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Effects of endurance exercise on mitochondrial efficiency, uncoupling and lipid oxidation in human skeletal muscle.

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ABSTRACT

During the last years the importance of muscle mitochondria, and mitochondrial function, not only for performance but also for health has been highlighted. The main function of the mitochondria is to produce ATP by oxidative phosphorylation (coupled respiration). In skeletal muscle a substantial part of the energy is lost in non-coupled reactions, it has been estimated that non-coupled respiration accounts for as much as 20-25% of the total energy expenditure. It is now almost 10 years since the discovery of uncoupling protein 3 (UCP3), but the functional role of UCP3 in non-coupled respiration is not completely understood. The aim of this thesis was to investigate mitochondrial efficiency (P/O ratio), mitochondrial fat oxidation, non-coupled respiration (state 4) and protein expression of UCP3 in response to exercise and training in human skeletal muscle.

In study I eight healthy subjects endurance trained for 6 weeks and 9 subjects performed one exercise session (75 min). In the cycling efficiency study II, and in the study on mitochondrial lipid oxidation III, 9 healthy trained and 9 healthy untrained men participated. In study IV mitochondrial function and reactive oxygen species (ROS) production was studied in 9 elite athletes after extreme exercise, 24 hours of cycling, running and paddling.

Endurance training increased whole body oxygen uptake (VO_2 peak) by 24% and muscle citrate synthase (CS) activity (marker of mitochondrial volume) by 47% ($P < 0.05$), but non-coupled respiration and UCP3 adjusted for mitochondrial volume were reduced ($P < 0.05$). One session of exercise did not affect non-coupled respiration or UCP3.

Cycling efficiency (expressed as work efficiency) was inversely related to protein expression of UCP3 ($r = 0.57$) and correlated to type 1 fibers ($r = 0.58$). Work efficiency was not influenced by training status or correlated to mitochondrial efficiency. UCP3 was 52% higher in the untrained men ($P < 0.05$). Mitochondrial capacity for fat oxidation was not influenced by training status, but related to fiber type composition. The hypothesis that mitochondrial fat oxidation is related to whole body lipid oxidation during low-intensity exercise was confirmed ($r = 0.62$).

Mitochondrial capacity for fat oxidation increased after 24 hours of exercise, whereas mitochondrial efficiency (P/O ratio) decreased. P/O ratio remained reduced also after 28 hours of recovery. Formation of ROS by isolated mitochondria increased after exercise. Non-coupled respiration (state 4), however, decreased and UCP3 tended to be reduced after recovery from ultra-endurance exercise ($P = 0.07$).

In conclusion: UCP3 does not follow exercise induced mitochondrial biogenesis. UCP3 is reduced by endurance training and lower in trained men compared with untrained men. Non-coupled respiration, measured in isolated mitochondria was reduced by endurance training and reduced after recovery from ultra-endurance exercise, but similar in trained and untrained men. In these studies UCP3 and non-coupled respiration follow the same pattern but are not correlated. Further studies are needed to understand the complex role of UCP3 in skeletal muscle metabolism.

This thesis is based on the following publications; they will be referred to by their roman number in the text. In addition, some unpublished result from the ultra-endurance study (IV) will be presented. The manuscript is under preparation and therefore not included in the thesis.

Shabalina IG, ***Fernström M****, Bakkman L, Tonkonogi M, Rozhdestvenskaya Z, Mattsson CM, Enqvist JK, Ekblom B and Sahlin K. Skeletal muscle mitochondrial ROS production in response to extreme endurance exercise in athletes.*

I. **Fernström M**, Tonkonogi M, and Sahlin K. Effects of acute and chronic endurance exercise on mitochondrial uncoupling in human skeletal muscle. *J Physiol* 554: 755-763, 2004.

II. Mogensen M, Bagger M, Pedersen PK, **Fernström M**, Sahlin K. Cycling efficiency in humans is related to low UCP3 content and to type 1 fibres but not to mitochondrial efficiency. *J Physiol*. 571: 669-81, 2006.

III. Sahlin K, Mogensen M, Bagger M, **Fernström M**, Pedersen PK. The potential for mitochondrial fat oxidation in human skeletal muscle influences whole body fat oxidation during low-intensity exercise. *Am J Physiol Endocrinol Metab*. Published online Aug 22, 2006.

IV. **Fernström M**, Bakkman L, Tonkonogi M, Shabalina IG, Rozhdestvenskaya Z, Mattsson CM, Enqvist JK, Ekblom B and Sahlin K. Reduced efficiency, but increased fat oxidation in mitochondria from human skeletal muscle after 24 hours ultra-endurance exercise. *In manuscript*.

CONTENTS

ABBREVIATIONS

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANT	Adenine nucleotide translocase
ATP	Adenosine triphosphate
BMI	Body mass index (kg/m^2)
BMR	Basal metabolic rate
CPT1	Carnitine palmitoyl transferase 1
CS	Citrate synthase
EPOC	Excess post-exercise oxygen consumption
ETC-activity	Electron transport chain activity
FA	Fatty acids
H_2O_2	Hydrogen peroxide
4-HNE	4-hydroxy-2-nonenal
IMM	Inner mitochondrial membrane
IMTG	Intramuscular triacylglycerol
LCFA	Long-chain fatty acids
MCFA	Medium-chain fatty acids
M-CoA	Malonyl coenzyme A
MEff	Mitochondrial efficiency = P/O ratio (i.e. ATP formed/oxygen consumed)
MFO	Mitochondrial fat oxidation (PC/Pyr)
% MHC 1	Myosin heavy chain 1, i.e. % slow twitch fibers
MPTP	Mitochondrial permeability transition pore
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxiribonucleic acid
NEFA	Non-esterified fatty acids
OxPhos	Oxidative Phosphorylation
PC	Palmitoyl carnitine

P/O ratio	ATP formed/oxygen consumed (i.e. MEff)
PPAR	Peroxisome proliferator-activated receptor
Pyr	Pyruvate
RCI	Respiratory control index
RER	Respiratory exchange ratio (reflecting whole body substrate oxidation)
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel
SEM	Standard error of the mean
State 3	Respiration coupled to ATP production
State 4	Respiration not coupled to ATP synthesis.
UCP1, 2 and 3	Uncoupling protein 1, 2 and 3
VO_2 peak	Peak oxygen uptake (whole body)
WE	Work efficiency

INTRODUCTION

During the last years the importance of mitochondria, and mitochondrial function, not only for performance but also for health has been highlighted (Hood & Joseph, 2005). It is well known that endurance exercise induces mitochondrial biogenesis (i.e. increased mass and improved function) to meet the increasing energy demand. Training also increases the capacity to oxidise fatty acids (FA) which will benefit health since many diseases of the modern society, such as type 2 diabetes and vascular diseases, are related to obesity, high levels of plasma FA and to an abnormal lipid metabolism.

The main function of human muscle mitochondria is to extract the energy from oxidised food and store it in the high-energy compound adenosine triphosphate (ATP). ATP is synthesised by phosphorylation of adenosine diphosphate (ADP). This process by which ATP is formed in the presence of oxygen is referred to as oxidative phosphorylation (OxPhos) or respiration coupled to ATP synthesis. The ATP synthesised is used for all the energy-consuming activities of the muscle cell. Only small amounts of ATP can bee stored, and there is a fine-tuned balance between the amount of ADP and ATP in all muscle fiber types.

In the electron transport chain protons are pumped from the mitochondrial matrix to the intermembrane space. This creates a proton (H^+) gradient and the gradient is used to drive the synthesis of ATP. Protons flows back to the matrix through ATP-synthase and ATP is formed.

Non-coupled respiration

Not all of the energy consumed will be stored as ATP. Instead a substantial part is lost in non-coupled reactions in which protons flow back over the inner mitochondrial membrane (IMM) circumventing ATP synthase, and the energy is lost as heat. Non-coupled respiration may occur by basal diffusion of protons through the IMM. By carrier mediated transport of protons to the matrix, or by transport of anions (FA^-) from the matrix by membrane proteins such as uncoupling proteins (UCP), or adenine nucleotide translocase (ANT). In some special situations non-specific pores can be formed, mitochondrial permeability transition pore (mtPTP). If PTP are opened the

mitochondria will be depolarised and oxidative phosphorylation inhibited (Halestrap *et al.*, 2002).

It has been estimated that non-coupled respiration accounts for as much as 20-25% of the total energy consumption (Rolfe & Brand, 1996). Skeletal muscle is the major tissue of the body, and therefore the level of mitochondrial uncoupling in this tissue will influence basal metabolic rate (BMR) considerably. Consequently, mitochondrial uncoupling in skeletal muscle is an interesting and important research field.

In 1985 the mitochondrial protein thermogenin was cloned, and later renamed to uncoupling protein 1 (UCP1). UCP1 is exclusively expressed in brown fat and the function is non-shivering thermogenesis. UCP1 helps rodents to resist cold, but it is not important in adult humans because brown fat disappears after infancy and the expression of UCP1 is minimal or not detectable (Rodriguez & Palou, 2004). In 1997 two proteins with amino acid sequences similar to UCP1 was identified and named uncoupling protein 2 (UCP2) (Fleury *et al.*, 1997) and uncoupling protein (UCP3) (Boss *et al.*, 1997). UCP2 has a broad tissue expression but UCP3 is predominantly expressed in skeletal and heart muscle. Because of their homology with UCP1 they became attractive candidates for research on weight regulation in humans. Both UCP2 and UCP3 mRNA are expressed in skeletal muscle but the amount of UCP3 is higher compared to UCP2 (Hjeltnes *et al.*, 1999) and only UCP3 has been detected as protein in human skeletal muscle (Nedergaard & Cannon, 2003). Many of the studies on human UCP3 have been performed on mRNA. UCP3 is coded from the nucleus, chromosome 11 (11q13), translated to a protein in the cytosol and then transported to the mitochondrial membrane. During the transport, a chaperone protein protects the protein. mRNA is the transcript whereas the protein is the biological active form, and therefore protein expression of UCP3 was studied in this work. There are several excellent studies on UCP1, and it has been stated that UCP1 has three functional states; Inhibited: binding of nucleotides (ATP, ADP, GTP or GDP) inhibits UCP1; Activated: fatty acids can overcome the inhibition by GDP and activate thermogenesis (Shabalina *et al.*, 2004); In the absence of an activator or inhibitor: UCP1 can function as a proton antiport (Criscuolo *et al.*, 2006). It is believed that UCP3 like UCP1 needs a specific activator to

perform its functional role (Brand & Esteves, 2005). Example of possible activators are fatty acids or superoxide ion (Echtay *et al.*, 2003).

