined with Fishers post hoc test. Possible correlations between two variables was tested with correlation analysis. Statistic significance was set to $p<0.05$. All data are presented as means ± SEM.

4.5.3 Study III – Obesity

Comparisons of respiration data between the three groups (*reference group A, reference group B, and obese subjects*) were conducted using the non parametric Kruskal-Wallis test. The location of significance was tested by a Wilcoxon Signed-Ranked-test. Statistical significance was set at $p<0.05$. The results are expressed as means ± SEM.

4.5.4 Study IV – Calorie reduction

Changes in variables from pre-operatively to six months post-operatively were tested using the Wilcoxon signed rank test. Statistical analyses were performed on effective pair (complete pre- and post- values for relevant variables). $P\leq0.05$ was considered as statistically significant. Data are presented as median and interquartile range.

5 RESULTS AND COMMENTS

5.1 ADAPTATIONS IN PERFORMANCE AND EFFECTS AT WHOLE BODY LEVEL

5.1.1 Study I – Influence of hypoxic compared to normoxic exercise

The maximal absolute workload (W_{max}) increased by 34% with training over the 4 weeks training period, irrespectively of oxygen availability during training (Figure 10a). Heart rate and ratings of perceived exertion for breathing (Borg scale) were significantly reduced (at the same absolute work rate) after the training period – independent of exercise conditions. The initial W_{max} , of which the training intensity was based on, was higher in normoxia than hypoxia in 7 of the 8 subjects. The difference, however, between the means of initial W_{max} was not statistically significant. Furthermore, heart rate during exercise at the same absolute workload was lower during normoxia compared with hypoxia before training and after 2 weeks of training, though after 4 weeks of training there was no change between training conditions (Figure 10b).

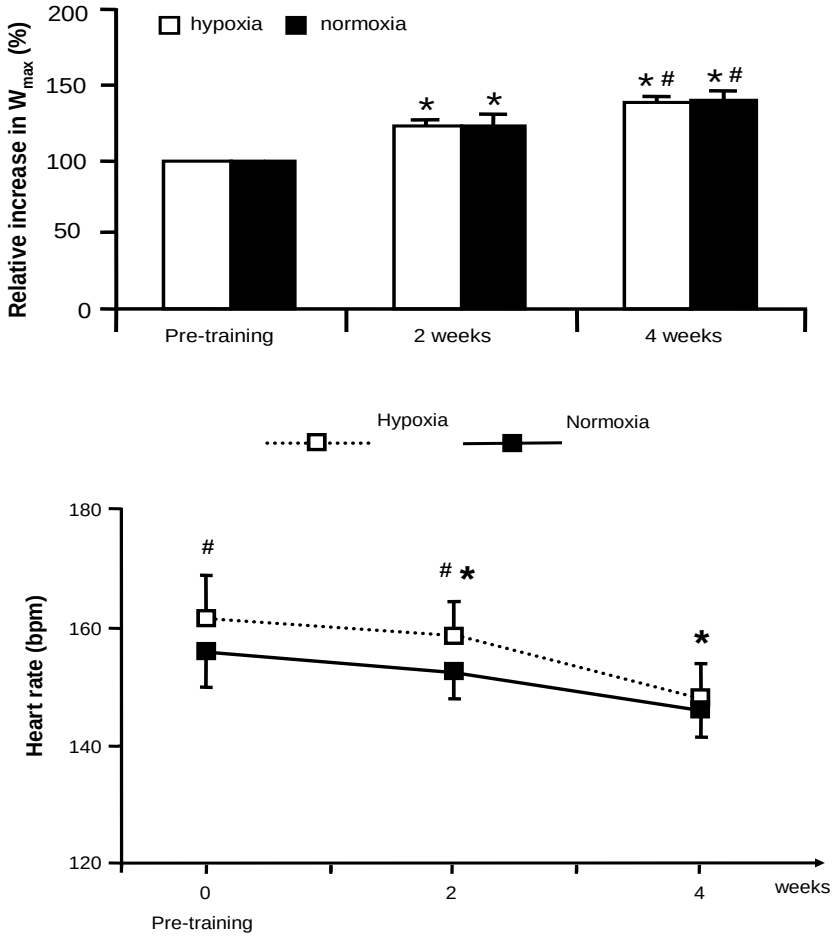

Figure 10. The effects of 2 and 4 weeks of one-legged cycling at hypoxic and normoxic conditions respectively, on.. (a) ..maximal power production (W_{max}), expressed as percentages of pre-training values, (b) ..heart rate measured at the same absolute work rate (113 +/- 9 W) for the individual and at similar relative intensity (90 ± 3% of hypoxic W_{max} and 85 ± 2% of normoxic W_{max}). Values are given as means ± SEM, (n=8), * $p<0.05$ vs. pre-training values. $^{\#}p<0.05$ hypoxic vs. normoxic training leg.

Seven out of the total eight subjects experienced the hypoxic training as harder. Their heart rate at hypoxic conditions was also increased compared to normoxia when exercising at the same absolute workload. However, our study could not demonstrate any beneficial functional outcome of hypoxic training compared to equivalent normoxic training in untrained subjects. These results are in agreement with several other studies [88-91], while some [1,37,92,93] have seen a more significant improvement in performance after hypoxic exercise than after normoxic exercise.

Our subjects were untrained. It is conceivable that the effects of training (it was a dramatic improvement in both heart rate and ratings of perceived exertion over the training period) exceed in importance of any additional effect of hypoxia. Thus, the results might differ in well trained athletes whose training effects have been almost maximized, compared to untrained subjects.

5.1.2 Study II – Influence of prolonged exercise

Before, immediately after exercise, and 28 hours post exercise, the whole body oxygen consumption was measured during cycling at a standardized work rate (40-50% of VO_2 peak). The oxygen consumption was 13% higher following exercise and remained 7% elevated after 28 hours recovery. This phenomenon is known as oxygen drift. Reduced mitochondrial efficiency is postulated as a potential mechanism [94,95].

Immediately after exercise, there was a four-fold increase of FA in plasma. The whole body lipid oxidation was also increased after exercise, demonstrated as a significant 6% reduction in RER. This reduction in RER corresponds to an increase in FA oxidation from 37% to 53%.

The performance during prolonged exercise is dependent on a high capacity to use FA as a fuel. Since the oxygen demand in vivo is about 10% higher with FA than with carbohydrate, the increased FA oxidation would correspond to an additional increase in post-exercise oxygen cost by approximately 2%. Both the levels of FA and RER returned to pre-exercise levels after 28 hours recovery.