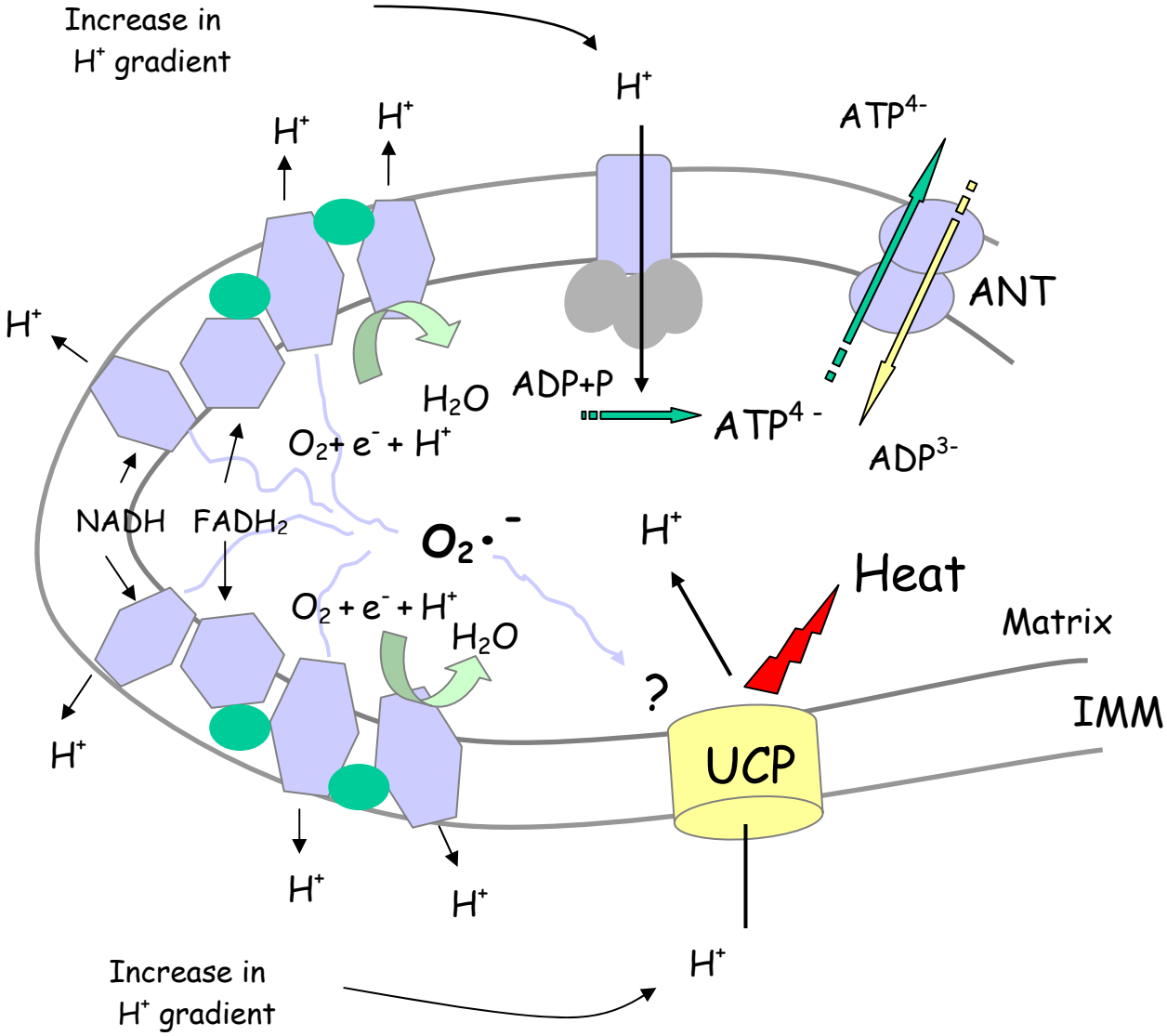

Figure 1. In the electron transport chain (ETC) electrons are transported to oxygen and protons are pumped over the inner mitochondrial membrane (IMM) to create the H gradient that is used to drive OxPhos. If the gradient is dissipated through a UCP less ADP is synthesised and energy is lost as heat.

What is the functional role of UCP3?

The functional role of UCP3 has been extensively debated for almost 10 years. There are still many questions that need to be resolved before the complex role of UCP3 in muscle mitochondrial metabolism is completely understood.

One important question about UCP3 is whether increased expression and activity influences non-coupled respiration and consequently increases BMR.

The most important argument against a main function for UCP3 in thermogenesis comes from studies showing increased UCP3 mRNA expression despite reduced metabolic rate in humans during starvation (Millet *et al.*, 1997). Protein expression of UCP3 is increased with starvation, but it is also increased with high fat diet. This inconsistency can be explained by the fact that UCP3 expression is regulated by the plasma level of non-esterified fatty acids (NEFA) (Dulloo *et al.*, 2001). NEFA are potent ligands for peroxisome proliferator-activated receptors (PPARs) which are known to be involved in the regulation of genes associated with lipid metabolism such as UCP3 (Solanes *et al.*, 2003). It has been suggested that UCP3 is a FA transport protein, and that effects on H^+ leak are secondary due to the charge of the FA anion. Regardless of the primary function of UCP3 the uncoupling capacity in skeletal muscle may still be important, since small changes in proton leak may influence BMR and the long-term body weight regulation. Support for a role of UCP3 in weight regulation of humans comes from a study of obese women. After 6 weeks of restricted diet all women were ranked according to weight loss. Weight loss was 43% greater, non-coupled (state 4) respiration 51% higher and UCP3 mRNA 25% higher in the most diet-responsive subjects (Harper *et al.*, 2002). Recently the same group published a study on rats, where they show that long-term caloric restriction increases UCP3 content, but decreases state 4 respiration in skeletal muscle (Bevilacqua *et al.*, 2005). The most direct evidence for uncoupling properties by UCP3 comes from the study by (Vidal-Puig *et al.*, 2000) showing decreased state 4 respiration in UCP3 knock–out mice.

Another question is whether increased expression and activity of UCP3 can reduce the formation of reactive oxygen species (ROS)?

One theory about UCP3 is that activation induces mild uncoupling and thereby reduces ROS production. According to the theory by (Brand *et al.*, 2004) increased ROS production leads to lipid peroxidation, and formation of aldehydes, such as 4-hydroxy-2-nonenal (4-HNE), 4-HNE can, according to the theory, function as an activator of UCP3 and induce mild uncoupling. This provides a feedback loop to control ROS production (Brand *et al.*, 2004). DNA in the mitochondria (mtDNA) is, in contrast to DNA in the nucleus, located close to the electron transport chain and lacks the protective sheet of histones that is characteristic for nuclear DNA (Irrcher *et al.*, 2003). Exposure to ROS can give rise to injured proteins, or lead to alterations in mtDNA; resulting in cell damage and apoptosis. The "mitochondrial theory of ageing" links increased ROS generation to ageing (Lenaz *et al.*, 2000). It has also been shown that the capacity of the mitochondria to produce ATP has an important role in the process of normal ageing (Trifunovic *et al.*, 2004).

Does UCP3 protect the mitochondria against lipotoxicity?

As mentioned previously it has been hypothesized that UCP3 has a role in lipid metabolism. It is suggested that UCP3 could function as a reversed transport protein, transporting long chain fatty acids (LCFA) out from the mitochondrial matrix. When fatty acid delivery to the mitochondria exceeds the oxidative capacity; LCFA may "flip-flop" over the membrane, become de-protonated and trapped in the matrix. FA anions cannot be metabolised and are toxic to proteins and lipids in the matrix. Therefore the hypothesis that UCP3 may function as a reversed carrier of fatty acid anions out from the mitochondria matrix was put forward (Himms-Hagen & Harper, 2001). It has also been shown that the expression of UCP3 is higher in glycolytic fibers (Hesselink *et al.*, 2001) and this has been used as an argument for a role of UCP3 in the protection against lipotoxicity. UCP3 may transport LCFA from the matrix in situations when the capacity to oxidise fat is insufficient, and this is more likely to occur in glycolytic fibers. The function of UCP3 in this case is to protect the mitochondria against

lipotoxicity (Schrauwen & Hesselink, 2004). This protection will occur at the expense of the ATP-production rate and may not be advantageous for the cell.

It has recently also been hypothesised that there is a role for UCP3 in glucose metabolism. In a study by (Criscuolo *et al.*, 2006) UCP3 as well as UCP1 can transport anions also in a non stimulated state, the authors suggest that UCP3 may function as a passive pyruvate transporter. If pyruvate is taken up to the matrix by the proton-pyruvate symport and not oxidised, pyruvate could be transported from the matrix by UCP3 and converted to lactate in the cytosol.

Pharmacological treatment to increase the expression and activity of UCP3 has been suggested as a tool to treat obesity. High plasma levels of FA characterize type 2 diabetics and a high expression of UCP3 would be expected. In contrast UCP3 is decreased in subjects with type 2 diabetes (Schrauwen *et al.*, 2005). Treatment by rosiglitazone, a new generation insulin sensitising drug, improves the disease and restores UCP3, despite the fact that the treatment also reduces plasma FA levels (Schrauwen *et al.*, 2005). If the function of UCP3 is to protect the mitochondria against accumulation of FA anions in the matrix, disturbances in this protection mechanism may contribute to development of type 2 diabetes (Schrauwen & Hesselink, 2004).

Mitochondrial lipid oxidation

As mentioned previously endurance exercise induces mitochondrial biogenesis which increases the capacity to oxidise both medium chain FA (MCFA) and long chain FA (LCFA). Lipid oxidation is influenced by several factors such as training status, diet and fiber type composition (Goedecke *et al.*, 2000). Increased levels of plasma FA will increase lipid oxidation (Harris *et al.*, 1973) although it is suggested that FA oxidation is controlled also at the mitochondrial level. Transport of LCFA into the mitochondria is dependent on the carnitine carrier system and carnitine palmitoyl transferase 1 (CPT1). This is not the case for MCFA and consequently MCFA oxidation increases during exercise at higher intensities when oxidation of LCFA is decreased (Sidossis *et al.*, 1997). CPT1 is inhibited by Malonyl-CoA (M-CoA), and influence of M-CoA on CPT1 can

explain the regulation of FA oxidation in rats during exercise (Winder *et al.*, 1989). Exercise activates AMP-activated protein kinase (AMPK), and AMPK in its turn inhibits acetyl-CoA carboxylase (ACC) which is the enzyme that catalyses the formation of M-CoA from Acetyl CoA (A-CoA). M-CoA is only modestly decreased by exercise in humans and it seems likely that other mechanisms than changes in AMPK or ACC is involved in the regulation of lipid utilization during exercise in humans (Kiens, 2006). Interestingly however activation of AMPK also induces transcription of UCP3 (Winder, 2001). Recently a fat transport protein, fatty acid translocase (FAT/CD36) was found in human mitochondria (Bezaire *et al.*, 2006). FAT/CD36 has been proposed to translocate to the mitochondria in response to exercise (Bonen *et al.*, 2004). It has also been shown that 120 min cycling profoundly increases FAT/CD36 in the mitochondrial membrane (Holloway *et al.*, 2006). FAT/CD36 may work in conjunction with CPT 1 to regulate transport and oxidation of LCFA in human skeletal muscle mitochondria during exercise.

Mitochondrial content and function differs in different fiber types. Type 1 fibers have the highest lipid oxidation capacity, which can be explained by increased mitochondrial volume and high levels of enzymes important for lipid oxidation. The supply of FA is also important and high IMTG stores in combination with more fatty acid binding proteins is important for the lipid oxidation capacity in type 1 fibers. Independent of mitochondrial volume state 3 respiration (respiration coupled to ATP synthesis) measured with lipid substrate (PC) in mitochondria isolated from rat soleus muscle (predominately type 1 fibers) is 45% higher than in extensor digitorum longus (EDL) (predominantly type 2 fibers) (Mogensen & Sahlin, 2005).

Effects of exercise and training on mitochondrial uncoupling

Endurance exercise is a strong activator of mitochondrial biogenesis. Six weeks of training increased VO_2 peak by 24% and CS-activity (marker of mitochondrial volume) by 47% in young healthy subjects (Fernstrom *et al.*, 2004). UCP3 mRNA is increased by one single bout of exercise (Pilegaard *et al.*, 2000), but the upregulation of mRNA is completely abolished if the subjects are allowed to ingest glucose during the training, to prevent increased plasma FA and/or increased FA oxidation. This indicates that the

increase in UCP3 mRNA is an effect of the increase in plasma FA due to endurance exercise (Schrauwen *et al.*, 2002a). Endurance trained subjects have lower levels of muscle UCP3 as compared to untrained (Hesselink *et al.*, 2003; Fernstrom *et al.*, 2004), even if they have high levels of muscle mitochondria. It has, however, also been shown that short-term training in rats increases UCP3 and it was concluded that UCP3 increases in parallel with other mitochondrial proteins during mitochondrial biogenesis (Jones *et al.*, 2003). In study I of this thesis this hypothesis was tested. Protein expression of UCP3 and non-coupled respiration was evaluated in 8 healthy volunteers before and after 6 weeks of endurance training. After exercise, oxygen consumption is elevated and this is referred to as excess post exercise oxygen consumption (EPOC) (Gaesser & Brooks, 1984). The slow component of EPOC increases energy expenditure for several hours and this may be important for the beneficial effects of exercise on weight regulation. The mechanism for the slow part of EPOC has not been fully understood. The hypothesis that EPOC partly is caused by uncoupling was also tested in study I. Metabolic rate, non-coupled respiration and protein expression of ANT and UCP3 was measured before and after 75 min cycling exercise in 9 healthy volunteers.