5.1.3 Study IV – Influence of calorie reduction

All obese subjects had normal HbA1c and blood lipids (TG, HDL and LDL) within the normal ranges when entering the study. Six months later HbA1c had decreased by 12% and TG by 22% while LDL and HDL had not changed significantly. The subjects reduced their weight by total of 24% over the six-month intervention period. This gave them a BMI of 29.6, and thus classifying them as overweight, thus no longer obese. There were no significant alterations in the number of steps taken between the pre- and post calorie reduction phase.

The achieved weight loss and the reduction in HbA1c were of the same magnitude as previously conducted research, using similar intervention time, has reached [96,97]. We expected weight loss to be associated with improvements in blood lipid levels and

lipoproteins – commonly found in earlier studies [98-100]. Though we found a significant decrease of TG levels after six months of calorie restriction, HDL- and LDL-levels remained unchanged. Our relatively short-term intervention may probably explain the lack of changes in blood lipid levels, compared with previous long-term studies [99,101]. In terms of HDL, the literature supports a sex difference, where women commonly do not exhibit any changes in HDL levels, whereas men's HDL increases by weight loss [102,103].

5.2 QUANTITATIVE ADAPTATIONS OF THE MITOCHONDRIA

5.2.1 Study I – Influence of hypoxic compared to normoxic exercise

It is well known that exercise increases the size and number of mitochondria within the muscle [33,82]. Increased mitochondrial content would be an efficient adaptation to maintain aerobic potential during reduced oxygen availability. We found a significant quantitative adaptation, measured as a 20% increase in CS, in the leg trained during normoxia, however, no change was detected in the leg trained during hypoxia (Figure 11).

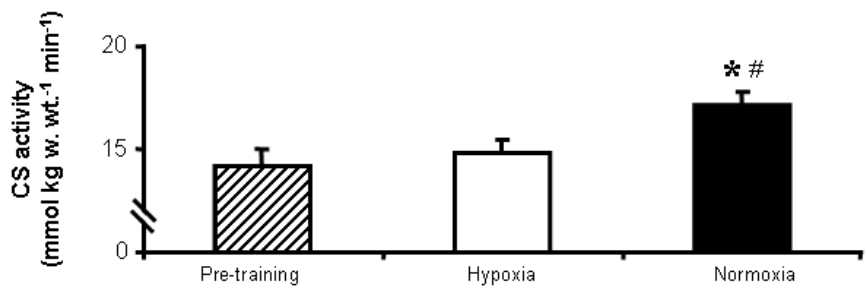

Figure 11. Effect of hypoxic and normoxic training on muscle CS activity. Values are given as means ± SEM (n=8). * $p<0.05$ vs. pre-training values # $p<0.01$ vs. hypoxic training.

In contrast to our hypothesis, the activity of CS was significantly higher after normoxic exercise than after hypoxic exercise. The absent increase in CS after hypoxic exercise could be related to the lower absolute work rate in hypoxia than normoxia (true for 7 of 8 subjects). A higher work rate is associated with a higher concentration of Ca^{2+}, which in turn is an important trigger of mitochondrial biogenesis [104]. This might be one explanation of our findings.

Another possible explanation for the lack of an increase in oxidative enzymes activity and mitochondrial respiratory function might be that hypoxia per se induces a catabolic condition, which impairs mitochondrial biogenesis. This catabolic condition may affect intracellular signaling and blunt the expected increase in mitochondrial enzyme activity.Indirectly this is supported by findings that prolonged exposure to hypoxia leads to a significant loss in body mass [105]. A reduced muscular oxidative capacity has previously been documented in subjects returning from a Himalayan expedition [106].

5.3 MITOCHONDRIAL RESPIRATORY CAPACITY

The oxidative capacity of skeletal muscle is determined not only by the total mitochondrial content but also of the respiratory activity of the mitochondria. For this thesis , we used models such as hypoxic exercise, prolonged exercise, obesity and calorie reduction to study how mitochondrial respiratory capacity is affected.

Table 3. An overview of mitochondrial state 3 respiration (I) effects of 4 weeks of one-legged exercise, at hypoxia and normoxia respectively, (II) effects of 24 hours of endurance exercise using palmitoyl-carnitine (PC) alone or in combination with pyruvate (PC + pyruvate), (III) in obese subjects compared to normal weight untrained subjects, (IV) changes following 6 months of surgically induced weight loss and calorie reduction. Values are expressed as a percentage of pre training/exercise/calorie reduction values or (for III) compared to normal weight, untrained subjects. In study I, respiration is related to muscle weight; in study II, respiration is related to mitochondrial protein content and in study III and IV respiration is expressed per CS. In study I, III and IV pyruvate was used as substrate. n.s. denotes non significant.

	Study I 4 weeks of one legged exercise		Study II 24 hours endurance exercise		Study III Obesity	Study IV Calorie reduction
	Normoxia	Hypoxia	PC	PC + pyruvate	Compared to normal weight	Changes after 6 months
State 3 respiration	+ 31%, $p<0.08$	+ 3%, n.s.	+ 39%, $p<0.05$	+ 12%, n.s.	-47% $p<0.01$	+ 69% $p\leq0.01$

5.3.1 Study I – Influence of hypoxic compared to normoxic exercise

Four weeks of hypoxic and normoxic one-legged training did not significantly affect the respiration rate (State 3) when it was expressed in relation to mitochondrial volume (per unit of CS). However, when the respiration was expressed relative to muscle weight, a tendency to an increase of approximately 30% in State 3 respiration was found after normoxic training but not after hypoxic training (Table 3).

To the best of our knowledge, this is the first time the qualitative aspects of mitochondrial adaptation to hypoxic training are evaluated. The increase in State 3 respiration rate after normoxic but not after hypoxic training adds additional support to the hypothesis that normoxic training might provide a more advantageous training stimulus than equivalent hypoxic training.

5.3.2 Study II – Influence of prolonged exercise

Maximal respiration following 24 hours of endurance exercise was evaluated with different substrates. State 3 respiration with PC increased by 39% post-exercise (Table 3), but was reversed to the pre-exercise value after 28 hours of recovery. With a combination of PC and pyruvate state 3 respiration was not changed (Table 3). The

relative mitochondrial FA oxidation (PC/(PC + pyruvate)) increased by approximately 28% after exercise but did not differ from the initial levels after 28 hours of recovery (Figure 12).