Influence of uncoupling on work efficiency

Work efficiency (WE) i.e. how much of the energy from nutrients that is transferred to external work is essential for work performance. Even small improvements in work efficiency may lead to major and important improvements in performance for top athletes. Work efficiency is influenced by anatomical, biomechanical and biochemical factors (Williams, 1985). The interesting question whether work efficiency can be improved by training has been tested in several studies with inconsistent results. It has also been shown in a number of studies that endurance trained persons have higher proportions of type 1 fibers than untrained (Fitzsimons *et al.*, 1990). The higher cycling efficiency (observed for example by (Kunstlinger *et al.*, 1985)) in endurance trained subject may therefore be a consequence of fiber type differences. (Coyle *et al.*, 1992). Oxidative phosphorylation (OxPhos) is the main process of ATP production and changes in the efficiency of OxPhos (mitochondrial efficiency: MEff) will therefore have a direct influence on work efficiency. MEff is measured during ADP stimulated

respiration (state 3) as the amount of ATP formed per consumed oxygen (P/O ratio). Previous studies from our laboratory have been unable to detect differences between trained and untrained subjects on mitochondrial efficiency (Tonkonogi *et al.*, 1997; Fernstrom *et al.*, 2004). It has been observed that cycling efficiency is inversely related to both mRNA and protein expression of UCP3 (Schrauwen *et al.*, 1999). Muscle content of UCP3 is reduced by endurance training and an inverse relation between VO_2 peak and UCP3 was observed in the study by (Schrauwen & Hesselink, 2003). The purpose of study II of this thesis was to test the hypothesis that cycling efficiency is influenced by differences in MEff, uncoupling (i.e. expression of UCP3) or by fiber type composition. For this purpose 8 well trained (VO_2 peak above 55 ml O_2/min/kg) and 8 untrained male subjects (VO_2 peak below 45 ml O_2/min/kg) were compared.

Mitochondrial lipid oxidation during low-intensity exercise

Several independent findings suggest that FA oxidation is controlled at the mitochondrial level, and it has recently been shown that whole body FA oxidation is correlated to mitochondrial FA oxidation (Holloway *et al.*, 2006). Lipid oxidation is dependent on fiber type composition. Type 1 fibers have higher activities of enzymes involved in FA oxidation (Essen *et al.*, 1975) and higher content of stored triglycerides (Pande & Blanchaer, 1971). Mitochondria isolated from rat soleus muscle (predominantly type 1 fibers) have higher rate of FA oxidation as compared to mitochondria isolated from EDL (extensor digitorium longus muscle) (predominantly type 2 fibers) (Mogensen & Sahlin, 2005). The role of mitochondrial characteristics for whole body lipid oxidation during low-intensity exercise was investigated in 8 well-trained (VO_2 peak 61 $\pm$ 1.6 ml O_2/min/kg) and 5 untrained male subjects (VO_2 peak 30 $\pm$ 2.5 ml O_2/min/kg). The purpose of study III was to test the hypothesis that whole body fat oxidation, measured as respiratory exchange ratio (RER), is related to lipid oxidation measured in mitochondria isolated from human skeletal muscle, and to investigate if the rate of mitochondrial FA oxidation is related to training status, fiber type composition and/or protein expression of UCP3.

Mitochondrial function during extreme exercise

Ultra-endurance exercise is a type of extreme exercise where the athletes perform exercise continuously for several days; with only short breaks for food and rest. Even though one of the beneficial effects of exercise is improved and possibly modified endogenous ROS defence (Gambelunghe *et al.*, 2001), ultra-endurance exercise is characterized by increased ROS generation. It is also possible that there are negative effects of extreme exercise on mitochondrial function. One aim of study IV was to evaluate the effect of 24 hours of exercise on MEff. 9 elite ultra endurance athletes (VO_2 peak 62 ml O_2/min/kg) performed cycling; running and paddling at fixed work rates (60% of their individual VO_2 peak) for 24 hours. In study III we could demonstrate that the relative FA oxidation measured in isolated mitochondria was correlated to whole body FA oxidation during low-intensity exercise (Sahlin *et al.*, 2006a). Performance during prolonged exercise is dependent on the ability to use FA as fuel and an increased mitochondrial FA oxidation would therefore be advantageous. Therefore it was hypothesized that the relative mitochondrial FA oxidation is increased in response to ultra-endurance performance.

UCP3 is increased during conditions of high plasma levels of FA. The adaptation is rapid and an increase in UCP3 protein has been observed in human muscle already after 36 hours of treatment with etamoxir, a compound that blocks CPT1 and interferes with FA metabolism (Schrauwen *et al.*, 2002b). According to the theory by (Brand *et al.*, 2004) increased ROS production can activate UCP3 and induce uncoupling. This concept recently has been questioned (Cannon *et al.*, 2006). UCP3 mRNA has been reported to be increased after exercise (Pilegaard *et al.*, 2000), but protein expression of UCP3 has not been shown to be changed by one exercise session. Ultra-endurance exercise is associated with prolonged exposure to high levels of plasma FA and elevated ROS production. This makes ultra-endurance exercise an interesting model for studies of UCP3 protein expression and mitochondrial uncoupling.

AIM

The general aim of this thesis work was to investigate:

Mitochondrial efficiency, mitochondrial lipid oxidation and protein expression of UCP3 and non-coupled respiration in response to endurance exercise in human skeletal muscle.

Especially the aim was:

- To assess the effects of one exercise session and of endurance training on non-coupled respiration and protein expression of UCP3.

- To test the hypothesis that cycling efficiency measured *in vivo* is inversely related to mitochondrial uncoupling measured *in vitro*.

- To test the hypothesis that mitochondrial capacity for fat oxidation influences whole body lipid oxidation during low-intensity exercise.

- To test the hypotheses that extreme exercise, ultra-endurance exercise for 24 hours decreases mitochondrial efficiency and increases mitochondrial capacity for fat oxidation.

- To investigate the effects of ultra-endurance exercise on mitochondrial ROS production, mitochondrial uncoupling and expression of UCP3 protein.

MATERIALS AND METHODS

Uncoupling, mitochondrial efficiency and lipid oxidation

Isolation of human skeletal muscle mitochondria and measurements of respiration in intact and respiring mitochondria is a complicated and difficult technique. All studies in this thesis define state 3 respiration as oxygen consumption during respiration coupled to ATP synthesis, and state 4 respiration as oxygen consumption during respiration not coupled to ATP phosphorylation. Respiratory control index (RCI) is the quotient of state 3 and state 4 respiration. Mitochondrial efficiency (MEff) is measured as the ratio between phosphorylated ATP (P) and consumed oxygen (O) (i.e. P/O ratio) during state 3. MEff has been measured with addition of high levels of ADP (maximal mitochondrial respiration), or with more physiological levels (sub-maximal mitochondrial respiration). To evaluate mitochondrial lipid oxidation palmitoyl-carnitine (PC) was used as substrate and for carbohydrate oxidation pyruvate (Pyr) was used. The relative FA oxidation was calculated as the ratio between state 3 respiration with PC and state 3 respiration with Pyr.

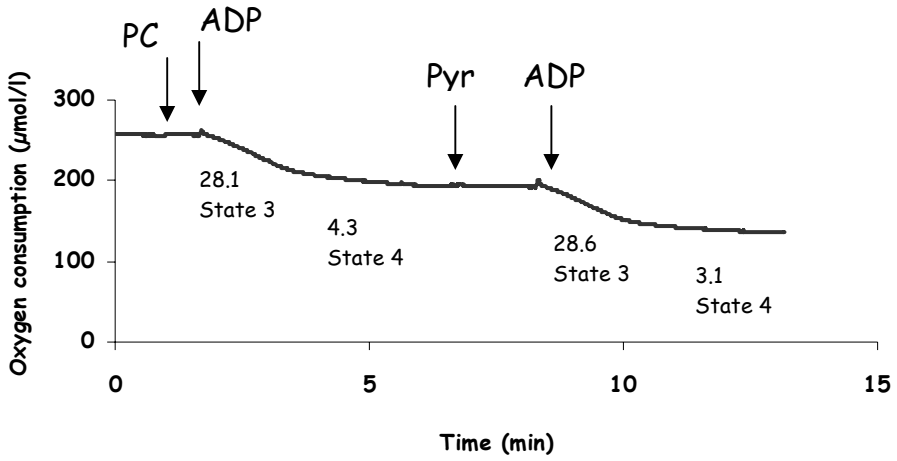

Figure 2. Mitochondrial oxygen consumption from a representative sample. PC = palmitoyl-carnitine + malate and ADP was added to initiate the first state 3 respiration, and Pyr = pyruvate + malate and ADP to initiate the second state 3.

Subject characteristics and exercise protocols

All subjects were informed about the procedure and risks involved in the experiment and they volunteered to participate by giving their written consent. The Ethics Committee of the Karolinska Institutet, Stockholm, Sweden, or the local ethics committee at the Odense University Hospital approved the experimental design of the studies; the design conforms to the Declaration of Helsinki.

In the endurance training program of study I, 8 healthy subjects 4 female and 4 male participated, and in the acute exercise test of study I, 9 young healthy subjects, 4 female and 5 male participated. In the cycling efficiency study III and the study on mitochondrial lipid oxidation III, 9 healthy trained and 9 healthy untrained men participated. In the ultra-endurance performance study IV, 9 healthy male elite ultra-endurance performance athletes participated.

	n	Age (years)	BMI (kg/m^2)	MHC 1 (%)	VO_2 peak (ml/min/kg)
I. Acute exercise	9	25 ± 1	24 ± 0.8	-	50 ± 2.1
I. Endurance training	8	26 ± 2	23 ± 1.2	-	38 ± 2.5
II & III. Efficiency study &	9 (trained)	25 ± 1	24 ± 2	46 ± 3	60 ± 1.4
Lipid oxidation study	9 (untrained)	24 ± 1	29 ± 2	43 ± 4	37 ±2.0
IV. Endurance exercise	9	28 ± 1	24 ± 0.7	56 ± 3	62 ± 1.7

***Table 1**: Subject characteristics for the studies are presented as mean ± SEM.*

Determination of VO_2 peak: All VO_2 peak tests where performed on ergometer cycle. The subjects started with an incremental sub-maximal exercise tests to estimate the VO_2 peak. After a brief rest the VO_2 peak test was performed. Workload was raised every minute to reach the estimated peak. Oxygen uptake was measured using an online system (AMIS 2001, Inovision A/S Odense, Denmark, (Study & IV) or (Oxycon Pro, Jaeger, Germany), (Study II & III). Heart rate was monitored continuously (Polar,

Kempele, Finland). The main criteria for the test were that that exhaustion occurred within 5-10 min, a levelling off in VO_2 was observed and that RER was higher than 1.10.

Endurance training (study I): Untrained subjects completed a six-week training program consisting of four sessions per week. Each training session was performed on ergometer cycle and lasted one our. The subjects exercised at 70% of VO_2 peak for the first 30 min and performed interval training for the remaining 30 min. Every second week, the workload was increased by 5% to account for the estimated increase in VO_2 peak. Two to three days following the last training session, the test of VO_2 peak was repeated. Muscle biopsies were taken from vastus lateralis before and after the training period (two days after the VO_2 peak test).

Single exercise session (study I): Two days after the VO_2 peak test the subjects came to the laboratory to perform the experiment. They cycled on an ergometer cycle for 75 min at an intensity of approximately 70% of their individual VO_2 peak. Expired air was collected online every 15 minutes and analysed for O_2 and CO_2 and on AMIS Cardiopulmonary Function Test System. RER (respiratory exchange ratio) was calculated from the expired O_2 and CO_2. Heart rate was registered continuously with a Polar sport tester (Polar Electro, Kemple, Finland). Muscle biopsies were taken from vastus lateralis before and after exercise and after 3h of recovery. Blood samples were taken from a forearm vein prior to the muscle biopsies before exercise and after 3h of recovery, the post exercise sample was taken during the last minutes of exercise.

Submaximal efficiency test (study II and III): At least 48 h after the VO_2 peak test, subjects performed submaximal tests at three different absolute work rates and at 80% of VO_2 peak. After adjusting the ergometer, the subjects rested on the ergometer cycle for 3 min, performed load-less pedalling 5 min, and cycled at 40W, 80W and 120W for 5 min at each work rate. After this the rate was decreased to 40W for 5 min before exercise at 80% of VO_2 peak for 10 min. Heart rate and oxygen uptake were measured at fixed work rates. The submaximal efficiency test was repeated two to four times separated by at least 48 h.