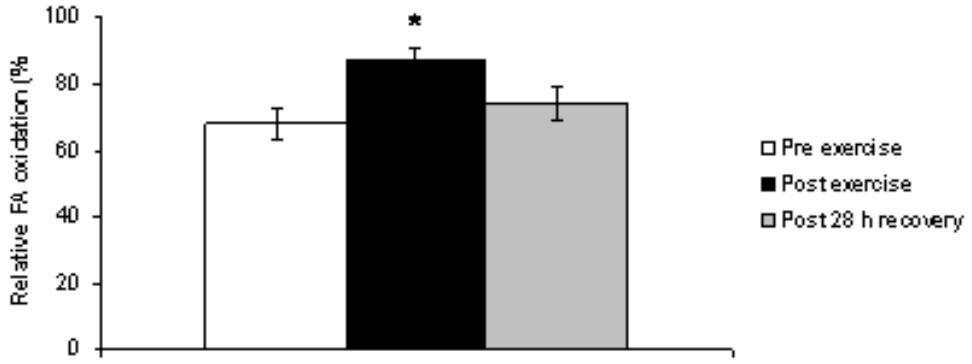

Figure 12. Effect of ultra endurance exercise on the relative rate of FA oxidation (PC/(PC + pyruvate)) observed in isolated mitochondria before. Values are mean ± SEM from 9 subjects. * = Significant difference ($P < 0.05$).

There is a correlation between relative FA oxidation during state 3 and whole body relative FA oxidation [107]. The observed shift in fuel utilization (recall that RER was decreased) might thus depend on an increased supply of FA (there was a fourfold increase in plasma FA) together with a mitochondrial increase in relative rate of FA oxidation. Increased fat oxidation as an adaptation to ultra-endurance exercise is a physiological advantage since it would spare the limited carbohydrate stores. However, the mechanism for this is unclear.

5.3.3 Study III – Comparing obese to normal weight subjects

The obese subjects showed a lower State 3 respiration per mitochondrial volume than the normal weight reference groups. Compared with normal weight, untrained subjects, State 3 respiration among the obese were 47% lower (Table 3), and compared with normal weight, trained subjects 71% lower.

These results support a role of mitochondrial respiratory capacity in weight regulation. A low capacity for fuel oxidation could play a role in the predisposition to obesity. However, based on the presented data we cannot establish whether the reduced oxidative capacity is a cause or consequence of the obesity.

5.3.4 Study IV – Influence of calorie reduction

Our findings of a decreased mitochondrial oxidative capacity in the skeletal muscle of obese subjects led us to study whether the mitochondrial function was normalized by weight loss, without any change in physical activity. This could potentially answer the question whether the reduced oxidative capacity is a causal factor or rather a consequence of the obesity.

The rate of state 3 respiration expressed per unit of CS increased over the six months of caloric restriction from median value of 20.60 nmol O_2 /min^{-1} U CS^{-1} to 34.89 nmol O_2 /min^{-1} U CS^{-1}, p= 0.01 (Table 3).

These results suggest that as weight normalizes, the respiratory capacity increases. Consequently the reduced capacity would be an effect of obesity rather than a casual factor.

5.4 MITOCHONDRIAL EFFICIENCY

To establish mitochondrial efficiency, P/O-ratio was measured in all four studies.

Table 4. An overview of mitochondrial efficiency measured as P/O-ratio (I) effects of 4 weeks of one-legged exercise, at hypoxia and normoxia respectively, (II) effects of 24 hours of endurance exercise using palmitoyl-carnitine (PC) alone, or in combination with pyruvate (PC + pyruvate), (III) in obese subjects compared to normal weight untrained subjects, (IV) changes following 6 months of surgically induced weight loss and calorie reduction. Values are expressed as a percentage of pre training/exercise/calorie reduction values or (for III) compared to normal weight, untrained subjects. In study I, III and IV pyruvate was used as substrate. n.s. denotes non significant.

	Study I 4 weeks of one legged exercise		Study II 24 hours endurance exercise		Study III Obesity	Study IV Calorie reduction
	Normoxia	Hypoxia	PC	PC + pyruvate	Compared to normal weight	Changes after 6 months
P/O-ratio	- 7%, n.s	± 0%	- 9%, p<0.05	- 6%, p<0.05	- 3% n.s.	+ 19% p=0.016

5.4.1 Study I – Influence of hypoxic compared to normoxic exercise

P/O-ratio was not altered by 4 weeks of exercise, neither at hypoxic nor at normoxic conditions (Table 4). P/O-ratio was calculated as the ratio between added ADP and consumed oxygen during respiration with pyruvate as substrate. These data are in accordance with previously published research [107-109] suggesting that P/O-ratio is a conservative parameter.

5.4.2 Study II – Influence of prolonged exercise

With ultra-endurance exercise on the other hand, the mitochondrial efficiency, or P/O-ratio, decreased both when using solely PC as substrate and with the combination of PC and pyruvate (Table 4). The efficiency remained reduced by 8% after 28 h of recovery with PC as substrate and by 7% using the combination of PC and pyruvate as substrates. The reduced efficiency increases the oxygen demand and might thus partly explain the oxygen drift post-exercise. An increased permeability of the inner

mitochondrial membrane, due to ROS formation or elevated FA, might explain the reduced efficiency.

5.4.3 Study III – Comparing obese to normal weight

Mitochondrial efficiency, i.e. P/O-ratio, was measured in obese subjects and compared with normal weight reference groups with various degrees of fitness levels, using pyruvate as substrate. Mitochondrial efficiency was not significantly different between the obese subjects and the lean untrained reference group (Table 4). However the P/O-ratio, was 13% higher in the trained subjects than in the untrained reference group, $p<0.05$.

It can be argued, that the difference in respiratory capacity between the obese and normal weight subjects might rather be a consequence of sedentary habits in the obese, than a difference depending on weight. If the respiratory data only reflect differences in the degree of activity level we should, in similar ways as between the trained and untrained group, have seen a distinction in P/O-ratio between the obese subjects and the untrained reference group.

5.4.4 Study IV – Influence of calorie reduction

We found that six months of calorie restriction significantly increased the P/O-ratio (Table 4), which indicate an enhanced efficiency of the mitochondria as a result of calorie reduction. An increased mitochondrial efficiency might well play a role in the reduced basal metabolism following calorie restriction.

5.5 MITOCHONDRIAL UNCOUPLING

Decreased efficiency could be a result of protons leaking back into the matrix via a mechanism that does not involve ATP synthase. The leakage reduces the proton gradient that drives ATP formation and uncouples respiration from oxidative phosphorylation. We measured uncoupled oxygen utilization as state 4 respirations in all four studies. UCP-3 may account for a significant portion of the state 4 respiration. In Study II, the prolonged exercise study; UCP-3 protein expression was measured in muscle homogenate.

Table 5. An overview of mitochondrial state 4 respiration (I) effects of 4 weeks of one-legged exercise, at hypoxia and normoxia respectively, (II) effects of 24 hours of endurance exercise using palmitoyl-carnitine (PC) alone or in combination with pyruvate (PC + pyruvate), (III) in obese subjects compared to normal weight untrained subjects, (IV) changes following 6 months of surgically induced weight loss and calorie reduction. Values are expressed as a percentage of pre training/exercise/calorie reduction values or (for III) compared to normal weight, untrained subjects. In study I, III and IV respirations are expressed per CS whereas in study II respiration is related to mitochondrial protein. In study I, III and IV pyruvate was used as substrate. n.s. denotes non significant.

	Study I 4 weeks of one legged exercise		Study II 24 hours endurance exercise		Study III Obesity	Study IV Calorie reduction
	Normoxia	Hypoxia	PC	PC + pyruvate	Compared to normal weight	Changes after 6 months
State 4 respiration	± 0%	- 13%, n.s.	+ 22%, $p<0.05$	+ 8%, n.s.	-35% $p=0.03$	+47% n.s.

5.5.1 Study II – Influence of hypoxic compared to normoxic exercise

The four weeks of one-legged training did not change state 4 respiration, neither at hypoxic nor at normoxic training conditions (Table 5).

5.5.2 Study II – Influence of prolonged exercise

State 4 respiration was higher post-exercise with PC (Table 5) but decreased below the pre-exercise value after 28 hours of recovery with both PC (-25%) and PC + pyruvate (-29%), $p<0.05$. Despite this reduction in state 4, the P/O-ratio was reduced. The observed efficiency loss can thus not be explained by an increase in uncoupled respiration.

The protein expression of UCP-3 tended to be lower 28 h post-exercise compared to pre exercise, which is in line with changes in state 4 respiration. Immediately post exercise there was no difference from initial values. No correlation was found between UCP3 and state 4 respirations. Since UCP3 is activated by ROS and FA [17,110] it is possible that the measurements in the isolated mitochondria do not reflect UCP3 induced uncoupling in vivo. UCP3 remained unchanged despite the increase in relative FA oxidation, thus, our data do not support a role of UCP3 in FA oxidation.

5.5.3 Study III – Comparing obese to normal weight subjects

Obese subjects exhibited an uncoupled respiration that was 65% of the uncoupling rate in the normal-weight untrained reference group (Table 5), and only 35% of the uncoupling rate measured among the trained, $p=0.0005$. A reduced uncoupling make more of the consumed energy available – which, if not expended, would eventually be stored as fat.

5.5.4 Study IV – Influence of calorie reduction

Animal studies have demonstrated a decrease in mitochondrial proton leakage which decreases oxygen consumption [111,112] following calorie reduction. We did not find a reduced state 4 respiration. On the contrary, we observed an increase (though not statistically significant) in state 4 respiration (Table 5). However, we could see a significant increase in ATP-generating respiration (state 3), resulting in a decrease of the relative contribution of uncoupled respiration. This implies that more energy becomes available. Again, if this energy is not consumed, it will eventually be stored as fat.

The only human study that currently exists in the field suggests that variations in uncoupling might explain differences in weight loss between individuals [113]. This support a role of mitochondrial uncoupling in weight regulation and in inter individual differences in the propensity for gaining or losing weight.

6 GENERAL DISCUSSION

6.1 METHODOLOGICAL CONSIDERATIONS

6.1.1 Choice of exercise model for studying hypoxia

The idea of providing hypoxia during exercise in study I (in contrast to the "*live high-train low model"*) was to alter the training stimulus and thereby the muscular adaptations. One-legged exercise do not stress oxygen transport in the same way as two legged exercise, thus it is not the appropriate model to study systemic effects of altitude training. We choose the one-legged exercise model since, in contrast to whole body work (with several large muscle groups involved), the rate of respiration are less likely to be limited by the circulation. With one-legged exercise it is more likely that the mitochondrial capacity, which we aimed to study, constitutes the limitation.

Furthermore, to evaluate muscular adaptations it is suitable to let the other leg serve as a control to the hypoxically trained leg. This removes the confounding effect of differences in trainability between subjects. It is well known that individuals respond differently to endurance training [114]. The one-legged exercise model gave us the possibility to separate the effect of hypoxic exercise conditions from inter-individual heterogeneity.

Most studies of hypoxic exercise have compared training at hypoxia and normoxia at the same absolute training intensity. Training at the same absolute work rate during hypoxia as normoxia will aggravate the stress on the metabolic, respiratory and circulatory systems [115]. A greater adaptation during hypoxic training is therefore to be anticipated. However, in reality the athlete does not maintain the same absolute intensity during hypoxic training, but rather the equal relative intensity in order to maintain the same exertion level. To isolate the effect of hypoxia, we found it more physiologically relevant to compare hypoxic and normoxic training when performed at the same relative intensity. However, since the athlete will experience a loss of efficiency due to the lower absolute training intensity at altitude, our "*live low-train high model*" is not as frequently used as the "*live high–train low*" approach.

Because the subjects were unaccustomed to one-legged exercise, it could be argued that any changes in W_{max} are rather an expression of technical improvement than enhanced performance. To assess the reliability of the measurements, a separate set of experiments were performed. Subjects unfamiliar with one-legged cycling conducted two W_{max} tests with 3-4 days in between. There were no differences in W_{max} between the tests. The contribution of technical improvement to the increase in W_{max} after the training period was thus likely to be negligible.

6.1.2 Reliability of received mitochondria

The vastus lateralis muscle, a portion of the quadriceps femoris muscle group, was chosen for muscle samples in all studies based on several reasons. First; in study I and

II, essential parts of the exercise protocols including all tests were performed on a cycle ergometer, where the quadriceps femoris is a major producer of the power output [116]. Second; a large body of data concerning oxidative function in this muscle can be found in the literature, facilitating comparisons with previous findings. The mixed fiber type composition, the trainability, and the accessibility of the vastus lateralis muscle were further arguments for choosing this muscle group.

The percutaneous muscle biopsy technique [83] is safe, rapid, and can be performed repeatedly. It allows for extraction of a small muscle sample with minimal discomfort and scarring [117]. Efforts were made to extract biopsies from the same location every time, to eliminate potential anatomic variations between subjects [118] and within the muscle [119]. For ethical reasons, the pre training data from each individual in the hypoxia study were obtained from one single biopsy (randomly from the leg that would be trained in hypoxia or normoxia). In one individual, a control biopsy from the other leg was sampled, without changing the results.