Determination of efficiency (study II): Efficiency can be expressed in a variety of ways. In this study gross efficiency (GE), work efficiency (WE) and delta efficiency (DE) was used. WE is defined as the work rate/(energy expenditure (EE) - Y intercept) expressed in percent. EE was calculated from the obtained oxygen uptake values, using the equation by Brouwer (1975), the equation takes substrate utilization (RER) into account. The Y intercept was used to compensate for EE during cycling at low loads that does not contribute to external work.

24 hours ultra-endurance exercise (study IV): The athletes arrived to the test lab in the morning after three days of standardised food intake, 4250 kcal/day, (52% CHO, 31% fat and 18% protein) and one night of starvation. Body composition was measured using the air displacement methodology (Dempster & Aitkens, 1995) on (BodPod S/T. Life measuring inc. USA). Before exercise start a polyethylene catheter was inserted in a forearm vein. The athletes performed 12 blocks of exercise (4 x running, 4 x kayaking and 4 x cycling) on a workload corresponding to 60% of their individual VO_2 peak. Each block consisted of 110 min of exercise followed by 10 min of rest. The subjects were allowed to eat standardise food during the 10 min breaks (59% carbohydrate, 29% fat and 12% protein), aiming to give each person 50% of the estimated energy expenditure. Blood samples were taken during cycling at standardised workload (60% of their individual VO_2 peak) pre-exercise, 28 hours post-exercise and during the last 15 minutes of the performance. Heart rate (HR) was recorded using a HR monitor (Polar S610i, Polar electro oy, Kempele, Finland). Exercise oxygen consumption (VO_2) was measured using the Douglas bag technique; the volume of expired air was measured with a Tissot Spirometer (WE Collins, MA, USA). Fractions of oxygen and carbon dioxide were determined using a Beckman S-3A and LB2 gas analyser (Beckman Instruments, Fullerton, USA). Respiratory exchange ratio (RER) was calculated from expired O_2 and CO_2. The post exercise biopsy was taken less than 30 minutes after the last block of exercise (i.e. cycling exercise). The pre-test biopsy was taken about one week before the experiment and the post-test biopsy was taken 28 hours after termination of exercise. All athletes ate standardized food with the same composition before the pre

test and the post-test biopsy, and they refrained from food 3 hours before the biopsy. Blood samples were taken at the same time points as the biopsies.

Muscle biopsies and isolation of mitochondria

All muscle biopsy samples were taken from the vastus lateralis muscle. After local anaesthesia (1-2 ml Carbocain; 20 mg/ml, Astra), an incision was made through the skin and fascia and the biopsy was taken using a modified Bergström Needle (Study I-III) or a Weil Blackesly conchotome (Wisex, Mölndal, Sweden) (Study nr :4). The biopsy was divided into portions, and the portion not used for isolation of mitochondria was frozen in liquid nitrogen and stored at -80° C. Mitochondria was isolated according to the method of (Tonkonogi & Sahlin, 1997). Muscle specimen was disintegrated with scissors and treated with 0.4 mg/ml protease (Sigma P-4789), followed by homogenisation and differential centrifugation. The final mitochondria pellet was resuspended in a buffer with 225 mM mannitol, 75 mM sucrose, 10 mM TRIS-base, 0.1 mM EDTA and 0.2% bovine serum albumin, pH 7.4 and kept on ice until analysis of respiratory activity.

Mitochondrial respiration

Oxygen consumption was measured using a Clark-type electrode (Hansatech DW1; Hansatech, King's Lynn, Norfolk, U.K.), at 25° C. Respiration was analysed in a medium containing 225 mmol/l mannitol, 75 mmol/l sucrose, 10 mmol/l Tris-base, 10 mmol/l, 10 mmol/l K_2HPO_4, 0.1 mmol/l EDTA, 0.08 mmol/l MgCl and 0.2% bovine serum albumin, pH 7.1.

Maximal respiration: Mitochondrial suspension was added to the reaction medium and coupled respiration (state 3) was initiated by the addition of 200-300 μM ADP. When all ADP was phosphorylated to ATP the respiratory rate returned to that prior of the addition of ADP i.e. non-coupled respiration (state 4). The respiratory control index (RCI) was calculated as the ratio between state 3 and state 4 respiration and P/O ratio was calculated as the amount of added ADP divided by the oxygen consumed. Respiration data have been corrected for electrode drift.

Submaximal respiration: After determination of the maximal respiration (state 3) the submaximal respiration was initiated by low rate ADP infusion. This was accomplished by

the use of a microdialysis pump (CMA/Microdialysis CMA/102, CMA/Microdialysis, Solna, Sweden) with a pumping rate ranging from 0.1 to 20 µl/min. The P/O ratio was calculated as the rate of infused ADP divided by the rate of oxygen consumption. The steady state respiration was corrected for the added oxygen by the ADP medium, electrode drift and for the effect of oxygen diffusion at different oxygen tensions.

Relative FA oxidation: In study IV respiration was measured on two occasions, first with 10µmol/l palmitoyl-carnitine (PC) + 2 mmol/l malate, and next with 5 µmol/l palmitoyl-carnitine and 4 mmol/l pyruvate (Pyr) + 2 mmol/l malate. The relative FA oxidation has been calculated as the ratio between state 3 respiration with PC and state 3 respiration with Pyr. After measurement of respiration the oxygraph solution was frozen in liquid nitrogen (N_2) and saved in - 80° C. Maximal electron transport chain (ETC) activity was measured after 2-3 days, by addition of 10 µg/ml alamethicin (Sigma A-3665), 450 µmol/l NADH and 2 µmol/l Cytocrome C.

Protein expression, citrate synthase (CS) activity and fatty acids (FA)

Protein expression: Portions of freeze-dried muscle were cleaned from blood, fat and connective tissue and homogenised in cold lyses buffer. (2 mM/l HEPES 1 mM/l EDTA, 5mM/l EGTA, 10mM/l $MgCl_2$, 50mM/l β glycerolphosphate, 1 mM/l $NaVO_4$, 2 mM/l dihiothretinol, 1% Triton X-100 and protease inhibitors pH 7.4). Protein concentration was determined in muscle homogenate and in isolated mitochondria, using Pierce protein assay (kit: 23223). The homogenate was used for determination of UCP3, ANT, CS activity and fiber type composition.

CS activity: CS was measured, using a technique described in (Tonkonogi *et al.*, 1997). Muscle homogenate was further diluted in phosphate buffer (50 mM) with 0.05% Triton-X, pH 7.4, and added to a 100 mM Tris buffer with 0.4 mM dithio-nitrobenzoic acid, pH 8.1. Finally oxalacetic acid was added to start the reaction before measuring the rate of absorbance change with a spectrophotometer at 412 nm.

Glucose: Glucose concentration was measured in whole blood with Accu-Chek, (Roche Diagnostics AB). In study IV plasma glucose was measured by the Synchron LX system

that determines glucose concentration by an oxygen rate method with Beckman oxygen electrode.

FA: Non-esterified fatty acids were measured in plasma by Wako NEFA C test kit. (Nr: 994-75409 D).

UCP3, ANT and fiber type distribution (% MHC 1)

UCP3: Muscle homogenate was solubilised in Laemmli sample buffer and denatured by boiling. 100 µg protein was added per lane on 12% polyacrylamid gels and separated by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) for 60 min at 135 V. The separated polypeptides were transferred to a PVDF membrane at 10 V for 60 min, and blocked in tris-buffered saline (TBS) with 5% non-fat milk. Membranes were incubated overnight with polyclonal antibody against UCP3 (Chemicon AB 3046), diluted 1:1000, washed and incubated with secondary antibody goat anti-rabbit (IgG-HRP, NO.sc-2030 Santa Cruz). The membrane was again washed and incubated with chemiluminescence detection reagent ECL, (No RPN 2106, Amersham). Finally, an X-ray film was exposed to the membrane for 10 min. The optical density of the bands was quantified by using Quantity One 1-D Analysing software (Bio-Rad). Samples where analysed in duplicates and the density of the bands where related to a standard sample, loaded on the same gel.

ANT: Denaturation was performed as for UCP3. 75µg of protein was added per lane on 12% polyacrylamid gels and separated by SDS-PAGE for 60 min at 135 V. Polypeptides were transferred to a PVDF membrane at 100 V for 120 min, and blocked in TBS with 5% non-fat milk. Membranes were incubated 2 h with polyclonal antibody against ANT (ANT-1, Q-18, No. sc-9300, Santa Cruz) diluted 1:200, washed and incubated with secondary antibody bovine anti-goat (IgG-HRP, No. sc-2350 Santa Cruz). After washing the membrane was incubated with chemiluminescence detection reagent ECL (No RPN 2106, Amersham), and exposed to a film for 50 min. The optical density of the bands was quantified by using Quantity One 1-D Analysing software (Bio-Rad).

Myosin heavy chain (MHC): % MHC 1 was analysed as previously described (Danieli Betto *et al.*, 1986) and modified for humans (Andersen & Aagaard, 2000). Briefly,

muscle homogenate (80 µl) was mixed with 200 µl of sample-buffer (10% glycerol, 5% beta–mercaptoethanol, 2.3% SDS, 62.5 mM Tris-base and 0.2% bromophenol blue at pH 6.8). The homogenate was denaturated by boiling at 100° C for 3 min and loaded on a 8% polyacrylamide SDS-PAGE gel (100:1 acrylamid:bisacrylamid), 30% glycerol, 67.5 mM tris-base, 0.4% SDS, and 0.1 M glycine). Bands were separated with electrophoresis at 80 V for at least 42 h at 4° C and MHC bands where made visible by Coomassie staining (Sigma B-0149). The gels were scanned on Gel Doc 2000 (Bio Rad). MHC 1 and MHC 2 bands where quantified using Quantity One 1-D Analysing software (Bio-Rad). MHC 1 is expressed in percent of total MHC.

Reactive oxygen species (ROS) and 4-hydroxy-2-nonenal (4-HNE) adducts

Superoxide production rate: Superoxide generation rate was assessed by measurement of H_2O_2 in the presence of superoxide dismutase (SOD). Mitochondrial H_2O_2 release was determined fluorometrically by measurement of oxidation of Amplex Red reagent coupled to the enzymatic reduction of H_2O_2 by horseradish peroxidase (HRP). Amplex Red reagent reacts with H_2O_2 in a 1:1 stoichiometric ratio to produce the brightly red fluorescent reaction product resorufin. Since HRP is a large protein that does not cross membranes, this assay only detects H_2O_2 that has been released from the mitochondria (i.e. the assay cannot measure H_2O_2 inside the mitochondria). 90 units/ml SOD was added to convert all superoxide into H_2O_2, a necessity since superoxide reacts rapidly with HRP, resulting in underestimation of the actual rate of H_2O_2 production

Mitochondria (0.15-0.22 mg/ml) were incubated at 37 °C in the same buffer as used for mitochondrial respiration (page. 20). All incubations also contained 5 µM Amplex Red, 12 units/ml HRP, 90 units/ml SOD, and 2 µg/ml oligomycin. The reaction was initiated by addition of 5 mM succinate with following addition of 3 µg/ml antimycin A. The increase in fluorescence at an excitation of 560 nm (slit 2.5 nM) and emission of 590 nm (slit 5 nM) was followed on a computer-controlled Hitachi F-2500 spectrofluorometer in 300 µl micro-cuvette for 10-15 min. The addition of 250 units/ml catalase decreased the fluorescence signal by ~99%. The rate of H_2O_2 generation was calculated as the change in fluorescence intensity during the linear increase. Appropriate corrections for

background signals were applied. The standard curves were generated using known amounts of H_2O_2 to calculate the rate of H_2O_2 production in pmol / min^{-1} /mg mitochondrial protein. Only freshly diluted H_2O_2 was used for measurements and the concentration of the stock solution was checked on spectrophotometer at 240 nm using coefficient of molar extinction equal 43.6.