High RCI values (between 7.6 and 15 in the four studies) and mitochondrial yield (~22% in study I) indicate that the obtained mitochondria were well preserved and functionally intact through the rather rough isolation procedure. A proteolytic enzyme was used in the isolation procedure to degrade the myofibrillar proteins in the muscle and free the mitochondria in-between the myofibrils (intermyofibrillar mitochondria). This method might be questioned since the protease is not specific and might also degrade the mitochondrial proteins. However, to isolate without proteolytic enzymes will mainly liberate mitochondria located just underneath the cell membrane (sub sarcolemmal mitochondria). This could lead to a misinterpretation of the result, since respiration in this subpopulation have been reported to be lower, yet with higher ability to alter its oxidative capacity than intermyofibrillar mitochondria [14].

It is difficult to establish whether a single biopsy could serve as an estimator for the muscular response. Good reasons for the choice of muscle exist and attempts to create a physiological milieu, standardized sampling, proteolytic treatment as well as a gentle isolation procedure were made. Still, it cannot be excluded that the mitochondrial function and adaptation would come across differently in another muscle or in vivo.

6.1.3 Assessment of mitochondrial density

In study I, II and IV we used CS activity to quantify mitochondrial volume. CS is a frequently used marker for Krebs cycle activity and mitochondrial density. Since CS activity increases immediately after exercise [32] (an increase that is not likely to reflect mitochondrial content) it cannot be used as a marker for mitochondrial density in the acute phase after exercise. In study I, 24-48 hours had passed from the end of the training period until the biopsies were taken. During that time frame, any acute effects are no longer present – the elevated CS is usually normalized within 3 hours. In study II, on the other hand; where biopsies were taken in the acute phase, all respiratory parameters were expressed per mg of mitochondrial protein instead of CS.

In study I, CS activity was determined in the freeze-dried muscle tissue in addition to the mitochondrial fraction. This method enabled us to express respiration per muscle weight and to compare respiratory data with other evaluations of training status [84]. Further, the combination of CS measurements in muscle and in mitochondrial fraction has the advantage of giving an estimation of the amount of *functioning* mitochondria within the muscle. This is in contrast to morphological techniques where quantification is complicated due to swelling of the mitochondria despite decreased activity [120] or damage. Hence, comparisons of our results with data from studies using morphological techniques should be made with caution.

To allow for comparisons with previous data from the reference groups in Study III, we used the same temperature, 25 degrees Celsius, at all CS measurements. In study IV, we used a temperature of 37 degrees Celsius, since this is physiologically more relevant. Enzyme activity is strongly dependent on the temperature. Respiration data expressed per CS from the obese subjects in study III is thus not consistent with pre-surgery values from study IV, even though they are reflecting the same respiration data.

6.1.4 Measurements of uncoupling

In the presented studies, state 4 respiration was used as a proxy for uncoupled respiration. This might be argued, is a simplification since state 4, at least theoretically, could be influenced by ATPase. If ATP is broken down to ADP by ATPase and then rephosphorylated, there will be an increase in state 4 respiration. During method development we found a low discrepancy in oxygen consumption rate before addition of ADP, and after conversion of ADP to ATP. This indicates clean preparations and most likely low levels of ATPase contamination.

6.1.5 Potential confounders explaining the impaired oxidative capacity among the obese compared to normal weight subjects

We found an impaired oxidative capacity in obese subjects compared to normal weight reference groups. The results support a role of mitochondrial respiratory capacity in weight regulation and inter-individual differences in the susceptibility to obesity. However, the mechanism is not clear. There could be a number of possible explanations for the reduced mitochondrial capacity (Figure 13). Unfortunately, we were not able to test the hypothesized reasons due to a limited amount of biopsy material. Below follows a discussion of the plausibility of the potential explanations.

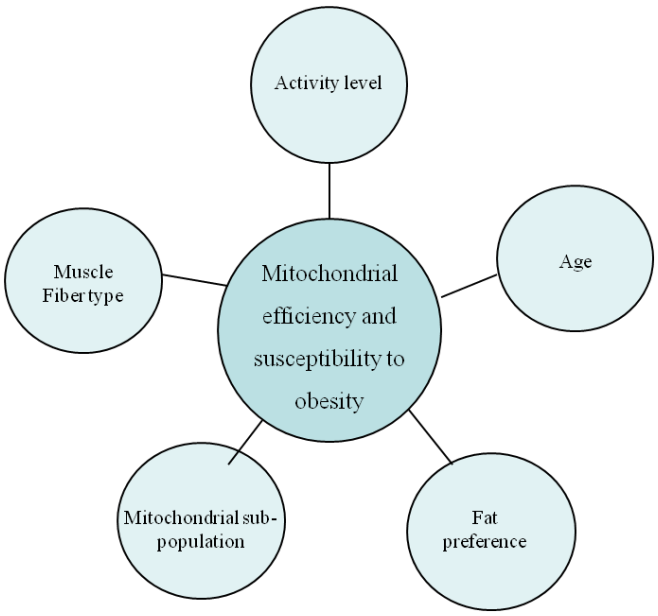

Figure 13. Factors that might affect mitohondrial respiratory capacity and susceptibility to obesity.

6.1.5.1 Age differences are likely to be of minor importance

In study III, the obese subjects are about 10 years older than the normal weight reference groups. It can therefore be argued that the reduced mitochondrial capacity is due to age differences. Whether or not age is associated with a reduction in skeletal muscle mitochondrial function is not entirely clear. A previous study found no differences in respiration of isolated mitochondria from older compared with younger subjects [9]. However, there are conflicting data from an in vitro study showing a decreased state 3 respiration in isolated mitochondria from elderly [121]. Additionally, another in vivo study reported a reduced rate of mitochondrial oxidative activity by approximately 40% in old subjects compared to young counterparts [122]. However, these differences were observed when age differences were considerable (more than 40 years) and are thus likely to be of minor importance in our study, where the difference is only about 10 years between the study subjects and the reference groups.

6.1.5.2 The results are most likely not only reflecting a sedentary lifestyle

Maximal oxygen consumption (VO_2 max) was used to assess training level in the normal weight reference groups, leading to a division into *trained* and *untrained*. However, we have no data of VO_2 for the obese subjects. All the obese subjects perceived themselves as inactive / low active. In our opinion, the self-assessments in combination with objective data from pedometer readings confidently support the classification as *untrained* for the obese subjects.

One can assume that individuals with a BMI of about 40 have an even lower activity level than the normal weight references, which are classified as *untrained*. It is therefore impossible to exclude that the difference in degree of activity is reflected in the respiratory data. However, we consider it unlikely, since we in such circumstances should have seen a difference in mitochondrial efficiency (P/O-ratio) between the obese subjects and the untrained reference groups - in the same way as we do when we compare normal weight reference groups with different degrees of activity.

Less than 5,000 steps/day is used as a “sedentary lifestyle index” [123] while 5,000-7,499 steps/day is typical of daily activity excluding sports/exercise. This level is considered "low active” [123]. The pedometer-determined physical activity level among the obese subjects was 6,600 steps per day and thus not extremely low. Therefore, it seems unlikely that the difference in mitochondrial capacity is due only to the degree of activity.

6.1.5.3 It is possible that a low percentage of type I fibers among the obese lead to a decreased mitochondrial capacity

The fiber-type distribution affects the metabolic capacity of skeletal muscle. Type I muscle fibers have a high oxidative capacity whereas type II fibers are more glycolytic [124]. Since type I muscle fibers have a higher capillary density and lipid storage capacity than type II fibers [125], they are more suitable to oxidize fat. There is a considerable interindividual variation in fiber type composition that appears to be genetically determined [125].

Fiber-type distribution in itself has been proposed as an etiological factor in the development of obesity. An inverse relationship between the fractions type I fiber and body fat has been observed [126]. It is possible that the decreased mitochondrial respiratory capacity among the obese subjects depends on a low percentage of type I fibers. A low degree of type 1 fibers may in turn lead to impaired fat oxidation. Histochemical analyses on fiber-type distribution have shown a low fraction of type I fibers and a higher fraction of type II fibers among subjects with abdominal adiposity [126-128].

We were not able to study the fiber-type composition among our subjects due to limited material. It can thus not be excluded that a reduced amount of type I muscle fiber-type explains the lower oxidative capacity observed among the obese. If so, causality remains to be explored. Fiber-type is considered a relatively conservative parameter [129]. Thus, maybe a low ratio of type I/type II fibers makes a person more susceptible to obesity.

6.1.5.4 The results might be reserved for one mitochondrial sub-population

There are two types of mitochondria; the subsarcolemmar mitochondria (SS-M), located just beneath the cell membrane and the intermyofibrillar mitochondria (IMF-M), located near the contractile unit in the muscle fibers. The IMF-M is characterized by a higher respiration rate and a higher protein synthesis [14]. In contrast, SS-M have been postulated to be critical for substrate transport, signal transduction and fatty acid oxidation [5].

In fact, it has been suggested that a disproportional reduction of SS-M activity might play a role in the pathogenesis of obesity. Measurements of ETC activity in SS-M demonstrate a three- to fourfold reduction in muscle of obese compared with lean subjects [130]. In animal experiments, high-fat fed rats showed lower state 3 and state 4 respiration in the SS-M. This could not be observed in IMF-M [131]. Because SS-M seems important for fat oxidation, it has been proposed that the reduced capacity of SS-M may contribute to the accumulation of intramyocellular lipids [132] leading to the obesity course.

We measured the decrease in respiratory capacity per cs, a marker of general mitochondrial volume. We made no distinction between subpopulations of mitochondria, again due to the limited amount of material. The decreased respiratory capacity observed among the obese subjects in our study might be reserved for the SS-M fraction. If so, our results are consistent with previous findings supporting capacity changes in the SS-M. Changes which in turn are important in obesity pathogenesis [131].

6.1.5.5 The discrepancy between the obese and normal weight might have been even more pronounced with fat as substrate

The lower rate of oxygen utilization, in both coupled and uncoupled respiration, was measured with pyruvate as substrate. Potentially, the outcome would have been different with fat as a substrate. One could imagine that obese individuals are likely to have a higher dietary fat intake than lean persons [133]. A higher fat intake would

result in elevated levels of free fatty acids in the circulation and thereby to increased fat oxidation in mitochondria [134]. A higher capacity to oxidize fat, and thus a higher relative fat oxidation, at the expense of pyruvate, is a plausible explanation for the impaired rate of respiration among the obese subjects.

However, there is considerable evidence against this theory. The capacity of skeletal muscle for uptake of fatty acid, measured as concentration of fatty acid binding protein and lipoprotein lipase, is equal to or elevated in obese compared with lean counterparts [135]. Instead there is a reduction in CPT activity measured among the obese [135], which restricted the flow of fatty acids into the mitochondria for oxidation. This will lead to a promotion of lipid accumulation and lipogenesis.

Measurements of substrate utilization suggest that obese compared with lean individuals exhibit a reduced fat oxidation and a higher dependence of carbohydrates, measured as higher respiratory quotients [136]. Overall, this contradicts the fact that the reduced oxidation we observed would be limited to carbohydrates. In fact, it might be argued that it might have been even more reduced with fat as fuel.

6.1.6 Gastric bypass as calorie reduction model

The reason for choosing gastric bypass as a model for calorie restriction was to achieve a guaranteed and significant calorie reduction within a limited timeframe. While conventional calorie-reducing programs are difficult to complete, calorie reduction is in principle guaranteed by the gastric bypass operation [79].

It can be argued that gastric bypass is not representative of calorie reduction in general. The mechanism of weight loss resulting from the operation is not solely attributed calorie reduction, but also to hormonal changes [77]. Several of the hormonal changes seen following gastric bypass are however associated with appetite regulation [77] and may indirectly lead to calorie reduction.

6.2 INTERPRETATIONS AND IMPLICATIONS

6.2.1 Does hypoxic exercise improve mitochondrial function in a manner beneficial for performance?

Hypoxic training is performed with the intention to improve performance. The theory behind the *"live low-train high"* model is to enhance the training stimuli, yet avoiding the negative effects observed with chronic altitude conditions, such as reduced mitochondrial content and catabolism [93].

In our study, seven of eight subjects experienced the hypoxic training more physically challenging than normoxic training. The heart rate was increased compared to normoxia, when exercising at the same absolute workload. However, our study with untrained subjects could not demonstrate any beneficial functional outcome of hypoxic training compared to equivalent normoxic training. These results are in agreement with

several studies [88-91], while others [1,37,92,93] indicate or confirm that performance improved after hypoxic exercise.

Our subjects were untrained. It is possible that the effect of training (we found a dramatic improvement in both heart rate and ratings of perceived exertion over the training period) exceeded the importance of any additional effect of hypoxia. The result might thus be different in well trained athletes, whose training effects have been almost maximized.