4-hydroxy-nonenal-adducts: For 4-HNE adducts detection, aliquots of freshly isolated mitochondrial suspension were stored at -80° C. Mitochondrial proteins were solubilised in Laemmli sample buffer with beta-mercaptoethanol and denatured by boiling. 16 µg protein was added per lane on 12% polyacrylamid gels (BioRad) and separated by SDS-PAGE for 180 min at 100 V. Protein samples were transferred by electroblotting to a PVDF membrane at 100 V for 180 min and blocked in TBS with 5% non-fat milk. Membranes were blotted overnight at 4°C with primary polyclonal antibodies (dilution 1:1000) to the 4-hydroxy-nonenal-adducts (Alpha Diagnostics). After incubation with primary antibodies, the membrane was washed three times in TBS buffer with 0.1% of Tween-20 (TBST) and incubated with secondary antibody conjugated with HRP (anti-goat, dilution 1:2000, Santa Cruz Bio Thechnology). The membrane was washed again in TBS and incubated with chemiluminescence detection reagent ECL (Amersham). Quantifications were performed with Quantity One 1-D Analysing software (Bio-Rad) using option profile trace with lane-based background subtraction.

Statistics

All reported values are presented as mean ± SEM, and significance has been set as $P< 0.05$. All data has been tested for normal distribution and both parametric and non-parametric statistics have been used. Groups have been compared using Students´s paired *t*-test, or Mann-Whitney *U* test. Correlation between variables has been tested with Sperman´s correlation analysis. In the acute exercise study repeated measures analysis of variance (ANOVA) was used, followed by *post hoc test* with Greenhouse-Geisser and Huynh-Feldt adjustments. In the cycling efficiency study and the study on fat oxidation significance was tested with a two-way ANOVA repeated measures,

followed by Student-Neuman-Keuls *post hoc test.* In the ultra-endurance study both one-way and two-way ANOVA repeated measures were used, followed by Fischers LSD *post-hoc test*.

Methodological considerations

Muscle biopsies: The technique of taking muscle biopsies with Bergström needle is frequently used. It is a safe method and complications are rare. All biopsies where taken from vastus lateralis. Vastus lateralis is commonly used for this type of studies, the risk of injury on big vessels and nerves are limited. All test and exercise protocols where performed on cycle ergometer and the quadriceps muscle is the major power producer during cycle exercise.

Mitochondria isolation: After the biopsy mitochondria was isolated according to the method of (Tonkonogi & Sahlin, 1997). The high RCI, (values from the acute exercise study: 9.2 ± 1.1 before exercise, 9.7 ± 0.5 after exercise and 8.3 ± 0.5 three hours post exercise) shows that the isolated mitochondria's had a high capacity to generate ATP. A general problem with measurements on isolated mitochondria is that all interactions between mitochondria and other cell structures are lost, it is also possible that there are functional differences between mitochondria with different locations in the cell (i.e. subsarcolemmal or intermyofibrillar mitochondria) and that the dissected mitochondria don't represent the whole muscle mitochondria population.

Non-coupled respiration and state 4 respiration: In these studies state 4 respiration measured in isolated mitochondria have been presented as non-coupled respiration. It could be argued that this is a simplification and that measurement of membrane potential and/or inhibition of ATP synthase by oligomycin should have been used to verify that state 4 respiration is equal to non-coupled respiration. Theoretically state 4 respiration is influenced by contamination of ATPase. This results in increased state 4 respiration due to rephosphorylation of ATP broken down by ATPase. The difference in oxygen consumption before addition of ADP and after conversion of ADP to ATP (state 4) was small (6.7% in study I), indicating clean preparations with low levels of ATPase

activity. In this type of studies when mitochondria from exercising humans are used, the limited time and material is the rationale for the study design.

Citrate synthase activity: CS activity was measured in the mitochondrial fraction and in muscle homogenate. CS activity was used as a marker of mitochondrial volume, and mitochondrial respiration was expressed per unit of CS. By using this approach mitochondrial respiration could be compared to other measurers of training status (Tonkonogi & Sahlin, 1997). In the acute exercise study muscle CS increased by 43% and remained elevated after 3 h recovery. CS is an allosteric enzyme, activated by ADP and measurements of CS activity should be performed, following one day without exercise. CS measured after exercise could not be used as a marker of mitochondrial density. Therefore, respiration was expressed per mg of mitochondrial protein in the acute exercise study and the ultra-endurance performance study.

Western blot for UCP3: Protein expression of UCP3 was measured using a commercially available antibody (Chemicon AB 3046). To make sure that the band of interest was UCP3 a biotynilated molecular weight marker was used (Fernstrom, 2004). According to the protocol to isolate mitochondria protease treatment with Nagarse was used, and nagarse is known to affect UCP3 content (Giacobino, 2001). In the preparation of muscle homogenate protease inhibitors was used. Measurement UCP3 in isolated mitochondria was therefore not possible in these preparations, and all measurements of UCP3 protein expression have been performed on muscle homogenate.

RESULTS

Effects of exercise and training on UCP3 and mitochondrial uncoupling

In the exercise study (I) 9 subjects cycled for 75 min at a work-rate corresponding to 70% of their individual VO_2 peak. Whole body oxygen consumption at rest was 17% higher 3h post-exercise compared to before exercise (0.28 ± 0.02 versus 0.24 ± 0.01 l/min) due to the slow phase of EPOC. Plasma FA increased 3-times after exercise and was further increased after three hours of recovery (P< 0.05), elevated FA is known to uncouple mitochondria, but the individual increase in FA was not correlated to the slow phase of EPOC. Protein expression of UCP3 and ANT was unchanged by exercise as well as mitochondrial respiration (Table 2).

	Pre-exercise	Post-exercise	3h post-exercise	Significance
UCP3	117 ± 29	109 ± 41	135 ± 46	n.s.
ANT	152 ± 25	169 ± 24	177 ± 22	n.s.
State 3	187 ± 16	201 ± 22	205 ± 20	n.s.
State 4	21 ± 3	24 ± 4	25 ± 3	n.s.

Table 2. *Effects of endurance exercise on muscle mitochondrial parameters. UCP3 and ANT (arbitrary units/mg protein), state 3 and state 4 respiration (nmol O_2/min/mg protein). Values are mean ± SEM from 9 subjects, P< 0.05.*

Endurance training for 6 weeks increased whole body oxygen uptake (VO_2 peak) by 24% (P< 0.05). At the muscle level citrate synthase (CS) activity was increased by 47%, (P< 0.05). In this study CS was used as a marker of mitochondrial volume. Mitochondrial state 3 respiration per weight of muscle increased by 40% (P< 0.05), but remained unchanged when expressed in relation to CS, whereas non-coupled respiration (state 4 respiration/mitochondria) expressed in relation to CS, decreased by training by –18% (P< 0.05. Fig 3). Protein expression of ANT was increased even more than CS (+96%. P< 0.05. Table 3). In contrast UCP3, expressed per muscle protein, was not significantly changed by training (table 3) and decreased by 53% (P< 0.05. Fig 3), when related to CS.

	Pre-training	Post-training	Significance
Muscle CS	21.2 ± 1.0	31.0 ± 1.5	P< 0.05
UCP3	142 ± 28	98 ± 18	n.s.
ANT	76 ± 20	149 ± 19	P< 0.05
State 3	65.7 ± 3.3	62.5 ± 2.4	n.s.
State 4	7.7 ± 0.6	6.3 ± 0.3	P< 0.05

Table 3. *Effects of 6 weeks endurance training on muscle mitochondrial parameters. Muscle citrate synthase (CS) activity (mmol/min/kg wet wt), UCP3 and ANT (arbitrary units/mg protein), state 3 and state 4 respiration (nmol O_2/min/unit of CS). Values are mean ± SEM from 8 subjects.*

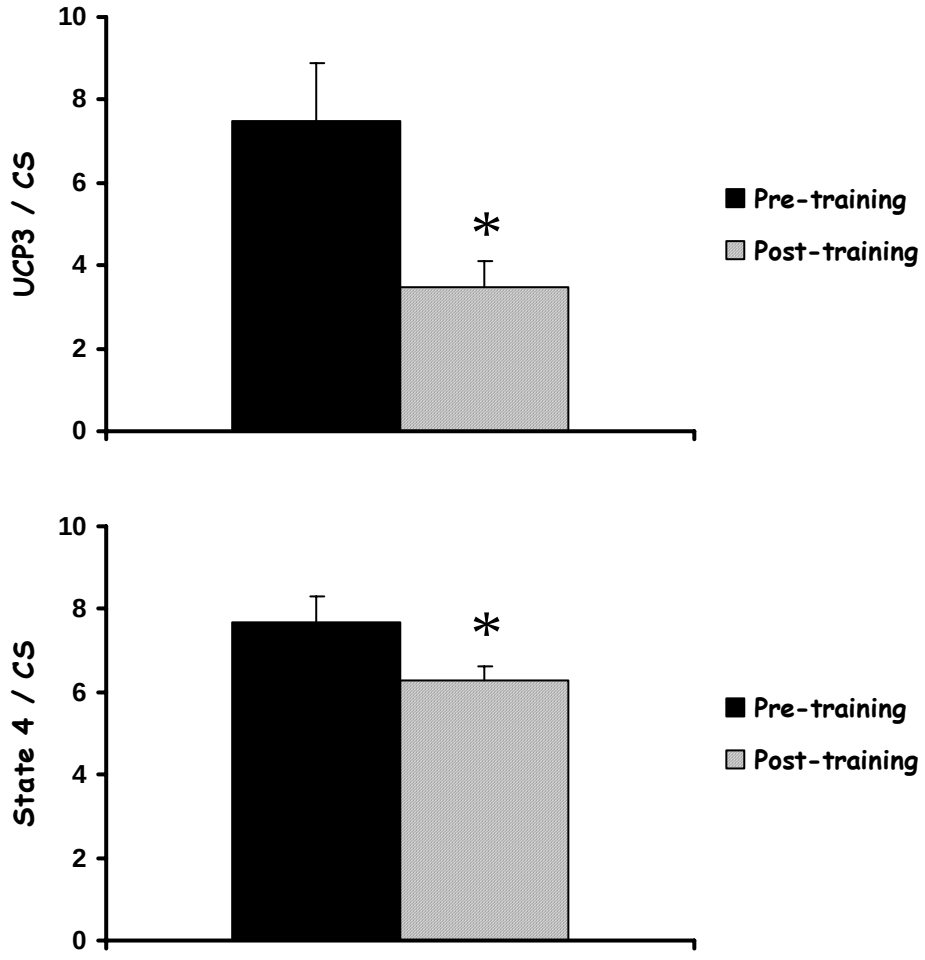

Figure 3. *Protein expression of UCP3 before training and after training (arbitrary units/mg muscle protein) related to CS-activity and state 4 respiration (nmol O_2/min/unit CS). Values are mean ± SEM, from 8 subjects.*

Influence of uncoupling on work efficiency

In the cycling efficiency study (II) two groups of subjects were compared, well-trained (VO_2 peak above 55 ml O_2/min/kg) and untrained subjects (VO_2 peak below 45 ml O_2/min/kg). Cycling efficiency at the whole body level was measured as work efficiency (WE). WE at 80w ranged from 25-30% but there was no significant difference between trained and untrained subjects in whole body cycling efficiency.

	Untrained	Trained	Significance
Muscle CS	19.7 ± 2.2	27.8 ± 0.7	P< 0.05
State 3	88.4 ± 8.9	88.7 ± 4.1	n.s.
State 4	7.2 ± 1.1	6.5 ± 0.4	n.s.
MEff (P/O ratio)	2.47 ± 0.20	2.50 ± 0.06	n.s.

Table 4. *Difference between untrained and trained subjects on muscle mitochondrial parameters. Muscle citrate synthase (CS) activity (mmol/min/kg wet wt), state 3 and state 4 respiration (nmol O_2/min/unit of CS). MEff the ratio between phosphorylated ATP and consumed oxygen during state 3. Values are mean ± SEM from 17 subjects, 8 untrained and 9 trained.*

The mitochondrial efficiency (MEff) was measured during maximal and sub-maximal (physiological) ADP addition. There was no difference between groups either at sub-maximal or at high ADP levels. When mitochondrial respiration was expressed per kg muscle mass there was a significant difference between the trained and untrained group, state 3 respiration was higher (P< 0.05). But when respiration was expressed per CS (Table 4) the difference disappeared. Untrained subjects had 52% more UCP3 protein per muscle weight compared to the trained group (P< 0.05. Fig. 4) and the difference was even more pronounced (3-times higher) when related to CS (i.e. UCP3 per mitochondrial volume). In contrast to previous findings (Hesselink *et al.*, 2001), there was no correlation between fiber type composition (% MHC 1) and protein expression of UCP3 in these subjects.

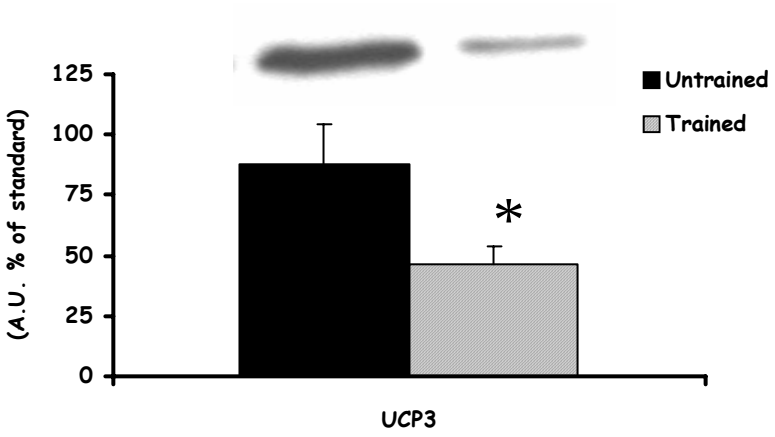

Figure 4. *Protein expression of UCP3 (arbitrary units % of standard) in 9 untrained and 9 trained subjects. Values are mean ± SEM. * = Significant difference (P< 0.05).*

Work efficiency was not correlated to mitochondrial efficiency, but there was a significant and positive correlation between work efficiency measured as WE at 80w and % MHC 1 (r= 0.58. P< 0.05). VO_2 peak was negatively correlated to UCP3 protein expression (r= -0.52. Fig. 5). In addition WE was negatively correlated to UCP3 protein expression both at 80W and 120W (r= -0.58. r= -0.57. P< 0.05). The correlation was most evident in the untrained subjects.

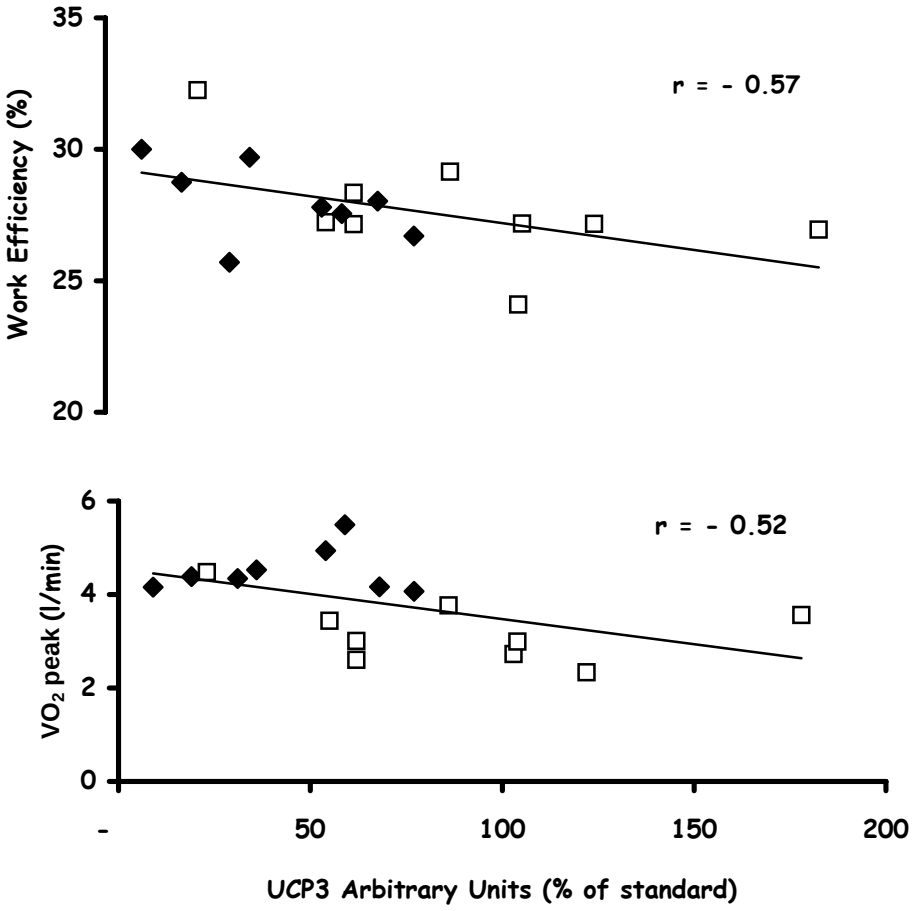

***Figure 5**. Correlation between work efficiency (WE at 80w) and UCP3 protein content (r= -0.57. P< 0.05) and between VO_2 peak and UCP3 (r= -0.52. P< 0.05). ♦ = Trained subjects (VO_2 peak >0.55ml/min/kg), □ = untrained subjects (VO_2 peak <0.55ml/min/kg). Values are mean ± SEM from 8 untrained and 9 trained subjects.*

Mitochondrial lipid oxidation during low-intensity exercise

In study III mitochondrial respiration was lower with PC than with Pyr but there was no difference in relative mitochondrial fat oxidation (MFO) between untrained and trained subjects. MFO however showed a large variation between subjects (ranged from 49-93%) and was correlated to % MHC 1 (r= 0.62. P< 0.05) but not to UCP3 protein (r= 0.30). A significant correlation between whole body relative substrate oxidation (RER)

and FA relative oxidation in isolated mitochondria (MFO) was observed, this was evident during exercise at 80W (r= -0.62. P< 0.05) (Fig 6) and at 120W (r= -0.71. P< 0.05), but not at rest. RER was not correlated to % MHC 1.

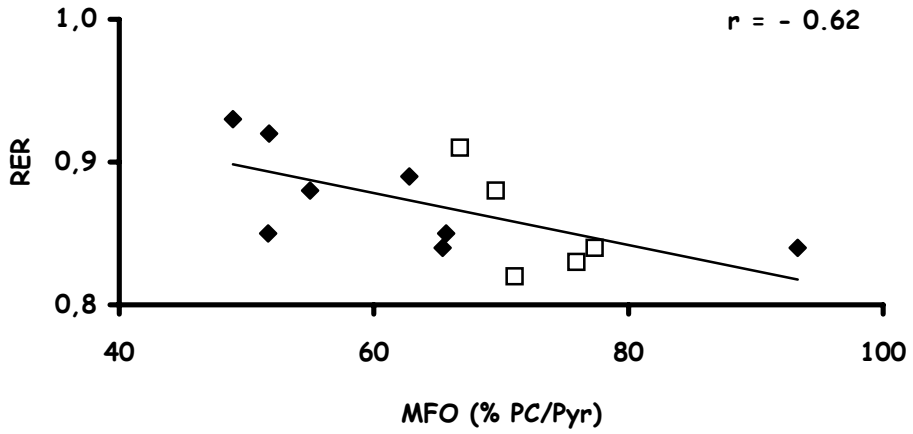

Figure 6. *Correlation between relative fat oxidation in vivo (RER) and in mitochondria (MFO), (r= -0.62. P< 0 .05). MFO was expressed as the ratio between state 3 respiration with PC and Pyr. ♦ = Trained subjects, □ = untrained subjects. Values are mean ± SEM from 8 untrained and 5 trained subjects.*

Mitochondrial function during extreme exercise

In the final study (IV) 9 subjects performed ultra-endurance exercise. The design of the study was similar to the first part of study I, except for the extreme duration of the exercise bout (24 hours) and the fact that all participants in the study belong to the Swedish and world elite of ultra-endurance performance athletes. At the whole body level oxygen consumption (measured during cycling at 60% of VO_2 peak) was increased at the end of exercise (+14%) and remained elevated 28h after recovery (+7%. P< 0.05). Plasma FA increased four-fold at the end of exercise (P< 0.05) and returned to the same level as pre-exercise after recovery, whereas plasma glucose remained unchanged by exercise. RER was reduced at the end of exercise (-6%. P< 0.05), indicating that whole body lipid oxidation was increased during exercise. The

relative lipid oxidation measured at the mitochondrial level was also increased by exercise (P< 0.05. Fig. 7). Mitochondrial state 3 respiration with PC was higher post-exercise (P= 0.05) and state 4 respiration with PC was elevated post-exercise (P< 0.05. Table 5).

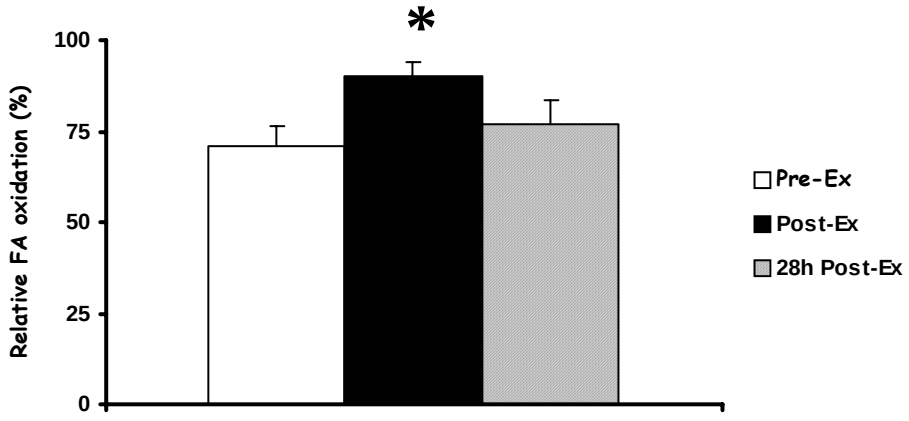

Figure 7. *Effect of ultra-endurance exercise on relative mitochondrial FA oxidation (MFO) in isolated mitochondria. Values are mean ± SEM from 9 subjects. * = Significant difference (P< 0.05).*

	Substrate	Pre-exercise	Post-exercise	28h Post-exercise	Significance
State 3	PC	65.8 ± 10.9	91.1 ± 7.5	70.0 ± 6.5	* #
	Pyr	93.1 ± 9.6	104.2 ± 6.3	93.8 ± 3.6	n.s.
State 4	PC	14.0 ± 1.6	17.1 ± 1.1	10.4 ± 0.7	* § #
	Pyr	10.2 ± 1.1	11.0 ± 0.6	7.0 ± 0.6	§ #
ETC-activity	NADH	358 ± 30	357 ± 24	312 ± 18	n.s.
MEff (%)	PC	100%	91 ± 2	92 ± 2	* #
	Pyr	100%	94 ± 2	93 ± 3	* #

Table 5. *Effects of ultra-endurance exercise on muscle mitochondrial parameters. State 3, state 4 and maximal ETC activity (nmol O_2/min/mg protein). MEff (i.e. produced ATP/consumed O_2). Values are mean ± SEM from 9 subjects. * = Difference between pre-ex and post-ex (P<*

0.05). § = Difference between post-ex and recovery P< 0.05). # = Difference between pre-ex and recovery (P< 0.05).