The favorable mitochondrial adaptation to exercise was blunted when training was performed at hypoxia, compared to the same relative intensity at normoxia. This absence of favorable muscular adaptation might have been caused by a lower absolute work rate than in normoxia. Another possibility might be a hypoxia-induced catabolic state impairing mitochondrial biogenesis.

Hypoxic training may thus be disadvantageous for muscle adaptation. The argument for the athlete to seek altitude for exercise is therefore solely restricted to improved oxygen transport. Possible improvements in performance after hypoxic training may occur *despite* rather than *due to* changes at the mitochondrial level.

6.2.2 Does prolonged exercise influence muscle mitochondrial function in an advantageous way for performance?

The performance during prolonged exercise is dependent on a high ability to use fat as fuel. An up regulation of the mitochondrial capacity to utilize fatty acids is therefore advantageous.

We could demonstrate an increased capacity of mitochondria to oxidize fatty acids with ultra-endurance exercise. This increase in fat oxidation was reflected at whole body level, which might be of advantage during prolonged exercise.

Improved fat burning capacity is a well-known consequence of endurance training. However, the explanation has so far been an increase in mitochondrial volume and subsequent increase in oxidative enzymes [26]. Our study proposes a simultaneous functional improvement of the mitochondria, which is likely to beneficial for performance.

6.2.3 Can reduced mitochondrial capacity and lower mitochondrial efficiency explain predisposition for obesity?

Many people feel that they can eat unrestrictedly without gaining weight, while others say they cannot pass a cheese sandwich before it sits around their waist. Obesity is the result of a prolonged imbalance between energy intake and energy expenditure. However, the degree of weight gain varies between individuals, despite equivalent overconsumption of energy [137,138]. Perhaps it is not quite as simple as eat less and exercise more.

We found a decreased mitochondrial oxidative capacity in skeletal muscle of obese subjects. A low capacity for fuel oxidation could play a role in the predisposition to obesity and explain why some people gain more weight than others – even though identical energy excess. On the other hand, the reduced oxidative capacity could also be a consequence of the obesity. If that is the case, mitochondrial function should be normalized by weight loss.

6.2.4 Can reduced mitochondrial capacity and lower mitochondrial efficiency explain weight loss difficulties?

Weight loss resulting from low calorie diets is often less than expected [54-56,139]. In addition, long-term success is low [52,53]. This suggests that differences and changes in metabolic efficiency and basal metabolism occur in obesity.

We found that six months of substantial calorie restriction increased the mitochondrial capacity for coupled, i.e. ATP-generating, respiration. However, the uncoupled respiration was not enhanced to the same extent, resulting in a higher mitochondrial efficiency. An increased mitochondrial efficiency could partly explain the reduced basal metabolism and thus the reduced inclination for weight loss at calorie restriction.

Our results propose that as weight normalizes, the respiratory capacity increases. Hence, it may be concluded that the reduced capacity among the obese is rather an effect of obesity than a casual factor. Simply put, it is very important to prevent obesity. Once there, obesity seems to cause impaired mitochondrial capacity that might impede weight loss.

6.3 FUTURE PERSPECTIVES

In the attempts to describe mitochondrial adaptations to different environmental conditions and metabolic demands this thesis presents some new knowledge. Explanations are suggested and we speculate on causes. However, more data illuminating the mechanisms behind our findings would be desirable. A limitation is that all experiments had to be run in real time and with limited material. These factors restrict the questions that can be answered.

The novel technology with micro plates appears to open up entirely new possibilities for measuring oxygen consumption in isolated mitochondria. This new equipment enables experiments to be run in parallel with very small amounts of muscle biopsies. As more experiments can be conducted on the same biopsies, it becomes possible to study, for instance, the differences in mitochondrial respiration with several different substrates. More specific measurements of respiration and uncoupling would give a clearer picture of muscle metabolism. Ideally, measurements should also be combined with calorimetric measurements to see if any differences and changes in muscle metabolism are reflected at a whole body level.

Based on our results, it seems particularly important to continue the study of the role of mitochondrial respiratory capacity in the course of obesity and its importance for weight control. There appears to be an adaptive mechanism preventing humans from becoming obese in an obesiogenic environment. This mechanism might also conserve the energy when we restrict energy intake. This adaptability also seems to vary between individuals. This is important to investigate. Increased knowledge about mitochondrial capacity and efficiency could provide a completely new focus in obesity treatment and dietary recommendations.

For future comparisons of mitochondrial capacity between obese and normal weight subjects, it would be desirable to have control subjects matched on age, gender and activity level. In this way we could rule out the possible effects of these parameters. Ideally, body composition measurements should also be included. Thereby, it is possible to isolate the effect of obesity alone.

The significance of muscle fiber type composition also remains to be investigated. Supplementation with fiber type assays may, for example, answer if the reduced mitochondrial function in the obese is associated with a low proportion of type 1 fibers. Such a correlation could lead to further emphasis on the importance of long-term endurance training to prevent and treat obesity. This idea is supported by data showing that type 2 fibers can be converted to type 1 fibers by prolonged and intensive aerobic endurance training [139]. It is also possible that changes in intramuscular fat (IMTG) affect mitochondrial function. To elucidate this possibility we have already scheduled additional analysis of histological measurements of intramuscular fat (IMTG) (manuscript IV).

Regarding the impact of calorie reduction, I would like to see the effect of other models than surgery, such as Very Low Calorie Diets and / or exercise programs. With the considerable and relatively rapid weight loss resulting from surgery, I hope to obtain results from further follow-ups. It would be interesting to know whether the trend of normalization of respiratory function persists even when the weight has stabilized. For future study designs; food diaries should also be included to allow relations of the results to calorie intake and substrate composition.

7 CONCLUSIONS

Favorable mitochondrial adaptations to exercise are absent when training is performed at hypoxia compared to the same relative intensity at normoxia. (Study I)

The mitochondrial capacity to oxidize fatty acids increases with prolonged exercise. (Study II)

Obese subjects exhibit a lower mitochondrial capacity compared to normal weight subjects. (Study III)

Calorie restriction in obese subjects increases the mitochondrial efficiency for energy production. (Study IV)

8 SAMMANFATTNING (SUMMARY IN SWEDISH)

Mitokondrierna har en central roll i kroppens ämnesomsättning och därmed också en avgörande betydelse för såväl viktreglering som fysisk prestationsförmåga. Energigivande näringsämnen från maten förbränns i cellernas mitokondrier, varvid energi frigörs och kan lagras i form av ATP. Nedbrytningen av ATP utgör i sin tur den omedelbara energikällan för cellens energikrävande processer, inklusive bildning och inlagring av fett.