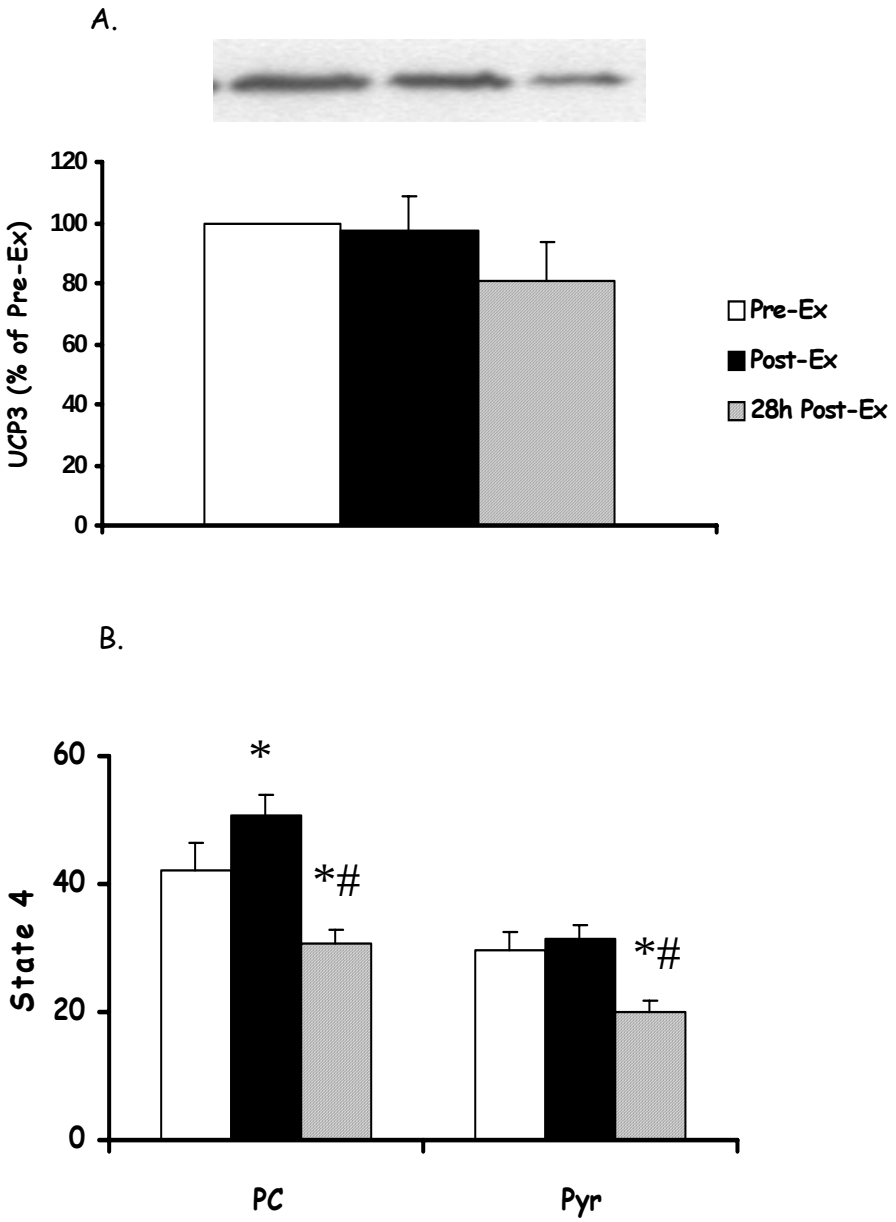

Figure 8. *A. Protein expression of UCP3 (arbitrary units). B. State 4 respiration (nmol O_2/min/mg protein). Pre-exercise, post-exercise and after recovery. Values are mean ± SEM from 9 subjects. * = Difference between pre-ex and post-ex or pre-ex and recovery (P< 0.05). # = Difference between pre-ex and recovery (P< 0.05).*

Interestingly, state 4 respiration with PC and Pyr was lower after recovery as compared both to pre-exercise and post-exercise, (P< 0.05. Fig. 8).

Maximal ETC-activity was unchanged by exercise. MEff however was decreased with both PC and Pyr after exercise (P< 0.05) and the decrease remained after 28 h of recovery. UCP3 protein expression was unchanged by exercise, but tended to be lower after recovery (P= 0.07).

Reactive oxygen species (ROS) was measured as the hydrogen peroxide (H_2O_2) production rate (succinate without rotenone, reversed electron flow). ROS production was significantly increased immediately after ultra-endurance exercise, but returned to a level similar as to pre-exercise after 28 hours of recovery (P< 0.05. Fig. 9). The lipid peroxide 4-hydroxy-2-nonenal (4-HNE) adducts was elevated by +40% after exercise but the increase was not significant. After recovery however the increase reached statistical significance (+68%. P< 0.05).

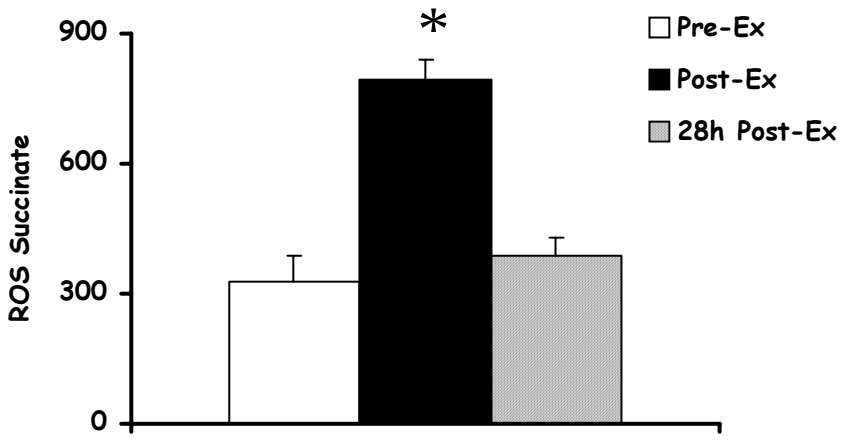

Figure 9. *Rate of superoxide production (pmol H_2O_2/min/mg protein) in the presence of succinate. Values are mean ± SEM from 8 subjects. * = Significant difference (P< 0.05).*

DISCUSSION

Uncoupling in response to exercise and training

The main finding from study I was that protein expression of UCP3 decreased after 6 weeks of endurance training. Expression of UCP3 does not as one would expect simply follow mitochondrial biogenesis. Mitochondrial mass measured as citrate synthase (CS) activity increased by 47% ($P < 0.05$), but the expression of UCP3 was despite the profound increase in mitochondrial volume, reduced. Non-coupled respiration (state 4) was also reduced ($P < 0.05$) after the training period but not correlated to UCP3. This is the first human longitudinal study of UCP3 protein expression. The finding that training decreases UCP3 is consistent with human cross-sectional studies showing lower levels of UCP3 in trained subjects compared with untrained (Schrauwen *et al.*, 1999; Russell *et al.*, 2003). In contrast, increased UCP3 protein expression has been shown in subjects with type 2 diabetes after 4 month of low-intensity exercise training (Fritz *et al.*, 2006). In line with this finding it has been shown that low levels of UCP3 in diabetic subjects, is restored by the insulin sensitizer drug roziglitation (Schrauwen *et al.*, 2006).

During exercise plasma FA is increased, but as mentioned previously lipid oxidation and the capacity for OxPhos is improved in trained subjects. Although FA was not measured in this study lower plasma levels after the training period could have influenced the expression of UCP3. Another possible explanation to the reduction of UCP3 is that endurance training induced a shift towards a more oxidative fiber type profile. UCP3 is expressed in lower levels the more oxidative the fiber is, with the lowest levels detected in heart muscle (Hoeks *et al.*, 2003). Against this explanation it could be argued that 6 weeks is too short to change fiber type profile and fiber type composition was not measured in the first study of this thesis.

One possible explanation to the reduced expression of UCP3 observed in study I could be that if UCP3 is responsible for the decreased non-coupled respiration (state 4) after training, down-regulation of UCP3 may be important to prevent excess heat production and waste of energy in trained subjects with high levels of mitochondria.

Provided that UCP3 and non-coupled respiration increased in parallel with mitochondrial biogenesis, this would increase BMR and consequently heat production by almost 50%.

Adenine nucleotide translocase (ANT) is an important and abundant protein in the IMM, mediating the exchange of ADP and ATP. ANT is also considered to contribute to non-coupled respiration (Tikhonova *et al.*, 1994). The difference in charge of ATP (4-) and ADP (3-) is bound to influence membrane potential especially during high ATP production rate. After 6 weeks of endurance training ANT protein expression was increased by 96% ($P < 0.05$), but when related to CS activity ANT was unchanged, and it can be concluded that the expression of ANT does indeed follow mitochondrial biogenesis.

One exercise session (75 min) had no effect on protein expression of UCP3 or ANT, nor on state 3 or state 4 respiration. Plasma FA increased 3-4 times after exercise and remained elevated also after recovery. Oxygen consumption, (excess post exercise oxygen consumption (EPOC)) was elevated by 17% in the recovery state, but not correlated to plasma FA, UCP3 or non-coupled respiration. The hypothesis that non-coupled respiration measured *in vitro* could explain the slow (and unexplained) part of EPOC could not be supported. It is, however, possible that UCP3 and non-coupled respiration was activated *in vivo* despite unchanged protein expression by intra muscular FA or ROS. If this was the case increased activity of UCP3 did not affect state 4 respiration, measured in isolated mitochondria. Increased FA oxidation due to high plasma levels of FA do contribute to increased BMR. Mitochondrial efficiency (MEff) is on average 10% lower with lipid oxidation as compared to CHO oxidation, but a shift towards lipid oxidation after exercise cannot explain an increase in oxygen consumption by 17%. Therefore it seems likely that substrate cycling or increased intracellular ATP demand after exercise was responsibly for the main part of the increased oxygen consumption during the recovery period.

We also demonstrated that cycling efficiency, measured as work efficiency (WE) was correlated to UCP3 protein and to fiber type composition i.e. % MHC 1. Protein expression of UCP3 was higher in untrained subjects, and when related to CS-activity

the expression of UCP3 was 3-times higher in untrained subjects. State 4 respiration when related to CS-activity was similar between trained and untrained men and not correlated to UCP3. As previously observed (Schrauwen & Hesselink, 2003) UCP3 is negatively correlated to maximal oxygen capacity (Fig 5) and this was confirmed in the present study.

Mitochondrial efficiency (P/O-ratio) and exercise

An unexpected finding from the second study was that cycling efficiency was similar in trained and untrained subjects. Mitochondrial efficiency (MEff) was also similar in trained and untrained subjects and not correlated to cycling efficiency. Mitochondrial respiration is measured during standardized conditions and it is possible that the absence of activation factors for UCP3, such as FA and ROS *in vitro*, explains why there is no correlation between MEff and UCP3 or between UCP3 and state 4 respiration, despite the fact that work efficiency was correlated to UCP3.

MEff is calculated during state 3 respiration and therefore probably not affected by non-coupled respiration. In a recent study by (Sluse *et al.*, 2006) it is suggested that UCP3 can be active also during state 3 respiration, and that the activity is regulated by the redox state of coenzyme Q. In study II MEff was measured during maximal and during sub-maximal mitochondrial respiration, with no difference between trained and untrained subjects, demonstrating that different expression, or activity of UCP3 did not affect MEff during state 3 in mitochondria isolated from exercising trained and untrained men.

The correlation between cycling efficiency and type 1 fibers may be explained by a higher mechanical efficiency in type 1 fibers. Most animal studies investigating the thermodynamic efficiency of muscle contraction have shown that type 1 fibers are more efficient than type 2 fibers (Smith *et al.*, 2005). In rat muscle there is no difference in mitochondrial efficiency between type 1 and 2 fibers either at sub-maximal or at maximal respiration (Mogensen & Sahlin, 2005). Cycling efficiency in humans is influenced also by the cadence. As work rate increases more type 2 fibers are

recruited and this is associated with decreased efficiency if the cadence is maintained constant.

Interesting to note is also that work efficiency (WE) was correlated to UCP3 when measured at 80 and 120w, but when work rate was raised to 80% of VO_2 peak, and the subjects started to depend on carbohydrate metabolism, the correlation disappeared.

In study IV mitochondrial efficiency (MEff) decreased after ultra-endurance exercise and remained reduced also after recovery. This is the first human study reporting reduced MEff after exercise. Previous studies from our group show that MEff is unchanged by high intensity exercise (Tonkonogi *et al.*, 1999), by intermittent static contractions to fatigue (Sahlin *et al.*, 2006b) and by endurance training (Tonkonogi *et al.*, 2000). The finding is difficult to explain. Elevated FA and oxidative stress (plasma FA was increased 4-fold and ROS production rate increased 2.5-fold post-ex) could theoretically increase uncoupling through UCP3 or ANT, or induce opening of mitochondrial permeability transition pores (mtPTP). MEff, however, is calculated during coupled respiration (state 3) and non-coupled respiration (state 4) was reduced after recovery, indicating tight coupling. The reason for the reduced mitochondrial efficiency may instead be that the efficiency of ETC or ATP-synthase was reduced, or simply reflect that mitochondrial proteins important for ATP synthesis were deteriorated by 24 hours of exercise. Creatine kinase (CK) a marker of damage on cellular membranes was increased (24 fold, $P<0.05$) after exercise.

Mitochondrial lipid oxidation

In study III the relative mitochondrial FA oxidation (MFO) was determined in trained and untrained men. MFO during low-intensity exercise was indeed correlated to whole body lipid oxidation and MFO was also correlated to type 1 fibers, but not different between untrained and trained subject. The difference between subjects was large (ranging from 49-93%) and could, except from fiber type distribution, be explained by preceding diet and training. In the present study training and diet was standardized, but only one day ahead of the test. Apart from lipid supply and lipid oxidation capacity muscle glycogen stores do also affect FA oxidation during exercise (Roepstorff *et al.*,

2004). As the rate of FA oxidation increases during exercise in parallel with increasing energy demand, FA oxidation reaches a peak at an intensity of 45-65% of VO_2 peak (Achten & Jeukendrup, 2003) and at high intensity exercise only CHO is oxidized. Accumulation of LCFA in the cytosol despite reduced lipid oxidation provides evidence that the limitation of lipid oxidation is in the transport of LCFA to the mitochondria or within the mitochondria itself (Kiens, 2006). Recently a well characterised key protein in human skeletal muscle lipid transport FAT/CD36 was found also in the mitochondria (Bezaire *et al.*, 2006). It is suggested that FAT/CD36 is located in intracellular pools and in similarity with glucose transport protein 4 (GLUT 4) can be recruited to the plasma membrane and facilitate LCFA uptake in response to exercise or insulin (Campbell *et al.*, 2004). It is also suggested that FAT/CD36 together with CPT1 can increase LCFA uptake to the mitochondria (Campbell *et al.*, 2004). The major finding of the study was that MFO at the mitochondria level influenced whole body lipid oxidation during low-intensity exercise.

A novel finding in study IV was that the capacity for mitochondrial FA oxidation increased after 24 hours of ultra-endurance exercise. State 3 respiration with PC and the relative mitochondrial lipid oxidation (PC/Pyr) was increased. The relative lipid oxidation, measured in isolated mitochondria increased by 34% indicating that mitochondrial lipid oxidation can be up regulated during exercise. CPT1 is considered to be a key control site for mitochondrial lipid oxidation. The function of CPT1 is to transport carnitine with or without a LCFA over the outer mitochondrial membrane. However, when palmitoyl carnitine (PC) is used as substrate, this regulatory step is bypassed. Fat/CD36 may facilitate lipid uptake and oxidation in muscle mitochondria, indeed it has been shown that 120 min cycling profoundly increases FAT/CD36 in the mitochondrial membrane, and that the increase correlates to mitochondrial and whole body lipid oxidation (Holloway *et al.*, 2006). FAT/CD36 was not measured in this study but may explain the observed increase in relative fatty acid oxidation observed after 24 hours of ultra-endurance exercise.

ROS production and non-coupled respiration in response to ultra-endurance exercise

Mitochondrial ROS production and lipid peroxidation was also studied in response to ultra-endurance exercise. A second manuscript from the ultra-endurance study: Skeletal muscle mitochondrial ROS production in response to extreme endurance exercise in athletes (Shabalina *et al.*, 2006) is under preparation and therefore not included in the thesis. As discussed previously state 4 respiration was reduced after recovery from ultra endurance-exercise, and this is consistent with the tendency of reduced expression of UCP3 (P= 0.07 vs. pre-exercise). One theory about the physiological function of UCP3 that has been extensively debated states that mild uncoupling limits the production of reactive oxygen species (ROS) (Brand & Esteves, 2005). UCP3 is activated by 4-hydroxy-2-nonenal (4-HNE) that originates from a cascade of lipid peroxidation reactions initiated by high levels of superoxide. This concept has recently been questioned (Cannon *et al.*, 2006). Extreme exercise is known to induce oxidative stress (Mastaloudis *et al.*, 2001). Our preliminary results show that in muscle samples taken after ultra-endurance exercise mitochondrial ROS production (H_2O_2) increased 2.5 fold but returned to a level similar as to pre-exercise after recovery (Fig. 9). Formation of the lipid peroxide 4-HNE adduct was unchanged after ultra-endurance exercise but increased by 70% in the recovery state. Despite the profound increase in oxidative stress, non-coupled respiration (state 4) measured *in vitro* was reduced and protein expression of UCP3 unchanged. The result does not support a role for UCP3 in the defense against ROS production after exercise in human skeletal muscle. Although the activity of UCP3 may have been increased *in vivo*, oxidative stress did not induce an increased expression of UCP3.

There are only a few articles published on mitochondrial function and extreme exercise. The influence of the polymorphism –55 C/T within the uncoupling protein 3 gene, however, has been studied in ultra-endurance triathlons. In the study by (Hudson *et al.*, 2004) it was hypothesised that UCP3 could be important for endurance performance and the 89 fastest and 89 slowest triathletes who completed either the 2000 or 2001 South African Ironman triathlon events were genotyped for the polymorphism. No

association was found between the –55 C/T polymorphism and ultra-endurance performance.

UCP3 and type 2 diabetes

Insulin resistance and impaired glucose and lipid metabolism characterize type 2 diabetes. It has also been suggested that impaired mitochondrial function contributes to the disease. In similarity with trained subjects, it has been shown that subjects with type 2 diabetes have low levels of UCP3 (Schrauwen *et al.*, 2001) and high levels of IMTG (van Loon & Goodpaster, 2006). This similarity is puzzling. Our group has recently shown that mitochondrial function is impaired in skeletal muscle of subjects with type 2 diabetes (Mogensen *et al.*, 2006). ADP stimulated respiration with pyruvate (state 3) was reduced as compared to in healthy well-matched controls. Unexpectedly however, no difference in UCP3 was observed. One difference between the two studies was the insulin levels. Insulin was not significantly higher in the diabetic subjects in the present study by Mogensen et al. High insulin levels indicate skeletal muscle insulin resistance and it has recently been shown that insulin signaling in skeletal muscle is important also for lipid uptake and metabolism in a similar way as for glucose uptake (Bouzakri *et al.*, 2006). It is also possible that the lower expression of UCP3 in diabetic subjects observed in the study by (Schrauwen *et al.*, 2001) simply reflects a role for UCP3 is in glucose metabolism. In contrast, result from this thesis work and from the study by Mogensen et al. indicates that both obese and diabetic subjects have higher levels of UCP3 as compared to trained and untrained young healthy subjects, unpublished observation. And this is in line with the observed tendency of increased UCP3 (P= 0.09) in aged humans compared with young subjects with the same degree of physical activity (Tonkonogi *et al.*, 2003).

General discussion

UCP3 and basal metabolic rate

From these studies it can clearly be stated that UCP3 protein expression is lower in trained subjects, and not increased by exercise. State 4 respiration was reduced by endurance training, and by ultra-endurance exercise, but similar in untrained and trained subjects. The lack of correlation between UCP3 and state 4 respiration however, does not support a direct role for UCP3 in non-coupled respiration and thereby weight regulation in young healthy endurance trained persons. It can, however, not be excluded that the activity of UCP3 and non-coupled respiration is different *in vivo*. The correlation between work efficiency and UCP3 is interesting and indicates that low levels of UCP3 improves work efficiency, although mitochondrial efficiency measured *in vitro* was unaffected. The correlation may also be explained by differences in fiber type composition since expression of UCP3 is lower in fast twitch fibers. The observation by (Harper *et al.*, 2002), that weight loss in response to caloric restriction depends on UCP3 expression is appealing. Although the correlation between UCP3, state 4 respiration and weight loss in the study by (Harper *et al.*, 2002) also could be explained by training status. VO_2 peak, fat free mass and fiber type distribution was not evaluated in the study and could have influenced the results.

UCP3 and lipotoxicity

UCP3 is increased when plasma FA is high and muscle mitochondria lipid oxidation is insufficient. This is also the basis for the lipotoxicity hypothesis, claiming that LCFA may "flip-flop" over the IMM, get trapped and needs to be transported from the matrix to avoid damage on mitochondrial proteins. UCP3 is increased both by high fat diet and starvation. High fat diet especially in sedentary subjects is a situation when muscle mitochondria are unable to consume the supplied amount of lipids. If plasma glucose and insulin also are elevated lipid oxidation will be inhibited and predominately glucose oxidized, and thereby FA not oxidized in the mitochondria might be re-esterificated and stored as intramuscular triacylglycerol. During starvation the physical activity level is reduced and ATP turnover in skeletal muscle is low. Furthermore UCP3 mRNA in human skeletal muscle is increased in persons with spinal cord injury, in these subjects,

as in healthy subjects, UCP3 is reduced by training (electrical stimulation) (Hjeltnes *et al.*, 1999). Patients with tetra- or paraplegia are characterized by low levels of mitochondria and low capacity to oxidize lipids and an almost complete shift towards fast twitch glycolytic fibers (Mohr *et al.*, 1997). They develop insulin resistance in skeletal muscle and have increased risk of type 2 diabetes.

In all these situations the FA load on the muscle mitochondria is high and the oxidation rate limited. From this perspective a role for UCP3 in the export of accumulating FA^- from the matrix or in the handling of not oxidized lipids seems likely, and supports the lipotoxicity theory.

Summary

Protein expression of UCP3 does not follow exercise induced mitochondrial biogenesis, UCP3 and non-coupled respiration (state 4) was decreased after endurance training when related to mitochondrial mass (CS-activity). One session of cycling exercise (75 min) did not affect expression of UCP3 or non-coupled respiration (study I).

Study II demonstrates that cycling efficiency is higher in subjects with a low level of UCP3. Cycling efficiency and mitochondrial efficiency (P/O ratio) was similar in trained and untrained subjects, positively correlated to type 1 fibers, and inversely related to protein expression of UCP3.

In study III the hypothesis that mitochondrial relative fat oxidation (MFO) measured during standardized conditions is correlated to whole body lipid oxidation during low-intensity exercise was confirmed. MFO was similar in trained and untrained men and correlated to fiber type composition.

Mitochondrial lipid oxidation increased after 24 hours of ultra-endurance exercise, whereas mitochondrial efficiency (P/O ratio) decreased and remained reduced after recovery. Study IV is the first study reporting reduced P/O ratio after exercise.

Mitochondrial ROS production increased after ultra-endurance exercise, and lipid peroxidation, measured as 4-HNE adducts was increased after recovery. Non-coupled (state 4) respiration, however, decreased with both PC and Pyruvate. The result

demonstrates that high ROS production after extreme exercise followed by lipid peroxidation did not increase non-coupled respiration measured *in vitro* or induce expression of UCP3 in skeletal muscle.

UCP3 protein expression is lower in trained subjects, and decreases after endurance training. Non-coupled (state 4) respiration was reduced by endurance training, and by ultra-endurance endurance exercise, but similar in trained and untrained subjects. In this thesis state 4 respiration and UCP3 follow the same pattern but are not correlated. Further studies are needed to understand the complex role of UCP3 in skeletal muscle metabolism.

Ideas for the future

If low levels of UCP3 protein in endurance-trained subjects reflect high capacity for lipid oxidation and predominately slow twitch fibers, it can be hypothesised that the regulation of UCP3 in skeletal muscle is different in strength-trained subjects.

- Despite much research on UCP3 and uncoupling in humans little is known about the effects of strength training on UCP3, MEff and lipid oxidation.

It is known that glucose intake during exercise inhibits the observed increase in UCP3 mRNA after exercise, and it has been concluded that increased UCP3 mRNA after one exercise session is related to increased levels of non-metabolised LCFA.

- Nothing is known about the effects of amino acids intake during or after exercise on uncoupling, protein expression of UCP3 and MEff.

The hypothesis that UCP3 can transport pyruvate in a non-stimulated state has not been evaluated in humans. If so the relation of UCP3 to insulin levels and glucose metabolism is important.

- It can be hypothesised that a GI-diet with low levels of carbohydrate and high dependence of lipids could influence uncoupling and protein expression of UCP3.

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