All mitokondriell oxidation leder emellertid inte till ATP-bildning, utan en del av energin som frigörs när näringsämnen oxideras omvandlas till värme. Det kan liknas vid tomgångsförbränning; en del av födans innehåll försvinner som värme och blir därmed inte tillgänglig för energikrävande processer och inte heller för inlagring av fett. Tomgångsförbränning utgör ett väsentligt bidrag till den grundläggande energiförbrukningen hos människa och är därmed avgörande för energiproduktion och viktkontroll.

I. Höghöjdsträning ger inte samma förbättring i mitokondriell förbränningskapacitet som motsvarande träning på havsnivå

Höghöjdsträning är ofta en integrerad del av träningsprocessen för elitaktiva inom uthållighetsidrott. Prestationsfördelarna är emellertid inte entydiga och mekanismen debatteras. Vi antog att höghöjdsträning (hypoxi, simulerat genom undertryck i en kammare) skulle medföra förbättrad förbränningskapacitet i mitokondrierna, jämfört med träning på samma relativa belastning vid normalt lufttryck (normoxi). Ett mer effektivt syreutnyttjande i mitokondrierna skulle kunna kompensera för den bristande syretillgången. Åtta otränade försökspersoner utförde enbenscykling i 30 minuter, fyra gånger i veckan under fyra veckor. Ena benet tränades vid normoxi och det andra vid hypoxi. Muskelbiopsier togs före och efter träningsperioden och analyserades avseende mitokondriell kvantitet och kvalitet. I motsats till vår hypotes visade sig den normoxiska träningen vara mer fördelaktig beträffande mitokondriell anpassning. Benet som tränats i normoxi ökade sin mitokondrievolym och tenderade att öka sin förbränningsaktivitet - anpassningar som uteblev i det hypoxitränade benet. Sammantaget visar vår studie att den träningsinducerade förbättringen i muskelns förbränningskapacitet (som observerades efter normoxisk träning) uteblir vid hypoxi. Höghöjdsträning kan således vara ofördelaktigt för muskulär träningsanpassning.

II. Mitokondriernas fettförbränningskapacitet ökar med långvarig uthållighetsträning

Ökad fettförbränningskapacitet är en välkänd effekt av uthållighetsträning. Genom att kroppen anpassar sig till att utnyttja fett som bränsle, även vid hög arbetsintensitet, räcker de begränsade kolhydratlagren längre. I delstudie II studerade vi om den ökade fettförbränningskapaciteten delvis kunde förklaras med en förbättring i mitokondriell funktion. Nio av Sveriges främsta manliga multisportare genomförde omväxlande

löpning, cykling och paddling på 60 % av sin maximala syreupptagningsförmåga under 24 timmar. Muskelbiopsier togs före och omedelbart efter det extrema uthållighetsarbetet, samt efter 28 timmars återhämtning. Resultaten visade att skelettmuskelmitokondrierna var mindre effektiva efter det extrema uthållighetsarbetet än före det påbörjades. Ineffektiviteten kvarstod efter drygt ett dygns återhämtning och skulle kunna vara en del av förklaringen till en kvarstående förhöjning av energiförbrukningen på helkroppsnivå efter avslutat arbete. Samtidigt uppreglerades mitokondriernas förmåga att utnyttja fett som bränsle omedelbart efter träningen, men återgick till utgångsnivån efter 28 timmars återhämtning. Den ökade fettförbränningen återspeglades på helkroppsnivå, vilket sannolikt är fördelaktigt vid långvarigt uthållighetsarbete.

III. Mitokondriernas förbränningkapacitet är lägre hos överviktiga än normalviktiga

Trots likvärdig överkonsumtion av energi, varierar viktökningen mellan olika individer. Det tyder på att den grundläggande ämnesomsättningen är olika effektiv hos olika personer. Eftersom mitokondrierna har en avgörande betydelse för den grundläggande ämnesomsättningen studerade vi mitokondriell förbränning i skelettmuskeln hos feta individer och jämförde med förbränningen hos normalviktiga referensgrupper med varierande fysisk aktivitetsnivå (tränade och otränade). Muskelbiopsier togs från låret på nio feta (BMI 40) försökspersoner i samband med att de genomgick överviktskirurgi. Mitokondrierna isolerades och analyserades med avseende på förbränning. Resulaten jämfördes sedan med nio normalviktiga otränade (BMI 24) och nio normalviktiga vältränade försökspersoner (BMI 24). De feta uppvisade en lägre förbränningskapacitet jämfört med båda referensgrupperna - vilket kan spela roll när det gäller benägenheten att utveckla fetma. I den här studien kan vi emellertid inte uttala oss om huruvida den lägre mitokondriella kapaciteten är en orsak eller verkan av övervikten.

IV. Ett minskat kaloriintag medför ökad mitokondriell effektivitet hos feta

I detta arbete testade vi en hypotes kring varför många som försöker gå ner i vikt misslyckas. Vid kaloribegränsning sänks den grundläggande ämnesomsättningen, vilket i sin tur försvårar fortsatt viktnedgång. Förklaringen till den reducerade ämnesomsättningen skulle kunna vara en sänkt tomgångsförbränning i muskulaturen. Vi studerade muskulaturens mitokondriella funktion hos feta patienter som remitterats till överviktskirurgi. I samband med kirurgin togs muskelbiopsier från låret hos 11 feta patienter (BMI 39). Mitokondrierna isolerades, varpå ATP-genererande förbränning och tomgångsförbränning analyserades. Ett halvår efter magsäckskirurgin följdes patienterna upp med en ny muskelbiopsi och samtliga analyser upprepades. Vid uppföljningen hade studiedeltagarna minskat i vikt med i genomsnitt 25.5 kg, vilket resulterade i ett BMI på i genomsnitt 30. Mitokondriernas kapacitet för ATP-genererande förbränning hade ökat med 69%, medan ökningen i tomgångsförbränning

inte var statistiskt säkerställd. Mitokondriernas effektivitet hade ökat med 19%. En ökad mitokondriell effektivitet skulle delvis kunna förklara sänkningen av den grundläggande energiförbrukningen och därmed den minskade benägenheten till viktförändring vid kaloribegränsning. Eftersom förbränningskapaciteten ökade med viktminskningen, är den lägre förbränningskapaciteten hos de feta (studie III) snarare en konsekvens av övervikten än en orsak till den.

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